Colorectal cancer-associated microbiota contributes to oncogenic epigenetic signatures

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Contributed by Philippe J. Sansonetti, September 27, 2019 (sent for review July 17, 2019; reviewed by Guido Grandi and Sven Pettersson)

Sporadic colorectal cancer (CRC) is a result of complex interactions between the host and its environment. Environmental stressors act by causing host cell DNA alterations implicated in the onset of cancer. Here we investigate the stressor ability of CRC-associated gut dysbiosis as causal agent of host DNA alterations. The epigenetic nature of these alterations was investigated in humans and in mice. Germ-free mice receiving fecal samples from subjects with normal colonoscopy or from CRC patients were monitored for 7 or 14 wk. Aberrant crypt foci, luminal microbiota, and DNA alterations (colonic exome sequencing and methylation patterns) were monitored following human fecal transfer. CRC-associated microbiota induced higher numbers of hypermethylated genes in murine colonic mucosa (vs. healthy controls’ microbiota recipients). Several gene promoters including SFRP1,2,3, PENK, NPY, ALX4, SEPT9, and WIF1 promoters were found hypermethylated in CRC but not in normal tissues or effluents from fecal donors. In a pilot study (n = 266), the blood methylation levels of 3 genes (Wif1, PENK, and NPY) were shown closely associated with CRC dysbiosis. In a validation study (n = 1,000), the cumulative methylation index (CMI) of these genes was significantly higher in CRCs than in controls. Further, CMI appeared as an independent risk factor for CRC diagnosis as shown by multivariate analysis that included fecal immunocenomic blood test. Consequently, fecal bacterial species in individuals with higher CMI in blood were identified by whole metagenomic analysis. Thus, CRC-related dysbiosis induces methylation of host genes, and corresponding CMIs together with associated bacteria are potential biomarkers for CRC.

Colon | Cancer | Microbiota | Gene methylation | Biomarker

Colorectal cancer (CRC) is among the most common malignancies worldwide with a high mortality rate and is believed to result from interactions between the host and long-term environmental exposures. Environmental chemicals have been associated with a higher incidence of various cancers (1) and may act as either carcinogens or tumor-promoting agents (2) by causing an accumulation of DNA mutations (3) and epigenetic changes in DNA within host cells (4). At a cellular level, the Wnt pathway has been generally accepted as important contributor (5). Nevertheless, a comprehensive assessment of all factors involved remains an extremely challenging endeavor, primarily due to the great variety of environmental stressors and the long-term exposure period of many years. However, significant associations linking diet to features of the microbiota and to CRC were reported recently (6, 7). Therefore, analyzing the microbiota may provide new insights into the effects of environmental exposures on CRC.

The human gut microbiota contains trillions of microorganisms and is critical for overall health, playing a fundamental role in interactions with environmental drivers (i.e., nutrition and medicine) (8). We have shown that alterations in the colonic luminal and adherent microbiota composition are associated with CRC (9, 10). Interestingly, it has been reported that microbiota dysbiosis increases the risk for CRC even in individuals with genetic predisposition to CRC, including constitutional mutations of pivotal genes (i.e., APC) (11).

We previously demonstrated that hypermethylation of the Wif1 promoter, the gene regulating the Wnt pathway, serves as surrogate diagnostic marker for early CRC (12).

The objective of this study was to assess the hypothesis that CRC-associated environmental factors may act by altering the composition of the gut microbiota and to investigate the underlying epigenetic pathways involved in CRC promotion. Thus, in a first step, we characterized the colonic luminal microbiota in humans and transferred it into germ-free mice in order to assess the

**Significance**

This study advances our appreciation and understanding of the role of colon dysbiosis in the pathogenesis of colorectal cancer. In a human pilot study of 266 individuals, greater epigenomic (methylation) DNA alterations correlated with CRC and microbiota composition. Beyond this correlative evidence, when germ-free mice received fresh feces from CRC patients and their healthy controls, the former animals developed colon epithelial renewal, more precancerous lesions, and increased tissue and blood DNA methylation in intestinal tissues. Confirmation was obtained in a larger cohort of 1,000 patients, indicating that CRC-associated dysbiosis may promote colon carcinogenesis via epigenome dysregulation. Gene methylation can therefore serve as a marker for CRC and likely for predicting efficacy of prebiotic supplementation in average-risk individuals.


Reviewers: G.G., University of Trento; and S.P., Lee Kong Chian School of Medicine.


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Data deposition: Data related to this paper are available from the European Nucleotide Archive (ENA) database (http://www.ebi.ac.uk/ena) under the accession nos. ERX3622297– ERX3622402, ERX3628496–ERX3628604, ERX3636180–ERX3636285, and PRUEB35144.

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This article contains supporting information online at www.pnas.org/lookup/supp/doi/10.1073/pnas.1912129116/-/DCSupplemental.

www.pnas.org/cgi/doi/10.1073/pnas.1912129116
potential effect of this microbiota as an environmental stressor for host DNA. Second, we investigated the links between dysbiosis and host gene methylated markers suggested by animal experiments in a pilot human study and validated in a large human cohort.

Results

Human Fecal Microbiota Transfer to Germ-Free Mice: Clinical and Routine Biochemical Parameters Among Groups. We performed human fecal microbiota transfer (FMT) to germ-free mice utilizing material from patient donors with CRC (n = 9) and healthy control donors (n = 9) as illustrated in **SI Appendix, Fig. S1**. In addition, mice received the carcinogen azoxymethane (AOM) or saline control for 10 wk in order to assess potential effects of chemically induced stress. As shown in **SI Appendix, Tables S1 A and B**, FMT had no effect on the general blood parameters of the recipient mice as no significant differences were observed among any of the groups (a total of 185 mice) at 7 and 14 wk. At 14 wk, mice that received both AOM and CRC microbiota (CRC-μ) showed a lower mean body weight compared to the normal microbiota (N-μ) controls. This was accounted to reduced food intake observed in the CRC-μ group when compared to the N-μ controls (1.5 ± 0.1 g/100 g food intake vs. 2.0 ± 0.1 g/100 g food intake, respectively; P < 0.01). Interestingly, this weight effect disappeared when mice were given PEG in their drinking water, which is known to prevent bacterial colonization of the gut (**SI Appendix, Fig. S2**). In line with this, the luminal bacterial load, adherence of luminal bacteria to the colonic mucosa, and short-chain fatty acid (SCFA) concentrations in the cecum were significantly lower in mice that received PEG as compared to the controls (**SI Appendix, Figs. S2 A and B**). These results suggest that human CRC FMT may induce systemic changes in mice mediated by the interaction of bacteria with the colonic mucosa.

Dysbiosis-Related Histological Alterations in Colonic Tissue After Human FMT in Mice. In order to delineate the predominant modifications occurring in murine colonic tissues following FMT, the number of aberrant crypt foci (ACF), i.e., clusters of enlarged thick crypts visualized by methylene blue at low magnification, were enumerated. Whereas histological features appeared normal in the group of mice receiving only N-μ, mild inflammation and ACF were seen at significant levels in the groups given CRC-μ, AOM, or both (Fig. 1 A–D, H, and I). After 7 or 14 wk, ACF numbers were highest in the group given CRC-μ + AOM, compared to all other groups, and were also significantly higher in the groups given CRC-μ alone or AOM alone, compared to the N-μ only group (Fig. 1 H and I). No ACFs were seen in mice given PEG, in which the microbiota was efficiently reduced in the mucus layer proximal to the mucosa. These latter samples were therefore not further examined. According to the colon length, numbers of ACFs became significantly higher in groups given CRC-μ versus N-μ, when only 20 mm of the left colonic mucosa was examined (13 ± 3 versus 5 ± 1; P < 0.05; Fig. 1 J).

Human FMT to Germ-Free Mice: Effect on Epithelial Proliferation and Gene Expression. Epithelial cell proliferation in the colonic mucosa was visualized by Ki67 staining (as illustrated in Fig. 1 G). At 7 wk following FMT, a significant increase of proliferation was observed in the groups given AOM as compared to all other groups (Fig. 1 H). However, at week 14, cell proliferation was significantly greater in the CRC-μ, as compared to N-μ recipients with AOM exerting an additional effect (Fig. 1 H and I), and ACFs increased with the rate of epithelial cell proliferation. These results are consistent with those reported after FMT from only 5 CRC patients as compared to 5 control individuals’ microbiota (13). Further, to investigate the involvement of Wnt and Notch pathways that control intestinal cell fate, mRNA of key mediators was quantified. qPCR analysis of mRNA from the colonic mucosa of recipient mice revealed an increase of 1.7-fold, 1.9-fold, and 1.9-fold expression levels of the transcription factors HES1, KLF4, and ELF3, respectively, but not of MATH1 in CRC-μ compared to N-μ recipients (**SI Appendix, Table S2**).

Human FMT to Germ-Free Mice: Mucosal Inflammation. Early after FMT (7 wk), a mild inflammatory response reflected by higher IL1β, IL6, and MIP2α levels and lower IL10, IL23, and INFγ levels was detected in CRC-μ recipient animals as compared to N-μ controls. AOM treatment tended to amplify this stimulatory effect as assessed by mRNA cytokine quantification (Fig. 1 J). In addition, semi-quantitative evaluation of mucosal myeloid cells along the entire histologically examined intestine (10 fields per sample, 3 samples per mouse) showed a trend toward increased numbers in human CRC-μ as compared to N-μ recipients but without reaching statistical significance at any time point (Fig. 1 K).

Microbiota Characterization Following Human FMT in Mice. To assess for the impact of the microbial communities on the observed differences in the mucosal phenotypes, murine fecal samples were analyzed by 16S rRNA gene sequencing. At baseline, the fecal microbiota of the CRC-μ (n = 9 donors) group contained a higher proportion of Fusobacteria, Parvimonas, Butyrivibrio, Gemella, and Akkermansia and lower proportions of Ruminococcus, Bifidobacterium, Eubacteria, and Lachnospira, as compared to stools from the N-μ (n = 9 donors) group (**SI Appendix, Table S3 and Fig. S3**).

During the follow-up, all groups (n = 6 per group and at each time point) showed a moderate decrease in Coccoides, Clostridium leptum, and Bifidobacterium on day 7 and stability in the community of bacteria afterward, as estimated by qPCR (**SI Appendix, Table S4**). Based on 16S rRNA gene sequencing of mouse stools (n = 6 in each group at each time point of the follow-up), 85% of OTUs remained unchanged over time. Overall, the bacterial species most associated with significant histopathological alterations, i.e., precancerous lesions (e.g., ACF), were Firmicutes, Clostridia, and Clostridiales. These histological changes were also associated with lower counts of genera with an antiinflammatory effect (e.g., Faecalibacterium and Eubacterium) and of butyrate-producing bacteria (Firmicutes species) (**SI Appendix, Fig. S4**). Interestingly, the abundance of Coprococcus was lower and of Bacteroides was higher in CRC-μ vs. N-μ. Covariations of these 2 genera with histological changes characterized by ACF numbers and with mRNA levels of the previously quantified transcription factors in mice colonic parameters were analyzed. Coinertia analysis revealed that these parameters were associated with variation of the fecal bacteria. The first component of the analysis showed a significant separation between N-μ and CRC-μ recipients. This first component was significantly associated linearly with Bacteroides and Coprococcus genera abundances in mice stools (**SI Appendix, Fig. S5**). These allowed a significant separation between N-μ and CRC-μ recipients regarding bacteria and mRNA levels of key transcription factors involved in Wnt and Notch pathways.

Human FMT to Germ-Free Mice Alters DNA.

**DNA mutations.** In order to analyze the potential of FMT to induce DNA modifications in the host, whole-genome sequencing of murine colonic mucosal tissues (n = 12, 3 in each group of recipients) was performed, covering 220,000 exons within 24,000 genes. Overall, the incidence of mutations at global exon/intron level was significantly higher in intestinal vs. spleen tissues. Results of the colonic mucosa showed a trend toward increased DNA alterations in exons or introns of CRC-μ compared to N-μ recipients, but this did not reach significance (Fig. 2 A). In addition, mice given CRC-μ and AOM showed the highest levels of DNA alterations in exons or introns showing a trend to an additive effect of CRC-μ as compared to N-μ. Interestingly, Principal Component Analysis (PCA) scatter diagrams separated.
that received CRC-μ or N-μ. Finally, an unsupervised analysis between mutated genes (including only selected genes as indicated above) and recipient status showed that changes in Wnt, PPARγ, and Notch pathway genes were associated with CRC-μ recipient, with an additive effect of AOM treatment (Fig. 2C and SI Appendix, Fig. S6 and Table S2).

**DNA methylation/demethylation.** We isolated colonic mucosal tissue from the colon (n = 24; 6 in each group) and evaluated epigenetic changes using the EPIC microarray to compare methylation rates in 63,987 probes reflecting 12,600 genes being expressed. For all probes pooled, both mean and median methylation rates decreased from the N-μ + NaCl group to the CRC-μ + NaCl and CRC-μ + AOM groups (Fig. 2E), with significantly lower methylation levels in the CRC-μ + AOM group than in the N-μ + NaCl group (P < 0.01). Overall, one third of the probes appeared unmethylated in all experimental conditions (<0.2 beta-value methylation mEPIC) in the colonic tissue after FMT (Fig. 2F). The number of methylated probes (>0.8) varied with N-μ, CRC-μ,
and AOM exposure, although the ratio of methylated/unmethylated probes was not significantly different (3.3% and 3.1%, respectively \([P = 0.25, \text{Fisher’s test}]\) \([\text{Fig. 2G}]\). The number of unmethylated probes was 11% higher in the CRC\(\mu\) + AOM group when compared to the N\(\mu\) + NaCl group \([P = 0.013]\), and the number of fully methylated probes was higher in CRC\(\mu\) and AOM recipients.

![DNA changes in mice after human FMT](image)

**Fig. 2.** DNA changes in mice after human FMT. Whole genome sequencing of total DNA extracted from colonic mucosa \((n = 12)\) and spleen samples \((n = 6)\) was performed. (A) All single-nucleotide polymorphisms (SNPs) in the colon and spleen samples are compared to the reference mouse genome (GRCm38), and mutation levels within gene segments are indicated. Mice given AOM showed the highest levels of DNA alterations in exons or introns with a trend of additive effect of CRC\(\mu\) as compared to N\(\mu\). (B) Distribution of animal subgroups according to the total gene mutations. DNA changes in single nucleotides with PCA scatter diagrams for colonic or spleen samples in the groups of mice. The groups are identified by the type of human microbiota received (CRC\(\mu\) or N\(\mu\) for CRC patients’ or controls’ stool, respectively) and type of treatment (AOM or NaCl for azoxymethane or saline, respectively). (C) Correlation circle of targeted gene mutations in the colonic mucosa and spleen tissues according to PCA. Vector length reflects targeted gene mutation weight in the first 2 component analyses; targeted mutated genes are indicated (those of Wnt pathway in red color). (D) When mutations in all Wnt genes were pooled together, rates of mutations were significantly higher in mice given AOM with an additive effect of CRC\(\mu\). The total number of mutations in Wnt pathway genes in both colonic mucosa and spleen (Sp) was the highest in the animals given the CRC\(\mu\) and AOM combination (see also SI Appendix, Fig. S6). The number of mutations was greater in colonic mucosa (but not in spleen tissues) with AOM combined with CRC microbiota compared to AOM combined with control microbiota. There was no significant effect in between colonic mucosa due to CRC\(\mu\) alone as compared to N\(\mu\) alone. Col, colonic mucosa; Sp, spleen. (E) DNA epigenetic changes were investigated by using mEPIC array \((39)\). The methylation level of probes \((n = 63,987)\) were estimated after bisulfite modification of DNAs. Changes based on the methylation of probes were investigated on DNAs from colon samples \((n = 16; 4\) mice from each experimental group): the level of methylated probes was quantified as reported \((39)\) and ranged from 0 (not methylated) to 1 (fully methylated). DNAs were classified as unmethylated if the methylation value was <0.2 and as hypermethylated if the methylation value was >0.799. Overall, mean and median values of all probes pooled \((n = 63,987)\) in each group of mice showed lowest values in the group of CRC\(\mu\) + AOM recipients and highest in control microbiota recipients. (F) Most of the probes were unmethylated in all animal groups; elevated numbers of both hypomethylated and hypermethylated probes were observed in the mice given CRC\(\mu\) + AOM. (G) Probes whose methylation level changed or remained unchanged under AOM in mice given N\(\mu\) or CRC\(\mu\). Mice receiving CRC\(\mu\) had a greater number of genes with changed methylation levels. (H) Number of hypermethylated probes in each group showing highest level in mice given CRC\(\mu\) and AOM combination compared to all other groups. **P < 0.01, *P < 0.05; NS, not significant.
Wnt represent the (Fig. 3 samples and were therefore subjected to further characterization. PENK controls (such as blood and stool (Fig. 3) and normal tissue effluent (n = 9)) in the mouse were also associated with CRC dysbiosis in humans, we tested the FMT in mice (Fig. 3) with normal colonoscopy in the validation study; in addition, FIT was performed on asymptomatic individuals who underwent colonoscopy, and we investigated whether positive FIT and CMI > 2 could be independent parameters for diagnosis of CRC (Table 1 and SI Appendix, Table S7).

A CMI > 2 correlated significantly with CRC in 999 individuals in the validation study. Interestingly, a tendency of CMI increase with age was noted; however, this observation did not

**Gene Methylation in Human Colonic Samples: From Bench to Bed.** To investigate whether the gene methylation patterns observed in the mouse were also associated with CRC dysbiosis in humans, we first investigated methylated genes in CRC tissues and effluents such as blood and stool (n = 9) and in normal tissue effluent controls (n = 9), both obtained from the same individuals as for the FMT in mice (Fig. 3A). In order to develop a quick and easy methylation test, panels of genes were selected, based on the difference between normal and CRC group being silenced vs. hypermethylated in the N-μ human FMT in the mouse colonic mucosa. Pooling of total genes with expected DNA alterations (i.e., hypermethylation, or mutation in exon) revealed the involvement of pathways implicated in cell growth, signal transduction, nucleic acid binding, protein synthesis, channel, and carrier protein (SI Appendix, Fig. S7).

**Fig. 3.** Identification of hypermethylated genes related to fecal microbiota in human. Overview on the strategy from experimental approach for the validation of gene methylated targets in humans based on microbiota donors (CRC patients or controls) in germ-free mice experiments. Methylated genes in CRC-associated tissues and fluids were identified based on their power for showing differences between normal colonoscopy individuals and CRC patients. (A) Human tissues and effluents were submitted to methylation gene array. Based on significant differences of methylation values in CpG probes between control (n = 9) and CRC patient (n = 9) donors, genes were selected according to the promoter segments hypermethylated in CRC patients. (B) Bidimensional (Right) and tridimensional (Left) distribution of genes regarding the difference in methylation values are indicated; in red color are indicated 7 selected more discriminant genes regarding CRC patients and controls. D, difference.
reach significance. By keeping specificity of both tests superior to 95%, the negative predictive value (mean, extremes) of CMI (set at ≥2) was better than FIT (set at >150 ng/mL): 84.09 [81.5 to 86.4] and 81.64 [77.5 to 85.3], respectively (SI Appendix, Table S10). Parvimonas genus was also more abundant in individuals with blood CMI > 2 vs. ≤2 in the current as well as in our pilot study. The whole metagenomic analysis showed 20 bacteria species, including several Parvimonas species, differed in abundance in patients with CMI > 2 (n = 53) and in those with CMI ≤2 (n = 90) (Table 3).

**Discussion**

The data reported here constitute evidence that the relative abundance of some bacterial taxonomic groups within the microbiota in CRC is significantly associated with methylation/demethylation of host genes. In our study, we show clear phenotypical differences between mice receiving fecal transplants from CRC patients vs. healthy controls. The CRC-μ group presented with lower fecal SCFA concentrations and had significant colonic mucosal changes including higher ACf numbers and marked epigenetic alterations independent of AOM. However, 2 findings in the mouse study indicated an additive effect of AOM to the CRC microbiota: 1) the number of DNA alterations was greater in colonic than in spleen tissues under AOM (Fig. 2D and G) and 2) the number of spleen tissue DNA alterations was similar in the CRC-μ and N-μ recipients. Interestingly, 16S rRNA gene sequencing of fecal microbiota from CRC patients and normal controls showed that CRC-associated dysbiosis was

<table>
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<tr>
<th>Characteristics</th>
<th>Reference</th>
<th>CRC (n = 187) (number (%) or mean ± SD)</th>
<th>No CRC (n = 812) (number (%) or mean ± SD)</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y (n = 981)</td>
<td></td>
<td>63 [57 to 71]</td>
<td>60 [53 to 68]</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Gender (n = 981)</td>
<td>Female</td>
<td>77 (41.2)</td>
<td>392 (48.8)</td>
<td>0.06</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td></td>
<td>25.31 [22.86 to 28.09]</td>
<td>25.31 [22.39 to 28.7]</td>
<td>0.96</td>
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<tr>
<td>History of GI cancer (n = 869)</td>
<td>Yes</td>
<td>7 (4.4)</td>
<td>31 (4.4)</td>
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</tr>
<tr>
<td>History of non-GI cancer (n = 868)</td>
<td>Yes</td>
<td>29 (18.4)</td>
<td>88 (12.4)</td>
<td>0.049</td>
</tr>
<tr>
<td>Family history of cancer (n = 866)</td>
<td>Yes</td>
<td>19 (11.7)</td>
<td>114 (16.4)</td>
<td>0.13</td>
</tr>
<tr>
<td>Diabetes (n = 980)</td>
<td>Yes</td>
<td>32 (17.1)</td>
<td>28 (16.3)</td>
<td>0.74</td>
</tr>
</tbody>
</table>

**Blood methylation test**

| Cumulative (Wif1 + PENK + NPY) methylation index | Continuous 0.16 [0.01 to 1.16] | 0.12 [0.01 to 0.40] | <0.001 |
| Single gene | Wif1 (n = 956) | >2 | 20 (11.4) | 21 (2.7) | <0.001 |
| PENK (n = 956) | >2 | 0.06 [0 to 0.49] | 0.07 [0 to 0.24] | 0.002   |
| NPY (n = 956) | >2 | 3 (1.7) | 3 (0.4) | 0.045   |
| CMI1 in multivariate analysis adjusted OR‡ [95%CI] | >2 | 11 (2.3) | 12 (1.5) | <0.001  |
| FIT limited to the Vatnimad subcohort (n = 468) | >150 ng³ | 4.92 [2.79 to 0.68] | 0.005   |
| FIT1 in multivariate analysis adjusted OR‡ [95%CI] | Continuous 61.66 [1 to 379] | 70 [9.67] | <0.001  |
| Negative | Not done | 8.69 [4.66 to 16.21] | <0.001  |
| Positive | Continuous 0.50 [0.33 to 0.75] | 0.24   |

**The cohort included 981 individuals from 2 different cohorts, of whom 468 were asymptomatic individuals enrolled via a mass CRC-screening program and 513 were symptomatic patients. Invasive carcinoma or carcinoma in situ of any aspect in the rectum or colon was more abundant in controls (Table 2 and SI Appendix, Table S8). A multivariate model including all individuals with and without neoplasia adjusted for age, gender, and FIT results showed CMI > 2 was independently associated with CRC (Table 1). Regarding CMI test and keeping with threshold for positivity set at ≥2, the sensitivity for the detection of CRC could reach 37% in the validation study. After stratification on Tumor-Nodes-Metastasis classification from the American Committee on Cancer (SI Appendix, Table S9), the CMI value levels increased significantly and linearly with increasing tumor stages.

**Dysbiosis and Associations with Methylation of Genes.** The 16S rRNA gene sequencing on stool samples (n = 513) indicated a great β-diversity in patients with CRC and in those with a CMI > 2 (P < 0.05). Furthermore, CMI levels were significantly correlated with dysbiosis. In the validation cohort, we hierarchically clustered bacterial phylotypes on the genus level based on the similarity of their dynamics in patients with CRC and in those with CMI > 2 (Fig. 4).

Interestingly, principal coordinate analysis on the microbiota when incorporating the diagnosis and the level of blood methylation test (Fig. 4A), separated into 2 main clusters which differed significantly between participants with versus without a CMI > 2, regardless of whether they did or did not have CRC (Fig. 4B). A single cluster differed significantly in abundance between controls with and without a CMI > 2 (P < 0.05, Wilcoxon signed-rank test; Fig. 4C).

Although microbiota composition varied across cohorts, several genera such as Parvimonas and Parasutterella were more abundant in CRC patients in both cohorts, whereas Eubacterium was more abundant in controls (Table 2 and SI Appendix, Table S10). Parvimonas genus was also more abundant in individuals with blood CMI > 2 vs. ≤2 in the current as well as in our pilot study. The whole metagenomic analysis showed 20 bacteria species, including several Parvimonas species, differed in abundance in patients with CMI > 2 (n = 53) and in those with CMI ≤2 (n = 90) (Table 3).
respectively) (Shaman c3bi platform; Institut Pasteur, http://shaman.c3bi.pasteur.fr/). A maximum likelihood phylogenetic tree was tested (171). Controls had no malignancy or significant polyp visible by full colonoscopy (n = 171). A maximum likelihood phylogenetic tree was tested (171).}

Cohort (173 asymptomatic individuals enrolled via a mass CRC-screening program and 165 patients from Vatnimad and symptomatic subcohorts, respectively) were recruited through mass screening and had early CRC at colonoscopy. However, the CRC patients had higher sensitivity rate in the pilot study likely due to advanced CRCs being at stage III or IV in this series (SI Appendix, Table S9). Indeed, CRCs in the validation study were consistent at an early stage (0, I, or II). Nevertheless, more than 50% of CRC patients with symptoms and more than 35% of asymptomatic individuals presenting with an early CRC in the validation study showed a CMI ≥ 2 contrasting with only 4% in individuals with normal colonoscopy.

In keeping with earlier reports and with 2 very recent meta-analyses (10, 13–16), microbiota from CRC patients in the current study contained higher proportions of Parvimonas, Butyrivibrio, Gemella, Fusobacteria, and Akkermansia contrasted with lower proportions of Ruminococcus, Bifidobacterium, Eubacteria, and Lachnospira, compared to human control microbiota. Despite some early changes after microbiota transfer in germ-free mice, possibly due to the FMT itself as reported (17), most of the human microbiota components subsequently remained stable over time. The mouse fecal bacteria that showed the greatest decline over time (SI Appendix, Fig. S4) were Clostridia and Clostridiales, as analyzed at the class or the genus levels which might account for food uptake and weight differences among mouse groups. Numerous species belonging to this bacterial community have been associated with an increase in food intake and up-regulated production of proinflammatory molecules (18, 19). SCFAs, the main end-products of butyrate-producing bacteria (19), have been shown to inhibit intestinal inflammation and modulate immune responses (20), maintain barrier function (21), decrease precancerous lesions due to DNA damage (22–24), and regulate DNA methylation (25).

Of interest, the observed dysbiosis was correlated with the histological and DNA findings in the animals while the putative role of dysbiosis and inflammation in the initiation of cancer in the colonic mucosa remained unclear. We used AOM as a co-factor together with microbiota that could induce DNA alterations (22, 25). DNA alterations were more numerous in the colonic mucosa than in splenic tissue, effects which were abolished in the colon by PEG in CRC-μ + AOM recipients. Furthermore, transcripts of factors such as HES, KLF4, and ELF3 involved in the Wnt and Notch pathways were more abundant in CRC-μ than in N-μ mice recipients, depending on higher Bacteroides and lesser Coprococcus in mouse feces after human FMT (SI Appendix, Fig. S5). Thus, our findings suggest that histological alterations in the colonic mucosa might be due to an imbalance in microbiota composition with CRC-μ being associated with greater DNA damage and/or gene methylation/demethylation changes in the colonic mucosa. Although there was globally a greater number of unmethylated probes in the CRC-μ + AOM group than in the N-μ group, few genes (i.e., Wif1) might be methylated after FMT. This is suggested by the observed human CRC-μ induced DNA methylation/demethylation imbalance in mouse colonic mucosa with an additive effect of AOM. These findings are consistent with previous evidence of gene expression silencing by gene methylation in overall one third of human tumors (5) and significant associations between abundance of Parvimonas micra and Bacteroides fragilis with highly methylated tumors (16). To evaluate this hypothesis, we confirmed that Parvimonas micra species was overabundant in the microbiota of those patients presenting a higher methylation gene index in the blood first by analyzing 16sRNA and then by using whole metagenome analysis for the confirmation at the species levels (Table 3). Thus, one would suggest that genera such as Parvimonas and Proteobacteria may use epigenetic pathways for adaptation to
Of interest was also
– Eubacterium as seen currently and reported by us and others
methylation was further investigated in random samples by
might have been obtained using plasma instead of serum. Thus,
serum and was negative in some CRC patients. Different results
may therefore have occurred. The blood CMI was performed on
the blood CMI was determined at a single laboratory, and bias
tations. First, in our prospective validation trial (NCT01270360),
as probiotics.
Bilophila was associated with
tumor tissue and blood (31, 32).
Our present results might be affected by various study limi-
tions. Second, our approach assumed a correlation between
classified as methylation/demethylation
Genus
Log2 fold change
Adjusted P values
Genus
Log2 fold change
Adjusted P values
Ruminococcus –1.3 4.37E-02 — —
Gemella –1.2 4.61E-02 — —
Parvimonas –3.4 1.10E-09 Parvimonas –2.0 1.16E-04
Parasutterella –1.5 4.47E-02 Parasutterella –1.4 1.72E-02
Mogibacterium 1.3 9.71E-03 Mogibacterium 1.2 5.41E-03
Butyrivibrio 1.0 2.18E-03 — —
Lactonifactor 1.0 2.35E-02 Megaphaera 1.3 1.10E-02
Oscillibacter 0.8 4.93E-02 Olsenella 1.6 1.64E-05
Howardella 1.1 4.16E-02 Howardella 1.1 1.10E-02
Abiotrophia 0.9 2.31E-02 Abiotrophia 0.9 1.03E-02
Eubacterium 2.5 1.49E-07 Eubacterium 2.3 4.64E-08
Acetitomaculum 0.8 4.16E-02 — —
Ezakiella 1.1 2.76E-03 Ezakiella 0.9 1.03E-02

Comparison between individuals according to blood CMI values ≤ 2 (negative) versus >2 (positive)

Coprococcus 1.1 4.717E-03 Coprococcus 1.26 4.0E-04
Gemella –1.7 3.02E-02 Dialister 2.68 3.1E-04
Parvimonas –2.3 4.10E-06 Parvimonas –2.39 6.04E-06
Peptostreptococcus –1.5 3.05E-5 Peptostreptococcus –1.6 6.0E-04
Oxalobacter –10.5 4.71E-04 Fusobacterium –1.8 2.11E-03
Acidaminococcus –1.9 3.05E-05 Acidaminococcus –2.61 1.0E-04
Howardella 1.9 3.06E-03 Howardella 1.1 1.10E-02
Eubacterium 2.5 1.49E-07 Enterococcus 2.7 5.9E-04
Acetitomaculum 0.8 4.16E-02 — —
Shewanella –11.2 1.13E-3 — —
Phenylobacterium 11.4 3.71E-03 — —

Table 2. Differences in fecal microbiota in the validation CCR2 cohort

Vatnimad (stage 0, I, and II)
Symptomatic (stage III and IV CRC)

The 16sRNA metagenomics was assessed after stool DNA extraction and subjected to 16S rRNA sequencing on the Illumina Miseq platform. Only those bacteria detected in at least 20% of individuals are indicated. Log2 is logarithmic value; P values are given after adjustment on age, gender, and BMI using Bonferroni-corrected Mann-Whitney U test. The cohort included 348 individuals of whom 173 were asymptomatic individuals enrolled via a mass CRC-screening program and 165 were symptomatic patients. Invasive carcinoma or carcinoma in situ of any aspect in the rectum or colon, CRC; controls, normal colonoscopy.

evironmental factors (4, 26–30), with methylation/demethylation as a pivotal mechanism (31, 32).

In our cohorts, the Prevotella genus, a leading source of interindividually gut microbiota variation associated with long-term fiber intake (8), was more abundant in individuals with CMI ≤ 2. Of interest, Parvimonas and Parasutterella were the bacteria most closely associated with an animal-based diet and were related to a high fat intake (8), both of which probably result in greater bile acid release and higher enteric deoxycholic acid concentrations. These 2 bacteria coexist with antiinflammatory bacteria such as Faecalibacterium and Eubacterium as seen currently and reported by us and others (10, 12–15). Of interest was also SFRP2 hypermethylation in tumor tissue and blood (SI Appendix, Fig. S10 A and C) which was also associated with Bilophila, another proinflammatory genus (SI Appendix, Tables S3 and S10), and that coexisted currently Faecalibacterium in the CRC microbiota. Increased abundance of the Bilophila genus was associated with SFRP2 gene promoter demethylation after black raspberry supplementation (33) used as probiotics.

Our present results might be affected by various study limitations. First, in our prospective validation trial (NCT01270360), the blood CMI was determined at a single laboratory, and bias may therefore have occurred. The blood CMI was performed on serum and was negative in some CRC patients. Different results might have been obtained using plasma instead of serum. Thus, methylation was further investigated in random samples by performing a digital PCR test known to be more sensitive than routine qPCR as previously reported (34). Even with dPCR test, sensitivity was higher in the symptomatic CRC patients likely due to the more advanced CRC in symptomatic patients as compared to asymptomatic CRC individuals. This would suggest a blood CMI positivity test in CRC patients with symptoms was likely due to the presence of tumor cells in the bloodstream rather than to systemic plasticity of peripheral blood cells such as mononuclear cells. A second limitation of this study is that only 3 genes were incorporated in the blood CMI. Additional genes such as SFRP2 might enhance sensitivity of the test and might yield different bacterial clustering patterns from those reported here. Furthermore, while viruses and CRC are still subject to discussion, we cannot rule out the hypothesis that in CRC patients, particular phages modulate the gut microbiota of patients, inducing a dysbiotic profile compared to healthy controls (35).

Nevertheless, our data present evidence for the association between a dysbiosis and CRC causing alterations in gene methylation. The cohort in which dysbiosis and gene methylation were investigated is the largest to date and strongly indicates a dysbiosis-induced imbalance in gene methylation and in bacterial species. We could identify bacteria species (Table 3) who were significantly associated with higher levels of methylation test (CMI > 2) by using whole metagenomic analysis. These measurements can now be proposed as markers for the effectiveness of and adherence to prebiotic and probiotic therapies.
Table 3. Species in the fecal microbiota in individuals with blood methylation test (negative versus positive)

<table>
<thead>
<tr>
<th>Species</th>
<th>Mean at baseline</th>
<th>Fold change</th>
<th>Log$_2$ fold change</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Faecalibacterium prausnitzii [1574]</td>
<td>2,480.5</td>
<td>2.2e + 00</td>
<td>1,159</td>
<td>0.042</td>
</tr>
<tr>
<td>Ruminococcus sp. SR1/5 [1621]</td>
<td>2,352</td>
<td>2.5e + 00</td>
<td>n = 19,924</td>
<td>0.004</td>
</tr>
<tr>
<td>Eubacterium hallii [1597]</td>
<td>1,942.6</td>
<td>2.2e + 00</td>
<td>1.14</td>
<td>0.045</td>
</tr>
<tr>
<td>Clostridium sp. L2-50 [1593]</td>
<td>1,306.6</td>
<td>3.8e + 00</td>
<td>1.945</td>
<td>0.004</td>
</tr>
<tr>
<td>Coprococcus comes [1616]</td>
<td>1,306.5</td>
<td>2.1e + 00</td>
<td>1.107</td>
<td>0.022</td>
</tr>
<tr>
<td>Dialister invisus [1259]</td>
<td>1,023</td>
<td>6.2e + 00</td>
<td>2.65</td>
<td>0.004</td>
</tr>
<tr>
<td>Coprococcus eutactus [1592]</td>
<td>941</td>
<td>2.9e + 00</td>
<td>1.569</td>
<td>0.042</td>
</tr>
<tr>
<td>Bacteroides eggertii [1097]</td>
<td>938</td>
<td>2.9e − 01</td>
<td>−1.745</td>
<td>0.003</td>
</tr>
<tr>
<td>Ruminococcus omnium [1619]</td>
<td>714</td>
<td>2.3e + 00</td>
<td>1.246</td>
<td>0.002</td>
</tr>
<tr>
<td>Clostridium bolteae [1598]</td>
<td>478</td>
<td>3.1e − 01</td>
<td>−1.686</td>
<td>0.002</td>
</tr>
<tr>
<td>Bacteroides sp. D2 [1094]</td>
<td>367.5</td>
<td>3.8e − 01</td>
<td>−1.384</td>
<td>0.042</td>
</tr>
<tr>
<td>Enterococcus faecalis [1363]</td>
<td>240</td>
<td>6.6e + 00</td>
<td>2.723</td>
<td>0.042</td>
</tr>
<tr>
<td>Mitsuokella multacida [1653]</td>
<td>220</td>
<td>2.3e − 01</td>
<td>−2.12</td>
<td>0.003</td>
</tr>
<tr>
<td>Parvimonas micra [1505]</td>
<td>211</td>
<td>2.1e − 01</td>
<td>−2.22</td>
<td>0.003</td>
</tr>
<tr>
<td>Peptostreptococcus stomatis [1530]</td>
<td>110.5</td>
<td>2.0e − 01</td>
<td>−2.26</td>
<td>0.002</td>
</tr>
<tr>
<td>Veillonella atypica [1260]</td>
<td>84.9</td>
<td>1.9e − 01</td>
<td>−2.34</td>
<td>0.005</td>
</tr>
<tr>
<td>Streptococcus equinus [1381]</td>
<td>60.66</td>
<td>1.8e − 01</td>
<td>−2.42</td>
<td>0.042</td>
</tr>
<tr>
<td>Gemella morbillorum [1302]</td>
<td>53</td>
<td>1.3e − 01</td>
<td>−2.921</td>
<td>0.00004</td>
</tr>
<tr>
<td>Parvimonas sp. oral taxon 110 [1506]</td>
<td>35.6</td>
<td>2.7e − 01</td>
<td>−1.95</td>
<td>0.006</td>
</tr>
<tr>
<td>Parvimonas sp. oral taxon 393 [1507]</td>
<td>35</td>
<td>3.2e − 01</td>
<td>−1.622</td>
<td>0.026</td>
</tr>
</tbody>
</table>

Stool samples were submitted to whole metagenomic sequencing of fecal bacteria DNA (controls, n = 61; CRC patients, n = 53) according to CMI test, and results were generated using Shiman C3bi from Institut Pasteur de Paris (http://shaman.c3bi.pasteur.fr/) (for methods, see ref. 9). Log$_2$ is logarithmic value; P values are given after adjustment on age, gender, and BMI. The blood test defined as negative (CMI < 2) versus positive (CMI > 2) is considered to compare abundances of bacteria species in the stool milieu. Fold changes are indicated in log values with minus meaning the bacteria is higher in CRC patients’ microbiota and plus meaning that the bacteria abundance was higher in controls.

Individuals, Materials, and Methods

Recruitment of Participants and Collection of Samples. Patients referred to University hospitals for colonoscopy were enrolled in several prospective cohorts. Effluents and tissues from participants were used for experimental, proof-of-concept, and a pilot study that included 266 individuals from a cohort named CCR1 (SI Appendix, Table S6). A second cohort, named CCR2 (SI Appendix, Table S7), was constituted for validation; this was composed of 2 subcohorts: one including only symptomatic (named Valihybritest study) and the second including only asymptomatic individuals (named Vatnimad) recruited from mass screening programs. All individuals underwent colonoscopy due to symptoms or due to a positive fecal blood test (FOBT). Both subcohort studies were registered on ClinicalTrials.gov (NCT01270360), and 1,000 consecutive colonoscopies were to be enrolled, among them 500 in-subcohort studies were registered on ClinicalTrials.gov (NCT01270360), and blood test defined as negative (CMI < 2) versus positive (CMI > 2) is considered to compare abundances of bacteria species in the stool milieu. Fold changes are indicated in log values with minus meaning the bacteria is higher in CRC patients’ microbiota and plus meaning that the bacteria abundance was higher in controls.

Studies of Microbiota from Human Participants.
The 16S rRNA gene and whole metagenomic sequencing on stools. Stool samples were collected in donors and patients during experimental and validations studies, respectively; samples of stool were collected and stored within 4 h for DNA extraction using the GNOME DNA Isolation Kit (MP Biomedicals) as previously described (9, 10, 12). After amplification by PCR of the V3 to V4 region of the 16S rRNA gene, sequencing was performed using a 250-bp paired-end sequencing protocol on the Illumina MiSeq platform. Raw FASTQ files were demultiplexed, quality-filtered using Trimmomatic (sliding windows of 2 with a quality score of 20), and merged using fasta-join from ea-utils (https://expressionanalysis.github.io/ea-utils/). Taxonomic assignations were performed using Qlime2 (no quality filtering; default parameters) (36) with the SILVA-123 database; OTU were constructed using UCLUST (threshold of 97% of similarity), Chimera Slayer for chimera removing, and SILVA 16S rRNA database (version 123) for taxonomical assignation. The intergroup high similarity and intragroup low similarity of microbiota were assessed by p-diversity, PCoA (generated by Qiime using unweighted unifrac metrics). We subjected study populations to 2 principle coordinates analyses, independently of other datasets, and we investigated separation of CRC microbiota from control microbiota in donors in the experimental study as well as in controls versus CRC patients in the clinical trials. All microbiome statistical tests where produced using the MetagenomeSeq packages or the Shaman Webserver that used the DESeq2 packages for differential expres-

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housekeeping gene and for normalization of DNA amounts) are reported elsewhere (SI Appendix, Table S12).

Quantitative methylation-specific PCR amplification and verification of the specificity. Bisulfite-converted universal human methylated DNA standard (Zymo Research) served as a calibrator and positive control and DNA-free distilled water as a negative control. Quantitative, single-gene methylation-specific PCR (Q-MS-PCR) and quantitative multiplex methylation-specific PCR (Q-MSP) were applied. The relative methylation level was determined using the \(2^{-\Delta\Delta Ct}\) formula. Briefly, for each PCR run, a KAPA PROBE master mix (Kapa Biosystems) was prepared with predefined concentrations of genes candidates.

Statistical analyses of blood CMI and FIT data in the validation cohort. With the type I error set at 0.05 and assuming that CRC would be found in 8% of asymptomatic and 25% of symptomatic individuals, to detect at least 60% of CRCs and 20% of polyps with 90% specificity in average-risk (asymptomatic) without CRC using Pearson \(\chi^2\) test for binary variables and Student’s \(t\) test for continuous variables. Blood methylation data (CMI and methylation of each of its 3 components, \(WIF1\), PENK, and \(NPY\)) and FIT data were handled as both binary and continuous variables. The following values were pre-defined to classify tests as positive: >2 for the CMI and >150 pg per device for the FIT.

To determine whether CMI and FIT were associated with CRC, we built a multivariate logistic regression model as described (37) adjusted for age, gender, and BMI and the adjusted odds ratios (ORs) and hazard ratios with their 95% confidence intervals (95%CIs) were computed. All tests were 2-tailed, \(P\) values < 0.05 were deemed significant. The statistical analysis software was Stat ET v15.0.

Studies from Experiments in Mice. Fresh stool samples were obtained from 9 females and 9 males in CCR1 cohort, for FIT to germ-free mice. They were 9 with CRC and 9 with normal colonoscopies (Table S11). FIT experiments. FITM was performed in male C57BL/6J germ-free 8-week-old mice (design of the experimental study in SI Appendix, Fig. S1). Mice were maintained in gnotobiotic isolators, ad libitum for 1 wk of acclimation to the laboratory conditions followed by FITM (day 0) then by 7 or 14 wk of follow-up, after which the mice were killed. The fecal microbiota donors (SI Appendix, Table S11) were 9 consecutive individuals with normal and 9 consecutive patients with CRC at colonoscopy from cohorts. They were considered in the current study as normal microbiota (N-μ) and CRC microbiota (CRC-μ) donors, were informed about the experimental study, and accepted giving additional stools. Blood and fecal methylation data were handled as both binary and continuous variables. The following values were pre-defined to classify tests as positive: >2 for the CMI and >150 pg per device for the FIT.

To determine whether CMI and FIT were associated with CRC, we built a multivariate logistic regression model as described (37), adjusted for age, gender, and BMI and the adjusted odds ratios (ORs) and hazard ratios with their 95% confidence intervals (95%CIs) were computed. All tests were 2-tailed, \(P\) values < 0.05 were deemed significant. The statistical analysis software was Stat ET v15.0.

Data Availability. The shotgun metagenomic sequencing data and the 16S rRNA amplicon sequencing data are available from the European Nucleotide Archive (ENA database (http://www.ebi.ac.uk/ena) under the accession number ERP005354. Data related to this paper are available from the European Nucleotide Archive (ENA) database (http://www.ebi.ac.uk/ena) under the accession nos. ERX3622297—ERX3624402, ER3628499—ER3628604, ER35396180—ER3536285, and PRJEB35144.

Acknowledgments. We thank all patients for their participation and physicians who invited them to participate; they are the following gastroenterologists: Drs. E. Zrihen, O. Percaux, J. Samama, M. Petit, Ph. Cattan, M. Cavicchi, Ch. Locher, G. Gattineau, M. Parieto, M. Mozer, A. Rosenbaum, Ph. Capelle, D. Levoir, Ph. Lebourgeois, Ph. De Land, E. Chanteloup, M. Simon, F. Mal, and F. Iglicki. We thank also Drs. J. Tran Van Thieu and M. L. Auriault for pathology analyses in human and animals; Prof. S. Loric for biochemistry analysis in mice; Prof. T. Simon, A. Touati, J. Tap, V. Jarrouse, A. Bado, and J. P. Fouret for animal experiments and help managing; A. P. Bado for revising the English; S. Peyvandi for assisting with the animal and molecular experiments; E. Guery and L. Segaux for their statistical contributions; C. Vialette for data managing; A. Caidia (Bioinformatics Core Laboratory) for 16S RNA analysis; and Catherine Philippe for SCFA analyses in mice. We thank all technicians and scientific consultants from Faculté de Médecine site Pitié Salpêtrière (Assistance Publique-Hôpitaux de Paris [APHP]), Unité Mixte de Service (UMS) 29 Omic Platform p3s for technical help on EPIC methylation array study in mice, and all members of clinical monitoring group from the Unité de Recherche Clinique de l’Est Parisien (URC-Est) Hôpital Saint-Antoine, APHP. We thank all the technicians from the Anaxem germ-free animal facility of the Micalis Institute for breeding the germ-free mice and carrying out FITM and gnotobiotic mice care and monitoring. We thank Dr. Abdoumohammaded Pezeshki and M. Sharifzad for assistance with the analysis of inflammation in mouse colon and Katja Brunner for editing the manuscript. Funding was provided by French Institute of Cancer and Ministry of Health (grant PHRC 2011-VatnimadAOM09268) and French Society of Gastroenterology (grant for fecal test screening). Ligue Nationale Contre le Cancer for fecal test screening and the Institut National du Cancer (INCA, Cancéropôle Ile de France, Grant for Microbiota and CRC), and National Institute of Health and Medical Research (INSERM) partially financed under the Institut Thématique Multi-Organisme program (ITMO). This work was sponsored by APHP, INSENM, ClinicalTrials.gov registration number is NCT 01270360.


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