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Altered Tissue Metabolites Correlate with Microbial Dysbiosis in Colorectal Adenomas

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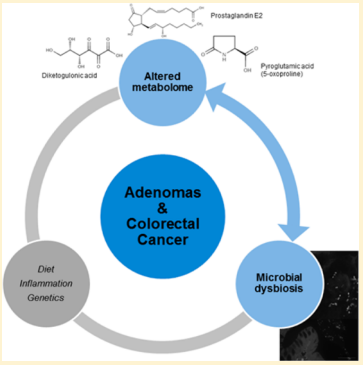
Supporting Information

ABSTRACT: Several studies have linked bacterial dysbiosis with elevated risk of colorectal adenomas and cancer. However, the functional implications of gut dysbiosis remain unclear. Gut bacteria contribute to nutrient metabolism and produce small molecules termed the "metabolome", which may contribute to the development of neoplasia in the large bowel. We assessed the metabolome in normal rectal mucosal biopsies of 15 subjects with colorectal adenomas and 15 nonadenoma controls by liquid chromatography and gas chromatography time-of-flight mass spectrometry. Quantitative real-time PCR was used to measure abundances of specific bacterial taxa. We identified a total of 274 metabolites. Discriminant analysis suggested a separation of metabolomic profiles between adenoma cases and nonadenoma controls. Twenty-three metabolites contributed to the separation, notably an increase in adenoma cases of the inflammatory metabolite prostaglandin E2 and a decrease in antioxidant-related metabolites 5-oxoproline and diketogulonic acid. Pathway analysis suggested that differential metabolites were significantly related to cancer, inflammatory response, carbohydrate metabolism, and GI disease pathways. Abundances of six bacterial taxa assayed were increased in cases. The 23 differential metabolites demonstrated correlations with bacteria that were different between cases and controls. These findings suggest that metabolic products of bacteria may be responsible for the development of colorectal adenomas and CRC.



KEYWORDS: *colorectal cancer, metabolome, microbiome, oxidative stress, diketogulonic acid*

INTRODUCTION

Colorectal cancer (CRC) is the third leading cause of cancer death in the United States, accounting for almost 9% of all cancer death in 2011.[1] This complex disease is characterized by a series of pathological stages with benign adenomas as intermediate precursors. The exact etiology of CRC is still unknown, but current literature suggests that diets low in fiber and high in red meat intake are among the significant risk factors for CRC incidence.[2,3] Diet may be responsible directly for CRC risk or may exert effects through the products of bacterial metabolism of unabsorbed dietary constituents in the colon. A number of studies have now linked the gastrointestinal (GI) microbiome to CRC development, although the mechanisms by which this occurs are not yet clear.[3−5]

The microbiome, which refers to the microorganisms that inhabit the human body in both normal and abnormal physiological states, has been important in our recent understanding of many disease processes. It has been implicated in the development of a number of conditions including skin diseases,[6] inflammatory bowel disease (IBD),[7,8] obesity,[9−11] and CRC.[3,12−14] The GI microbiota are responsible for the cometabolism of many food products. Byproducts of host dietary metabolism as well as these products of bacterial metabolism contribute to the GI metabolome. Bacterial fermentation of proteins and amino acids occurs primarily in the distal colon, and several metabolites of this fermentation process are thought to have negative effects on colon health and could potentially lead to disease.[15] Bacterial metabolism of carbohydrates can generate compounds such as short-chain fatty acids that are potentially beneficial.[15] Additionally, metabolites from host cells can also preferentially select for the growth of certain bacteria in the GI tract.[16]

Metabolomics is defined as "the science of examining the unique chemical fingerprint of a cell, tissue, or organ by measuring the global variation of metabolites present."[17] The group of metabolites produced during metabolic processes is known as the metabolome.[17] The metabolome is the ultimate

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physiological response of biological systems to genetic or environmental changes and as such has generated much interest as a tool for better understanding these physiological responses in many disease states.[18] As early as 1989, experiments in the colon adenocarcinoma cell line HT29 demonstrated that metabolic changes occur in CRC.[19] More recently, alterations in the metabolome have been reported in IBD, fulminant type 1 diabetes (FT1DM), hepatomas, and breast tumors.[18,20,21] A few studies have found changes in metabolites in the colon tissue, urine, serum, and feces of patients with CRC and animal models of CRC.[21−28] Results from studies on the metabolome and CRC have varied in tissue type assayed, methods for metabolome detection, and identity of detected metabolites. Although many perturbed metabolic pathways, such as glucose and fatty acid metabolism, have been demonstrated by these studies, further investigation is needed to better understand the metabolome in CRC. To our knowledge, there have been no studies that have evaluated the metabolome in relation to gut bacterial dysbiosis and human colorectal neoplasia.

The relationship between the microbiome and the metabolome is just starting to be understood. We hypothesize that an altered metabolome caused by microbial dysbiosis contributes to the development of adenomas and CRC. In the current study, we characterized the rectal mucosal metabolome in subjects with and without colorectal adenomas. We also assessed the correlation between the metabolome and GI microbiome in these subjects. Investigating the concurrent changes in metabolites and the microbiome provides an important opportunity to begin to understand their physiological interplay and their role in colorectal adenomas and cancer.

EXPERIMENTAL SECTION

Participants and Collection of Rectal Biopsies

Participants (15 subjects with adenomas and 15 adenoma-free control subjects) were randomly selected from persons age 30 years or older who underwent screening colonoscopy at the University of North Carolina Hospitals (Chapel Hill, NC). The methods of this study have been previously described.[3] Eligible subjects gave written informed consent to provide colorectal biopsies and a phone interview that included questions about diet and lifestyle. Information on diet was obtained from a comprehensive, validated, quantitative food-frequency questionnaire developed at the National Cancer Institute. At the time of the colonoscopy, the research assistant measured waist−hip ratio (WHR) and weight and height to determine body mass index (BMI). Inclusion criteria included visualization of the entire colon and a good preparation to avoid the misclassification of cases and controls. Exclusion criteria included colitis (either ulcerative, Crohn's, radiation or infectious colitis, chronic inflammatory illnesses), previous colonic or small bowel resection, previous colon adenomas or colon cancer, sigmoidoscopy or incomplete colonoscopies, familial polyposis syndrome, or antibiotic use within the last 3 months.

Specimen collection was carried out as previously described.[3,5] In brief, normal rectal mucosal biopsies were collected for each patient at approximately 10−12 cm from the anal verge, immediately after inserting the scope. The biopsies from all subjects were collected from normal rectal mucosa and not adenomas as the adenomas were used for clinical diagnosis. For subjects that had adenomas, the location, size, and number of adenomas were recorded during the procedure. The normal rectal mucosal biopsies were rinsed in sterile phosphate-buffered saline to reduce contamination with fecal matter and snap-frozen in liquid nitrogen on site and transferred to −80 °C until processing for DNA extraction and metabolome analyses. A pathologist examined all pathologic specimens to confirm adenoma case status and recorded the number of polyps, size, location, and histology. Subjects with confirmed adenomatous polyps were classified as cases and those without adenomas as controls. This study was approved by the Institutional Review Board at the UNC School of Medicine (study no. 05-3138).

Metabolomics

The methods for metabolome characterization in tissue biopsies are similar to those performed by Liao et al.[29] A ∼10 mg colon biopsy was used for a two-step metabolite extraction with 4-chlorophenylalanine (used as an internal standard). First, the samples were extracted with 200 μL of methanol: chloroform (3:1) with homogenization in 1.5 mL of screw cap tubes for 3 min using 1 mm inner diameter beads in a Bullet Blender (Next Advance, USA). After centrifugation, the supernatant was transferred to a new tube. The remainder was extracted with 200 μL of methanol, and the supernatant was combined with the first step extraction supernatant.

The samples were first analyzed by liquid chromatography time-of-flight mass spectrometry (LC−TOFMS, Agilent Corporation, Santa Clara, CA). After LC−TOFMS analysis, the samples were derivatized with *N*,*O*-bis(trimethylsilyl)-trifluoroacetamide (BSTFA) and analyzed by gas chromatography time-of-flight mass spectrometry (GC−TOFMS, Leco Corporation, St. Joseph, MI). The metabolic profiling procedures were performed according to previous publications.[23,30] Two samples failed in the derivatization process in GC−TOFMS analysis and were excluded from the data set. Metabolite annotation was performed by comparing the mass spectrum and retention time to our in-house reference library (University of Hawaii Cancer Center, Honolulu, HI) and NIST library (NIST, Gaithersburg, MD) for GC−TOFMS generated data or Human Metabolome Database (HMDB, http://www.hmdb.ca/) for LC−TOFMS generated data.

DNA Isolation

Bacterial genomic DNA was extracted as previously reported.[4] In brief, two rectal biopsies per subject (10−20 mg per biopsy) were processed with bead-beating and homogenization in lysozyme (30 mg/mL; Sigma, St. Louis, MO) followed by extraction with the Qiagen DNeasy Blood & Tissue kit (Qiagen, Valencia, CA cat. no. 69581) as per manufacturer's protocol. The DNA samples were stored in aliquots at −20 °C.

DNA from the positive control strains *Lactobacillus* sp., *Escherichia coli*, *Bifidobacterium* sp., *Clostridium* sp., *Bacteroides* sp., and Eubacteria (universal) was isolated using The UltraClean Microbial DNA isolation kit (MO BIO Laboratories, Carlsbad, CA, cat no. 12224-50) per manufacturer's instructions. Positive control bacterial strains were cultured as previously described.[31]

Quantitative Real-Time PCR (qPCR)

In recent publications, we reported that the mucosal microbiome is altered in adenoma subjects compared with controls.[3−5] The abundances of some specific bacteria identified in these previous studies, including *Lactobacillus* sp., *Escherichia coli*, *Bifidobacterium* sp., *Clostridium* sp., *Bacteroides* sp., and Eubacteria (universal), were evaluated by qPCR with primers that amplify 16S rDNA (Supplemental Table 1 in the Supporting Information). Each qPCR was carried out in duplicate in a final volume of 25 μL and contained 1× Fast-SYBR Green Master Mix

Table 1. Characteristics of Study Subjects

characteristic[a]	case (n = 15)[b]	control (n = 15)[b]	p value[c]
age (mean, s.e.)	54.3 ± 1.1	55.0 ± 1.1	0.68
sex (% male)	40	27.7	0.44
body mass index (mean, s.e.)	27.71 ± 1.3	27.4 ± 1.6	0.89
waist−hip ratio (mean, s.e.)	0.90 ± 0.02	0.86 ± 0.02	0.21
calories (mean, s.e.)	2085.7 ± 205.3	1741.3 ± 124.6	0.16
dietary fat (mean, s.e.)	80.5 ± 10.8	63.9 ± 6.6	0.21
dietary fiber (mean, s.e.)	25.2 ± 2.8	21.4 ± 1.6	0.26

[a]s.e. = standard error of the mean. [b]cases = with adenoma; controls = without adenoma. [c]p value was calculated from Student's t test.

(Applied Biosystems, Grand Island, NY, cat no. 4385612), 0.5 μM of each primer, and 50 ng of purified DNA. PCR conditions were as follows: 10 min at 95 °C, followed by 40 cycles of 95 °C for 15 s, 20 s at the appropriate annealing temperature (Supplemental Table 1 in the Supporting Information), and 72 °C for 1 min. Melting curve analysis of the PCR products was conducted following each assay to confirm that the fluorescence signal originated from specific PCR products and not from primer-dimers or other artifacts. PCR reactions were carried out on a Biorad CFX 384 Real-Time System with a C1000 Touch Thermal Cycler (Biorad, Hercules, CA).

To generate a standard curve, we amplified the target 16S rRNA from a positive control strain by PCR. Each resulting amplicon was inserted into a vector using the TOPO TA Cloning Kit (Invitrogen, Grand Island, NY, cat. no. K4500-01) as per manufacturer's instructions, and the number of copies was calculated as previously reported to generate the standard curve.[5] The copy number of each specific bacteria in biopsy samples was calculated as previously reported using the appropriate standard curve.[5]

Statistical Analyses

Analysis of the metabolome data was conducted using SIMCA-P software (Umetrics, Umea, Sweden). Principle component analysis (PCA) was first performed to check the outliers and the separation tendency. A further supervised partial least-squares-discriminant analysis (PLS-DA) was performed. Differential metabolites were selected from the PLS-DA model using a combination of variable importance in the projection (VIP) value >1 and p value (Student's t test) < 0.1. The VIP estimates the importance of each variable in the PLS-DA model; variables with a VIP score >1 are important in the model. A p value of <0.1 was chosen because it allows a broader interrogation of the data that can be investigated further in the future. Wilcoxon tests were used to compare the differential metabolites while adjusting for multiple testing.[32] Unpaired, two-tailed Student's t test with Welch's correction for unequal variances was used to compare qPCR data between cases and controls. Unconditional logistic regression models and Spearman's correlation were used to assess the association between metabolites and specific bacteria in adenoma cases and nonadenoma controls using JMP (SAS, Cary, NC). We examined the association between metabolites, bacteria, and adenomas while adjusting for potential covariates such as BMI, WHR, age, and gender.

The Ingenuity Pathway Analysis (IPA) Program (Ingenuity Systems, Qiagen, Redwood City, CA) was used for functional and pathway analysis to sort dysregulated metabolites into biologically relevant functional pathways.

RESULTS

Characteristics of study subjects are shown in Table 1. Adenoma cases and nonadenoma controls showed comparable general characteristics. Several risk factors evaluated showed no significant differences between cases and controls; however, cases trended toward having elevated caloric intake, dietary fat, and dietary fiber consumption compared with controls. Among cases with adenomas, 60% had small adenomas (1−5 mm), 33.33% had medium adenomas (6−10 mm), and 6.67% had large adenomas (>10 mm). The mean number of adenomas per subject was 2 (range 1−7), while the location of adenomas was 46.67% distal (splenic flexure, descending colon, sigmoid, rectosigmoid, and rectum), 33.33% proximal (cecum, ascending colon, hepatic flexure and transverse colon), and 20% both locations.

Altered Mucosal Biopsy Metabolome in Adenoma Cases

We identified a total of 274 metabolites by GC−TOFMS and LC−TOFMS (Supplemental Table 2 in the Supporting Information). PCA was first performed to check the outliers and the separation tendency (Figure 1A). A further supervised PLS-DA using two components (R^2X = 0.394, R^2Y_{cum} = 0.511, Q_{cum}^2 = 0.132, p value = 0.0079) was performed, resulting in some separation tendencies between adenoma cases and nonadenoma controls (Figure 1B). Of the detected metabolites, 23 metabolites were found to be significantly different between adenoma cases and nonadenoma controls, with most metabolites having a lower concentration in case subjects (Table 2).

Detected Metabolites Correlate Differentially with Bacteria between Cases and Controls

We recently reported that the mucosal microbiome is altered in adenoma subjects compared with controls, with increased microbial diversity and richness as well as altered abundance of select bacterial taxa.[3−5] The abundances of some specific bacteria identified in these previous studies were quantified from normal rectal mucosal biopsies of adenoma cases and nonadenoma controls (Figure 2). Cases showed a general trend of increased abundance of bacteria; *Bifidobacterium* sp. and Eubacteria abundances were significantly increased in cases compared with controls (p = 0.0021 and 0.0036, respectively).

We also examined the relationship between the 23 differential metabolites and specific bacteria taxa in adenoma cases and nonadenoma controls. The correlation patterns between the metabolites and specific bacteria differed markedly for cases and controls (Figure 3). In particular, the direction of correlation between the metabolites and bacteria are altered between cases and controls, suggesting that the bacteria and metabolites are interacting differently depending on adenoma status (Figure 3). We then evaluated the correlation between specific bacteria and each metabolite and examined those that were the most highly correlated with each metabolite. Strikingly, all differential metabolites except dihydroceramide were most highly correlated with different bacteria between cases and controls (Supplemental Table 3 in the Supporting Information). However, while

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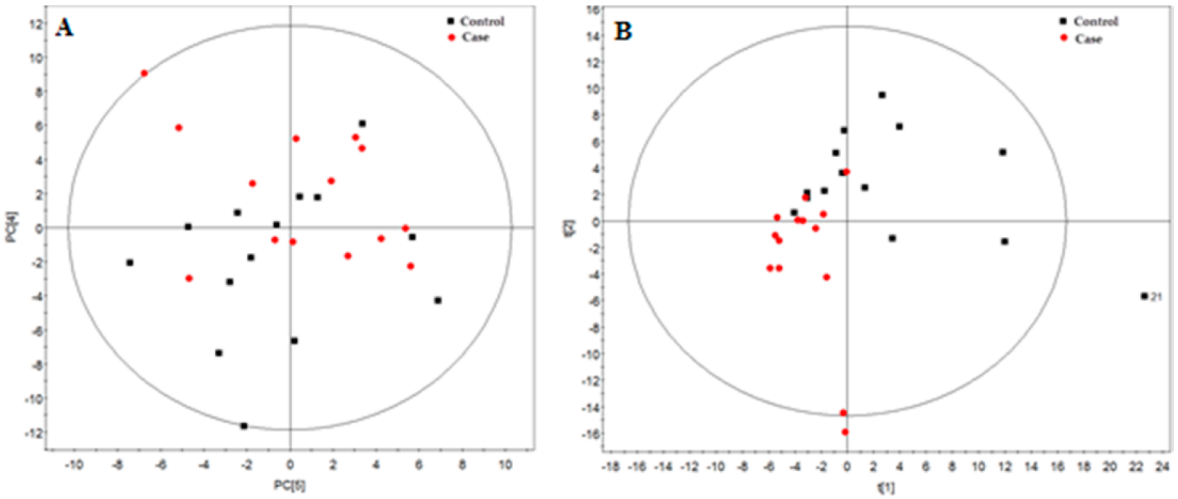

Figure 1. (A) PCA scores plot and (B) PLS-DA scores plot of adenoma cases and nonadenoma controls (PLS-DA using two components; $R^2X = 0.394$, $R^2Y_{cum} = 0.511$, $Q_{cum}^2 = 0.132$, p value = 0.0079).

Table 2. Differential Metabolites between Adenoma Cases and Non-Adenoma Controls

rank	compound name	VIP[a]	p value[b]	FDR (0.15)[c]	fold change[d]	detection[e]	identification[f]
1	galactose	2.45	0.0096	0.0424	−2.81	GC−TOFMS	NIST
2	13,14-dihydro-15-keto-PGE2	2.23	0.0198	0.0424	−1.91	LC−ES+	HMDB
3	5-oxoproline	2.21	0.0215	0.0424	−1.56	GC−TOFMS	Std
4	2,4-diaminobutyric acid	2.07	0.0319	0.0424	−1.25	LC−ES+	HMDB
5	pentadecanoic acid	1.97	0.0425	0.0424	−1.28	GC−TOFMS	NIST
6	5-hydroxyindoleacetic acid	1.96	0.0433	0.0424	−1.98	LC−ES−	HMDB
7	phosphoric acid, 2-aminoethanol	1.93	0.0473	0.0424	2.51	GC−TOFMS	NIST
8	dihydroceramide	1.92	0.0480	0.0424	−2.05	LC−ES+	HMDB
9	ornithine	1.89	0.0519	0.0424	−2.49	GC−TOFMS	Std
10	linoleic acid	1.88	0.0539	0.0424	1.79	LC−ES−	HMDB
11	petroselinic acid	1.86	0.0565	0.0424	1.78	LC−ES−	HMDB
12	LysoPC (18:2(9Z,12Z))	1.85	0.0575	0.0424	2.59	LC−ES+	HMDB
13	myo-inositol	1.84	0.0595	0.0424	−1.36	GC−TOFMS	NIST
14	diketogulonic acid	1.83	0.0602	0.0424	−8.06	LC−ES−	HMDB
15	prostaglandin E2	1.82	0.0615	0.0424	1.54	LC−ES−	HMDB
16	methionine	1.76	0.0722	0.0425	−2.37	GC−TOFMS	Std
17	2-aminobutyric acid	1.74	0.0756	0.0425	−2.18	GC−TOFMS	Std
18	oleamide	1.72	0.0797	0.0425	−1.95	LC−ES+	HMDB
19	glycine	1.69	0.0842	0.0425	−1.41	GC−TOFMS	Std
20	maltitol	1.69	0.0849	0.0425	−2.08	GC−TOFMS	NIST
21	2-phenylglycine	1.68	0.0862	0.0425	−1.89	LC−ES+	Std
22	2-phenylacetamide	1.66	0.0905	0.0426	−2.66	LC−ES+	HMDB
23	N6-acetyl-L-lysine	1.62	0.0996	0.0448	−1.66	LC−ES+	Std

[a]Variable importance in the projection (VIP) was obtained from PLS-DA with a threshold of 1. [b]p value was calculated from Student's t test with a threshold of 0.1. [c]All metabolites were discriminant ($p < 0.05$) with an FDR of 15%. [d]Fold change with a value larger than one indicates a relatively higher concentration in the case samples, while a value less than one means a relatively lower concentration as compared with control samples. [e]Detection methods used were liquid chromatography coupled to time-of-flight mass spectrometry with positive or negative electrospray (LC−ES+ and LC−ES−, respectively) or gas chromatography time-of-flight mass spectrometry (GC−TOFMS). [f]Metabolites were identified by our in-house library (Std), NIST library (NIST), or HMDB database (HMDB).

dihydroceramide was most highly correlated with Eubacteria in both cases and controls, the correlation was inverse for cases and direct for controls (Supplemental Table 3 in the Supporting Information).

Differential Metabolites Are Associated with Pathways Related to Cancer, Gastrointestinal Disease, Inflammation, and Carbohydrate Metabolism

We performed a supervised pathway analysis with IPA to analyze the functional pathways most perturbed by the 23 differential metabolites observed for adenoma cases and nonadenoma controls (Table 2). The resulting pathways were significantly ($p < 0.02$) related to cancer, the inflammatory response, carbohydrate metabolism, and general GI diseases (Figure 4).

DISCUSSION

In the current study, we characterized the metabolome in the normal rectal mucosa of subjects with and without colorectal adenomas. We also assessed the relationship between the metabolome and specific bacteria taxa in these subjects.

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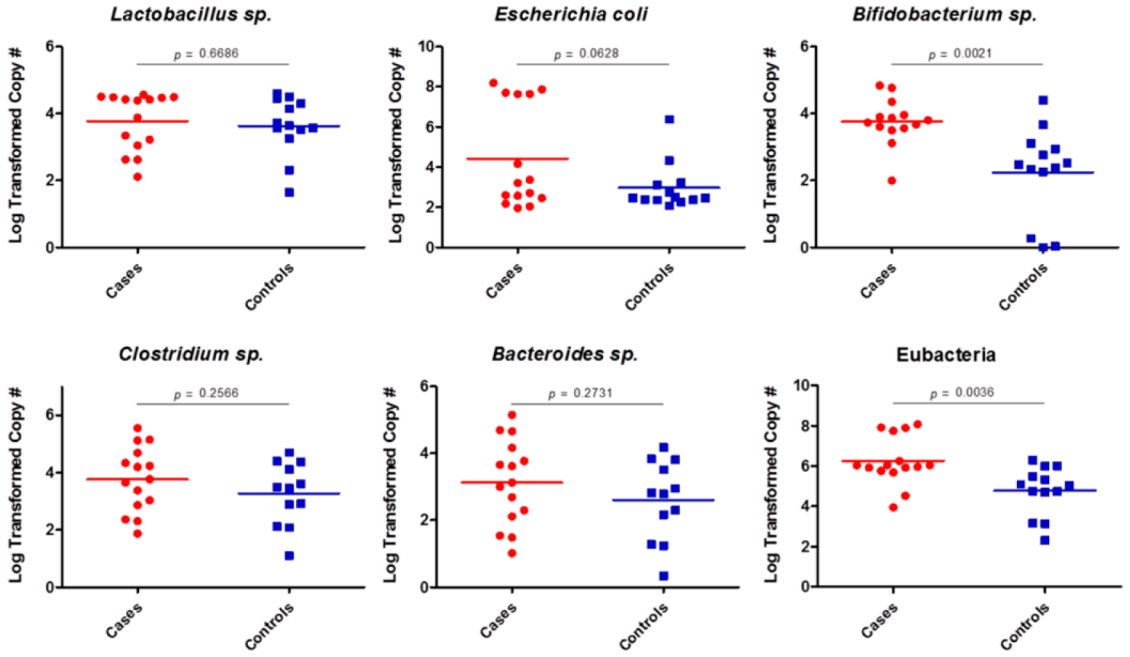

Figure 2. Quantitative real-time PCR of select bacteria from mucosal biopsies of adenoma cases and nonadenoma controls. *P* values were calculated from Student's *t* test.

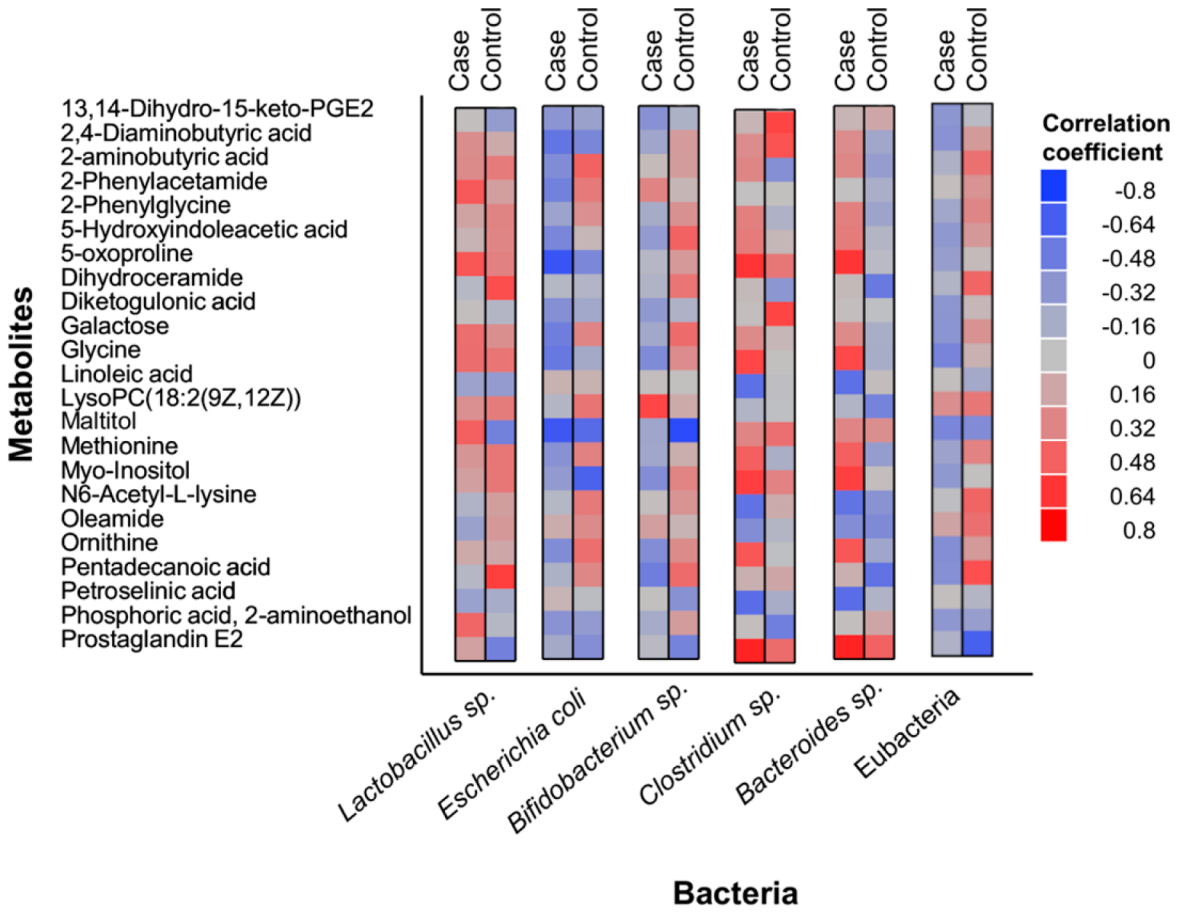

Figure 3. Correlation between differential metabolites and bacterial taxa in adenoma cases and nonadenoma controls.

We found that the metabolome was significantly different in subjects with adenomas compared with nonadenoma control subjects. Interestingly, the association between metabolites and bacteria differed depending on adenoma status, suggesting a potential role for the interaction of the metabolome and microbiome in the development of CRC. To our knowledge, this is the first report to directly relate mucosal metabolites with bacteria in the development of colorectal adenomas and cancer.

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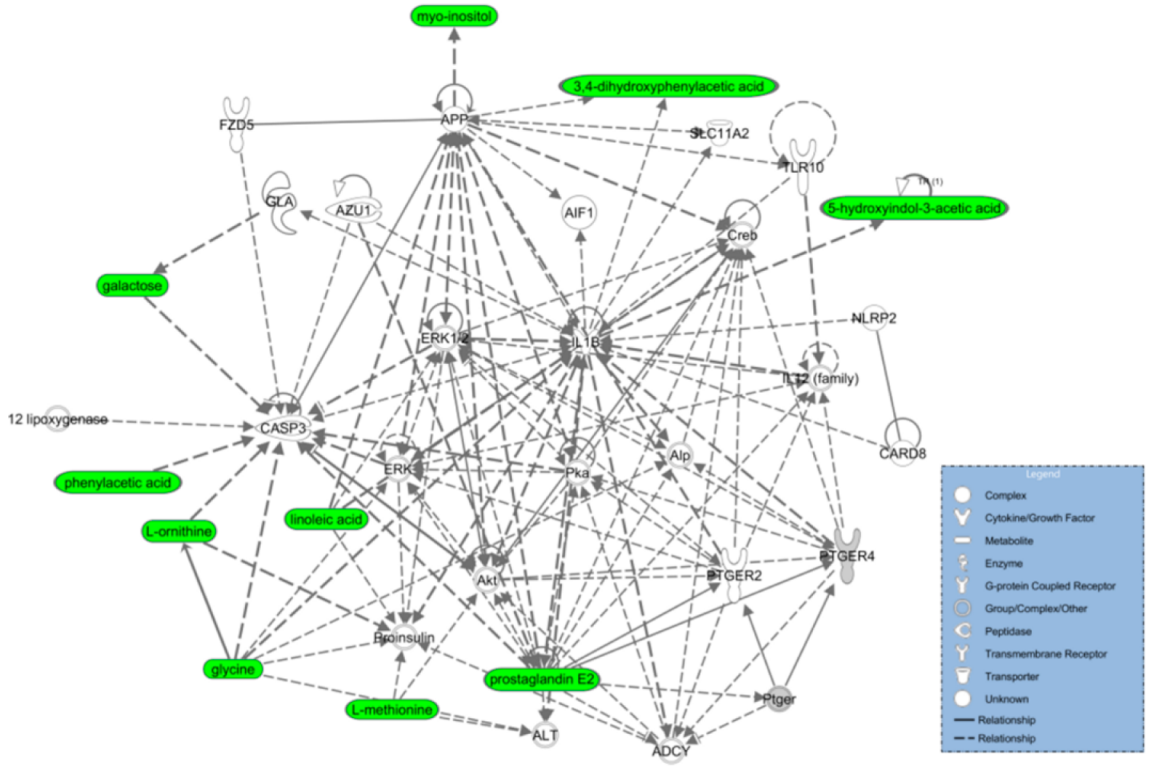

Figure 4. Pathway analysis of 23 differential metabolites with IPA.

We evaluated the tissue metabolome in normal rectal mucosa of adenoma cases and nonadenoma controls in the current study to better understand the metabolic changes in CRC. We detected 274 metabolites by GC−TOFMS and LC−TOFMS, from which 23 metabolites were found to be differentially expressed in adenoma cases. In this study, we used the PLS-DA model to select metabolites to investigate and describe interesting differences between these two groups and their relation to bacteria. We observed decreased metabolites related to nucleotide metabolism, such as glycine, and increased levels of fatty acids, such as linoleic acid, in adenoma cases compared with controls. Few other studies have reported an altered tissue metabolome in CRC, primarily decreased tricarboxylic acid (TCA) cycle metabolites and lipid-derived metabolites in cancer tissue and increased glycolytic pathways.[25,27,28,33−35] While studies have observed no association between the serum metabolome and stage of CRC (stages I−IV),[23,36] the differences in metabolism between CRC and adenomas, which are intermediate precursors, may explain the contradictions between our observations and others in the literature. Future studies with larger sample sizes that explore models to discriminate between cases and controls may provide additional insights.

Pathway analysis of the 23 differential metabolites between adenoma cases and nonadenoma controls showed that they were significantly related to inflammation, GI disease, and cancer pathways. Prostaglandin E2 (PGE2) was a key metabolite in this pathway analysis that was significantly increased in adenoma cases in our study. PGE2 is a prostaglandin that is synthesized from arachidonic acid, an omega-6-fatty acid, by cyclooxygenase enzymes (COX1 and COX2). PGE2 and COX2 have been implicated in the development of malignant tumors. PGE2 can promote tumor extension[37] and growth by activating proliferation, migration, aberrant apoptosis, and angiogenesis.[38] Nonsteroidal anti-inflammatory drugs (NSAIDs), which inhibit COX enzymes, have been shown to reduce the risk of CRC.[39] PGE2 has also been linked to cellular proliferation in CRC[40,41] and CRC metastasis.[42] Our observation that PGE2 is increased in the normal mucosa of patients with adenomas is consistent with this metabolite's role in CRC, lending validity to the functional importance of the metabolites reported in our study. Furthermore, the observed increase in PGE2 highlights the importance of inflammatory processes early in the development of adenomas and CRC.

Among the 23 differential metabolites in this study, two metabolites related to oxidative stress, 5-oxoproline and diketogulonic acid, were significantly decreased in adenoma cases compared with nonadenoma controls. 5-Oxoproline is a byproduct of glutathione synthesis[43] and has been proposed as a biomarker of glutathione status.[44] Glutathione is an endogenously synthesized molecule that is a key cellular antioxidant; when concentrations of glutathione are depleted, cells are susceptible to DNA damage that can lead to carcinogenesis.[44] Glutathione has also been implicated in growth-factor-mediated chemoresistance of colon cancer cells.[45] In other studies investigating the metabolome, 5-oxoproline was reported as a metabolite marker in fulminant type 1 diabetes[20] and was dysregulated in the urine of patients with CRC.[22] Decreased levels of 5-oxoproline reported in the current study suggest that glutathione levels may be decreased in adenoma cases, giving insight into the potential role that oxidative stress plays in the development of colorectal adenomas.

Diketogulonic acid, a metabolite of ascorbic acid (vitamin C) degradation, was also decreased in adenoma cases.[46] Like glutathione, ascorbic acid is an antioxidant that protects cells against oxidative stress from reactive oxygen species.[46] Ascorbic acid cannot be synthesized by human cells and must be obtained from the diet and is typically metabolized by bacteria in the GI tract.[46] Intake of ascorbic acid has been disputed as protective

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against CRC,[47,48] and very little is known about the role of diketogulonic acid in CRC. In our subjects, there was no difference in ascorbic acid intake (dietary and supplemental) between cases and controls (data not shown), yet decreased levels of diketogulonic acid were observed in cases. This suggests that ascorbic acid metabolism is altered in the rectal tissue of subjects with adenomas, which we propose may contribute to decreased antioxidant capacity of ascorbic acid and increased oxidative stress. Taken together, these data indicate an important role for oxidative stress and antioxidant metabolism in the development of colorectal adenomas.

The interdependence of ascorbic acid metabolism and bacteria in the GI tract is one example of many ways in which the microbiome and metabolome interact in health and disease. Along with vitamin metabolism, fermentation of dietary proteins and amino acids by bacteria occurs in the GI tract and is a significant way in which humans break down food. Additionally, metabolites from host and bacterial cells can preferentially select for the growth of certain bacteria in the GI tract.[16,49] In 2008, Li et al. published the first article reporting a correlation between cometabolites and microbes in humans,[50] and the production of certain metabolites by different bacteria has been further detailed. Since then, Marcobal et al. demonstrated in specifically reconstituted gnotobiotic mice that bacterial communities could drive major changes in the host metabolomic profile.[51] Many studies have implicated dysbiosis or dysregulation of the metabolome in CRC, yet to the authors' knowledge, none have directly correlated the microbiome and metabolome to colorectal adenomas or cancer.

In the current study, we examined the abundance of specific bacteria taxa observed in our previous studies to investigate the relationship of the bacteria to differential metabolites. Significant increases in the abundances of *Bifidobacterium* sp. and Eubacteria (universal) were observed, while the abundances of *Escherichia coli*, *Clostridium* sp., and *Bacteroides* sp. were also increased but the results did not reach statistical significance. These findings are generally consistent with our prior reports.[3−5] However, our observation that *Bifidobacterium* sp. were more abundant in the tissue of patients with colorectal adenomas was surprising. *Bifidobacterium* is a genus of lactic-acid-producing bacteria that is generally viewed as probiotic, yet the relationship between *Bifidobacterium* sp. and CRC has been inconclusive. *Bifidobacterium adolescentis* has been demonstrated to inhibit proliferation of colon cancer cell lines in vitro,[52] and some studies have shown that administration of lactic-acid-producing bacteria is able to reduce the occurrence of CRC.[53] Chen et al. reported lower levels of *Bifidobacterium* sp. in tumor tissue compared with matched normal mucosa of patients with CRC.[54] On the other hand, *Bifidobacterium* sp. have been reported to be increased in the tissue of patients with adenomas.[3] Furthermore, higher levels of the lactic-acid bacterial genus *Lactobacillus* were associated with increased risk for CRC.[13] Reasons for these conflicting results are unclear, but the relationship between lactic-acid-producing bacteria and CRC clearly warrants further investigation to determine the functional results of microbial dysbiosis in adenoma and cancer pathogenesis.

To assess the physiological implications of the observed microbial dysbiosis in our studies, we investigated the relationship between specific bacteria and differential metabolites in adenoma cases and nonadenoma controls. We report correlations that are significantly different based on disease status, which may suggest that the interplay between bacteria and metabolites is important in the development of CRC. *E. coli* contains a specific metabolic pathway for the degradation of ascorbic acid to diketogulonic acid.[46] However, *E. coli* is actually inversely correlated with diketogulonic acid in our data. Additionally, the correlation is quite similar between cases and controls, indicating that the difference seen in diketogulonic acid between cases and controls, although not related to *E. coli*, may involve other bacteria that we did not measure. Diketogulonic acid is instead most highly correlated with Eubacteria in the cases (inverse correlation) and *Clostridium sp.* in the controls (direct correlation). The different bacteria as well as different directions of correlation suggest that these bacteria or others may contribute to the disparate metabolism of ascorbic acid in adenoma cases. Additionally, the correlation of many of the 23 differential metabolites with Eubacteria indicates that there are other bacteria not specifically assayed that are likely playing an important role in shaping the microbiome. Furthermore, the host diet can alter the gut microbiota functionality, which can result in an altered metabolome.[51] Although we controlled for dietary calories, fiber, and fat intake, other dietary parameters such as red meat intake could impact the microbiome and metabolome. Future studies will aim to elucidate the association between the metabolome and a wider variety of bacteria in a larger subject cohort.

While the current study contributes novel insights into the role of metabolites, microbiota, and their interplay in the development of adenomas and CRC, there are several considerations we recognize as important in the interpretation of these results. The current study used a small cohort from the same geographic region (North Carolina, USA). It is also important to consider the sample type assayed and the comparisons made between samples when interpreting the current study in relation to other reports in the literature. We assayed normal mucosal biopsies from patients with and without adenomas in the current study. Other studies have focused on serum[23,36] or urine[22,55] from patients with and without CRC or tumor tissue and matched normal mucosa only from patients with fully developed CRC.[25,28,33−35] We previously reported a field effect for apoptosis in the normal tissue of patients with adenomas,[39] and our current data suggest that there is a similar field effect on the metabolome. Therefore, the relationships drawn between cases and controls in the current study are different than those in other studies that compare tumor tissue to matched normal tissue without comparison with subjects without disease.

In summary, our findings demonstrate that in addition to bacterial dysbiosis the mucosal metabolome is altered in subjects with colorectal adenomas. Differential metabolites were related to pathways of inflammation, cancer, and GI disease, and they may play a role in the development of CRC by lessening the antioxidant capabilities of the gut metabolome. The correlation between the altered metabolome and microbiota in the normal rectal mucosal tissue of subjects with colorectal adenomas is an exciting observation and one that warrants further investigation to elucidate the specific interactions between microbes and metabolites. It may allow for a better understanding of the pathogenesis of CRC and may lead to identification of novel markers for diagnostics and therapeutics as well as improved outcomes for patients with CRC.

■ ASSOCIATED CONTENT

Supporting Information

Primer sequences and amplification temperatures used for quantitative real-time PCR, a table of all metabolites identified

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in the current study, and the bacteria most highly correlated with each differential metabolite in adenoma cases and nonadenoma controls. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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ABBREVIATIONS

ADCY, adenylate cyclase; AIF1, allograft inflammatory factor 1; Alp, alkaline phosphatase; ALT, alanine aminotransferase; APP4, amyloid beta (A4) precursor protein; AZU1, azurocidin 1; CARD8, caspase recruitment domain family, member 8; CASP3, caspase 3; CRC, colorectal cancer; Creb, cyclic AMP response element-binding protein; ERK, extracellular signal-regulated kinase; ERK1/2, extracellular signal-regulated kinase 1/2; FZD5, frizzled family receptor 5; GLA, galactocidase alpha; IL1B, interleukin 1, beta; IL12 (family), interleukin 12 (family); NLRP2, NOD-like receptor family, pyrin domain containing 2; Ptger, prostaglandin E receptor; PTGER2, prostaglandin E receptor 2 (subtype EP2); PTGER4, prostaglandin E receptor 4 (subtype EP4); SLC11A2, solute carrier family 11 (proton-coupled divalent metal ion transporters), member 2; TLR10, toll-like receptor 10

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