Research Article

Cancer Prevention Research

Urinary Metabolites of Prostanoids and Risk of Recurrent Colorectal Adenomas in the Aspirin/Folate Polyp Prevention Study (AFPPS)

Veronika Fedirko[1], Patrick T. Bradshaw[2], Jane C. Figueiredo[3], Robert S. Sandler[4], Elizabeth L. Barry[5], Dennis J. Ahnen[6], Ginger L. Milne[7], Robert S. Bresalier[8], and John A. Baron[9]

Abstract

Aspirin has been shown to protect against colorectal neoplasms; however, the optimal chemopreventive dose and underlying mechanisms are unclear. We aimed to study the relationship between prostanoid metabolites and aspirin's effect on adenoma occurrence. We used data from the Aspirin/Folate Polyp Prevention Study, in which 1,121 participants with a recent adenoma were randomized to placebo or two doses of aspirin (81 or 325 mg/d) to be taken until the next surveillance colonoscopy, anticipated about 3 years later. Urinary metabolites of prostanoids (PGE-M, PGI-M, and dTxB$_2$) were measured using liquid chromatography/mass spectrometry or GC/NICI-MS in 876 participants near the end of treatment follow-up. Poisson regression with a robust error variance was used to calculate relative risks and 95% confidence intervals. PGE-M, PGI-M, and dTxB$_2$ levels were 28%, 37%, and 60% proportionately lower, respectively, in individuals who took 325 mg of aspirin compared with individuals who took placebo (all $P < 0.001$). Similarly, among individuals who took 81 mg of aspirin, PGE-M, PGI-M, and dTxB$_2$ were, respectively, 18%, 30%, and 57% proportionately lower compared with placebo (all $P < 0.005$). None of the metabolites or their ratios were statistically significantly associated with the risk of adenoma occurrence. The effect of aspirin in reducing adenoma risk was independent of prostanoid levels. Aspirin use is associated with lower levels of urinary prostanoid metabolites. However, our findings do not support the hypothesis that these metabolites are associated with adenoma occurrence, suggesting that COX-dependent mechanisms may not completely explain the chemopreventive effect of aspirin on colorectal neoplasms. *Cancer Prev Res; 8(11); 1061–8. ©2015 AACR.*

Introduction

There is strong evidence from observational studies and randomized clinical trials that aspirin and other nonsteroidal anti-inflammatory drugs (NSAID) are protective against colorectal neoplasms (1–5); however, the dose–response relationship and underlying antineoplastic mechanisms are not clear. Pooled results from four clinical trials with 2,967 participants randomly assigned to doses of aspirin from 81 to 325 mg daily showed a borderline statistically significant 15% decrease in any adenoma occurrence, and a statistically significant 29% decrease in advanced adenoma occurrence with higher dose aspirin supplementation ($\geq$300 mg/d; ref. 6). Interestingly, in a combined analysis of the two trials (refs. 2 and 5; including ours, ref. 2) that tested higher (300 or 325 mg/d) and lower ($\leq$160 mg/d) doses of aspirin versus placebo, lower dose aspirin showed statistically significantly greater risk reduction for all colorectal adenomas than higher dose aspirin (6).

Prostanoids, important mediators of human physiology, are a subclass of eicosanoids consisting of the prostaglandins, the prostacyclins, and the thromboxanes. They are generated from the precursor arachidonic acid by cyclooxygenase COX enzymes: COX-1, constitutively expressed in the luminal gastrointestinal tract and responsible for the production of cytoprotective prostaglandins; and COX-2, inducible by a variety of factors such as growth factors and mitogens, and a central element of the inflammatory response (7). The increased activity of COX-2 plays an important role in colorectal carcinogenesis (8, 9); the involvement of COX-1 has been less investigated, but also appears to be involved (10). Of the prostaglandins, prostaglandin E$_2$ (PGE$_2$) is likely to be the primary mediator of COX procarcinogenic effects (8).

Thromboxane A$_2$ (TXA$_2$) and prostacyclin (PGI$_2$) are other prostanoids, both well known to regulate cardiovascular homeostasis: TXA$_2$ stimulates vasoconstriction and platelet aggregation,

[1]Department of Epidemiology, Rollins School of Public Health, Winship Cancer Institute, Emory University, Atlanta, Georgia. [2]Department of Nutrition, Gillings School of Global Public Health, University of North Carolina, Chapel Hill, North Carolina. [3]Department of Preventive Medicine, Keck School of Medicine, University of Southern California, Los Angeles, California. [4]Department of Medicine, University of North Carolina, Chapel Hill, North Carolina. [5]Department of Epidemiology, Geisel School of Medicine at Dartmouth, Lebanon, New Hampshire. [6]Department of Veterans Affairs Medical Center, Denver, Colorado. [7]Division of Clinical Pharmacology, Vanderbilt University, Nashville, Tennessee. [8]Department of Gastrointestinal Medicine and Nutrition, The University of Texas MD Anderson Cancer Center, Houston, Texas. [9]Department of Medicine, University of North Carolina School of Medicine, Chapel Hill, North Carolina.

Corresponding Authors: Veronika Fedirko, Department of Epidemiology, Rollins School of Public Health, Winship Cancer Institute, Emory University, 1518 Clifton Road, Atlanta, GA 30322. Phone: 404-712-8332; Fax: 404-727-8737; E-mail: vfedirk@emory.edu; and John A. Baron, jabaron@med.unc.edu

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whereas prostacyclin PGI_2 promotes vasodilatation and inhibits platelet aggregation (8). In addition to their cardiovascular effects, both PGI_2 and TXA_2 appear to affect carcinogenesis. TXA_2 activation of platelets leads to the release of mediators of carcinogenesis, and TXA_2 itself seems to act as a procarcinogenic factor (10–13). On the other hand, PGI_2 seems anticarcinogenic (14): lower levels are seen in neoplastic tissue (15–17), *in vitro* and *in vivo* studies suggest interference with carcinogenesis (12, 18), and studies of the prostacyclin synthase gene point to antineoplastic effects as well (19, 20).

Low doses of aspirin are sufficient to reduce PGE_2 levels in the colorectal mucosa, with no additional increase in inhibition with doses above 81 mg/d (21–25). There have been no studies of the effects of aspirin on PGI_2 or TXA_2 in the large bowel mucosa, but higher doses are generally required to substantially modulate systemic prostacyclin production (26–29), while lower doses are effective in inhibiting platelet TXA_2, and increasing the ratio of PGI_2 to TXA_2 (30–32). If prostacyclin has antineoplastic properties, aspirin doses above 81 mg would then lose preventive potency from the inhibition of this potentially antineoplastic prostanoid.

To understand the role of PGE_2, PGI_2, and TXA_2, in colorectal carcinogenesis, we conducted secondary analyses in the Aspirin/Folate Polyp Prevention Study (AFPPS; refs. 2, 33), a clinical trial of aspirin and/or folic acid for the prevention of occurrence of colorectal adenomas. Our aims were to assess the effect of two doses of daily aspirin intake on urinary prostanoid metabolites, to investigate whether urinary prostanoid metabolites are associated with risk for recurrent adenoma diagnosed during treatment, and to examine whether a hypothesized aspirin dose-dependent PGE_2 to PGI_2 trade-off plays a role in colorectal adenoma development.

Materials and Methods

Design

These data were collected as part of the AFPPS, a randomized, double-blind, placebo-controlled, three-by-two factorial trial testing whether oral aspirin (81 or 325 mg daily) or folic acid (1 mg daily) reduces the risk of new colorectal adenomas (clinicaltrials.gov identifier: NCT00272324; refs. 2, 33).

Recruitment, randomization, treatment, and follow-up

Details of subject eligibility, recruitment, randomization, treatment, and follow-up, and study outcomes have been previously described (2, 33). Briefly, patients with a recent history of colorectal adenomas were recruited between 1994 and 1998 from nine clinical centers in the United States and Canada. Eligible subjects were between 21 and 80 years of age, in good health, had a complete colonoscopy within 3 months before enrollment with no known polyps left in the bowel, and had received a recommendation for a 3-year follow-up colonoscopy by their regular medical practitioner.

At enrollment, eligible subjects completed a questionnaire regarding demographics, medical history, and lifestyle habits. All participants were asked to avoid the use of aspirin, NSAIDs, and supplements containing folate for the duration of active treatment. Each subject underwent a three-month, single-blind run-in period on 325 mg of aspirin per day. Only subjects with at least 80% compliance and no adverse effects of aspirin were randomly assigned to receive aspirin placebo, low-dose aspirin (81 mg/d), or high-dose aspirin (325 mg/d), and to receive folate placebo or

folate (1 mg/d). A total of 1,021 full factorial subjects were randomized, as well as 100 "aspirin-only" subjects who were recruited before the folic acid component of the study was added.

By protocol, participants were to remain on study treatment until their anticipated surveillance colonoscopy, about three years after the qualifying exam. Every 4 months during study treatment subjects completed a questionnaire regarding compliance with study agents, use of medications and supplements, large bowel endoscopy, and medical events. In addition to a baseline blood sample, blood and spot urine samples were obtained late in the third year of participation, and stored at $-70°C$. Compliance in the initial 3-year treatment period was excellent: 87% to 95% of subjects took study pills at least 6 days per week during those three years. All study aspirin treatment ended on September 28, 2001.

Study outcomes

The primary outcome, adenoma occurrence, was determined by colonoscopy and confirmed by pathology review. All lesions removed from the large bowels of study subjects were reviewed by a single study pathologist. Polyps were classified as adenomatous, hyperplastic, serrated, or other polyps; the degree of dysplasia and the extent of villous component in each adenoma were recorded. Low-risk adenomas were defined as solitary adenomatous polyps < 1 cm in greatest diameter with tubular histology. Advanced adenomas were defined as adenomatous polyps with an estimated diameter of ≥ 1 cm, or at least 25% villous component, any high-grade dysplasia, or invasive cancer. "High risk findings" were one or more advanced adenomas or two or more low risk adenomas (for comparability with previous studies; refs. 34, 35).

Laboratory measurements

Catabolism of PGE_2 results in an end metabolite, 11-α-hydroxy-9,15-dioxo-2,3,4,5-tetranor-prostane-1,20-dioic acid (PGE-M), that is excreted in the urine and stable for prolonged periods when stored at $-70°C$ (36). Measurement of urine PGE-M is a better measure of systemic PGE_2 production than plasma measurements (37, 38) because PGE_2 in plasma is rapidly metabolized in the lungs and consequently may not accurately reflect endogenous prostaglandin production (39). We measured urinary PGE-M using liquid chromatography/mass spectrometry (LC/MS) as previously described (40) on a Waters Acquity UPLC coupled to a Thermo Scientific Quantum Vantage triple quadruple mass spectrometer.

2,3-dinor-6-keto-$PGF1\alpha$ (PGI-M), a major urinary metabolite of prostacyclin PGI_2, was measured using gas chromatography-negative-ion chemical ionization mass spectrometry (GC/NICI-MS) as previously described (41) on an Agilent 5973 Inert Mass Selective Detector coupled with an Agilent 6890n Network GC system (Agilent Labs). 11-dehydro-thromboxane B_2 ($dTxB_2$), a major urinary enzymatic metabolite of TXA_2, was measured using GC/NICI-MS as previously described (42) using the same instruments as described above for PGI-M.

PGE-M, PGI-M, and $dTxB_2$ were successfully measured in 871, 816, and 854 samples, respectively, in the Eicosanoid Core Laboratory at Vanderbilt University (Nashville, TN). The lower limit of detection of PGE-M and PGI-M was 15 pg/mg creatinine, and of $dTxB_2$, 20 pg/mg creatinine. Interday and intraday variability was less than 10% for all prostanoids assays. Quality control samples, from a pooled urine collection, were included with each batch analyzed. Between-batch coefficients of variation were 8.38%, 5.92%, and 7.32%, for the PGE-M, PGI-M, and $dTxB_2$

Table 1. Baseline selected characteristics of the study participants with at least one biomarker measurement

Baseline characteristics	Placebo (N = 296)	Aspirin 81 mg/d (N = 296)	Aspirin 325 mg/d (N = 284)	P-value
Age, yrs, mean (SD)	57.5 (9.8)	57.2 (9.4)	57.6 (4.5)	0.88
Male sex, n (%)	189 (63.9)	193 (65.2)	178 (62.7)	0.83
White, n (%)	253 (85.5)	260 (87.8)	250 (88.0)	0.14
BMI, kg/m^{2a}, mean (SD)	27.3 (4.5)	27.3 (4.5)	27.7 (4.8)	0.91
Overweight, n (%)	140 (47.5)	142 (48.1)	130 (45.8)	0.89
Obese, n (%)	63 (21.4)	60 (20.3)	70 (24.7)	
Current smoker, n (%)	41 (13.9)	40 (13.6)	41 (14.5)	0.56
Alcohol drinker, n (%)	199 (67.3)	189 (63.9)	193 (68.0)	0.62
Multivitamin use, n (%)	104 (35.1)	111 (37.5)	96 (33.8)	0.40
Family history of colorectal polyps, n (%)	62 (26.8)	72 (29.2)	77 (34.1)	0.13
Adenoma characteristics (at baseline)[b]				
Number, mean (SD)	1.6 (0.9)	1.6 (1.0)	1.6 (1.1)	0.41
Advanced adenomas, n (%)[c]	84 (36.1)	80 (34.9)	89 (39.6)	0.69
Proximal location, n (%)	136 (54.4)	135 (54.0)	133 (55.0)	0.74
Folic acid treatment group, n (%)	149 (51.0)	147 (49.7)	143 (50.7)	0.98

[a]N = 1 in the placebo, and N = 1 in the aspirin 81 mg/d group had missing BMI data.
[b]Data available for 268, 257, 243 participants in the placebo, aspirin 81 mg/d, and aspirin 325 mg/d treatment groups, respectively.
[c]Data available for 233, 229, and 229 in the placebo, aspirin 81 mg/d, and aspirin 325 mg/d treatment groups, respectively.

assays, respectively. Urinary prostanoid metabolite levels were expressed as pg/mg of analyte per mg of urinary creatinine. Urinary creatinine was measured using a chemical assay based on Jaffe reaction according to the manufacturer's instructions (Enzo Life Sciences). Laboratory staff was blinded to the treatment group assignment of urine samples and the identity of quality control samples included in the study. Samples from each treatment group were randomly included in every batch.

Statistical analyses

Levels of urinary biomarkers were natural log transformed to improve normality. Values below limit of detection (LOD) were replaced by LOD/2 [n = 4 (0.5%) for PGE-M, n = 61 (7.5%) for PGI-M, and n = 4 (0.5%) for dTxB$_2$]. Fisher exact tests (for categorical variables) and ANOVA (for continuous variables) were used to compare randomized treatment groups. Correlations between biomarkers were calculated using Spearman correlation coefficient (ρ). We used modified Poisson regression with a robust variance estimate to calculate the relative risks (RR) and 95% confidence intervals (CI) of having at least one adenoma (43). Covariates in multivariable models included age (continuous), sex, clinical center, number of lifetime adenoma before randomization (continuous), follow-up time (continuous), and aspirin treatment group assignment (placebo, 81 mg/d, or 325 mg/d). In secondary analyses, we further adjusted for randomized folic acid treatment, first-degree family history of colorectal cancer (yes/no), body mass index (<25, 25–30, $\geq$ 30 kg/m^2) at study enrollment, alcohol use (yes/no), smoking status (never/former/current). Inclusion of these covariates in the models did not substantially change the results; therefore, only the most parsimonious models are presented. Adjusted estimates are presented for one or more adenomas, low-risk adenomas, or advanced adenomas, and high-risk findings. The ratio of PGE-M to PGI-M was used as an indicator of trade-off between systemic PGE$_2$ and PGI$_2$ production. As PGE$_2$ and TXA$_2$ are hypothesized to be procarcinogenic, and PGI$_2$ possibly anticarcinogenic, we used the ratio of (PGE-M + dTxB$_2$) to PGI-M as an indicator of the overall balance between potentially pro- and anticarcinogenic effects of prostanoids. The P value for trend was calculated using the continuous biomarker levels entered linearly into the models. An interaction by aspirin treatment assignment (placebo, 81 mg/d, or 325 mg/d)

was assessed by including the cross product of the treatment variable and urinary biomarker, and evaluated using the Wald test. To compare our results with previous findings (35), we conducted an analysis of aspirin treatment effects within low (first tertile) and high (second and third tertiles) PGE-M levels. All statistical tests were two-sided. Statistical analysis was conducted using Stata (version 12.1, Stata Corp).

Results

There were 1,121 individuals randomized to aspirin or placebo, and 1,084 (97%) underwent a follow-up examination. A total of 876 participants (80.8% of with a follow-up exam) had at least one urinary prostanoid metabolite measurement; 328 had one or more recurrent adenoma at the end of treatment follow-up. The baseline characteristics of the subjects in the three aspirin treatment arms with at least one biomarker measurement are shown in Table 1. Participants with missing urine measurements (n = 208) were comparable with those included in the analysis (n = 876) with respect to baseline characteristics and treatment randomization (data not shown). There were no statistically significant differences among the three arms with regard to demographic and lifestyle factors at the study entry. The three treatment arms were also similar with regard to the percentage of subjects who were randomly assigned to folic acid treatment.

Among all study participants, urinary PGE-M levels were weakly correlated with urinary PGI-M ($\rho = 0.16$, $P < 0.001$) and dTxB$_2$ ($\rho = 0.30$, $P < 0.001$), with a somewhat stronger correlation between urinary PGI-M and dTxB$_2$ ($\rho = 0.41$, $P < 0.001$). Similar correlations were found in each treatment group (data not shown). Urinary PGE-M, PGI-M, and dTxB$_2$ differed by sex, age, study center, and smoking status (Supplementary Tables S1 and S2). Aspirin treatment was associated with lower urinary prostanoid metabolites levels in a dose-dependent manner (Fig. 1). Urinary PGE-M levels were proportionately 28% and 18% lower in participants who took 325 mg and 81 mg of aspirin, respectively, compared with participants who took placebo. Similar but weaker dose-dependent patterns were observed for PGI-M and dTxB$_2$. However, the ratios of PGE-M to PGI-M, and of (dTxB$_2$ + PGE-M) to PGI-M did not differ substantially by aspirin treatment
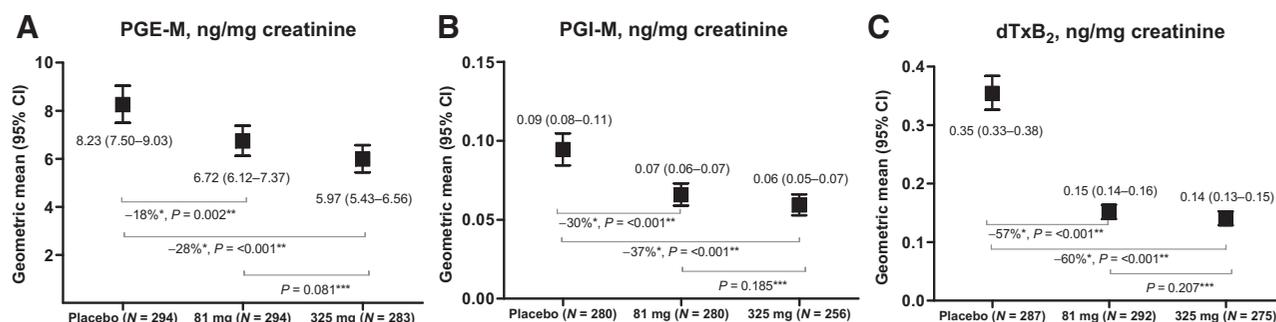
Figure 1.
Urinary PGE-M (A), PGI-M (B) and $dTxB_2$ (C) levels by aspirin treatment group, the AFPPS. *, the proportional difference in geometric means was calculated as [(treatment group) − placebo group)/placebo group]*100%; **, P value versus placebo; ***, P value versus 81 mg.

group (Supplementary Table S3). Comparable aspirin-associated decreases in the levels of urinary prostanoid metabolites were observed in the data further stratified by the folic acid treatment arm (Supplementary Table S4).

There was no evidence that urinary PGE-M, PGI-M, or $dTxB_2$ concentrations (or their ratios) were associated with risk for any adenoma: RR quartiles 2 through 4 were each close to 1.0, with narrow CIs. Urinary PGE-M, PGI-M, and $dTXB_2$ also had no material associations with low-risk adenomas or high-risk findings (Table 2). In contrast, the PGE-M, PGI-M, and to a lesser extent $dTXB_2$, showed nonsignificant suggestions of decreasing RR trends with increasing urinary levels for advanced adenomas (Table 2). The ratios of PGE-M to PGI-M, and of ($dTxB_2$ + PGE-M) to PGI-M were unassociated with risk of any adenomas class (Supplementary Table S5). None of the interactions of urinary prostanoid levels with aspirin treatment group were

statistically significant (all $P > 0.1$). We repeated the analyses in the placebo group only and obtained similar findings (Supplementary Table S6).

In an exploratory analysis, in which we stratified the study participants according to PGE-M levels (Fig. 2), aspirin treatment was associated with a significant reduction in any and high-risk adenoma occurrence among participants with low PGE-M (<5.34 ng/mg creatinine), but not among participants with high PGE-M ($\geq$5.34 ng/mg creatinine) concentrations; however, neither of the interactions were statistically significant. A similar pattern was suggested for low-risk adenoma, whereas for advanced adenoma the aspirin treatment effects did not seem to differ by PGE-M levels. Analyses stratified by sex (Supplementary Tables S7 and 8) suggested that among women, higher levels of PGE-M may be positively associated with adenoma occurrence; however, the results were based on a very small number of events and no

Table 2. Association of adenoma occurrence during study treatment with urinary PGE-M, PGI-M, and $dTxB_2$ levels, the AFPPS

Biomarker/quartile range	Any adenoma		Low-risk adenoma[a]		High-risk findings[a]		Advanced adenoma[a]	
	Cases/ total N	RR[b] (95% CI)	Cases/ total N	RR[b] (95% CI)	Cases/ total N	RR[b] (95% CI)	Cases/ total N	RR[b] (95% CI)
PGE-M, ng/mg creatinine[c]								
<4.57	71/207	1.00 (ref)	36/172	1.00 (ref)	35/171	1.00	21/205	1.00 (ref)
4.57–7.37	77/207	1.00 (0.78–1.29)	39/169	1.07 (0.72–1.61)	38/168	0.94 (0.63–1.41)	22/204	0.93 (0.51–1.71)
7.37–11.38	86/210	1.00 (0.78–1.28)	45/169	1.08 (0.72–1.60)	41/165	0.91 (0.61–1.35)	16/207	0.59 (0.31–1.11)
$\geq$11.39	93/211	1.07 (0.83–1.37)	48/166	1.19 (0.80–1.77)	45/163	0.92 (0.61–1.38)	16/205	0.56 (0.29–1.08)
P_{trend}		0.92		0.53		0.21		0.12
PGI-M, ng/mg creatinine[c]								
<0.049	70/196	1.00 (ref)	33/159	1.00 (ref)	37/163	1.00 (ref)	18/194	1.00 (ref)
0.049–0.082	82/194	1.14 (0.90–1.45)	41/153	1.30 (0.88–1.93)	41/153	1.10 (0.75–1.60)	20/190	1.01 (0.55–1.87)
0.082–0.124	76/194	1.05 (0.82–1.36)	38/156	1.21 (0.81–1.82)	38/156	0.97 (0.65–1.47)	19/192	0.93 (0.49–1.79)
$\geq$0.125	80/196	1.04 (0.81–1.35)	47/163	1.40 (0.94–2.09)	33/149	0.77 (0.51–1.16)	13/192	0.56 (0.28–1.15)
P_{trend}		0.92		0.22		0.48		0.16
$dTxB_2$, ng/mg creatinine[c]								
<0.107	68/199	1.00 (ref)	31/162	1.00 (ref)	37/168	1.00 (ref)	18/196	1.00 (ref)
0.107–0.194	96/209	1.33 (1.04–1.69)	56/169	1.79 (1.22–2.62)	40/153	1.12 (0.75–1.67)	22/207	1.04 (0.55–1.93)
0.195–0.346	79/206	0.98 (0.74–1.30)	42/169	1.27 (0.83–1.95)	37/164	0.78 (0.50–1.21)	19/200	0.60 (0.29–1.24)
$\geq$0.347	77/204	0.94 (0.69–1.28)	34/161	1.15 (0.70–1.88)	43/170	0.72 (0.45–1.15)	16/199	0.46 (0.22–1.00)
P_{trend}		0.93		0.86		0.89		0.44

[a]Low-risk adenoma defined as solitary adenomatous polyp < 1 cm in greatest diameter and tubular or unknown histology (high-risk adenoma excluded from the analysis). High-risk adenoma defined as adenomatous polyp $\geq$1 cm in greatest diameter and/or tubulovillous, villous or high-grade dysplasia, histology or multiple ($\geq$2) adenomatous polyps of any size or histology (low-risk adenoma excluded from the analysis). Advanced adenoma defined as adenomatous polyp with an estimated diameter of $\geq$1 cm, or at least 25% villous component, any high-grade dysplasia, or invasive cancer.
[b]Adjusted for age (continuous), sex, center (categorical), number of previous adenomas (continuous), follow-up time (continuous) and aspirin treatment group (placebo, 81 mg, 325 mg).
[c]P values for interaction for aspirin treatment group were: for any adenoma, P PGE-M = 0.14, P PGI-M = 0.40, and P dTxB2 = 0.50; for low-risk adenoma, P PGE-M = 0.13, P PGI-M = 0.22, and P dTxB2 = 0.40; for high-risk adenoma, P PGE-M = 0.53, P PGI-M = 0.45, and P dTxB2 = 0.63; and for advanced adenoma, P PGE-M = 0.62, P PGI-M = 0.33, and P dTxB2 = 0.32.
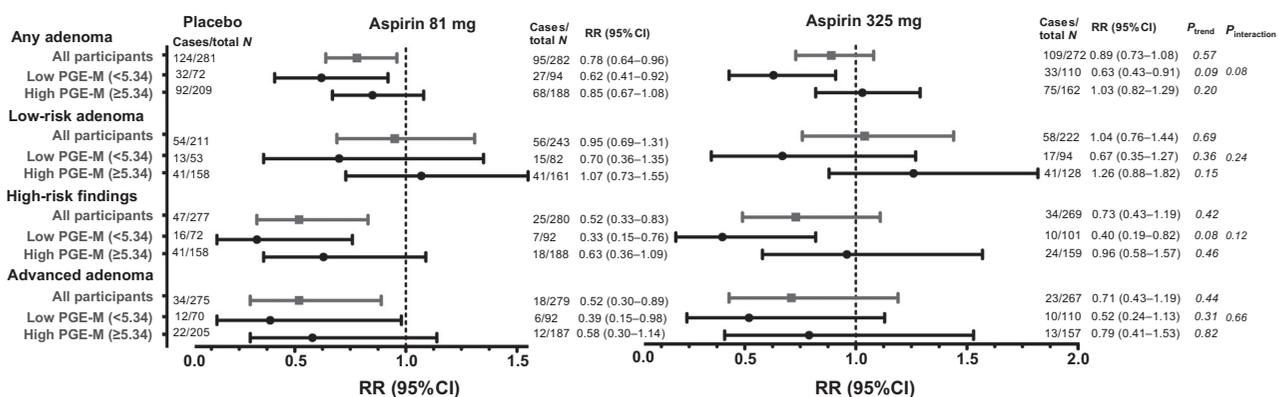
Figure 2.
Association of adenoma occurrence during study treatment within categories of urinary PGE-M levels by aspirin treatment group, the AFPPS. All models were adjusted for age (continuous), sex, center (categorical), number of previous adenomas (continuous), and follow-up time (continuous). Placebo was the reference group. Low PGE-M category = tertile 1; High PGE-M category = tertile 2 and tertile 3.

statistically significant multiplicative interactions by sex were found.

Two participants in the low-dose aspirin group and three in the high-dose aspirin group were classified as aspirin resistant based on $dTxB_2 > 1.5$ ng/mg creatinine (>67.9 ng/mmol of creatinine; refs. 44, 45). Among the 5 participants, one did not take the study treatment (0% compliance). The exclusion of these subjects from the analysis had no effect on the risk estimates (data not shown).

Discussion

In this analysis of individuals participating in a randomized clinical trial, aspirin treatment was associated with lower levels of urinary prostanoids in a dose-dependent manner: participants who took high-dose aspirin had the lowest concentrations of PGE-M, PGI-M, and $dTxB_2$ compared with participants who took low-dose of aspirin or placebo. None of the urinary prostanoid metabolites or their ratios were significantly associated with risk of any measure of adenoma risk.

The levels of COX-derived PGE_2 and its synthase are increased in colorectal neoplasia as compared with histologically normal tissue (16, 46, 47). PGE_2 has been shown to inhibit apoptosis, promote cellular proliferation, angiogenesis, and migration, and activate *Wnt* signaling (48, 49). In previous studies, the major urinary metabolite of PGE_2, PGE-M, has been strongly directly associated with the risk of colorectal cancer (50) and (contrary to our findings) with multiple and/or advanced adenoma (RR ranging from 1.66 to 2.19 comparing the highest vs. lowest quartiles; refs. 34, 35). Conversely, in our study, the association pattern for PGE-M with advanced adenomas was nonsignificantly suggestive of an inverse association. In agreement with our results, previous studies have not found an association with single small tubular adenoma (34, 35) or any adenoma (35). There are no previous data regarding the associations of metabolites of PGI_2 and thromboxane with risk of colorectal cancer or adenomas of any type.

Our data suggested that aspirin treatment was associated with a significant reduction in adenoma occurrence in participants with low PGE-M, measured near the end of treatment. This is expected, as both adenoma occurrence and low PGE-M levels are associated with aspirin use and so are logically associated with each other.

Trial participants on aspirin tended to have low PGE-M and also had the lowest adenoma risk. On the other hand, participants with high PGE-M tended to be placebo subjects (or aspirin subjects resistant to treatment) and had a higher risk for adenomas. Bezawada and colleagues (35) also investigated this issue, and reported the opposite pattern: stronger aspirin/NSAIDs associations in women with higher urinary PGE-M. To the extent that their samples were truly baseline (taken before starting aspirin), their data are logical and do not conflict with ours. However, to the extent their data were taken "on treatment" (during aspirin/NSAID use) the two studies disagree. It is not clear whether their samples were truly baseline as they were collected at the same time as information on aspirin/NSAID use.

Thromboxane is essential to platelet activation, and the antiplatelet effect of aspirin through inhibition of thromboxane synthesis seems to be relevant to its antineoplastic actions (11). It has been proposed that activated platelets promote COX-2 upregulation in adjacent cells of the colorectal mucosa at sites of mucosal injury via paracrine lipid (e.g., TXA_2) or protein (e.g., interleukin-1β) mediators (11). There may also be direct effects of TXA_2 itself, as mucosal levels stimulate proliferation, promote metastases, and mediate COX-2–dependent angiogenesis (13, 51–53). Elevated levels of TXA_2 and thromboxane synthase in colorectal neoplasms have been reported (13, 17), and colon cancer cells that overexpress the TXA_2 synthase gene grow faster and exhibit more abundant vasculature (12). In our study, higher levels of $dTxB_2$, a major urinary metabolite of TXA_2, were not associated with the risk of any adenoma. In contrast to our initial hypothesis, the association pattern for $dTxB_2$ was suggestive of an inverse association for advanced adenoma at the end of study follow-up among all and placebo-only participants.

Prostacyclin PGI_2, a prostaglandin downstream of COX-1 and COX-2 (54), has important roles in cardiovascular homeostasis, and may also influence carcinogenesis. Expression of PGI_2 seems reduced in lung cancers (15), and administration of a PGI_2 analog improves endobronchial dysplasia in former smokers (18). Colon cancer cells that overexpress the prostacyclin synthase gene (*PTGIS*) grow more slowly and with less developed vasculature than control inoculants (12). Epigenetic inactivation of the *PTGIS* is common in colorectal cancer (19), and reduced tumor tissue levels of PGI_2 compared with adjacent normal mucosa have been

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reported (16, 17). Furthermore, a polymorphism associated with reduced activity of the *PTGIS* gene has been associated with an increased risk of colorectal adenomas and a blunting of the protective effect of NSAIDs (20). Our results show that PGI-M, a major urinary metabolite of PGI_2, was not associated with any adenoma, but, in agreement with our initial hypothesis, higher levels of PGI-M were nonsignificantly inversely associated with advanced adenoma.

Although numerous epidemiologic studies and several randomized clinical trials reported that aspirin is protective against colorectal neoplasms (1–5); it is unknown exactly how it exerts its antineoplastic effects, and why the lower aspirin dose seems more effective in preventing colorectal adenomas. The main proposed mechanism so far includes inhibition of COX enzymes. We also hypothesized that a trade-off between procarcinogenic PGE_2 and/or TXA_2, and putatively anticarcinogenic PGI_2 may explain a protective effect of lower dose aspirin. However, our findings did not suggest that differential inhibition of prostanoids explains the paradoxical aspirin effects, nor support the COX-dependent antineoplastic effects of aspirin, as urinary prostanoid metabolites were not associated with risk for colorectal adenoma occurrence. It is possible that COX-independent effects of aspirin are involved, including inhibition of PPARδ (55) and oxidative DNA damage (56), modulation of polyamine metabolism (57) and *Wnt* signaling (58), activation of the NF-κB signaling pathway resulting in apoptosis (59), increase in leukotriene production as a result of shuttling of free arachidonic acid between COXs and lipoxygenases pathways (58), and activation of the NSAID-activated gene (*NAG-1*; ref. 60), which is a member of the TGF-β family that has proapoptotic and antitumorigenic activities (61).

This study has several limitations. First, participants in this analysis had a previous history of at least one colorectal adenoma, potentially limiting the generalizability of the data. In particular, it is possible that the colorectal mucosa that has had prior adenoma may already have altered prostanoid signaling [e.g., a deficiency in 5-hydroxyprostaglandin dehydrogenase (15-PGDH)], which may efface the differences in levels between those who do, and do not, have a recurrent adenoma on a later colonoscopy. Second, we did not measure the prostanoids concentrations at the study entry, as we did not collect urinary samples at that time. However, the on-treatment measurements better reflect the subjects' metabolic milieu during the period the adenomas were forming. Third, the ratios of PGE-M and $dTxB_2$, individually and in combination, to PGI-M may not adequately capture trade-off between systemic productions of these prostanoids. Fourth, treatment effects of aspirin on the prostanoid synthesis in the colon mucosa are unclear, as we did not measure the expression of prostanoids in the colorectal tissue. Finally, we had a limited sample size to investigate risk of advanced adenoma occurrence.

The strengths of this study include the randomized, placebo-controlled trial design; high protocol adherence by study participants; the high follow-up rate; and the systemic collection of risk factor information at baseline and follow-up intervals as well as outcomes at the end of treatment. Other strength of this study is that we measured stable biomarkers of systemic production of prostanoids using highly sensitive assays in one laboratory. Finally, this study is the first human study to investigate the association between PGI-M and $dTxB_2$ and risk of colorectal adenoma.

Overall, in this study, we found that low- and high-dose aspirin is associated with lower levels of urinary prostanoid metabolites in a dose-dependent manner. However, our findings provided no evidence that urinary prostanoid metabolites are associated with reduced risk of colorectal adenoma occurrence. In agreement with our original hypothesis, we observed a possible inverse association of metabolites of the possibly anticarcinogenic prostanoid PGI_2 with advanced adenoma occurrence. However, similar inverse associations were observed for other potentially procarcinogenic prostanoids. Studies of colorectal neoplasms that measure changes in both systemic and tissue-specific levels of prostanoids in response to aspirin treatment are needed to confirm our results.

Disclosure of Potential Conflicts of Interest

D.J. Ahnen is a consultant/advisory board member for Cancer Prevention Pharmaceuticals. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: D.J. Ahnen, J.A. Baron
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): R.S. Sandler, E.L. Barry, D.J. Ahnen, G.L. Milne, R.S. Bresalier
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): V. Fedirko, P.T. Bradshaw, J.C. Figueiredo, E.L. Barry, R.S. Bresalier, J.A. Baron
Writing, review, and/or revision of the manuscript: V. Fedirko, P.T. Bradshaw, J.C. Figueiredo, R.S. Sandler, E.L. Barry, D.J. Ahnen, G.L. Milne, R.S. Bresalier, J.A. Baron
Study supervision: D.J. Ahnen, R.S. Bresalier, J.A. Baron

Acknowledgments

Grant Support

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Urinary Metabolites of Prostanoids and Risk of Recurrent Colorectal Adenomas in the Aspirin/Folate Polyp Prevention Study (AFPPS)

Veronika Fedirko, Patrick T. Bradshaw, Jane C. Figueiredo, et al.

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