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A Combined Proteomics and Mendelian Randomization Approach to Investigate the Effects of Aspirin-Targeted Proteins on Colorectal Cancer

Aayah Nounu[1,2], Alexander Greenhough[2,3], Kate J. Heesom[4], Rebecca C. Richmond[1], Jie Zheng[1], Stephanie J. Weinstein[5], Demetrius Albanes[5], John A. Baron[6], John L. Hopper[7,8], Jane C. Figueiredo[9,10], Polly A. Newcomb[11,12], Noralane M. Lindor[13], Graham Casey[14], Elizabeth A. Platz[15], Loïc Le Marchand[16], Cornelia M. Ulrich[17], Christopher I. Li[18], Fränzel J.B. van Duijnhoven[19], Andrea Gsur[20], Peter T. Campbell[21], Víctor Moreno[22,23,24,25], Pavel Vodicka[26,27,28], Ludmila Vodickova[26,27,28], Hermann Brenner[29,30,31], Jenny Chang-Claude[32,33], Michael Hoffmeister[29], Lori C. Sakoda[18,34], Martha L. Slattery[35], Robert E. Schoen[36], Marc J. Gunter[37], Sergi Castellví-Bel[38], Hyeong Rok Kim[39], Sun-Seog Kweon[40,41], Andrew T. Chan[42,43,44,45,46,47], Li Li[48], Wei Zheng[49], D. Timothy Bishop[50], Daniel D. Buchanan[51,52,53], Graham G. Giles[54,55], Stephen B. Gruber[56], Gad Rennert[57,58,59], Zsofia K. Stadler[60], Tabitha A. Harrison[18], Yi Lin[18], Temitope O. Keku[61], Michael O. Woods[62], Clemens Schafmayer[63], Bethany Van Guelpen[64,65], Steven Gallinger[66], Heather Hampel[67], Sonja I. Berndt[5], Paul D.P. Pharoah[68], Annika Lindblom[69,70], Alicja Wolk[71,72], Anna H. Wu[73], Emily White[18,74], Ulrike Peters[18,74], David A. Drew[75], Dominique Scherer[76], Justo Lorenzo Bermejo[76], Ann C. Williams[2], and Caroline L. Relton[1]

ABSTRACT

Background: Evidence for aspirin's chemopreventative properties on colorectal cancer (CRC) is substantial, but its mechanism of action is not well-understood. We combined a proteomic approach with Mendelian randomization (MR) to identify possible new aspirin targets that decrease CRC risk.

Methods: Human colorectal adenoma cells (RG/C2) were treated with aspirin (24 hours) and a stable isotope labeling with amino acids in cell culture (SILAC) based proteomics approach identified altered protein expression. Protein quantitative trait loci (pQTLs) from INTERVAL ($N = 3{,}301$) and expression QTLs (eQTLs) from the eQTLGen Consortium ($N = 31{,}684$) were used as genetic proxies for protein and mRNA expression levels. Two-sample MR of mRNA/protein expression on CRC risk was performed using eQTL/pQTL data combined with CRC genetic summary data from the Colon Cancer Family Registry (CCFR), Colorectal Transdisciplinary (CORECT), Genetics and Epidemiology of Colorectal Cancer (GECCO) consortia and UK Biobank (55,168 cases and 65,160 controls).

Results: Altered expression was detected for 125/5886 proteins. Of these, aspirin decreased MCM6, RRM2, and ARFIP2 expression, and MR analysis showed that a standard deviation increase in mRNA/protein expression was associated with increased CRC risk (OR: 1.08, 95% CI, 1.03–1.13; OR: 3.33, 95% CI, 2.46–4.50; and OR: 1.15, 95% CI, 1.02–1.29, respectively).

Conclusions: MCM6 and RRM2 are involved in DNA repair whereby reduced expression may lead to increased DNA aberrations and ultimately cancer cell death, whereas ARFIP2 is involved in actin cytoskeletal regulation, indicating a possible role in aspirin's reduction of metastasis.

Impact: Our approach has shown how laboratory experiments and population-based approaches can combine to identify aspirin-targeted proteins possibly affecting CRC risk.

[1]Medical Research Council (MRC) Integrative Epidemiology Unit, Bristol Medical School, University of Bristol, Bristol, United Kingdom. [2]School of Cellular and Molecular Medicine, University of Bristol, Bristol, United Kingdom. [3]Centre for Research in Biosciences, The Faculty of Health and Applied Sciences, The University of the West of England, Bristol, United Kingdom. [4]Proteomics Facility, Faculty of Life Sciences, University of Bristol, Bristol, United Kingdom. [5]Division of Cancer Epidemiology and Genetics, National Cancer Institute, National Institutes of Health, Bethesda, Maryland. [6]Department of Medicine, University of North Carolina School of Medicine, Chapel Hill, North Carolina. [7]Centre for Epidemiology and Biostatistics, Melbourne School of Population and Global Health, The University of Melbourne, Melbourne, Victoria, Australia. [8]Department of Epidemiology, School of Public Health and Institute of Health and Environment, Seoul National University, Seoul, South Korea. [9]Department of Medicine, Samuel Oschin Comprehensive Cancer Institute, Cedars-Sinai Medical Center, Los Angeles, California. [10]Department of Preventive Medicine, Keck School of Medicine, University of Southern California, Los Angeles, California. [11]Public Health Sciences Division, Fred Hutchinson Cancer Research Center, Seattle, Washington. [12]School of Public Health, University of Washington, Seattle, Washington. [13]Department of Health Science Research, Mayo Clinic, Scottsdale, Arizona. [14]Center for Public Health Genomics, University of Virginia, Charlottesville, Virginia. [15]Department of Epidemiology, Johns Hopkins Bloomberg School of Public Health, Baltimore, Maryland. [16]University of Hawaii Cancer Center, Honolulu, Hawaii. [17]Huntsman Cancer Institute and Department of Population Health Sciences, University of Utah, Salt Lake City, Utah. [18]Public Health Sciences Division, Fred Hutchinson Cancer Research Center, Seattle, Washington. [19]Division of Human Nutrition and Health, Wageningen University & Research, Wageningen, the Netherlands. [20]Institute of Cancer Research, Department of Medicine I, Medical University Vienna, Vienna, Austria. [21]Behavioral and Epidemiology Research Group, American Cancer Society, Atlanta, Georgia. [22]Oncology Data Analytics Program, Catalan Institute of Oncology-IDIBELL, L'Hospitalet de Llobregat, Barcelona, Spain. [23]CIBER Epidemiología y Salud Pública (CIBERESP), Madrid, Spain. [24]Department of Clinical Sciences, Faculty of Medicine, University of Barcelona, Barcelona, Spain. [25]ONCOBEL Program, Bellvitge Biomedical Research Institute (IDIBELL), L'Hospitalet de Llobregat, Barcelona, Spain. [26]Department of Molecular Biology of Cancer, Institute of Experimental Medicine of the Czech Academy of Sciences, Prague, Czech

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Introduction

Colorectal cancer is the fourth most common cancer worldwide (1). Observational studies as well as randomized controlled trials (RCT) using aspirin for the prevention of vascular events have shown that aspirin use is associated with a decrease in colorectal cancer incidence and mortality (2–5). This was primarily thought to be through the acetylation of the COX enzymes thereby inhibiting their action (6). These enzymes are involved in the COX/prostaglandin E2(PGE2) signaling pathway, which is frequently upregulated in colorectal cancer, driving many of the hallmarks of cancer (7, 8).

Evidence for COX-independent mechanisms have also emerged, such as the prevention of NFκB activation, inhibition of the extracellular-signal–regulated kinase (ERK) signaling pathway, cell-cycle progression inhibition, and possible induction of autophagy (7, 9). An aspirin derivative that does not inhibit COX reduced the mean number of aberrant crypt foci (an early lesion in colorectal carcinogenesis) in a mouse model of colorectal cancer more than aspirin itself (10). Furthermore, aspirin was able to inhibit proliferation and induce apoptosis in COX-2–negative colon cancer cell lines as well as reducing angiogenesis in 3D assays where COX inhibitors showed no effect (11–13). Clinically, aspirin has been shown to reduce tumor recurrence in phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PIK3CA)-mutant cancer, whereas rofecoxib (a COX-2 selective inhibitor) showed no effect (14) and has also been shown to improve survival in patients with human leukocyte antigen (HLA) class I antigen expression, regardless of COX-2 expression (15). There are now a significant number of studies that indicate the mechanism behind the action of aspirin on colorectal cancer risk is still not fully understood and that multiple mechanisms are involved (16).

In conventional epidemiologic studies, it is often difficult to determine causality due to limitations of confounding and reverse causation. While RCTs can overcome these limitations, they are generally limited to assessing the causal role of health interventions or pharmaceutical agents on disease outcomes, rather than understanding biological mechanisms. Furthermore, in the context of cancer, RCTs for cancer primary prevention are not always feasible, as they require long-term follow-up for the cancer to develop. Mendelian randomization (MR) is an epidemiologic method that applies a similar notion of randomization as in the RCT to evaluate causality. In MR, genetic variants (most commonly single-nucleotide polymorphisms; SNPs) are used to proxy an exposure of interest (17). As genetic variants are randomly assorted at conception, an individual's genetic makeup is unlikely to be influenced by exposures later on in life, thus reducing the possibility of confounding and reverse causation (18). These SNPs instrumenting exposure instruments can then be used to test for association with an outcome of interest.

Republic. [27]Institute of Biology and Medical Genetics, First Faculty of Medicine, Charles University, Prague, Czech Republic. [28]Faculty of Medicine and Biomedical Center in Pilsen, Charles University, Pilsen, Czech Republic. [29]Division of Clinical Epidemiology and Aging Research, German Cancer Research Center (DKFZ), Heidelberg, Germany. [30]Division of Preventive Oncology, German Cancer Research Center (DKFZ) and National Center for Tumor Diseases (NCT), Heidelberg, Germany. [31]German Cancer Consortium (DKTK), German Cancer Research Center (DKFZ), Heidelberg, Germany. [32]Division of Cancer Epidemiology, German Cancer Research Center (DKFZ), Heidelberg, Germany. [33]University Medical Centre Hamburg-Eppendorf, University Cancer Centre Hamburg (UCCH), Hamburg, Germany. [34]Division of Research, Kaiser Permanente Northern California, Oakland, California. [35]Department of Internal Medicine, University of Utah, Salt Lake City, Utah. [36]Department of Medicine and Epidemiology, University of Pittsburgh Medical Center, Pittsburgh, Pennsylvania. [37]Nutrition and Metabolism Section, International Agency for Research on Cancer, World Health Organization, Lyon, France. [38]Gastroenterology Department, Hospital Clínic, Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), Centro de Investigación Biomédica en Red de Enfermedades Hepáticas y Digestivas (CIBEREHD), University of Barcelona, Barcelona, Spain. [39]Department of Surgery, Chonnam National University Hwasun Hospital and Medical School, Hwasun, Korea. [40]Department of Preventive Medicine, Chonnam National University Medical School, Gwangju, Korea. [41]Jeonnam Regional Cancer Center, Chonnam National University Hwasun Hospital, Hwasun, Korea. [42]Division of Gastroenterology, Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts. [43]Channing Division of Network Medicine, Brigham and Women's Hospital and Harvard Medical School, Boston, Massachusetts. [44]Clinical and Translational Epidemiology Unit, Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts. [45]Broad Institute of Harvard and MIT, Cambridge, Massachusetts. [46]Department of Epidemiology, Harvard T.H. Chan School of Public Health, Harvard University, Boston, Massachusetts. [47]Department of Immunology and Infectious Diseases, Harvard T.H. Chan School of Public Health, Harvard University, Boston, Massachusetts. [48]Department of Family Medicine, University of Virginia, Charlottesville, Virginia. [49]Division of Epidemiology, Department of Medicine, Vanderbilt-Ingram Cancer Center, Vanderbilt Epidemiology Center, Vanderbilt University School of Medicine, Nashville, Tennessee. [50]Leeds Institute of Cancer and Pathology, University of Leeds, Leeds, United Kingdom. [51]Colorectal Oncogenomics Group, Department of Clinical Pathology, The University of Melbourne, Parkville, Victoria, Australia. [52]University of Melbourne Centre for Cancer Research, Victorian Comprehensive Cancer Centre, Parkville, Victoria, Australia. [53]Genetic Medicine and Family Cancer Clinic, The Royal Melbourne Hospital, Parkville, Victoria, Australia. [54]Cancer Epidemiology Division, Cancer Council Victoria, Melbourne, Victoria, Australia. [55]Precision Medicine, School of Clinical Sciences at Monash Health, Monash University, Clayton, Victoria, Australia. [56]Department of Preventive Medicine & USC Norris Comprehensive Cancer Center, Keck School of Medicine, University of Southern California, Los Angeles, California. [57]Department of Community Medicine and Epidemiology, Lady Davis Carmel Medical Center, Haifa, Israel. [58]Ruth and Bruce Rappaport Faculty of Medicine, Technion-Israel Institute of Technology, Haifa, Israel. [59]Clalit National Cancer Control Center, Haifa, Israel. [60]Department of Medicine, Memorial Sloan Kettering Cancer Center, New York, New York. [61]Center for Gastrointestinal Biology and Disease, University of North Carolina, Chapel Hill, North Carolina. [62]Discipline of Genetics, Memorial University of Newfoundland, St. John's, Canada. [63]Department of General Surgery, University Hospital Rostock, Rostock, Germany. [64]Department of Radiation Sciences, Oncology Unit, Umeå University, Umeå, Sweden. [65]Wallenberg Centre for Molecular Medicine, Umeå University, Umeå, Sweden. [66]Lunenfeld Tanenbaum Research Institute, Mount Sinai Hospital, University of Toronto, Toronto, Ontario, Canada. [67]Division of Human Genetics, Department of Internal Medicine, The Ohio State University Comprehensive Cancer Center, Columbus, Ohio. [68]Department of Public Health and Primary Care, University of Cambridge, Cambridge, United Kingdom. [69]Department of Clinical Genetics, Karolinska University Hospital, Stockholm, Sweden. [70]Department of Molecular Medicine and Surgery, Karolinska Institutet, Stockholm, Sweden. [71]Institute of Environmental Medicine, Karolinska Institutet, Stockholm, Sweden. [72]Department of Surgical Sciences, Uppsala University, Uppsala, Sweden. [73]University of Southern California, Preventative Medicine, Los Angeles, California. [74]Department of Epidemiology, University of Washington School of Public Health, Seattle, Washington. [75]Massachusetts General Hospital and Harvard Medical School, Clinical and Translational Epidemiology Unit, Boston, Massachusetts. [76]Institute of Medical Biometry and Informatics, University of Heidelberg, Heidelberg, Germany.

Note: Supplementary data for this article are available at Cancer Epidemiology, Biomarkers & Prevention Online (http://cebp.aacrjournals.org/).

Corresponding Author: Aayah Nounu, Oakfield House, Oakfield Grove, Clifton, Bristol, UK, BS8 2BN. E-mail: an0435@bristol.ac.uk

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More recently, the increase in genome-wide association studies for molecular traits has identified SNPs that are associated with protein and mRNA expression levels, thereby providing protein quantitative trait loci (pQTL) and expression quantitative trait loci (eQTL; refs. 19, 20), which may be used to investigate the causal mechanism of drug targets on disease risk (21). Such methods can complement laboratory experiments to better understand the mechanism of action of drugs on cancer growth and progression.

Because of evidence showing that aspirin may prevent adenoma formation (22) and adenomas being the precursors of most colorectal cancers (23), we focused on a colorectal adenoma cell line (RG/C2) in this study and identified altered protein expression in relation to aspirin treatment. Findings were then taken forward into an MR analysis to investigate which proteins targeted by aspirin may be causally implicated in reducing risk of colorectal cancer incidence, thereby providing insight into alternative mechanisms/pathways for the action of aspirin.

Materials and Methods

Cell culture experiments

The S/RG/C2 (referred to as RG/C2 henceforth whereby the prefix "S" denotes that they are from a sporadic tumor; RRID:CVCL_IQ11) colorectal adenoma cell line was derived in the Colorectal Tumour Biology group and is described in detail elsewhere (24). These cells express WT full-length *APC* (25) as well as wild-type *KRAS* and *PIK3CA* (26), but express mutant *TP53* (25–27). RG/C2s were cultured in DMEM (Life Technologies) and supplemented with 20% FBS (Life Technologies), L-glutamine (2 mmol/L; Life Technologies), penicillin (100 U/mL; Life Technologies), streptomycin (100 μg/mL; Life Technologies) and insulin (0.2 U/mL; Sigma-Aldrich). Cells were *Mycoplasma* tested (Mycoalert Plus Mycoplasma Detection Kit; Lonza Group) before generation of proteomic data and experiments were performed within 10 passages. Aspirin (Sigma-Aldrich) was dissolved in fresh growth medium and diluted to form concentrations of 2 mmol/L and 4 mmol/L. Concentrations of aspirin between 0.1–2 mmol/L are known to be typical therapeutic ranges *in vivo* (13). While the 2 mmol/L dose is similar to clinically relevant doses of aspirin, we also treated with 4 mmol/L to identify more consistent and apparent effects of the drug.

Generation of proteomic data—SILAC approach

A stable isotope labeling with amino acids in cell culture (SILAC) approach was carried out on RG/C2 cells treated with 0 mmol/L, 2 mmol/L and 4 mmol/L aspirin for 24 hours. Control cells (0 mmol/L aspirin) were cultured with an L-arginine and L-lysine (light labeling), 2 mmol/L treated cells were cultured with $^{2}H_4$-lysine and $_{13}C_6$-arginine (medium labeling) and 4 mmol/L treated cells were cultured with $^{15}N_2{}^{13}C_6$-lysine and $^{15}N_4{}^{13}C_6$-arginine (heavy labeling; Cambridge Isotope Laboratory). These methods were based on the SILAC-based mass spectrometry approach by Trinkle-Mulcahy and colleagues (2008; ref. 28).

Cells were cultured with aspirin and the isotopes for 24 hours before extracting protein lysates. This experiment was carried out in duplicate. Lysates from the three conditions were pooled in a 1:1:1 ratio, separated by SDS-PAGE and then subjected to in-gel tryptic digestion. The resulting peptides were analyzed by LC-MS using an LTQ Orbitrap Velos mass spectrometer (Thermo Fisher Scientific) and the mass spectral data analyzed using Proteome Discoverer software v1.4 (Thermo Fisher Scientific). Details of SILAC labeling and proteomics have been published previously (29) and are mentioned in the Supplementary Methods. To determine proteins whose expression is altered due to aspirin treatment, we applied a threshold of a 1.4-fold change between 4 mmol/L/control and 2 mmol/L/control, as suggested previously (30). Results were also limited to a variability of <100% and a peptide count of at least 2.

Statistical analyses

Two-sample MR

To assess the effect of protein/mRNA expression of aspirin targets on risk of colorectal cancer, we used a two-sample MR approach. First, SNPs were identified to instrument/proxy for protein/mRNA expression of the proteins shown to be altered in cell culture. SNP associations were then obtained for CRC risk before two-sample MR was carried out to identify how increases in protein/mRNA expression (pQTLs/eQTLs) (sample 1) affected risk of colorectal cancer (sample 2) using the statistical methods described below.

Genetic predictors for protein and gene expression

Protein quantitative trait loci (pQTL) were obtained from the INTERVAL study (19). The original study is comprised of about 50,000 individuals within a randomized trial conducted to evaluate the effect of varying intervals between blood donations and how this affects outcomes such as quality of life (31). Relative protein measurements were taken using SOMAscan assays for 3,622 plasma proteins in a subset of 3,301 participants, randomly chosen. All participants provided consent before joining the study and ethics were approved by the Research Ethics Service (11/EE/0538; ref. 19). Genotyping and imputation (using a combined 1000 Genomes Phase 3-UK10K as the reference panel) of these individuals provided measures for 10,572,814 variants that passed quality control and were taken forward in a GWAS analysis to identify pQTLs for the measured proteins (details of quality control are mentioned elsewhere; ref. 19). pQTLs were extracted from summary level data and used to instrument/proxy a SD change in protein expression (19). To adjust for multiple testing, a Bonferroni correction ($0.05/10{,}572{,}814 = 4.72 \times 10^{-9}$) was applied and pQTLs below this *P* value threshold were used to proxy for protein expression in our analysis (32).

In the absence of a relevant pQTL for the protein of interest, an equivalent mRNA expression GWAS was used instead. Summary level data for expression quantitative trait loci (eQTL) were extracted from the eQTLGEN consortium consisting of 31,684 individuals from 37 datasets, of which 26,886 samples were from whole blood and 4,798 from peripheral blood mononuclear cells (PBMC). Because of the differing methods for genotyping between the studies, variants for each transcript ranged between 2,337–31,684 variants (20). For this reason, a Bonferroni correction threshold was adjusted depending on the number of variants measured for each transcript (0.05/number of variants; ref. 32). eQTLs were standardized and meta-analyzed through a Z-transformation, therefore eQTL effect sizes are reported as SD changes (20).

Although *cis* (within 1 Mb of the gene transcription start sit) associations are more likely to play a role in regulating gene/protein expression due to their close proximity to the gene start site and influencing binding affinity of regulatory proteins (33), we used both *cis*- and *trans*-QTLs in this analysis to instrument/proxy for expression. Once suitable pQTLs/eQTLs were identified, linkage disequilibiurm (LD) clumping at an R^2 of 0.001 was carried out to remove SNPs that are inherited together and so that only the SNP most strongly associated with the mRNA/protein expression within a 10,000 kb window was used.

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Genetic association for colorectal cancer

Genetic association summary statistics for colorectal cancer, comprising 55,168 colorectal cancer cases and 65,160 controls, were obtained from the Colon Cancer Family Registry (CCFR), Colorectal Transdisciplinary (CORECT) and Genetics and Epidemiology of Colorectal Cancer (GECCO) consortia and UK Biobank (34–36). Quality control procedures have been described elsewhere (34). Written informed consent was provided by all participants and ethics for each study were approved by research ethics committee or respective institutional review boards (34).

Evaluating the association of mRNA/protein expression on colorectal cancer

Analyses were carried out in R version 3.2.3 using the MR-Base TwoSampleMR R package (github.com/MRCIEU/TwoSampleMR; ref. 37), which allows the formatting, harmonization, and analysis of summary statistics. The package reassigns alleles so that the effect allele has a positive association with the exposure and so represents an increase in protein/mRNA expression. In turn, allele harmonization ensures that the same allele (that predicts increased expression) is the effect allele in the outcome dataset as well. In the case of palindromic SNPs (represented by either A/T or G/C on both the forward and reverse alleles) these were also harmonized where possible based on allele frequencies. If allele frequencies for the effect allele and the other allele were similar, thus making harmonization difficult, these SNPs were dropped from the analysis (37).

Separate MR analyses were carried for *cis* and *trans* pQTLs as well as *cis*- and *trans*-eQTLs. For proteins with just one pQTL or eQTL, Wald ratios (SNP–outcome estimate SNP–exposure estimate) were calculated to give a causal estimate for risk of colorectal cancer per SD increase in mRNA/protein expression. Where more than one QTL was available as an instrument/proxy for the exposure (mRNA/protein levels), a weighted mean of the ratio estimates weighted by the inverse variance of the ratio estimates [inverse-variance weighted (IVW) method] was used (38).

When one genetic variant used to proxy for an exposure is invalid, for example, due to horizontal pleiotropy (where a genetic variant affects the outcome through an alternative exposure/pathway of interest; ref. 17), then the estimator from the IVW method becomes biased (39). As a sensitivity analysis, alternative MR methods were used when more than 2 SNPs were available as instruments for mRNA/protein expression (MR Egger, simple mode, weighted mode, and weighted median; refs. 37, 40, 41). Unlike the IVW method, the MR Egger method is not constrained to pass through an effect size of 0, thereby allowing the assessment of horizontal pleiotropy through the y-intercept (39, 42). The weighted median approach is useful as it allows a consistent estimate even if 50% of the SNPs proxying protein/mRNA expression are invalid instruments (41) and the mode estimate also provides a consistent causal–effect estimate even if the majority of the instruments are invalid, as the estimate depends on the largest number of similar instruments (40).

Results

Mendelian randomization of gene/protein expression and risk of colorectal cancer identified in aspirin-treated human adenoma cells

To investigate the early changes that could reduce cancer risk, we investigated the proteome of aspirin-treated adenoma-derived cells to identify new targets of aspirin that may alter the risk of colorectal cancer by combining these proteomic results with an MR analysis. After applying a filtering threshold based on fold change and variability in expression, we identified 125 proteins whose expression appeared to be regulated by aspirin treatment (**Fig. 1**; Supplementary Table S1), although 5 were uncharacterized from mass spectrometry and therefore excluded from the analysis.

Of the 120 proteins, expression of 28 proteins was measured in the INTERVAL study, of which 12 proteins had pQTLs that were below the Bonferroni significance threshold ($0.05/10{,}572{,}814 = 4.73 \times 10^{-9}$). From these 12 proteins, *cis* pQTLs were available for 3 proteins and *trans* pQTLs for 10 proteins (Supplementary Table S2). In the absence of available pQTLs, eQTLs for the transcripts of the identified proteins were used instead. Of the 108 proteins with no pQTLs available, expression of 89 mRNAs were measured in the eQTLGen consortium, of which 77 proteins had eQTLs that were below the Bonferroni significance threshold. From these 77 proteins, *cis* eQTLs were available for 71 proteins and *trans* eQTLs were available for 37 proteins (Supplementary Table S3). In total, there were 318 unique SNPs proxying for protein and mRNA expression, of which outcome summary statistics were available for 305 SNPs to test for association between 99 mRNA/proteins against risk of colorectal cancer.

Using the datasets summarized in **Table 1**, two-sample MR analysis using the Wald ratio or IVW method was conducted to test the effect of increased mRNA/protein expression on the risk of colorectal cancer incidence using *cis* and *trans* pQTLs (Supplementary Table S4) as well as *cis* and *trans* eQTLs (Supplementary Table S5). In total, 99 proteins were tested for association with colorectal cancer incidence. To correct for multiple testing, a Bonferroni-adjusted threshold of significance was applied ($0.05/99 = 5.05 \times 10^{-4}$), but we also considered associations of a nominal significance ($P < 0.05$) to identify possible pathways and mechanisms of aspirin's action. Overall, 1 protein with *cis* eQTLs and 2 with *trans* eQTLs were associated with colorectal cancer incidence at $P < 5.05 \times 10^{-4}$ and a further 3 proteins with *cis* eQTLs, 1 with a *trans* eQTL and 1 instrumented by a *trans* pQTL were associated with colorectal cancer incidence at a $P < 0.05$.

Increased mRNA expression of Human Leukocyte Antigen A (*HLA-A*) and mini chromosome maintenance 6 (*MCM6*) instrumented by *cis* eQTLs were found to be associated with an increased risk of colorectal cancer incidence (OR 1.28; 95% CI, 1.04–1.58; *P*: 0.02 and OR 1.08; 95% CI, 1.03–1.13; *P*: 9.23×10^{-4} per SD increase in mRNA expression, respectively). An SD increase in mRNA expression of fatty acid desaturase 2 (*FADS2*) and DNA polymerase delta subunit 2 (*POLD2*) instrumented by *cis* eQTLs was associated with a decrease in risk of colorectal cancer incidence (OR 0.94; 95% CI, 0.90–0.97; *P*: 2.50×10^{-4} and OR 0.84; 95% CI, 0.75–0.94; *P*: 1.17×10^{-3}, respectively; **Fig. 2**; **Table 2**). For *FADS2* and *POLD2*, results were consistent using other MR methods (weighted median, weighted mode, and simple mode) and the MR Egger test shows no evidence of pleiotropy (Supplementary Table S6; Supplementary Fig. S1). From the *cis* eQTL analysis, only results for *FADS2* survived the Bonferroni significance threshold.

Proteins instrumented by *trans* eQTLs include ribonucleoside-diphosphate reductase subunit M2 (*RRM2*), stathmin-1 (*STMN1*) and lipin 1 (*LPIN1*). An increase in *RRM2* was estimated to increase the risk of cancer incidence (OR 3.33; 95% CI, 2.46–4.50; *P*: 6.25×10^{-15} per SD increase in mRNA expression), whereas an increase in *STMN1* and *LPIN1* was associated with decreases in the risk of colorectal cancer incidence (OR 0.72; 95% CI, 0.54–0.97; *P*: 0.03 and OR 0.40; 95% CI, 0.32–0.50; *P*: 5.50×10^{-16} per SD increase in mRNA expression, respectively). From the *trans* eQTL analysis, results for *RRM2* and *LPIN1* both survived the Bonferroni significance threshold.

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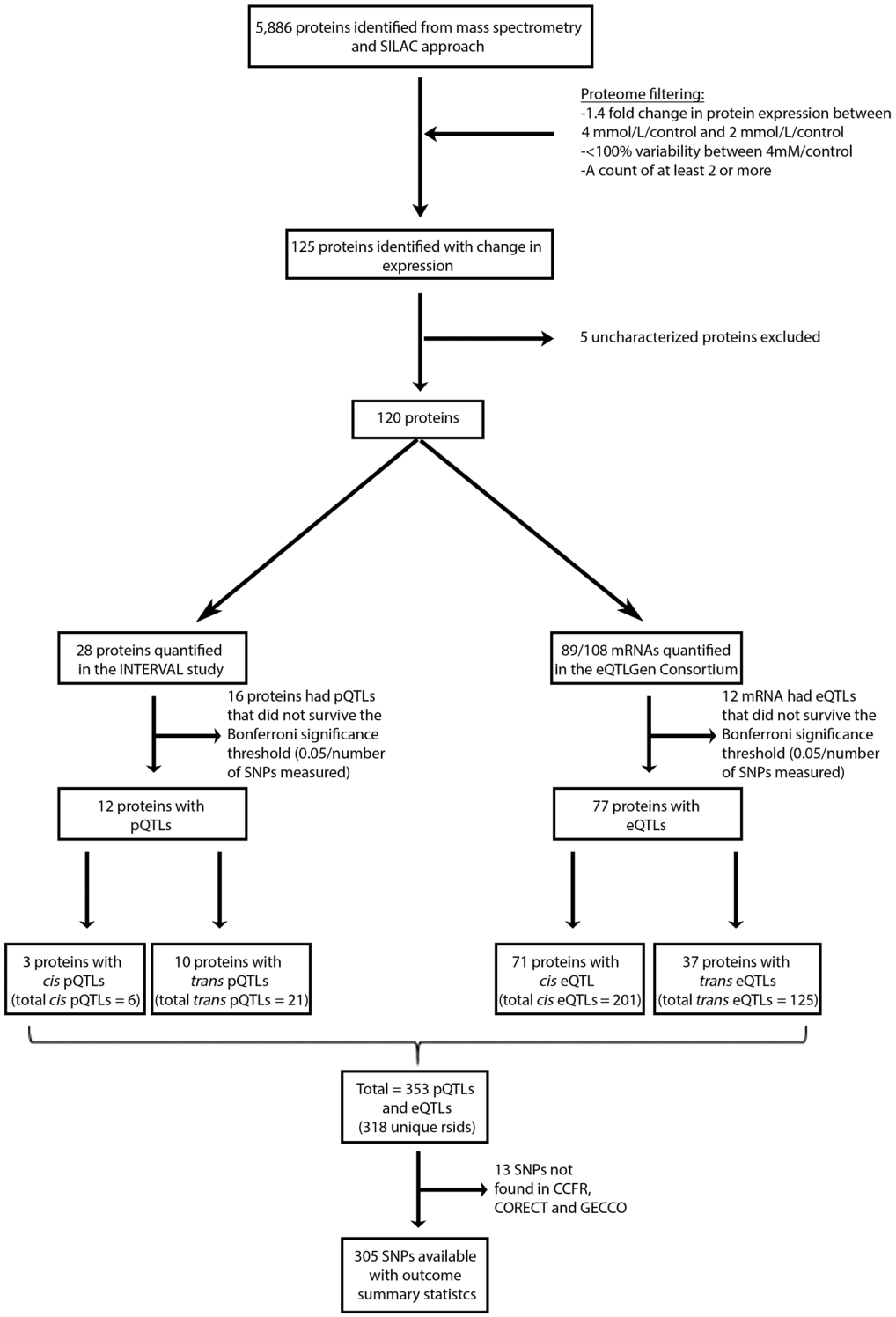

Figure 1.

Flow diagram of SNP selection. A total of 5,886 proteins were identified using the SILAC proteomic approach. After applying a threshold, 125 proteins appear to be regulated by aspirin treatment, of which 5 were uncharacterized proteins and were therefore excluded from the analysis. In total, 12 proteins and 77 mRNAs had been quantified and had pQTLs/eQTLs below the Bonferroni significance threshold. Overall, summary statistics for 353 pQTLs and eQTLs were available, of which summary statistics for 305 of the SNPs were also present in the CCFR, CORECT, and GECCO consortia.

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Table 1. Datasets used in the Mendelian randomization analysis.

Exposure/outcome	Trait	Consortia	*N*	Source	Ref
Exposure	Protein levels	INTERVAL	3,301	Plasma	19
Exposure	mRNA levels	eQTLGEN	31,684	Whole blood ($N = 28,886$) and PBMCs ($N = 4,798$)	20
Outcome	CRC incidence	GECCO[a]	55,168 cases and 65,160 controls	Whole blood	34–36

Note: The table shows the exposure and outcome datasets used in the two-sample MR analysis.
Abbreviations: CRC, colorectal cancer; PBMC, peripheral blood mononuclear cell.
[a]GECCO summary data consist of the Colon Cancer Family Registry (CCFR), Colorectal Transdisciplinary (CORECT), and Genetics and Epidemiology of Colorectal Cancer (GECCO) consortia and UK Biobank.

For proteins instrumented by pQTLs, ADP ribosylation factor interacting protein 2 (ARFIP2) proxied using a *trans* pQTL conferred an increased risk of colorectal cancer incidence (OR 1.15; 95% CI, 1.01–1.29; *P*: 0.03 per SD increase in protein expression).

Overall, the directions of effects between *HLA-A, MCM6, RRM2*, and ARFIP2 and colorectal cancer risk obtained from our MR analysis concur with those anticipated given the protective role of aspirin on colorectal cancer and the effect of aspirin treatment on expression of these proteins. Aspirin reduces the protein expression of HLA-A, MCM6, RRM2, and ARFIP2 (fold change in protein expression with 4 mmol/L aspirin treatment compared with control: 0.55, 0.65, 0.36, and 0.69, respectively; **Table 2**) and aspirin intake is associated with a decreased risk of colorectal cancer (2–4). Our MR analysis shows that increased expression of these proteins is associated with an increased risk of colorectal cancer incidence. Taken together, our results indicate that a possible mechanism through which aspirin decreases the risk of colorectal cancer incidence is through the downregulation of HLA-A, MCM6, RRM2, and ARFIP2. The direction of effect was less consistent for the other 4 proteins (FADS2, POLD2, STMN1, and LPIN1) showing opposite results to what we would expect based on the proteomic results (**Table 2**).

Discussion

Evidence for the use of aspirin in the prevention of colorectal cancer is increasing (2–5). However, the mechanism through which it functions is still not fully understood. By combining both a proteomic-based approach as well as an MR analysis, our results provide mechanistic insights into how aspirin could decrease the risk of colorectal cancer.

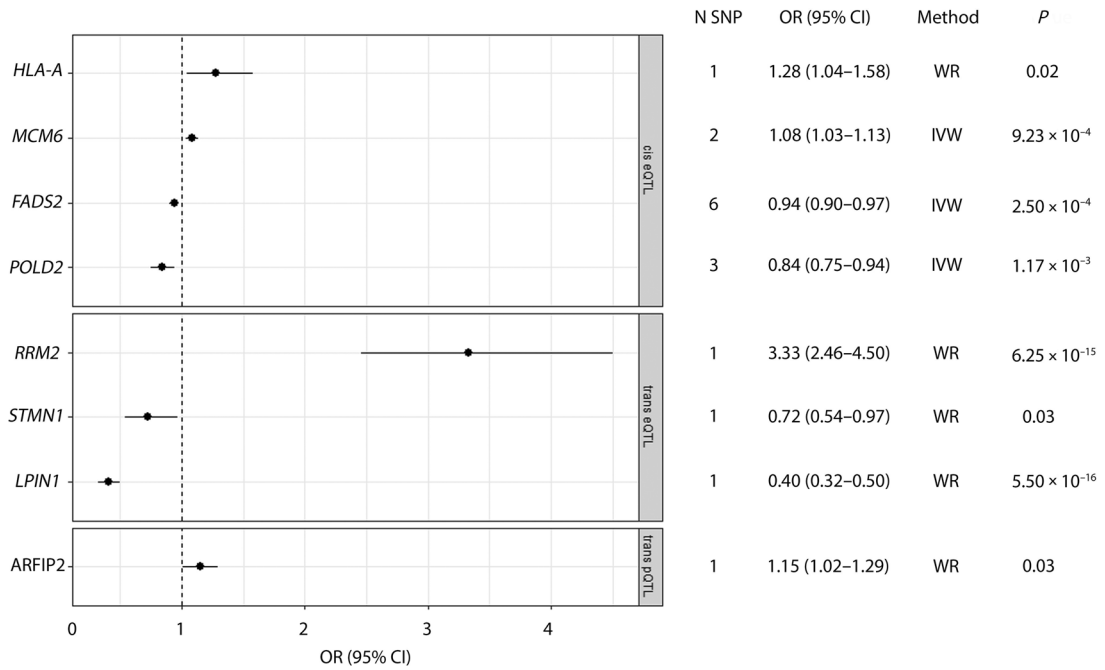

Figure 2.
Forest plot of mRNA/protein associations with colorectal cancer incidence at $P < 0.05$. The upper box presents results using *cis* eQTLs, followed by *trans* eQTLs, and finally *trans* pQTLs. Each dot on the plot represents the change in OR of colorectal cancer incidence per SD increase in mRNA/protein expression, and the horizontal lines on either side of the dot represent the 95% CIs. The dotted line represents a null association between expression and cancer incidence. The number of SNPs used as instruments as well as the OR, the method, and *P* value of association are also reported. Abbreviations: N SNP, number of SNPs; IVW, inverse-variance weighted; WR, Wald ratio.

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Table 2. MR results of the 8 proteins associated with colorectal cancer incidence.

					Association of predicted expression with CRC risk					Fold change of protein expression in response to aspirin		
Gene	Instrument	N SNP	Variance explained R^2 (%)	Method	OR	LCI	UCI	*P*	Effect on CRC risk	2 mmol/L vs. Control	4 mmol/L vs. Control	Effect on protein expression
FADS2	*cis* eQTL	6	2.29	IVW	0.94	0.90	0.97	2.5×10^{-4}	↓	0.61	0.26	↓
MCM6	***cis* eQTL**	**2**	**3.85**	**IVW**	**1.08**	**1.03**	**1.13**	$\mathbf{9.23 \times 10^{-4}}$	↑	**0.59**	**0.65**	↓
POLD2	*cis* eQTL	3	0.05	IVW	0.84	0.75	0.94	1.73×10^{-3}	↓	0.54	0.35	↓
HLA-A	***cis* eQTL**	**1**	**5.95**	**WR**	**1.28**	**1.04**	**1.58**	**0.02**	↑	**0.55**	**0.64**	↓
LPIN1	*trans* eQTL	1	0.08	WR	0.40	0.32	0.50	5.50×10^{-16}	↓	0.65	0.64	↓
RRM2	***trans* eQTL**	**1**	**0.19**	**WR**	**3.33**	**2.46**	**4.50**	$\mathbf{6.52 \times 10^{-15}}$	↑	**0.33**	**0.36**	↓
STMN1	*trans* eQTL	1	0.04	WR	0.72	0.54	0.97	0.03	↓	0.47	0.61	↓
ARFIP2	***trans* pQTL**	**1**	**0.09**	**WR**	**1.15**	**1.01**	**1.29**	**0.03**	↑	**0.67**	**0.69**	↓

Note: The table shows the inverse-variance weighted (IVW) or Wald ratio (WR) results for the 7 proteins associated with CRC incidence. The results indicate the change in OR of CRC incidence per unit increase in mRNA or protein expression (z-score or SD, respectively). Results that are consistent with aspirins' effect on protein expression (i.e., aspirin decreases protein expression and increasing levels of protein are associated with increased risk of colorectal cancer) are in bold font. Abbreviations: N SNP, number of SNPs; LCI, lower confidence interval; UCI, upper confidence interval; SE, standard error; IVW, inverse-variance weighted; WR, Wald ratio.

Using a SILAC-based proteomics approach, 120 proteins appear to be regulated at 24 hours by 4 mmol/L and 2 mmol/L aspirin treatment. Genetic variants (pQTLs and eQTLs) were identified and used to proxy for protein and mRNA expression levels of the identified proteins to test for evidence of a causal effect on colorectal cancer incidence. When no pQTL was available for a protein, eQTLs were used instead.

Overall, 4 *cis* eQTLs, 3 *trans* eQTLs, and 1 *trans* pQTL were associated with cancer incidence at a $P < 0.05$. Increased expression of *HLA-A* and *MCM6* proxied by *cis* eQTLs were associated with an increase in the risk of colorectal cancer incidence and an increase in *RRM2* and ARFIP2 (proxied by a *trans* eQTL and *trans* pQTL, respectively) also conferred an increased risk. Therefore, suppressing the expression of these four proteins could decrease the risk of CRC. As the proteomic results showed that aspirin treatment decreases the expression of these proteins, this could be a potential mechanism by which aspirin reduces the risk of colorectal cancer. However, only results for *RRM2* survive the Bonferroni significance threshold, indicating that further studies are required to verify these results.

The proteins MCM6 and RRM2 are both involved in repair of DNA damage. MCM6 is part of a helicase complex involved in unwinding DNA and is involved in repair of double stranded breaks (DSB) in homologous recombination through interaction with RAD51. This interaction is required for chromatin localisation and formation of foci for DNA damage recovery (43). Likewise, RRM2 is part of a protein complex called ribonucleotide reductase, that catalyzes the biosynthesis of dNTPs and is therefore required for DNA replication and damage repair (44).

Cancer cells commonly lose the DNA damage response, which results in the accumulation of mutations that may be oncogenic (45). Because of this, tumor cells end up relying on a reduced number of repair pathways and are therefore more sensitive to inhibition of DNA damage repair pathways when compared with normal cells which have full capability of DNA repair (46). Drugs that target these other pathways have been shown to selectively kill the cancer cells which is known as synthetic lethality (47, 48). It may be that by reducing the expression of DNA repair proteins, which combined with DNA damage response proteins that are already mutated during tumor progression, aspirin can induce cell death in the developing tumor cells reducing the risk of developing cancer.

The MR results for the proteins ARFIP2 and HLA-A also concur with our SILAC proteomic results. ARFIP2 is a protein previously shown to play a role in membrane ruffling and actin polymerization, therefore regulating the actin cytoskeleton (49). The remodeling of the actin cytoskeleton is known to be involved in cancer metastasis (50). This is of particular interest as aspirin reduces the odds of colorectal adenocarcinoma metastasis by 64% (OR, 0.36; 95% CI, 0.18–0.74; ref. 51) and this may be through the reduction in ARFIP2 expression. With regards to HLA-A expression and cancer risk, results from a cohort study showed that aspirin was more chemopreventative in tumors that expressed HLA class I antigen [which includes HLA-A, HLA-B, and HLA-C; rate ratio (RR) 0.53; 95% CI, 0.38–0.74] and this association was no longer apparent in tumors that lacked expression of this protein (15). Our MR analysis showed that an increase in *HLA-A* was associated with increased colorectal cancer risk, and that aspirin may reduce this risk through a reduction in HLA-A expression; however, further investigation is required before any conclusions can be drawn.

Our MR analysis results also showed that increased mRNA expression of *FADS2, POLD2, LPIN1*, and *STMN1* all decreased the risk of colorectal cancer, indicating that decreased expression increases the risk of cancer. Our proteomic results showed that aspirin decreases the expression of these proteins and aspirin is known to decrease cancer risk. The exact meaning behind the inconsistencies in direction of effect is unclear, but may be related to the dosage used in this study. A randomized trial of aspirin to prevent adenomas showed that lower doses reduced adenoma risk more than higher doses, suggesting that lower doses of aspirin may affect mRNA/protein expression differently than higher doses (52, 53). Furthermore, the genetic instruments used to proxy for 1SD in *POLD2, LPIN1*, and *STMN1* expression explain little of the variance in mRNA expression (0.05%, 0.08%, and 0.04%, respectively) indicating that SNPs that explain more of the variance are required before any conclusions can be made.

Further limitations also exist in our analysis. First, the exact correlation between eQTLs and pQTLs has not been fully determined. Second, it is difficult to interpret results using *trans* eQTLs and pQTLs without clear confirmation that these SNPs directly influence the gene/protein expression. It may be that they indirectly influence expression, for example, *trans* eQTLs may regulate gene expression by affecting

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expression of a nearby *cis* gene, which is in fact a transcription factor that is regulating the expression of the *trans* gene (54). Third, both the pQTL and eQTL associations were carried out using blood plasma, whole blood samples or PBMCs (19, 20); therefore, these SNPs estimate changes in gene and protein expression in circulating immune cells or plasma proteins, respectively. Our SILAC approach identified cellular proteins affected by aspirin treatment; however, the pQTLs used in this analysis proxied levels of plasma proteins. We believe that if the expression of cellular proteins is affected by aspirin, then this in turn will affect the amount of protein secreted into the plasma. We acknowledge that pQTLs for cellular proteins in colorectal tissue would be more appropriate for this analysis; however, studies that have measured cellular pQTLs are small and involve lymphoblastoid cell lines, rather than primary tissues of interest (55, 56). Also, the specificity of eQTLs/pQTLs for specific tissues is unclear. As found by the Genotype-Tissue Expression (GTEx) study, *cis* eQTLs are either shared across tissues or are specific to a small number of tissues (57). Therefore, the use of these eQTLs and pQTLs measured in the blood may not be fully suitable as proxies for mRNA and protein expression in the epithelium of the colon and rectum.

Furthermore, the units for the eQTLs and pQTLs represent SD changes in expression, making interpretation of the results difficult. However, we can interpret the direction of effect as well as the statistical significance of the association (*P* values) for these analyses. Moreover, pQTLs and eQTLs could not be identified for 20 of the proteins found to be regulated by aspirin in our proteomic approach, therefore we could not test the association of their expression with colorectal cancer risk. Finally, apart from the association of *FADS2* with colorectal cancer incidence, the other associations proxied by *cis* eQTLs found by our study are not below the Bonferroni threshold of significance ($P \leq 4.63 \times 10^{-4}$).

MR is commonly used to proxy for a drug's effect on risk of various outcomes after identification of its target. Genetic variants that predict lower function of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase are commonly used to investigate the effect of lowering LDL cholesterol via the use of statins on outcomes such as ovarian cancer, Alzheimer disease, or coronary heart disease (58–60). These studies involve investigation of a drug's effect via a known target on an outcome. However, this approach would be difficult to apply in the case of drugs with pleiotropic targets such as aspirin. Therefore, to identify all possible targets of aspirin, a proteomic approach was first applied and targets that may affect risk of cancer were identified through using MR. To our knowledge, this is the first study that combines basic science and MR to generate hypotheses of a drug's mechanism of action in cancer.

Further experiments need to be conducted to confirm the effect of aspirin on gene and protein expression and the consequent effect this may have on hypothesized pathways such as DNA repair before definitive conclusions can be made. However, the potential of this unbiased approach to gain mechanistic insight is clear, allowing hypothesis driven research will better inform the clinical use of aspirin for the prevention of colorectal cancer.

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Authors' Contributions

A. Nounu: Conceptualization, formal analysis, investigation, methodology, writing–original draft, writing–review and editing. **A. Greenhough:** Conceptualization, data curation, investigation, methodology, writing–review and editing. **K.J. Heesom:** Resources, data curation, writing–review and editing. **R.C. Richmond:** Supervision, methodology, writing–review and editing. **J. Zheng:** Methodology, writing–review and editing. **S.J. Weinstein:** Data curation, writing–review and editing. **D. Albanes:** Data curation, writing–review and editing. **J.A. Baron:** Data curation, writing–review and editing. **J.L. Hopper:** Data curation. **J.C. Figueiredo:** Data curation. **P.A. Newcomb:** Data curation. **N.M. Lindor:** Data curation. **G. Casey:** Data curation. **E.A. Platz:** Data curation, writing–review and editing. **L. Le Marchand:** Data curation, writing–review and editing. **C.M. Ulrich:** Data curation. **C.I. Li:** Data curation. **F.J.B. van Duijnhoven:** Data curation, writing–review and editing. **A. Gsur:** Data curation. **P.T. Campbell:** Data curation. **V. Moreno:** Data curation. **P. Vodicka:** Data curation. **L. Vodickova:** Data curation. **H. Brenner:** Data curation, writing–review and editing. **J. Chang-Claude:** Data curation. **M. Hoffmeister:** Data curation. **L.C. Sakoda:** Data curation, writing–review and editing. **M.L. Slattery:** Data curation. **R.E. Schoen:** Data curation. **M.J. Gunter:** Data curation. **S. Castellví-Bel:** Data curation, writing–review and editing. **H.R. Kim:** Data curation. **S.-S. Kweon:** Data curation. **A.T. Chan:** Data curation, writing–review and editing. **L. Li:** Data curation. **W. Zheng:** Data curation. **D.T. Bishop:** Data curation. **D.D. Buchanan:** Data curation. **G.G. Giles:** Data curation. **S.B. Gruber:** Data curation. **G. Rennert:** Data curation. **Z.K. Stadler:** Data curation. **T.A. Harrison:** Data curation. **Y. Lin:** Data curation. **T.O. Keku:** Data curation. **M.O. Woods:** Data curation. **C. Schafmayer:** Data curation. **B. Van Guelpen:** Data curation. **S. Gallinger:** Data curation. **H. Hampel:** Data curation. **S.I. Berndt:** Data curation. **P.D.P. Pharoah:** Data curation. **A. Lindblom:** Data curation. **A. Wolk:** Data curation, writing–review and editing. **A.H. Wu:** Data curation. **E. White:** Data curation, writing–review and editing. **U. Peters:** Data curation, writing–review and editing. **D.A. Drew:** Data curation. **D. Scherer:** Conceptualization, writing–review and editing. **J.L. Bermejo:** Conceptualization. **A.C. Williams:** Conceptualization, supervision, funding acquisition, writing–review and editing. **C.L. Relton:** conceptualization, supervision, funding acquisition, writing–review and editing.

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