

Metabolomics Analysis of Aspirin's Effects in Human Colon Tissue and Associations with Adenoma Risk

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ABSTRACT

Although substantial evidence supports aspirin's efficacy in colorectal cancer chemoprevention, key molecular mechanisms are uncertain. An untargeted metabolomics approach with high-resolution mass spectrometry was used to elucidate metabolic effects of aspirin treatment in human colon tissue. We measured 10,269 metabolic features in normal mucosal biopsies collected at colonoscopy after approximately 3 years of randomized treatment with placebo, 81 or 325 mg/day aspirin from 325 participants in the Aspirin/Folate Polyp Prevention Study. Linear regression was used to identify aspirin-associated metabolic features and network analysis was used to identify pathways and predict metabolite identities. Poisson regression was used to examine metabolic features associations with colorectal adenoma risk. We detected 471 aspirin-associated metabolic features. Aside from the carnitine shuttle, aspirin-associated metabolic pathways were largely

distinct for 81 mg aspirin (e.g., pyrimidine metabolism) and 325 mg (e.g., arachidonic acid metabolism). Among aspirin-associated metabolic features, we discovered three that were associated with adenoma risk and could contribute to the chemopreventive effect of aspirin treatment, and which have also previously been associated with colorectal cancer: creatinine, glycerol 3-phosphate, and linoleate. The last two of these are in the glycerophospholipid metabolism pathway, which was associated with 81 mg aspirin treatment and provides precursors for the synthesis of eicosanoids from arachidonic acid upstream of cyclooxygenase inhibition by aspirin. Conversely, carnitine shuttle metabolites were increased with aspirin treatment and associated with increased adenoma risk. Thus, our untargeted metabolomics approach has identified novel metabolites and pathways that may underlie the effects of aspirin during early colorectal carcinogenesis.

Introduction

Chemoprevention has the potential to reduce the global burden of cancer and aspirin is one of the most promising agents on the horizon, but a deeper understanding of the molecular underpinnings is central to moving forward (1, 2). Substantial evidence from preclinical, observational, and clinical studies supports the use of aspirin as a preventive agent for colorectal cancer (3, 4). Aspirin has shown efficacy in reducing the risk of both preinvasive adenomas and colorectal cancer. This evidence basis recently led to the U.S. Preventive Services Task Force recommendation for the use of aspirin to prevent

colorectal cancer in addition to cardiovascular disease among those at increased risk of cardiovascular disease (5). Despite this, the key molecular mechanisms responsible for aspirin's chemopreventive effects are not clearly understood (3, 6–8). Aspirin incorporates two bioactive components in one molecule, a reactive acetyl moiety and a salicylate group, and is well known for its pleiotropic effects (9). Its best-characterized pharmacologic activity is the irreversible acetylation and inhibition of the cyclooxygenases (COX-1 and COX-2), which are responsible for the initial step in the conversion of arachidonic acid into prostaglandins and related eicosanoids. However, there is also evidence for COX-independent mechanisms that could modify carcinogenesis (7, 8, 10, 11). The identification of key targets in colorectal tissue that mediate aspirin's protective effects may help to optimize its use for colorectal cancer chemoprevention.

We previously conducted the Aspirin/Folate Polyp Prevention Study, a randomized trial of aspirin (81 or 325 mg/day) for the prevention of colorectal adenomas among individuals with a history of adenomas (12). In this trial, the 81 mg/day dose reduced risk of any adenoma by 19% and advanced lesions by 41%, whereas there were statistically non-significant risk reductions for the 325 mg/day dose (4% and 17%, respectively; ref. 12). In prior targeted analyses in this study population, we were unable to link aspirin-induced changes in inflammation markers in blood plasma or eicosanoid products of COX-1/COX-2 in urine to its

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chemopreventive effects in the colorectum (13, 14). In this work, we used an untargeted high-resolution metabolomics approach to investigate the effects of aspirin on low molecular weight molecules (metabolites) in normal colon tissue biopsies to identify key metabolic features and pathways involved in the chemopreventive effects of aspirin in the colorectum. While previous targeted analyses of eicosanoids have been performed, to our knowledge this is the first untargeted metabolomics analysis of aspirin's effects in normal human colonic mucosa.

Materials and Methods

Clinical trial study population and design

The Aspirin/Folate Polyp Prevention Study was a multicenter, double-blind, placebo-controlled, randomized trial to assess the chemopreventive effects of aspirin and folic acid in individuals with a recent history of colorectal adenomas, as described in detail previously (NCT00272324; refs. 12, 15). The study was approved by institutional review boards at all participating institutions, and all study participants provided written informed consent. Participants were enrolled from eight clinical centers in the United States and one in Canada. Recruitment began in July 1994 and ended in March 1998. Eligible participants were 21 to 80 years old with no history of colorectal cancer or familial colorectal cancer syndromes but with a recent colorectal adenoma and no remaining polyps after a complete colonoscopy within 3 months before study enrollment. At enrollment, participants completed a questionnaire regarding demographic and health factors and were asked to avoid using aspirin and other NSAID for the duration of study treatment. Following a 3-month run-in period, eligible participants who took at least 80% of their study pills were randomized in a 3 × 2 factorial design to aspirin (placebo, 81 mg/day, or 325 mg/day) and folic acid (placebo or 1 mg/day). Every 4 months during the study, participants completed questionnaires regarding adherence to study pill taking, medications used, and medical events. Treatment ended and adenoma occurrence was assessed at a surveillance colonoscopy approximately 3 years from the preenrollment colonoscopy.

All lesions removed from the large bowels of study participants were reviewed by a single study pathologist who was blinded to treatment assignments. Advanced adenomas were defined as those with cancer, high-grade dysplasia, more than 25% villous features, or an estimated diameter of at least 1 cm as assessed by the endoscopist. High-risk findings were defined as the occurrence of at least one advanced adenoma or multiple (3 or more) adenomas of any type.

Collection and selection of colon tissue biopsies

Normal colon mucosal biopsies were collected at the year 3 (end-of-treatment) colonoscopy from a subset of study participants who provided additional informed consent for the purpose of prior research, as described previously (16, 17).

Before their colonoscopy, participants were instructed to discontinue study treatment (aspirin or placebo) for 1 week to minimize bleeding risk per standard of care. Using biopsy forceps, tissue specimens were taken from the normal mucosa of the proximal colon, approximately 5 cm above the ileocecal valve. The specimens (approximately 10 mg) were put into freezer tubes and immediately frozen in liquid nitrogen or an ethanol/dry ice slurry prior to storage at $\leq -70^{\circ}\text{C}$. Within a year of collection, specimens were shipped on dry ice to the Dartmouth study biorepository for storage at $\leq -70^{\circ}\text{C}$ and were never thawed prior to the present analysis. Subsequently, frozen tissue samples were shipped on dry ice to the Emory Department of Medicine Clinical Biomarkers Laboratory and maintained at $\leq -70^{\circ}\text{C}$ prior to metabolomics analysis.

Selection of participants with colon biopsies for inclusion in the current work was as follows (Fig. 1A). Among 1,121 randomized participants, normal tissue biopsies were collected from 768 participants. For the present research, of 592 participants with biopsies still available, we excluded 26 due to missing data on adenoma outcomes. Furthermore, to maximize the separation between the placebo control and aspirin treatment groups, we excluded participants with NSAID use >24 days/year at baseline or >2 days/month during study participation, or with $<85\%$ adherence to study pills, leaving 325 participants for analysis in the work described here.

High-resolution metabolomics analyses and data processing

The global metabolic effects of aspirin in colon tissue were assessed using dual liquid chromatography coupled with high-resolution mass spectrometry with dual ionization to maximize detection of diverse metabolites. Methods for sample preparation and metabolomics analyses were similar to those described previously (18–20). Briefly, batches of 40 experimental samples were prepared daily by the addition of 50 μL water, 200 μL acetonitrile, and 5 μL of a mixture containing 14 stable isotope internal standards. Samples were homogenized for a few seconds using an Active Motif EpiShear probe sonicator, incubated on ice for 30 minutes, and centrifuged at 14,000 rpm at 4°C for 10 minutes. The resulting supernatant (100 μL) was removed and six technical replicates (10 μL each) were analyzed for each sample: three using C18 chromatography (Higgins Analytical, 50 × 2.1 mm column) and three using HILIC chromatography (Waters Xbridge BEH Amide, 50 × 2.1 mm column). Mass spectral data were collected with a 5-minute gradient on a Dionex UliMate 3000 rapid separation liquid chromatography system coupled with a Thermo Orbitrap Fusion Tribrid mass spectrometer (Thermo Fisher Scientific) at a resolution of 120,000 and operated in negative or positive electrospray ionization mode with C18 or HILIC chromatography, respectively. Each of the colon tissue samples were identified only with an eight-digit barcode and analyzed in random order by a metabolomics analyst blinded to each sample's treatment classification. Reference samples analyzed

included pooled human plasma samples from the National Institute of Standards and Technology (NIST; SRM #1950) and from Equitech Bio. In addition, a colon tissue reference sample was made using normal tissue removed at surgery from

an anonymous donor. Reference samples of each type were included at the beginning, middle and end of each analytical batch for quality control purposes including evaluating instrument stability. Average pairwise Pearson

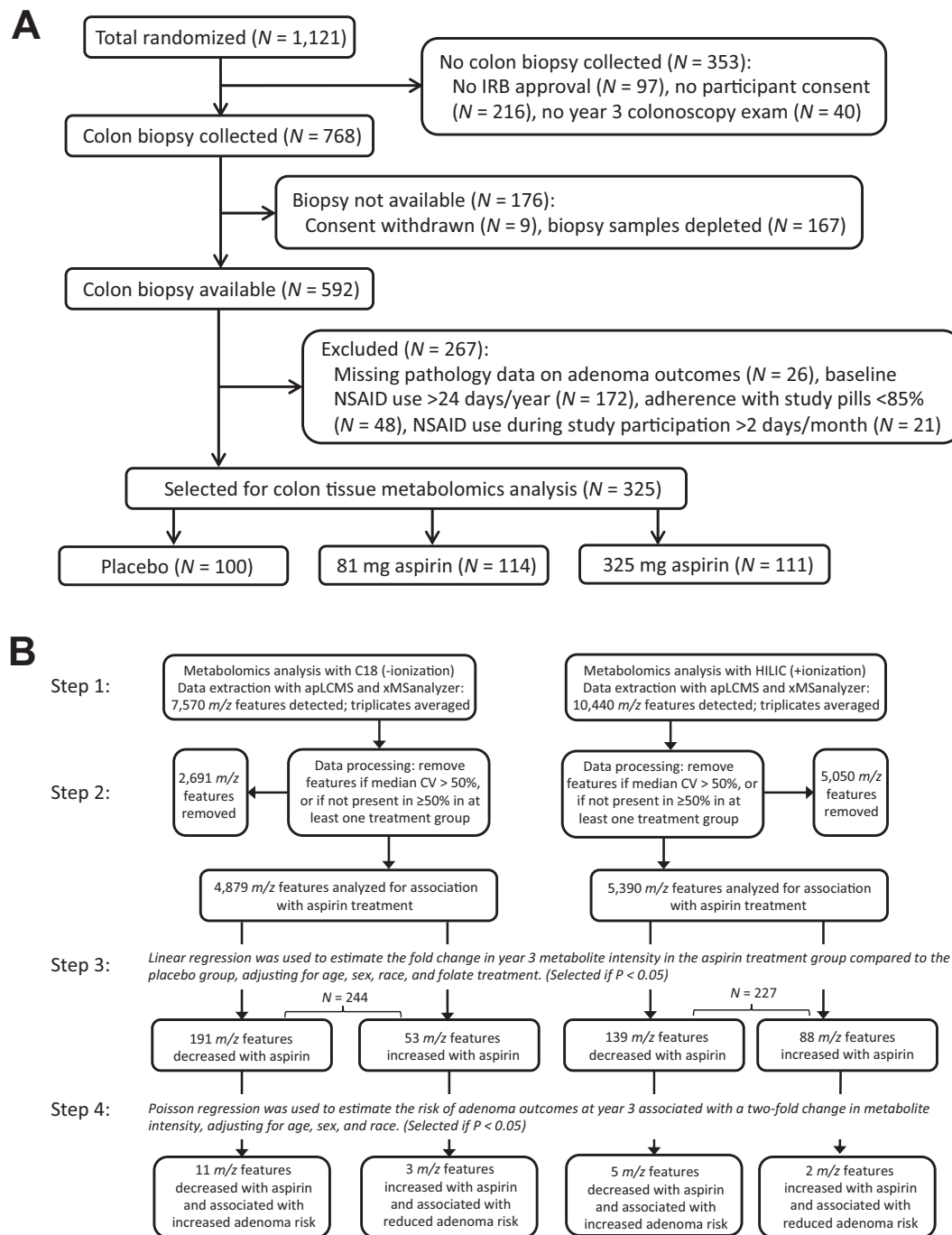


Figure 1.

Flow charts. **A**, Selection of participants for inclusion in colon tissue metabolomics analysis. **B**, Workflow for metabolomics data analysis. In step 3, separate linear models were run for metabolic features associated with low (81 mg) or high (325 mg) doses of aspirin treatment versus placebo, and total numbers of metabolic features associated with either dose aspirin are shown. In step 4, separate Poisson regression models were run for three types of adenoma outcomes (any adenoma, advanced adenoma, or high-risk findings) and total numbers of metabolic features associated with any of these three outcomes are shown in the last row. These metabolic features ($N = 21$ in total) are detailed in **Table 3** and may contribute to the chemopreventive effect of aspirin treatment to reduce adenoma outcomes.

correlation coefficients for all features across all pooled plasma and colon reference samples were, respectively, 0.96 and 0.98 using HILIC+ and 0.94 and 0.84 using C18– analyses.

Raw data files were extracted using apLCMS v6.3.3 (21) with modification by xMSanalyzer v2.0.7 (22), with each unique metabolic feature (ion) defined by accurate mass (± 5 ppm) mass-to-charge ratio (m/z) and retention time. Batch correction was performed using Combat (23) and ion intensity values were averaged across triplicates. Extraction of mass spectral data initially yielded 7,570 and 10,440 metabolic features in the C18 and HILIC datasets, respectively (Fig. 1B, step 1). Samples with mean overall pairwise Pearson correlation $<70\%$ between replicates were excluded from the analyses: 4 and 0 samples were excluded from the C18 and HILIC datasets, respectively. Metabolic features were excluded due to high variability between triplicates (median coefficient of variation $>50\%$) or low abundance (undetectable in $>50\%$ of samples in all three treatment groups), leaving a total of 10,269 features to be analyzed, 4,879 and 5,390 in the C18 and HILIC datasets, respectively (Fig. 1B, step 2).

Detection of aspirin catabolites in blood plasma samples

To validate adherence to randomized aspirin treatments, raw intensities of two common aspirin catabolites (salicylic acid and salicyluric acid) were assessed in participants' baseline and year 3 plasma samples collected as described previously (16, 17). Notably, the colon tissue biopsies could not be used for this purpose because they were collected a week after treatment cessation, as described above. The year 3 blood samples analyzed here were collected while participants were still on study treatment a median of 17 days (interquartile range 11–35 days) prior to their year 3 end-of-treatment colonoscopy. Briefly, nonfasting blood collected in 7-mL EDTA Vacutainer brand tubes was immediately put on ice and then centrifuged at $1,100 \times g$ for 10 minutes at 4°C . The plasma fraction was removed and stored frozen at $\leq -20^\circ\text{C}$ for up to 12 months prior to shipment on dry ice to the Dartmouth study biorepository for storage at $\leq -70^\circ\text{C}$. Of the 325 participants selected for the tissue metabolomics analysis as described above, 293 had paired baseline and year 3 plasma samples for this analysis of aspirin catabolites. Of those 586 samples, 25% (149) had never been thawed previously, 73% (430) had been thawed once, and 1% (7) had been thawed twice. Samples selected were shipped on dry ice to the Emory Department of Medicine Clinical Biomarkers Laboratory and maintained at $\leq -70^\circ\text{C}$ prior to metabolomics analysis.

Methods for plasma sample preparation and metabolomics analyses were similar to those described previously (18–20). Briefly, batches of 20 experimental samples were prepared daily by thawing on ice and removal of 50 μL and then addition of 100 μL of acetonitrile containing 2.5 μL of internal standards. The samples were kept on ice for 30 minutes followed by centrifugation at 14,000 rpm at 4°C for 10 minutes. The resulting supernatant (100 μL) was removed, and three technical replicates (10 μL each) were analyzed for each sample

using C18 chromatography (Thermo Accucore C18, 100×2.1 mm column). Mass spectral data were collected with a Thermo QExactive High Field mass spectrometer (Thermo Fisher Scientific) operated in positive electrospray ionization mode at a resolution of 120,000. Each of the plasma samples were identified with an eight-digit barcode and paired sets of baseline and year 3 samples from the same participant were analyzed in a single batch in random order by a metabolomics analyst blinded to their treatment classification. Data files were extracted as described for the tissue data. Identities of aspirin catabolites were confirmed at level 1 per the metabolomics standards initiative (MSI; ref. 24) by co-elution using tandem mass spectrometry (MS/MS) relative to authentic standards: $m/z = 139.0389$, salicylic acid [M + H] and $m/z = 196.0604$, salicyluric acid [M + H]. For each of the three treatment groups, the differences in aspirin catabolite intensities between paired baseline and year 3 plasma samples were assessed using Wilcoxon signed-rank tests and visualized with box plots.

Statistical analyses

Data from tissue samples was normalized by dividing the ion intensity of each metabolic feature by the total ion intensity for all features in the sample. Zero intensity values were replaced by the minimum value for that feature across all samples divided by 2 prior to normalization. Normalization naturally leads to extremely small values for the ion intensities that may cause instability in analyses when these are \log_2 -transformed prior to regression modelling. Hence, the ion intensity values were multiplied by an arbitrary large constant 10^9 , to ensure that all \log_2 values were greater than 0. The multiplication has no impact on the resulting P values.

To identify metabolic features in colon tissue associated with aspirin treatment, we used multivariable linear regression with the \log_2 -transformed ion intensity as the dependent variable and the randomized aspirin treatment assignment as the predictor variable with adjustment for age, sex, race (coded as a binary variable: non-Hispanic white vs. other), and folate treatment assignment. Separate models were used for assessing associations with 81 mg/day aspirin versus placebo or 325 mg/day aspirin versus placebo in intention-to-treat analyses. Results were visualized using Manhattan and volcano plots. Metabolic pathways associated with aspirin treatment were identified using *Mummichog* v2.0.6 (25), a set of algorithms specifically designed for high-throughput metabolomics that utilizes the collective power in metabolic networks to help resolve the ambiguity in metabolite prediction in a data-driven analysis.

Aspirin-associated metabolic features were subsequently assessed for their associations with adenoma outcomes using multivariable Poisson regression models for binary data with common outcomes to estimate risk ratios (RR) and 95% confidence intervals (CI) for associations with two-fold changes in ion intensity, adjusting for age, sex, and race. Three separate models were used to assess associations with risk of at least one adenoma, advanced adenoma (those with cancer,

high-grade dysplasia, more than 25% villous component, or an estimated diameter of at least 1 cm) or high-risk findings (at least one advanced adenoma or at least three adenomas of any type).

The number of independent estimates in our analysis is unknown because multiple metabolic features detected on our platform can map to one metabolite, and metabolites in a pathway are correlated. FDR q values were computed using the Benjamini–Hochberg method with a 0.2 threshold for significance (26). In regression analyses, aspirin-associated metabolic features were selected and prioritized using a two-sided P value threshold of <0.05 without adjustment for multiple testing to avoid type II error. This approach, common in metabolomics studies, supports the discovery of novel metabolites associated with aspirin treatment and enriches input information for pathway analyses (25, 27). P -values in pathway analyses were calculated using permutation tests with sampling from Gamma distributions to account for clustered data; enriched metabolic pathways were selected using a *Mummichog* scoring threshold of $P < 0.05$ to avoid type I error (25, 27). In all other analyses (e.g., changes in aspirin catabolite intensities in plasma), two-sided $P < 0.05$ were considered statistically significant. Analyses were conducted in R v3.5.1 and SAS v9.4.

Metabolite annotation and identification

Metabolite identification was performed by comparing accurate mass and retention time to authentic standards in

an in-house library run under identical conditions using MS/MS. Additional tentative annotations were assigned using *Mummichog* v2.0.6 (25) and *xMSannotator* v1.3.2 (28) with Human Metabolome Database v3.5. *xMSannotator* uses a multistep approach based on m/z , retention time, isotopes, adducts, correlation across samples, and network and pathway associations to assign database matches into different categories: high, medium, or low confidence. Metabolite identities were classified per MSI criteria (24): level 1 (confirmed by MS/MS and co-elution with authentic standards), level 2 (tentative annotation using *xMSannotator* with medium or high confidence), and level 3 (tentatively characterized chemical class), and level 4 (accurate mass or no database match). Features that were redundant (multiple adducts), implausible, or with suspect retention time were excluded.

Results

Population characteristics

Characteristics of the 325 participants with colon tissue biopsies included in the present analyses were similar across the three aspirin treatment groups (Table 1). The mean age at enrollment was approximately 58 years, 34% were female, and 87% were non-Hispanic whites. Risk factors for colorectal neoplasia were similar across the treatment groups, including body mass index, smoking status, alcohol use, and family history of colorectal cancer. Overall, 53% of these participants

Table 1. Characteristics of randomized participants selected for colon tissue metabolomics study, by aspirin treatment group ($N = 325$).

	Placebo ($N = 100$)	81 mg Aspirin ($N = 114$)	325 mg Aspirin ($N = 111$)	P^d
Baseline characteristics:				
Age at enrollment, mean \pm SD	57.8 \pm 9.2	57.7 \pm 8.1	58.7 \pm 8.6	0.61
Female, N (%)	37 (37%)	37 (33%)	37 (33%)	0.76
Race/ethnicity, N (%)				0.49 ^e
White, Non-Hispanic	86 (86%)	97 (85%)	100 (90%)	
Black, Non-Hispanic	4 (4.0%)	5 (4.4%)	1 (0.9%)	
Hispanic	6 (6.0%)	7 (6.1%)	3 (2.7%)	
Other	4 (4.0%)	5 (4.4%)	7 (6.3%)	
Body mass index, kg/m ² , mean \pm SD	27.2 \pm 4.6	27.3 \pm 4.1	27.9 \pm 4.8	0.53
Current smoker, N (%)	13 (13%)	11 (9.7%)	14 (13%)	0.69
Alcohol use, N (%)	62 (66%)	71 (65%)	72 (67%)	0.95
Family history of colorectal cancer, N (%)	33 (42%)	35 (39%)	37 (45%)	0.71
Study and follow-up characteristics:				
Allocated to folate treatment, N (%)	48 (48%)	63 (55%)	60 (54%)	0.53
Study follow-up time, mean \pm SD ^a	32.7 \pm 2.6	32.6 \pm 2.3	32.7 \pm 2.3	0.95
≥ 1 adenoma at year 3 colonoscopy	33 (33%)	39 (34%)	47 (42%)	0.30
≥ 1 advanced adenoma at year 3 colonoscopy ^b	6 (6.0%)	8 (7.0%)	13 (11.7%)	0.27
High-risk findings at year 3 colonoscopy ^c	9 (9.0%)	11 (9.7%)	16 (14.4%)	0.38
Paired baseline and year 3 plasma samples analyzed for aspirin metabolites, N (%)	90 (90%)	105 (92%)	98 (88%)	0.63

Note: N 's for missing data for alcohol use: 6 placebo, 4 aspirin (81 mg), 3 aspirin (325 mg); for family history of colorectal cancer: 21 placebo, 24 aspirin (81 mg), 29 aspirin (325 mg).

^aTime in months from enrollment to year 3 end of treatment colonoscopy.

^bAdvanced adenomas are those with cancer, high-grade dysplasia, $>25\%$ villous component, or diameter ≥ 1 cm.

^cHigh risk refers to ≥ 1 advanced adenomas or ≥ 3 adenomas of any type.

^d P values for comparisons among the three treatment groups: Pearson chi-square tests for categorical variables and analysis of variance likelihood ratio chi-square tests for continuous variables.

^e P value using Fisher exact test.

were randomly assigned to receive folate supplementation. Study treatment lasted 32.7 ± 2.4 months on average. Overall, 37% of participants had at least one adenoma at their year 3 colonoscopy, 8% had at least one advanced adenoma, and 11% had high-risk findings.

Plasma levels of aspirin catabolites: salicylic acid and salicyluric acid

Among the 325 participants with colon biopsy samples, 293 (90%) also had paired baseline and year 3 plasma samples (Table 1) that were used to validate adherence to randomized treatment by analysis of two common aspirin catabolites: salicylic acid and salicyluric acid (Supplementary Fig. S1). Intensities of salicylic acid and salicyluric acid statistically significantly increased between baseline and year 3 among participants in the two aspirin treatment groups ($P = 1.9 \times 10^{-6}$ and $P = 4.9 \times 10^{-12}$, respectively for 81 mg aspirin; $P = 7.9 \times 10^{-13}$ and $P = 2.2 \times 10^{-17}$, respectively, for 325 mg aspirin) but not among participants in the placebo group. There was a small decrease in salicyluric acid ($P = 0.02$) in the placebo group at year 3 compared with baseline that was likely due to modest use of aspirin pre-enrollment (although we excluded those with baseline NSAID use >24 days/year) and subsequent avoidance of personal aspirin use during study participation.

Colon tissue metabolic features and pathways associated with aspirin treatment

Of 10,269 metabolic features analyzed, 471 were associated with aspirin treatment (81 or 325 mg/day): 244 and 227 in the C18 and HILIC datasets, respectively (Fig. 1B, step 3; Fig. 2). The majority of metabolic features decreased with aspirin treatment: 330 (70%; 191 and 139 in the C18 and HILIC datasets, respectively; Fig. 2). Only 22 features (4.7%) were associated with both doses of aspirin treatment (14 and 8 in the C18 and HILIC datasets, respectively; Fig. 2). Following correction for multiple testing, only one feature was statistically significantly associated with both low- and high-dose aspirin treatment: m/z 233.1185 ($q = 0.11$ and 9.8×10^{-5} , respectively). This was annotated as multiple forms of Pterosin, compounds detected in some types of vegetables.

Metabolic pathways associated with 81 mg versus 325 mg aspirin treatment were largely distinct (Fig. 3). The three pathways with the lowest P values associated with 81 mg aspirin were involved in energy metabolism from fatty acids (carnitine shuttle), nucleotide (pyrimidine) metabolism, and amino acid (alanine and aspartate) metabolism. In contrast, the three pathways with the lowest P values associated with 325 mg aspirin were related to arachidonic acid metabolism, including prostaglandin formation from arachidonate, and leukotriene metabolism. Only the carnitine shuttle pathway was associated with both doses of aspirin treatment.

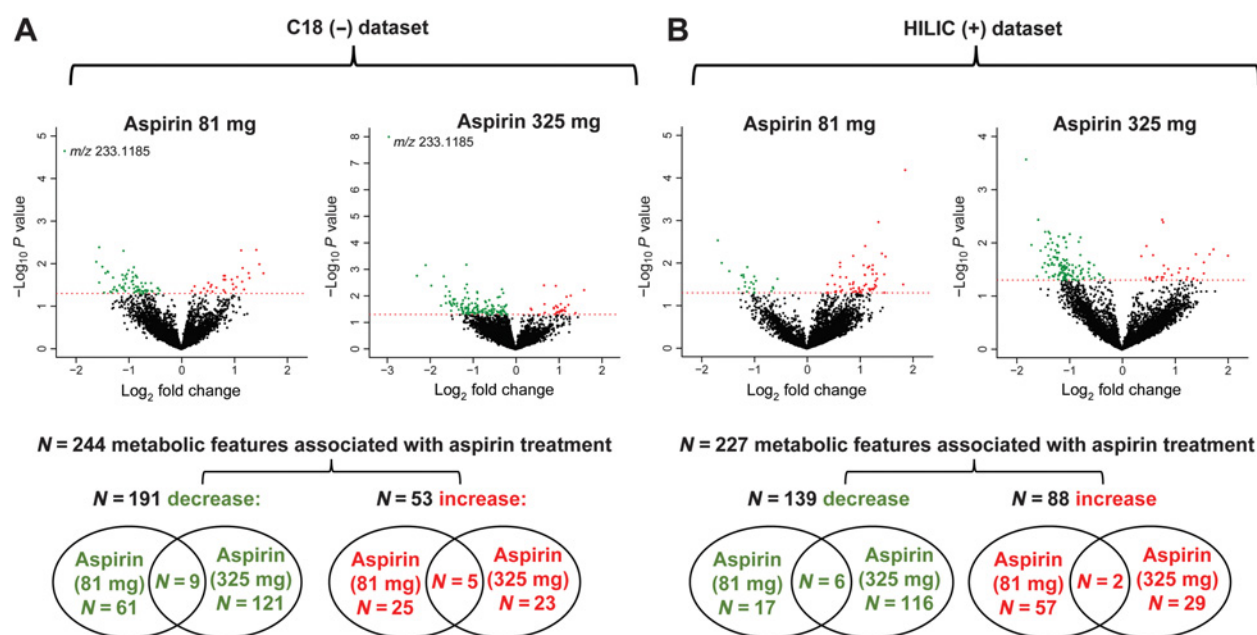
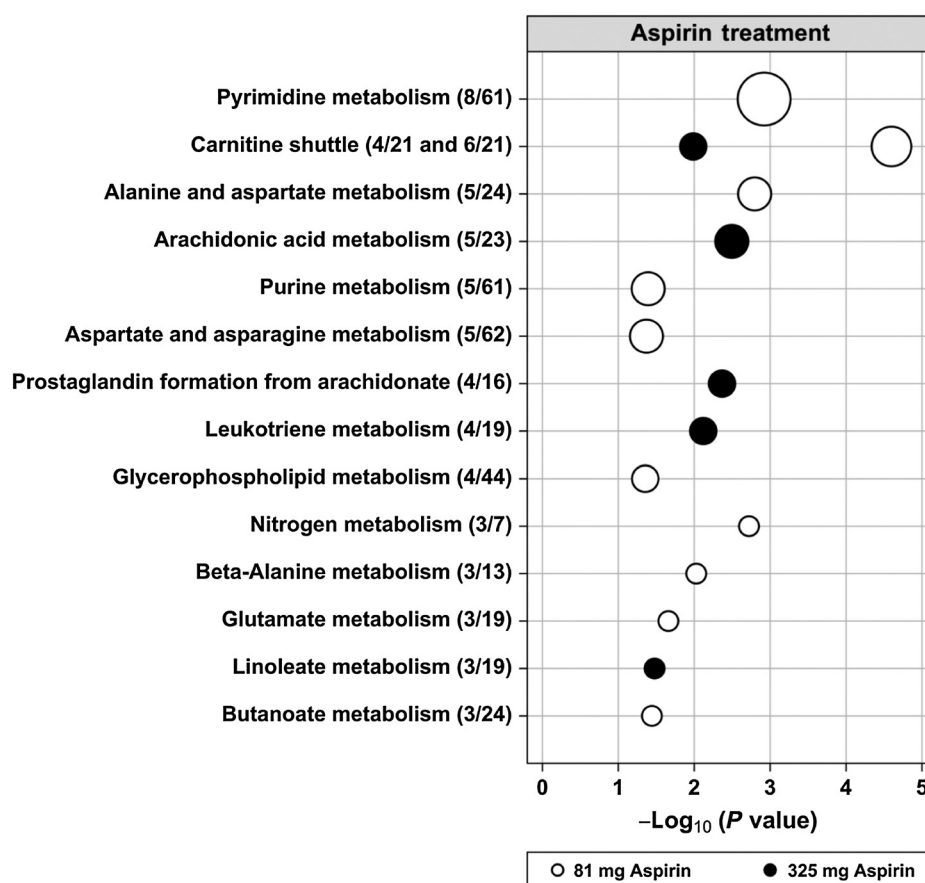


Figure 2.

Colon tissue metabolic features associated with aspirin treatment. **A**, C18 (-) dataset. **B**, HILIC (+) dataset. Linear regression was used to estimate the change in year 3 ion intensities in the aspirin-treated group compared with the placebo group, adjusting for age, sex, race, and folate treatment. Volcano plots depict for each metabolic feature the magnitude of the change on the x-axis (\log_2 fold change with aspirin treatment vs. placebo) and the statistical significance on the y-axis ($-\log_{10} P$ value for the association with aspirin treatment). Color-coded metabolic features statistically significantly increased (red) or decreased (green) with aspirin treatment ($P < 0.05$). One feature significant at a FDR ($q < 0.2$) is indicated in the C18 volcano plots as m/z 233.1185. Venn diagrams indicate overlap of significant metabolic features across the two aspirin treatment groups (81 or 325 mg) for those that either decrease or increase with treatment.

Figure 3.

Dysregulated metabolic pathways associated with aspirin treatment in colon tissue. The vertical axis represents the pathways (circles) with the radius representing the number of hits (significant metabolite features). The horizontal axis represents the negative \log_{10} of the gamma adjusted P values for each pathway with at least three hits. The solid circles are for 81 mg aspirin and the open circles are for 325 mg aspirin treatment. In parentheses next to each pathway name is the number of hits divided by the pathway size (total number of features detected in the pathway).



Putative or confirmed identities for 20 metabolic features in the aspirin-associated pathways along with their fold changes with aspirin treatment are shown in **Table 2**. As expected, levels of several putative arachidonic acid metabolites were substantially reduced with aspirin treatment. Metabolic features with the largest fold changes with aspirin treatment were: an 80% decrease (0.20-fold change) in the putative prostaglandin metabolite α -hydroxy-9,15-dioxoprostanoate with 325 mg aspirin ($m/z = 351.2180$, $P = 0.002$) and a 2.68-fold increase in the confirmed carnitine shuttle metabolite arachidyl carnitine with 81 mg aspirin ($m/z = 456.4043$, $P = 0.006$).

Association of colon tissue metabolic features with adenoma risk

Aspirin-associated metabolic features were assessed for their associations with adenoma risk to identify those that could contribute to the chemopreventive effects of aspirin (**Fig. 1B**, step 4; **Table 3**). There are only two categories of metabolic features that could contribute to aspirin's chemopreventive effects: (i) those that increased with aspirin treatment and were also associated with a reduction in adenoma risk, or (ii) those that decreased with aspirin treatment and were also associated with an increase in adenoma risk. In total, we discovered 21

metabolic features meeting these criteria, of which seven have putative or confirmed identities (**Table 3**; Supplementary Table S1).

The 20 metabolic features in aspirin-associated pathways in **Table 2** were also assessed for their associations with adenoma risk (**Table 2**). Findings for glycerol 3-phosphate ($m/z = 171.0069$) and linoleate ($m/z = 261.2224$) are included in this table (in addition to **Table 3**) because they were identified as components of the glycerophospholipid metabolism pathway. However, there was no evidence that any of the other metabolic features from the pathway analyses could contribute to the chemopreventive effects of aspirin because either: (i) they were not statistically significantly associated with adenoma risk, or (ii) they exhibited associations that were in a direction that would increase rather than reduce adenoma risk with aspirin treatment. These include putative arachidonate pathway metabolites (e.g., prostaglandin C2, leukotriene A4) that decreased with aspirin treatment but were not associated with adenoma risk. Also, some putative or confirmed carnitine shuttle metabolites (e.g., L-palmitoylcarnitine, octadecenoyl carnitine, stearoylcarnitine) increased with aspirin treatment and were associated with increased, rather than reduced, adenoma risk.

Table 2. Metabolic features in pathways associated with aspirin treatment: fold changes with aspirin and associations with adenoma outcomes.

m/z	RT	Col	FC (95% CI) ^a	FC (95% CI) ^b	RR (95% CI) ^b	RR (95% CI) ^b	RR (95% CI) ^b	RR (95% CI) ^b	Putative identity ^d	Adduct ^d	MSI level ^d
A. Metabolic features that decreased with aspirin treatment:											
88.012	19.3	C18-	0.74 (0.56-0.98)	0.87 (0.74-1.03)	0.94 (0.91-1.02)	0.95 (0.83-1.12)	0.89 (0.83-1.02)	0.87 (0.83-0.99)	Pyruvate	M(C13)-H	1
103.0591	18.7	C18-	0.74 (0.55-1.00)	1.07 (0.91-1.25)	0.94 (0.91-1.01)	1.17 (0.93-1.35)	1.17 (0.93-1.35)	0.87 (0.83-0.99)	2-Aminobutyrate	M(C13)-H	1
156.0288	26.4	C18-	0.49 (0.26-0.90)	0.68 (0.39-1.17)	1.02 (0.98-1.05)	0.97 (0.91-1.05)	0.96 (0.92-1.03)	0.96 (0.92-1.03)	N-Acetyl-L-aspartate	M-H2O-H	1
171.0069	18.4	C18-	0.57 (0.34-0.96)	0.78 (0.51-1.21)	1.02 (0.97-1.06)	1.10 (1.00-1.14)	1.12 (1.02-1.15)	1.12 (1.02-1.15)	Glycerol 3-phosphate	M-H	1
173.0569	24.2	C18-	0.56 (0.33-0.97)	0.79 (0.50-1.25)	1.00 (0.97-1.03)	0.93 (0.89-1.02)	0.92 (0.90-1.00)	0.92 (0.90-1.00)	5,6-Dihydrouracil	M+-CH3COO	2
191.0679	19.9	C18-	0.54 (0.29-0.99)	0.86 (0.51-1.44)	1.02 (0.98-1.05)	1.04 (0.94-1.12)	0.99 (0.93-1.06)	0.99 (0.93-1.06)	L-Asparagine	M+-CH3COO	1
231.1358	20.2	C18-	0.38 (0.18-0.83)	1.24 (0.65-2.36)	1.01 (0.98-1.03)	1.03 (0.96-1.08)	1.02 (0.97-1.07)	1.02 (0.97-1.07)	Spermic acid 2	M-H	2
319.2253	35	HILIC+	0.77 (0.43-1.38)	0.48 (0.24-0.96)	1.00 (0.98-1.03)	1.00 (0.93-1.08)	0.97 (0.93-1.03)	0.97 (0.93-1.03)	Leukotriene A4	M+-H	2
335.2205	30.2	HILIC+	0.81 (0.46-1.43)	0.43 (0.21-0.84)	0.98 (0.96-1.01)	1.04 (0.96-1.10)	0.97 (0.93-1.03)	0.97 (0.93-1.03)	Prostaglandin C2 ^e	M+-H	2
336.2241	25.6	HILIC+	0.76 (0.42-1.38)	0.42 (0.21-0.83)	0.99 (0.97-1.02)	1.06 (0.96-1.12)	1.01 (0.95-1.07)	1.01 (0.95-1.07)	12,13-DHOME	M+-Na	2
351.2180	23	C18-	0.66 (0.27-1.63)	0.20 (0.08-0.54)	0.98 (0.97-1.01)	0.98 (0.94-1.03)	0.96 (0.94-1.01)	0.96 (0.94-1.01)	Alpha-hydroxy-9,15-dioxoprostanate ^{e,f}	M-H	2
370.2323	21.2	C18-	0.34 (0.16-0.70)	0.31 (0.15-0.64)	1.00 (0.98-1.03)	1.01 (0.94-1.07)	1.00 (0.95-1.06)	1.00 (0.95-1.06)	Thromboxane B2 ^g	M(C13)-H	2
B. Metabolic features that increased with aspirin treatment:											
261.2224	244.8	C18-	1.35 (1.02-1.78)	0.92 (0.61-1.38)	0.95 (0.93-1.00)	0.92 (0.85-1.04)	0.94 (0.87-1.06)	0.94 (0.87-1.06)	Linoleate	M-H2O-H	1
400.3402	24.8	HILIC+	1.30 (0.92-1.83)	1.36 (1.01-1.84)	1.22 (1.06-1.24)	1.41 (0.92-1.75)	1.36 (0.95-1.60)	1.36 (0.95-1.60)	L-Palmitoyl carnitine	M+-H	1
426.3568	24.8	HILIC+	1.39 (0.95-2.04)	1.50 (1.08-2.10)	1.18 (1.04-1.20)	1.23 (0.90-1.48)	1.25 (0.95-1.44)	1.25 (0.95-1.44)	Octadecenoyl carnitine ^e	M+-H	2
428.3716	25	HILIC+	1.24 (0.91-1.70)	1.38 (1.08-1.76)	1.22 (1.04-1.26)	1.43 (0.94-1.75)	1.44 (0.99-1.67)	1.44 (0.99-1.67)	Stearoyl carnitine	M+-H	1
448.3429	23.9	HILIC+	1.83 (1.06-3.15)	1.03 (0.53-2.00)	1.05 (0.99-1.08)	1.00 (0.92-1.09)	1.04 (0.94-1.12)	1.04 (0.94-1.12)	C20:4 carnitine ^e	M+-H	2
450.3572	24.5	HILIC+	2.49 (1.18-5.26)	1.58 (0.71-3.51)	1.02 (0.99-1.04)	1.03 (0.95-1.09)	1.03 (0.96-1.09)	1.03 (0.96-1.09)	Dihomo-gamma-linolenyl carnitine ^e	M+-H	2
456.4043	25.3	HILIC+	2.68 (1.34-5.35)	1.65 (0.79-3.46)	1.03 (0.99-1.05)	0.99 (0.93-1.06)	1.01 (0.95-1.06)	1.01 (0.95-1.06)	Arachidyl carnitine	M+-H	1
476.3729	24.5	HILIC+	2.30 (1.04-5.07)	2.64 (1.21-5.78)	1.01 (0.99-1.03)	1.08 (0.99-1.13)	1.06 (0.98-1.10)	1.06 (0.98-1.10)	Adrenyl carnitine	M+-H	2

Note: Only metabolic features from pathways associated with aspirin treatment in **Fig. 3** are included.

Abbreviations: CI, confidence interval; Col, chromatography column (C18 or HILIC) and ionization mode (- or +); FC, fold change; 12,13-DHOME, 12,13-hydroxyoctadec-9(Z)-enoate; MSI, Metabolomics Standards Initiative; RR, risk ratio; RT, retention time in seconds.

^aLinear regression was used to estimate the fold change in year 3 ion intensities in the aspirin treatment group compared with the placebo treatment group, adjusting for age, sex, race, and folate treatment. Bolding indicates statistically significant ($P < 0.05$).

^bPoisson regression was used to assess the association of risk of adenoma outcomes with a two-fold change in ion intensity, adjusting for age, sex, and race. Bolding indicates statistically significant ($P < 0.05$).

^cAny adenoma refers to ≥ 1 adenoma of any type; advanced refers to adenomas with cancer, high-grade dysplasia, $>25\%$ villous component, or diameter ≥ 1 cm; high-risk refers to ≥ 1 advanced adenoma or ≥ 3 adenomas of any type.

^dFor putative identities shown, the adduct form and MSI identification confidence level are indicated in the columns to the right.

^eFor these metabolic features, an additional adduct form was detected but is not shown to reduce redundancy in the table.

^fIn pathway analysis, two metabolic features in the Prostaglandin Formation from Arachidonate pathway were associated with any aspirin treatment (combined analysis of 81 and 325 mg), but not either dose alone.

Table 3. Metabolic features that may contribute to the chemopreventive effects of aspirin treatment: fold changes with aspirin and associations with adenoma outcome.

m/z	RT	Col	FC (95% CI) ^a 81 mg Aspirin	FC (95% CI) ^a 325 mg Aspirin	RR (95% CI) ^b Any adenoma ^c	RR (95% CI) ^b advanced ^c	RR (95% CI) ^b High-risk ^c	Putative identity ^d	Adduct ^d	MSI level ^d
A: Metabolic features that decreased with aspirin treatment compared with placebo, and were also associated with increased risk of adenoma outcomes:										
133.9746	290.8	HILIC+	0.90 (0.55–1.45)	0.48 (0.26–0.89)	1.03 (0.99–1.06)	1.15 (1.03–1.19)	1.11 (0.99–1.16)			
138.5112	108.8	HILIC+	0.64 (0.42–0.97)	0.50 (0.31–0.82)	1.01 (0.97–1.05)	1.18 (1.01–1.24)	1.15 (1.02–1.19)			
171.0069	18.4	C18–	0.57 (0.34–0.96)	0.78 (0.51–1.21)	1.02 (0.97–1.06)	1.10 (1.00–1.14)	1.12 (1.02–1.15)	Glycerol 3-phosphate	M-H	1
197.1899	20.8	HILIC+	0.75 (0.54–1.04)	0.58 (0.39–0.86)	1.07 (0.99–1.10)	1.19 (1.02–1.25)	1.21 (1.05–1.24)			
201.1247	19.9	C18–	0.55 (0.32–0.95)	1.04 (0.70–1.55)	1.02 (0.97–1.05)	1.10 (1.01–1.13)	1.11 (1.02–1.13)	Leucyl-Alanine	M+Na-2H	2
398.2864	242.7	C18–	0.89 (0.44–1.80)	0.46 (0.22–0.97)	1.05 (1.01–1.06)	1.11 (1.01–1.14)	1.08 (1.00–1.12)			
435.313	215.3	C18–	0.61 (0.37–0.99)	1.20 (0.81–1.78)	1.06 (0.99–1.09)	1.28 (1.05–1.33)	1.08 (0.94–1.19)	Varanic acid	M-H	2
439.2982	219.1	C18–	0.68 (0.48–0.99)	0.92 (0.70–1.22)	1.10 (0.97–1.17)	1.48 (1.05–1.64)	1.41 (1.05–1.52)			
460.2833	208.1	C18–	0.67 (0.48–0.96)	0.89 (0.76–1.03)	1.10 (0.99–1.16)	1.46 (1.02–1.66)	1.31 (0.99–1.47)	LysoPE (C18:1)	M-H2O-H	2
516.0552	16.3	C18–	0.77 (0.39–1.54)	0.46 (0.22–0.94)	1.05 (1.01–1.06)	1.03 (0.96–1.09)	1.01 (0.96–1.07)			
527.3094	198.2	C18–	0.56 (0.34–0.91)	1.16 (0.91–1.48)	1.07 (0.98–1.11)	1.18 (1.02–1.24)	1.04 (0.92–1.15)			
589.5188	291.4	C18–	0.84 (0.62–1.15)	0.68 (0.47–0.97)	0.99 (0.95–1.05)	1.28 (1.01–1.40)	1.06 (0.92–1.18)	Polyoxyethylene dioleate	M-H	2
591.8592	253	C18–	0.61 (0.26–1.41)	0.34 (0.15–0.81)	1.04 (1.00–1.05)	1.03 (0.96–1.08)	1.04 (0.98–1.08)			
634.3312	222.3	C18–	0.82 (0.56–1.19)	0.62 (0.40–0.95)	1.00 (0.95–1.05)	1.26 (1.00–1.39)	1.20 (1.01–1.28)			
714.3432	50.3	HILIC+	0.78 (0.41–1.50)	0.48 (0.24–0.99)	1.05 (1.00–1.06)	1.08 (0.98–1.14)	1.07 (0.99–1.11)			
816.4432	295.7	HILIC+	0.98 (0.43–2.22)	0.43 (0.19–0.99)	1.04 (1.00–1.05)	0.99 (0.94–1.05)	0.99 (0.95–1.04)			
B: Metabolic features that increased with aspirin treatment compared with placebo, and were also associated with decreased risk of adenoma outcomes:										
115.0632	35.9	HILIC+	1.17 (0.94–1.46)	1.29 (1.05–1.59)	0.91 (0.88–1.00)	0.88 (0.78–1.07)	0.90 (0.79–1.08)	Creatinine	M+H	1
118.0589	17.3	C18–	1.43 (1.04–1.95)	1.15 (0.81–1.63)	0.92 (0.90–0.98)	0.97 (0.84–1.14)	0.97 (0.87–1.10)			
167.0379	175.1	C18–	1.74 (1.08–2.81)	1.91 (1.24–2.96)	0.97 (0.94–1.02)	0.88 (0.86–0.98)	0.89 (0.87–0.97)			
261.2224	244.8	C18–	1.35 (1.02–1.78)	0.92 (0.61–1.38)	0.95 (0.93–1.00)	0.92 (0.85–1.04)	0.94 (0.87–1.06)	Linoleate	M-H2O-H	1
681.9263	281	HILIC+	1.29 (0.50–3.34)	2.93 (1.17–7.34)	0.97 (0.96–1.00)	0.99 (0.95–1.04)	0.99 (0.95–1.04)			

Note: Only metabolic features associated with at least one dose of aspirin treatment ($P < 0.05$, bold type) and also with risk of at least one of the adenoma outcomes ($P < 0.05$, bold type) in the opposing direction are presented.

Abbreviations: CI, confidence interval; Col, chromatography column (C18 or HILIC) and ionization mode (– or +); FC, fold change; LysoPE, lysophosphatidylethanolamine; MSI, Metabolomics Standards Initiative; RR, risk ratio; RT, retention time in seconds.

^aLinear regression was used to estimate the fold change in year 3 ion intensity in the aspirin treatment group compared with the placebo group, adjusting for age, sex, race, and folate treatment.

^bPoisson regression was used to assess the association of risk of adenoma outcomes at year 3 with a two-fold change in ion intensity, adjusting for age, sex, and race.

^cAny adenoma refers to ≥ 1 adenoma of any type. Advanced refers to adenomas with cancer, high-grade dysplasia, $>25\%$ villous component, or diameter ≥ 1 cm. High-risk refers to ≥ 1 advanced adenoma or ≥ 3 adenomas of any type.

^dFor putative identities shown, adduct form and MSI identification confidence level are indicated in the columns to the right.

Discussion

In this untargeted metabolomics analysis of colon tissue, we found that top metabolic pathways associated with 81 mg/day aspirin treatment primarily involved energy, nucleotide and amino acid metabolism, and were largely distinct from pathways associated with 325 mg/day aspirin treatment, which primarily involved pathways related to arachidonic acid metabolism, including prostaglandin and leukotriene metabolism. However, the carnitine shuttle pathway was associated with both doses of aspirin treatment. On the basis of associations with adenoma risk, we discovered several metabolites in colon tissue involved in energy metabolism that may contribute to the chemopreventive effects of aspirin during early colorectal carcinogenesis, including glycerol 3-phosphate, creatinine, and linoleate. Conversely, we also discovered several carnitine shuttle metabolites that may be associated with increased risk of carcinogenesis with aspirin treatment.

Interestingly, the three confirmed metabolites linked to reductions in carcinogenesis with aspirin treatment have all previously been associated with colorectal cancer risk: glycerol 3-phosphate, creatinine, and linoleate (Supplementary Table S1). In cross-sectional studies, linoleate in stool (29–33) and blood (34) and creatinine in urine (35) were inversely associated with cancer status. In studies of colorectal cancer versus normal mucosal tissue, creatinine was lower in tumors (30, 36), whereas glycerol 3-phosphate was higher (37). The directions of these associations were all consistent with our findings for aspirin treatment and adenoma risk, that is, aspirin treatment increased linoleate and creatinine levels, and reduced glycerol 3-phosphate levels, as would be expected given chemopreventive effects of aspirin. Furthermore, two of these metabolites (glycerol-3-phosphate and linoleate) are in the glycerophospholipid metabolism pathway, which was associated with treatment with 81 mg aspirin. Glycerophospholipids provide precursors for the synthesis of eicosanoids from arachidonic acid upstream of cyclooxygenase inhibition by aspirin (38). Also, linoleic acid in stool has been associated with specific gut microbial profiles (29, 33), and the gut microbiome has been linked to colorectal cancer risk (39). Thus, a novel hypothesis is that the mechanism for the chemopreventive effect of aspirin may involve modification of the gut microbiome.

There have been a few previous studies of the metabolic effects of aspirin in humans. In one trial, 40 healthy adults were randomized to 325 mg aspirin treatment or placebo for 60 days in a crossover design (40). Among 363 metabolites that were analyzed in plasma, aspirin treatment reduced levels of 2-hydroxyglutarate, a putative oncometabolite associated with epigenetic dysregulation in myeloid malignancies. In our colon tissue specimens, although 325 mg aspirin treatment appeared to be associated with slightly reduced 2-hydroxyglutarate levels (fold change = 0.88, $P = 0.08$), 81 mg aspirin treatment was not (fold change = 0.98, $P = 0.83$) and changes in 2-hydroxyglutarate levels were also not significantly associated with adenoma outcomes. Also, in a cross-sectional study of 58 individuals,

a targeted metabolomic analysis of the colon mucosa biopsied at routine colonoscopy showed decreased levels of eicosanoids with aspirin use (41), in agreement with our findings. Finally, in analyses of up to 156 participants in the Hereditary and Phenotype Intervention Heart Study who were treated with 81 mg aspirin for 14 days, serum levels decreased for 25 of 30 oxylipids measured as well as linoleic acid and arachidonic acid (42–44). The decrease in linoleic acid detected in serum is opposite what we observed in colon tissue with long-term aspirin treatment and short-term cessation of treatment.

Our findings have a number of interesting implications. Modulation of energy metabolism appears to be a key factor in aspirin's chemopreventive effects in colon tissue. It is well known that transformed cells reprogram energy metabolism to carry out aerobic glycolysis, a phenomena called the Warburg effect (45). Aspirin may be able to suppress this reprogramming via effects on the AMPK and mTOR signaling pathways (11). Also, the metabolic changes that we detected appear to be relatively long-lasting because they were detected in normal colon mucosal biopsies collected at colonoscopies a week after cessation of aspirin treatment.

Interestingly, aspirin-associated metabolic features and pathways differed for the two aspirin doses used (81 and 325 mg) and some of the metabolic changes caused by aspirin treatment might increase rather than decrease risk. These two observations may help to explain the lack of a typical dose-response relationship observed in our clinical trial where the lower 81 mg dose appeared more efficacious than the 325 mg dose in preventing adenomas (12). For example, increases in some carnitine shuttle metabolites appeared greater for the 325 mg dose as compared with the 81 mg dose, and these increases were associated with increased adenoma risk rather than reduced risk. Our findings are in agreement with recent evidence in cultured cells that aspirin increases mitochondrial fatty acid oxidation via stimulation of the carnitine shuttle (46). Potentially this increase in energy production from fatty acids may increase carcinogenesis. Ultimately, this finding may have important implications for optimizing the dose of aspirin used in chemoprevention. It is noteworthy that similar unusual dose responses were seen for associations of aspirin use with ovarian cancer risk in the Nurses' Health Studies, where low dose (≤ 100 mg) aspirin was inversely associated with risk but the 325 mg dose was not associated with risk (47), and in another colorectal adenoma prevention trial (48).

Although we detected reductions in putative eicosanoids metabolites downstream of cyclooxygenase inhibition, especially for the 325 mg aspirin dose, we did not find evidence that these changes contribute to the chemopreventive effects of aspirin because changes in these metabolites were not associated with adenoma risk. This is in agreement with null findings from our prior targeted analysis in which the effect of aspirin in reducing adenoma risk was independent of urinary prostanoid levels (14). Thus, despite previous research implicating cyclooxygenase inhibition (4), our findings do not provide evidence for a role of these metabolites in the chemopreventive effects of

aspirin in the normal mucosa at early stages of colorectal carcinogenesis. This is perhaps not surprising since COX-2 levels may not be elevated in non-neoplastic tissue.

This work has important strengths. We examined the effect of aspirin in individuals who were randomly assigned to daily treatment with two defined doses of aspirin (81 or 325 mg) or a placebo control for approximately 3 years. We analyzed the effects of aspirin in the relevant target tissue (colon). We linked our metabolomics findings to prospective data on adenoma outcomes. Finally, we carefully selected individuals for this metabolomics analysis to minimize aspirin exposure in the placebo control group.

There are also some limitations. Colon tissue biopsies were only collected at the end of treatment and a week after treatment cessation. However, we measured increases in two common aspirin catabolites (salicylate and salicylic acid; ref. 9) in plasma samples collected just prior to treatment cessation compared with those collected at baseline to confirm treatment adherence and lack of exposure in the placebo group. There was probably some variability in the amount of mucosal tissue in the biopsies, which were not weighed. However, data were normalized during statistical analyses to address this limitation. Also, 16 of 21 metabolic features discovered that may contribute to aspirin's protective effects were unidentified, a common limitation inherent in current untargeted metabolomics analyses (49, 50). Nevertheless, the three metabolites with confirmed identities have previously been associated with colorectal cancer, and two of these are involved in the biosynthesis of eicosanoids from arachidonic acid upstream of cyclooxygenase inhibition by aspirin. Finally, we did not adjust for multiple comparisons in the present exploratory analysis. However, the strength of the untargeted approach to discover novel aspirin-associated pathways and metabolites outweighs this limitation and identifying and validating the metabolic features discovered here should be a priority in future work.

In conclusion, our metabolomics analysis of normal colon mucosa from individuals who underwent long term aspirin treatment suggests that the modulation of energy metabolism and the effect of cyclooxygenase inhibition on arachidonic acid precursors may be key to aspirin's chemopreventive efficacy in early colorectal carcinogenesis. Conversely, aspirin's effects on carnitine shuttle metabolism may increase carcinogenesis and

could be important for understanding dose-dependent effects of the drug.

Disclosure of Potential Conflicts of Interest

E.L. Barry reports grants from NIH/NCI during the conduct of the study. J.A. Baron reports grants from NCI during the conduct of the study, as well as has a patent for chemopreventive use of aspirin issued (held with Dartmouth College). D.P. Jones reports grants from Emory University (received NIH grant support) during the conduct of the study. No potential conflicts of interest were disclosed by the other authors.

Data Sharing

These data are available at the NIH Common Fund's National Metabolomics Data Repository (NMDR) website, the Metabolomics Workbench, <https://www.metabolomicsworkbench.org>, where it has been assigned Project ID PR000730. The data can be accessed directly via its Project DOI: 10.21228/M89X1C. This work was supported by NIH grant, U2C-DK119886.

Authors' Contributions

E.L. Barry: Conceptualization, resources, supervision, funding acquisition, writing-original draft and project administration. **V. Fedirko:** Conceptualization, project administration, writing-review and editing. **K. Uppal:** Data curation, software, methodology, writing-review and editing. **C. Ma:** Formal analysis, writing-review and editing. **K. Liu:** Investigation, writing-review and editing. **L.A. Mott:** Data curation, formal analysis, visualization, writing-review and editing. **J.L. Peacock:** Formal analysis, writing-review and editing. **M.N. Passarelli:** Visualization, writing-review and editing. **J.A. Baron:** Conceptualization, resources, funding acquisition, writing-review and editing. **D.P. Jones:** Conceptualization, resources, supervision, funding acquisition, project administration, writing-review and editing.

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