Colorectal cancer-associated microbiota contributes to oncogenic epigenetic signatures

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\textsuperscript{2}Published under the PNAS license.


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, ERX3622499–ERX3622964, ERX336180–ERX336208, and PRIEB6154.


Contributed by Philippe J. Sansonetti, September 27, 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 feces 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 immunoc hemical 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.

\textbf{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.


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

\textbf{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 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, meaning that FMT induced a reduction in the microbial 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, Fig. S2). 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. 1G). At 7 wk following FMT, a significant increase of proliferation was observed in the groups given AOM as compared to all other groups (Fig. 1H). 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. 1J). In addition, semiquantitative 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. 1K).

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, Porphyromonas, Butyribacterium, 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 (i.e., 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 versus 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 variations 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 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. 2A). In addition, mice given CRC-μ and AOM showed the highest levels of DNA alterations in exons or introns showing a trend to an aditive effect of CRC-μ as compared to N-μ. Interestingly, Principal Component Analysis (PCA) scatter diagrams separated
animal subgroups according to the total DNA changes in single nucleotides in the colonic mucosa or spleen samples suggesting a link between gene mutation and type of microbiota (N or CRC) or treatment (saline or AOM) given to mice (Fig. 2 B and C).

In order to investigate the local oncogenic potential of CRC-μ, we went on to perform in-depth analysis of the following selected gene pathways (number of genes analyzed): Wnt and β-catenin (19 genes), Notch (4 genes), PPAR (3 genes), SMAD (2 genes), TGF-β (2 genes), ACRV (2 genes), DKK (4 genes), TCF (2 genes), MYC (1 gene), and SOCS (1 gene). Changes were most prominent in pivotal Wnt pathway genes (with indel and single nucleotide polymorphisms in 11 Wnt genes), with no significant differences between the CRC-μ and N-μ groups. When all DNA mutations were pooled, however, the number of DNA changes was highest in the CRC-μ + AOM recipients vs. the N-μ recipients (Fig. 2D). Again, DNA changes were significantly more numerous in colonic than in spleen tissues. Interestingly, in the spleen, DNA changes were similar among AOM-exposed animals 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 \(P = 0.25\), 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

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. (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.
(Fig. 3). In summary, human CRC-μ induced greater epigenetic alterations in murine colonic mucosal tissues when compared to N-μ. The ratios of methylation-shifted probes (methylated to unmethylated and vice versa) were higher in the CRC-μ group than in the N-μ group, with AOM. When we limited the analysis to in silico specific EPIC probes, two-thirds of the probes with hypermethylation levels (0.8 or more) in the CRC-μ + AOM group were unmethylated (<0.2) in the N-μ group, corresponding to 46 genes in the CRC-μ + AOM group being silenced vs. N-μ group. In contrast, various probes in Wnt and Notch gene families appeared unmethylated in the CRC-μ + AOM group but were methylated in the N-μ group. Overall, significant DNA epigenetic rather than mutation changes in several genes were associated with CRC-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).

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-μ + AOM group being silenced vs. N-μ group. Among the various genes classified as hypermethylated in the individual samples (tissue, stool, and blood) from the same individuals, only 8 (Wnt1-regulating gene and SEPT9, SFRP1,2,3, PENK, NPY, and ALX4 genes) of these were common to all samples and were therefore subjected to further characterization (Fig. 3B and SI Appendix, Figs. S8–S11). We selected Wnt1 to represent the Wnt pathway and NPY and PENK, 2 other tissue CRC-associated hypermethylated genes, for methylation testing, as at least either one was found hypermethylated in all tumor tissues. The sum of the methylation levels of the 3 selected genes was found to serve best when discriminating between effluents from normal and cancer individuals (details in SI Appendix). Finally, to conceive easy and reproducible biological testing for large cohort screening, a housekeeping gene plus selected genes (i.e., ALB, WIF1, NPY, and PENK) were combined in a multiplex procedure for qPCR measurement in blood. A cumulative methyl index (CMI) was determined in blood samples from participants with normal colonoscopy or with a cancer diagnosed (32 patients with CRC and 46 with extra colonic cancer; SI Appendix). To validate its performance, CMI assessment was performed in a pilot study including 266 individuals (SI Appendix) from a well-characterized cohort designed as CCR1 (SI Appendix) and in a final validation study including 999 individuals (Table 1) from a second cohort designed as CCR2 (SI Appendix).

Validation of a CMI. Demographic, clinical characteristics, and results of CMI were compared between CRC patients and those with normal colonoscopy in the validation study; in addition, FIT was performed in those 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). 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
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 = CRC; controls = normal colonoscopy.

Student’s t test and x2 test for continuous and binary variables, respectively.

Positive cutoffs were 2.0 for the CMI and 150 ng/mL for the FIT; OR, odds ratio; 95%CI, 95% confidence interval.

Concentration of hemoglobin for FIT is given per device.

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. 4), 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).

**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. 2 D 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
Materials, and Methods (Shaman c3bi platform; Institut Pasteur, http://shaman.c3bi.pasteur.fr). A maximum likelihood phylogenetic tree was tested (Individuals, Materials, and Methods). Brown to red colors indicate negative CMI results (≤2), and blue colors indicate positive CMI results (>2). Note that diversity was less in the group with a positive CMI compared to the group with a negative CMI.

significantly associated with hypermethylation of several gene promoters, including NPY and PENK from the brain gut system and Wif1 from the Wnt pathway. A CMI test constructed from these genes and performed in blood detected more (vs. controls) CRC patients even in asymptomatic individuals (n = 32) who were recruited through mass screening program and had early CRC at colonoscopy. However, the CMI showed 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 consistently 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, Butyribrio, Gemella, Fusobacteria, and Akkermansia contrasting 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
environmental factors (4, 26–30), with methylation/demethylation as a pivotal mechanism (31, 32).

In our cohorts, the *Prevotella* genus, a leading source of interindividual 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 coexclude 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, Table S3 and S10*), and that coexcluded 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 2. Differences in fecal microbiota in the validation CCR2 cohort

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<th>Adjusted P values</th>
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<td>Parvimonas</td>
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<td>1.10E-09</td>
<td>Parvimonas</td>
<td>−2.0</td>
<td>1.16E-04</td>
</tr>
<tr>
<td>Parasutterella</td>
<td>−1.5</td>
<td>4.47E-02</td>
<td>Parasutterella</td>
<td>−1.4</td>
<td>1.72E-02</td>
</tr>
<tr>
<td>Mogibacterium</td>
<td>1.3</td>
<td>9.71E-03</td>
<td>Mogibacterium</td>
<td>1.2</td>
<td>5.41E-03</td>
</tr>
<tr>
<td>Butyrviribrio</td>
<td>1.0</td>
<td>2.18E-03</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lactonifactor</td>
<td>1.0</td>
<td>2.35E-02</td>
<td>Megasphaera</td>
<td>1.3</td>
<td>1.10E-02</td>
</tr>
<tr>
<td>Oscillospira</td>
<td>0.8</td>
<td>4.93E-02</td>
<td>Olsenella</td>
<td>1.6</td>
<td>1.64E-05</td>
</tr>
<tr>
<td>Howardella</td>
<td>1.1</td>
<td>4.16E-02</td>
<td>Howardella</td>
<td>1.1</td>
<td>1.10E-02</td>
</tr>
<tr>
<td>Abiotrophia</td>
<td>0.9</td>
<td>2.31E-02</td>
<td>Abiotrophia</td>
<td>0.9</td>
<td>1.03E-02</td>
</tr>
<tr>
<td>Eubacterium</td>
<td>2.5</td>
<td>1.49E-07</td>
<td>Eubacterium</td>
<td>2.3</td>
<td>4.64E-08</td>
</tr>
<tr>
<td>Acetitomaculum</td>
<td>0.8</td>
<td>4.16E-02</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ezakiella</td>
<td>1.1</td>
<td>2.76E-03</td>
<td>Ezakiella</td>
<td>0.9</td>
<td>1.03E-02</td>
</tr>
</tbody>
</table>

The 16S rRNA 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.
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 (proof-of-concept, and a pilot study that included 266 individuals from a recruitment of participants and collection of samples."

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 Shaman C3bi from Institut Pasteur de Paris (http://shaman.c3bi.pasteur.fr) (for methods, see ref. 9). Log, 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.

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, 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. 5R15 [1621]</td>
<td>2,252</td>
<td>2.5e + 00</td>
<td>1.304</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 invius [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 eggertthii [1097]</td>
<td>938</td>
<td>2.9e − 01</td>
<td>−1.745</td>
<td>0.003</td>
</tr>
<tr>
<td>Ruminococcus obeum [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 multicaida [1653]</td>
<td>220</td>
<td>2.3e − 01</td>
<td>−2.12</td>
<td>0.003</td>
</tr>
<tr>
<td>Parvimonas mira [1505]</td>
<td>211</td>
<td>2.1e − 01</td>
<td>−2.22</td>
<td>0.003</td>
</tr>
<tr>
<td>Peptostreptococcus stomatica [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 Shaman C3bi from Institut Pasteur de Paris (http://shaman.c3bi.pasteur.fr) (for methods, see ref. 9). Log, 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.

For a deep identification of bacteria species in the current study, DNA samples were submitted to whole metagenomic analyses as previously described (18).

Studies of Methylation from Human Participants.

DNA isolation and bisulfite conversion. DNAs from colorectal tissues (QIAamp DNA Mini Kit; Qiagen), blood, and stool samples (QIAamp DNA stool Mini Kit; Qiagen) were extracted using the ZR Serum DNA kit (Ozyme) according to the manufacturer’s protocol. The DNA samples were then exposed to sodium bisulfite at 50 °C in the dark for 16 h (EZ DNA Methylation kit; Zymo Research) to convert unmethylated cytosine nucleotides into uracil nucleotides (subsequently converted to thymidine nucleotides during PCR cycling) without changing the methylated cytosines as detailed elsewhere (10, 12).

DNA methylation using Illumina Golden Gate methylation bead arrays. The GoldenGate Methylation Cancer Panel I (Illumina) was used to probe 500 ng of each bisulfite-converted DNA sample of human (n = 18, 9 CRC patients and 9 controls) including tissue (n = 18), stool (n = 18), and blood (n = 18); the stool samples were used for FMT to germ-free mice (SI Appendix, Table S11). Methylation levels ranged from 0 to 100.0% were used for the calculation of the ratio of the methylated signal intensity. The strategy for hierarchical clustering of gene candidates is further described in Fig. 3 and SI Appendix, Figs. S8–S11. Briefly, comparisons across tissue, stool, and blood samples identified genes with CpG loci methylation levels in the promoter above the expected number. The CMI was computed by addition of the methylation values of the 3 genes generated by the discovery study (characteristics of individuals in the pilot study in SI Appendix, Table S12). Primers targeting all genes (including albumin gene-ALB, devoid of CpG sites and used as a 16S rRNA database (version 123) for taxonomical assignment. 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 producing the MetagenomeSeq packages or the Shaman Webserver that used the DESeq2 packages for differential expression analyses; therefore, every statistical analysis for microbiome have been corrected according to gender, age, and BMI and adjusted for multiple testing.

For a deep identification of bacteria species in the current study, DNA samples were submitted to whole metagenomic analyses as previously described (18).
housekeeping gene and for normalization of DNA amounts) are reported elsewhere (SI Appendix, Table S12).

Quantitative methylation-specific PCR (QM-MSP) and quantitative multiplex methylation-specific PCR (QM-MSP) were applied. The relative methylation level was determined using the 2^(-ΔΔCt) 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) populations, 1,000 participants (with at least 400 in each sub-cohort) were needed. Characteristics of study populations were described using number (%) for qualitative variables and mean ± SD for quantitative variables.

The main endpoint was identifying those individuals with advanced neoplasia (invasive carcinoma or carcinoma in situ or speciﬁc carcinoma on any aspect in the rectum or colon, roughly called CRC). Demographic, clinical, blood methylation, and FIT data were compared between patients with and without CRC using Pearson’s r2 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 predefined to classify tests as positive: >2 for the CMI and >150 ng 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, and P values < 0.05 were deemed signiﬁcant. The statistical analysis software was Statate 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 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. First, the microbiota transfer, and carrying out FMT and gnotobiotic mice monitoring group from the Unité de Recherche Clinique de l’Est Parisien (URC-Est) Hôpital Saint-Antoine, APHP. We thank all the technicians 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 scientists 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 Unite 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 FMT and gnotobiotic mice care and monitoring. We thank Dr. Abdulmohammad Pezeshki and M. Sharifian-Boroujeni for assistance with the analysis of infection in mouse colons and Katja Brunner for editing the manuscript. Funding was provided by French Institut of Cancer and Ministry of Health (grant PHRC 2011-VatnimadAMOM09268) 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, Cancéropole Île 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, INSERM, ClinicalTrials.gov registration number is NCT 01270360.

Data Availability. The shotgun metagenomic sequencing data and the 16S RNA amplicon sequencing data are available from the European Nucleotide Archive (ENA) database (http://www.ebi.ac.uk/ena) under the accession number ERPO05534. Data related to this paper are available from the European Nucleotide Archive (ENA) database (http://www.ebi.ac.uk/ena) under the accession nos. ERX622297–ERX622402, ERR3628499–ERR3628604, ERS393180–ERS3936285, 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. Peciraux, J. Samama, M. Petit, Ph. Cattan, M. Cavichiu, Ch. Locher, G. Gattineau, M. Parieto, M. Mozer, A. Rosenbaum, Ph. Capelle, D. Levoir, F. Maille, Ph. Lebourgés, Ph. De Land, E. Chanteloup, M. F. Mal, and F. Igluki. We thank also Drs. J. Tran Vanthieu and M. A. L. Auriault for pathology analyses in human and animals; Prof. S. Loric for biochemistry analysis in mice; Prof. T. Simon, A. Touati, J. Tap, J. Jarrouse, A. Bodo, and J. P. Fougé for animal experiments and help managing. And 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 Unite 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 FMT and gnotobiotic mice care and monitoring. We thank Dr. Abdulmohammad Pezeshki and M. Sharifian-Boroujeni for assistance with the analysis of infection in mouse colons and Katja Brunner for editing the manuscript. Funding was provided by French Institut of Cancer and Ministry of Health (grant PHRC 2011-VatnimadAMOM09268) 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, Cancéropole Île 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, INSERM, ClinicalTrials.gov registration number is NCT 01270360.

21. K. B. Islam et al., Bile acid is a host factor that regulates the composition of the cecal microbiota in rats. Gastroenterology 141, 1773–1781 (2011).