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1 **Colorectal cancer-associated microbiota contributes to oncogenic epigenetic signatures**

2

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6

7 **Running title:** *Methylation Pathway-Related CRC Dysbiosis*

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39

40 **Abstract**

41

42 Sporadic colorectal cancer (CRC) is a result of complex interactions between the host and its
43 environment. Environmental stressors act by causing host-cell DNA alterations implicated in
44 the onset of cancer. Here, we investigate the stressor ability of CRC-associated gut dysbiosis
45 as causal agent of host DNA alterations. The epigenetic nature of these alterations was
46 investigated in humans and in mice. Germ-free mice receiving fecal samples from subjects
47 with normal colonoscopy or from CRC patients were monitored for 7 or 14 weeks. Aberrant
48 crypt foci, luminal microbiota and DNA alterations (colonic exome sequencing and
49 methylation patterns) were monitored following human feces transfer. CRC-associated
50 microbiota induced higher numbers of hypermethylated genes in murine colonic mucosa (vs.
51 healthy controls' microbiota recipients). Several gene promoters including SFRP1,2,3,
52 PENK, NPY, ALX4, SEPT9, WIF1 promoters were found hypermethylated in CRC but not
53 in normal tissues or effluents from fecal donors. In a pilot study (n=266), the blood
54 methylation levels of three genes (*Wif1*, *PENK* and *NPY*) were shown closely associated
55 with CRC dysbiosis. In a validation study (n=1000), the cumulative methylation index
56 (CMI) of these genes was significantly higher in CRCs than in controls. Further, CMI
57 appeared as an independent risk factor for CRC diagnosis as shown by multivariate analysis
58 that included fecal immunochemical blood test. Consequently, fecal bacterial species in
59 individuals with higher CMI in blood were identified by whole metagenomic. Thus, CRC-
60 related dysbiosis induces methylation of host genes and corresponding CMI together with
61 associated bacteria are potential biomarkers for CRC.

62

63

64

65 **Significance Statement**

66 This study advances our appreciation and understanding of the role of colon dysbiosis in the
67 pathogenesis of colorectal cancer. In a human pilot study of 266 individuals, greater
68 epigenomic (methylation) DNA alterations correlated with CRC and microbiota
69 composition. Beyond this correlative evidence, when germ-free mice received fresh feces
70 from CRC patients and their healthy controls, the former animals developed colon epithelial
71 renewal, more precancerous lesions, and increased tissue and blood DNA methylation in
72 intestinal tissues. Confirmation was obtained in a larger cohort of 1000 patients, indicating
73 that CRC-associated dysbiosis may promote colon carcinogenesis via epigenome
74 dysregulation. Gene methylation can therefore serve as a marker for CRC and likely for
75 predicting efficacy of prebiotic supplementation in average-risk individuals.

76

77

78 **Key words:** Colon. Cancer. Microbiota. Gene methylation. *Wif1*, *NPY*, *PENK* Biomarker.

79 **Introduction**

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

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

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

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

104 free mice in order to assess the potential effect of this microbiota as an environmental
105 stressor for host DNA. Secondly, we investigated the links between dysbiosis and host gene
106 methylated markers suggested by animal experiments in a pilot human study and validated in
107 a large human cohort.

108

109

110 **RESULTS**

111

112 **Human fecal microbiota transfer (FMT) to germ-free mice: clinical and routine** 113 **biochemical parameters among groups.**

114 We performed human fecal microbiota transfer (FMT) to germ-free mice utilizing
115 material from patient donors with CRC (n=9) and healthy control donors (n=9) as illustrated
116 in “SI Appendix, Fig. S1”. In addition, mice received the carcinogen azoxymethane (AOM)
117 or saline control for 10 weeks in order to assess potential effects of chemically induced
118 stress. As shown in “SI Appendix, Tables S1A-S1B”, FMT had no effect on the general
119 blood parameters of the recipient mice as no significant differences were observed among
120 any of the groups (a total of 185 mice) at 7 and 14 weeks. At 14 weeks, mice that received
121 both AOM and colorectal cancer microbiota (CRC- μ) showed a lower mean body weight
122 compared to the normal microbiota (N- μ) controls. This was accounted to reduced food
123 intake observed in the CRC- μ group when compared to the N- μ controls (1.5 ± 0.1 g/100 g
124 food intake vs. 2.0 ± 0.1 g/100 g food intake, respectively; $P<0.01$). Interestingly, this weight
125 effect disappeared when mice were given PEG in their drinking water, which is known to
126 prevent bacterial colonization of the gut (SI Appendix, Fig. S2). In line with this, the luminal
127 bacterial load, adherence of luminal bacteria to the colonic mucosa and short-chain fatty acid
128 (SCFA) concentrations in the caecum were significantly lower in mice that received PEG as

129 compared to the controls (SI Appendix, Fig. S2A-S2B). These results suggest that human
130 CRC FMT may induce systemic changes in mice mediated by the interaction of bacteria with
131 the colonic mucosa.

132

133 **Dysbiosis-related histological alterations in colonic tissue after human FMT in mice**

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

147

148 **Human fecal microbiota transfer (FMT) to germ-free mice: effect on epithelial** 149 **proliferation and gene expression**

150 Epithelial-cell proliferation in the colonic mucosa was visualized by KI67 staining (as
151 illustrated in Figure 1G). At 7 weeks following FMT, a significant increase of proliferation
152 was observed in the groups given AOM as compared to all other groups (Figure 1H).
153 However, at week 14 cell proliferation was significantly greater in the CRC- μ , as compared

154 to N- μ recipients with AOM exerting an additional effect (Figure 1H-I) and ACFs increased
155 with the rate of epithelial-cell proliferation. These results are consistent with those reported
156 after FMT from only 5 CRC patients as compared to 5 control individuals' microbiota (13).
157 Further, to investigate the involvement of *Wnt* and *Notch* pathways that control intestinal cell
158 fate, mRNA of key mediators was quantified. qPCR analysis of mRNA from the colonic
159 mucosa of recipient mice revealed an increase of 1.7-fold, 1.9-fold, and 1.9-fold expression
160 levels of the transcription factors *HES1*, *KLF4*, and *ELF3*, respectively but not of *MATH1* in
161 CRC- μ compared to N- μ recipients (SI Appendix, Table S2).

162

163 **Human fecal microbiota transfer (FMT) to germ-free mice: mucosal inflammation**

164 Early after FMT (7 weeks), a mild inflammatory response reflected by higher IL1 β ,
165 IL6, and MIP2 α levels and lower IL10, IL23 and INF γ levels, was detected in CRC- μ
166 recipient animals as compared to N- μ controls. AOM treatment tended to to amplify this
167 stimulatory effect as assessed by mRNA cytokine quantification (Figure 1J). In addition,
168 semi-quantitative evaluation of mucosal myeloid cells along the entire histologically-
169 examined intestine (ten fields per sample, 3 samples/mice) showed a trend towards increased
170 numbers in human CRC- μ as compared to N- μ recipients, but without reaching statistical
171 significance at any timepoint (Figure 1K).

172

173 **Microbiota characterization following human FMT in mice**

174 To assess for the impact of the microbial communities on the observed differences in
175 the mucosal phenotypes, murine fecal samples were analyzed by 16S rRNA gene
176 sequencing. At baseline, the fecal microbiota of the CRC- μ (n = 9 donors) group contained a
177 higher proportion of *Fusobacteria*, *Parvimonas*, *Butyrivibrio*, *Gemella*, and *Akkermansia*,

178 and lower proportions of *Ruminococcus*, *Bifidobacterium*, *Eubacteria* and *Lachnospira*, as
179 compared to stools from the N- μ (n=9 donors) group (SI Appendix, Table S3 and Fig. S3).

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

199

200 **Human FMT to germ-free mice alters DNA**

201 **DNA mutations.** In order to analyze the potential of FMT to induce DNA modifications in
202 the host, whole-genome sequencing of murine colonic mucosal tissues (n=12, three in each

203 group of recipients) was performed, covering 220,000 exons within 24,000 genes. Overall,
204 the incidence of mutations at global exon/intron level was significantly higher in intestinal vs
205 spleen tissues. Results of the colonic mucosa showed a trend toward increased DNA
206 alterations in exons or introns of CRC- μ compared to N- μ recipients, but this did not reach
207 significance (Figure 2A). In addition, mice given CRC- μ and AOM showed the highest
208 levels of DNA alterations in exons or introns showing a trend to an additive effect of CRC- μ
209 as compared to N- μ . Interestingly, PCA scatter diagrams separated animal subgroups
210 according to the total DNA changes in single-nucleotides in the colonic mucosa or spleen
211 samples suggesting a link between gene mutation and type of microbiota (N or CRC) or
212 treatment (Saline or AOM) given to mice (Figure 2B-C).

213 In order to investigate the local oncogenic potential of CRC- μ , we went on to perform in-
214 depth analysis of the following selected gene pathways (number of genes analyzed): Wnt
215 and β -catenin (19 genes), Notch (4 genes), PPAR (3 genes), SMAD (2 genes), TGF- β (2
216 genes), ACRV (2 genes), DKK (4 genes), TCF (2 genes), MYC (1 gene), SOCS (1 gene).
217 Changes were most prominent in pivotal *Wnt* pathway genes (with indel and single
218 nucleotide polymorphisms in 11 *Wnt* genes), with no significant differences between the
219 CRC- μ and N- μ groups. When all DNA mutations were pooled, however, the number of
220 DNA changes was highest in the CRC- μ + AOM recipients vs. the N- μ recipients (Figure
221 2D). Again, DNA changes were significantly more numerous in colonic than in spleen
222 tissues. Interestingly, in the spleen, DNA changes were similar among AOM-exposed
223 animals that received CRC- μ or N- μ . Finally, an unsupervised analysis between mutated
224 genes (including only selected genes as indicated above) and recipient status showed that
225 changes in *Wnt*, *PPAR γ* , and *Notch* pathway genes were associated with CRC- μ recipient,
226 with an additive effect of AOM treatment (Figure 2C, SI Appendix, Fig. S6 and Table S2).

227

228 **DNA methylation/demethylation.** We isolated colonic mucosal tissue from the colon (n=24;
229 6 in each group) and evaluated epigenetic changes using the EPIC microarray to compare
230 methylation rates in 63,987 probes reflecting 12,600 gene being expressed. For all probes
231 pooled, both mean and median methylation rates decreased from the N- μ +NaCl group to the
232 CRC- μ +NaCl and CRC- μ +AOM groups (Figure 2E), with significantly lower methylation
233 levels in the CRC- μ /AOM group than in the N- μ +NaCl group ($P<0.01$). Overall, one third of
234 the probes appeared unmethylated in all experimental conditions (<0.2 beta-value
235 methylation mEPIC) in the colonic tissue after FMT (Figure 2F). The number of methylated
236 probes (>0.8) varied with N- μ , CRC- μ , and AOM exposure, although the ratio of
237 methylated/unmethylated probes was not significantly different (3.3% and 3.1%,
238 respectively ($P=0.25$, Fisher's test)) (Figure 2G). The number of unmethylated probes was
239 11% higher in the CRC- μ +AOM group when compared to the N- μ +NaCl group ($p=0.013$)
240 and the number of fully methylated probes was higher in CRC- μ and AOM recipients
241 (Figure 2H). In summary, human CRC- μ induced greater epigenetic alterations in murine
242 colonic mucosal tissues when compared to N- μ . The ratio of methylation-shifted probes
243 (methylated to unmethylated and vice versa) were higher in the CRC- μ group than in the N-
244 μ group, with AOM. When we limited the analysis to *in silico* specific EPIC probes, two
245 thirds of the probes with hypermethylation levels (0.8 or more) in the CRC- μ +AOM group
246 were unmethylated (<0.2) in the N- μ group, corresponding to 46 genes in the CRC- μ +AOM
247 group being silenced vs. N- μ group. In contrast, various probes in *Wnt* and *Notch* gene
248 families appeared unmethylated in the CRC- μ +AOM group but were methylated in the N- μ
249 group. Overall, significant DNA epigenetic rather than mutation changes in several genes
250 were associated with CRC- μ human FMT in the mouse colonic mucosa. Pooling of total
251 genes with expected DNA alterations (i.e. hypermethylation, or mutation in exon) revealed

252 the involvement of pathways implicated in cell growth, signal transduction, nucleic acid
253 binding, protein synthesis, channel and carrier protein (SI Appendix, Fig. S7).

254

255 **Gene methylation in human colonic samples: from bench to bed**

256 To investigate whether the gene methylation patterns observed in the mouse were also
257 associated with CRC dysbiosis in humans, we first investigated methylated genes in CRC
258 tissues and effluents such as blood and stool (n=9) and in normal tissue effluent controls
259 (n=9), both obtained from the same individuals as for the FMT in mice (Figure 3A). In order
260 to develop a quick and easy methylation test, panels of genes were selected, based on the
261 difference between “normal” and “CRC” in various effluents and tissues. Among the various
262 genes classified as hypermethylated in the individual samples (tissue, stool, blood) from the
263 same individuals, only 8 (*Wif1*-regulating gene and *SEPT9*, *SFRP1,2,3*, *PENK*, *NPY*, and
264 *ALX4* genes) of these were common to all samples and were therefore subjected to further
265 characterization (Figure 3B and SI Appendix, Fig. S8-S11). We selected *Wif1* to represent
266 the *Wnt* pathway and *NPY*, *PENK*, two other tissue CRC-associated hypermethylated genes
267 for methylation testing, as at least either one was found hypermethylated in all tumor tissues.
268 The sum of the methylation levels of the three selected genes was found to serve best when
269 discriminating between effluents from normal and cancer individuals (details in SI
270 Appendix). Finally, to conceive easy and reproducible biological testing for large cohort
271 screening, a housekeeping gene plus selected genes (i.e. *ALB*; *WIF1*; *NPY*; *PENK*) were
272 combined in a multiplex procedure for qPCR measurement in blood. A cumulative methyl
273 index (CMI) was determined in blood samples from participants with normal colonoscopy or
274 with a cancer diagnosed (32 patients with CRC and 46 with extra colonic cancer; see SI
275 Appendix). To validate its performance, CMI assessment was performed in a pilot study
276 including 266 individuals (SI Appendix, Table S5) from a well-characterized cohort

277 designed as CCR1 (SI Appendix, Table S6) and in a final validation study including 999
278 individuals (Table 1) from a second cohort designed as CCR2 (Table S7). The specificity
279 and the sensitivity of CMI>2 in blood, was 95% and 59% respectively in the pilot study
280 when those with positive Fecal Occult Blood Test (FOBT) in stool were 97% and 43%,
281 respectively. In the validation study half of individuals were enrolled through mass CRC-
282 screening programs and had a new fecal immunochemical test to directly measure human
283 hemoglobin in stools (FIT) and all had a blood with a threshold of CMI> 2 for positivity.

284

285 **Validation of a cumulative methylation index (CMI)**

286 Demographic, clinical characteristics and results of CMI were compared between CRC
287 patients and those with normal colonoscopy in the validation study; in addition, FIT was
288 performed in those asymptomatic individuals who underwent colonoscopy and we
289 investigated whether positive FIT and CMI>2 could be independent parameters for diagnosis
290 of CRC (Table 1 and SI Appendix, Table S7).

291 A CMI>2 correlated significantly with CRC in 999 individuals in the validation study.
292 Interestingly, a tendency of CMI increase with age was noted, however this observation did
293 not reach significance. By keeping specificity of both tests superior to 95%, the negative
294 predictive value (mean, extremes) of CMI (set at >2) test was better than FIT (set at >150
295 ng/mL): 84.09 [81.5-86.4] and 81.64 [77.5-85.3], respectively (SI Appendix, Table S8). A
296 multivariate model including all individuals with and without neoplasia adjusted for age,
297 gender and FIT results, showed CMI>2 was independently associated with CRC (Table 1).
298 Regarding CMI test and keeping with threshold for positivity set at ≥ 2 , the sensitivity for
299 the detection of CRC could reach 37% in the validation study.

300 After stratification on TNM-ICAA classification (SI Appendix, Table S9), the CMI
301 value levels increased significantly and linearly with increasing tumor stages.

302

303 **Dysbiosis and associations with methylation of genes**

304 16S rRNA gene sequencing on stool samples (n=513) indicated a great β -diversity in
305 patients with CRC and in those with a CMI>2 ($P<0.05$). Furthermore, CMI levels were
306 significantly correlated with dysbiosis. In the validation cohort, we hierarchically clustered
307 bacterial phylotypes on the genus level based on the similarity of their dynamics in patients
308 with CRC and in those with CMI>2 (Figure 4).

309 Interestingly, Principal Coordinate Analysis on the microbiota when incorporating the
310 diagnosis and the level of blood methylation test (Figure 4A), separated into two main
311 clusters which differed significantly between participants with versus without a CMI>2,
312 regardless of whether they did or did not have CRC (Figure 4B). A single cluster differed
313 significantly in abundance between controls with and without a CMI>2 ($P<0.05$, Wilcoxon
314 signed-rank test; Figure 4C).

315 Although microbiota composition varied across cohorts, several genera such as
316 *Parvimonas* and *Parasutterella* were more abundant in CRC patients in both cohorts,
317 whereas *Eubacterium* was more abundant in controls (Table 2 and SI Appendix, Table S10).
318 *Parvimonas* genus was, also, more abundant in individuals with blood CMI>2 vs. ≤ 2 in the
319 current as well as in our pilot study. The whole metagenomic analysis showed twenty
320 bacteria species, including several *Parvimonas* species, differed in abundance in patients
321 with CMI>2 (n=53) and in those with CMI ≤ 2 (n= 90) (Table 3).

322

323 **DISCUSSION**

324 The data reported here constitute the first evidence that the relative abundance of some
325 bacterial taxonomic groups within the microbiota in CRC is significantly associated with
326 methylation/demethylation of host genes. In our study, we show clear phenotypical

327 differences between mice receiving fecal transplants from CRC patients vs. healthy controls.
328 The CRC- μ group presented with lower fecal short-chain fatty acid (SCFA) concentrations
329 and had significant colonic mucosal changes including higher ACF numbers and marked
330 epigenetic alterations independent AOM. However, two findings in the mouse study indicated
331 an additive effect of AOM to the CRC microbiota: (i) the number of DNA alterations was
332 greater in colonic than in spleen tissues under AOM (Figure 2D, 2G), and (ii) the number of
333 spleen-tissue DNA alterations was similar in the CRC- μ and N- μ recipients. Interestingly,
334 16S rRNA gene sequencing of fecal microbiota from CRC patients and normal controls
335 showed that CRC-associated dysbiosis was significantly associated with hypermethylation of
336 several gene promoters, including *NPY* and *PENK* from the brain gut system and *Wif1* from
337 the *Wnt* pathway. A CMI test constructed from these genes and performed in blood detected
338 more (vs. controls) CRC patients even in asymptomatic individuals (n=32) who were
339 recruited through mass screening program and had early CRC at colonoscopy. However, the
340 CMI showed higher sensitivity rate in the pilot study likely due to advanced CRCs being at
341 stage III or IV in this series (SI Appendix, Table S9). Indeed, CRCs in the validation study
342 were consistently at an early stage (0, I or II). Nevertheless, more than 50% of CRC patients
343 with symptoms and more than 35% of asymptomatic individuals presenting with an early
344 CRC in the validation study showed a $CMI \geq 2$ contrasting with only 4% in individuals with
345 normal colonoscopy.

346 In keeping with earlier reports and with two very recent meta-analyses (10, 13-16),
347 microbiota from CRC patients in the current study contained higher proportions of
348 *Fusobacterium*, *Parvimonas*, *Butyrivibrio*, *Gemella*, *Fusobacteria*, and *Akkermansia*
349 contrasting with lower proportions of *Ruminococcus*, *Bifidobacterium*, *Eubacteria*, and
350 *Lachnospira*, compared to human control microbiota. Despite some early changes after
351 microbiota transfer in germ-free mice, possibly due to the FMT itself as reported (17), most

352 of the human microbiota components subsequently remained stable over time. The mouse
353 fecal bacteria that showed the greatest decline over time (SI Appendix, Fig. S4) were
354 *Clostridia* and *Clostridiales*, as analyzed at the class or the genus levels which might account
355 for food uptake and weight differences among mouse groups. Numerous species belonging
356 to this bacterial community have been associated with an increase in food intake and
357 upregulated production of proinflammatory molecules (18-19). SCFAs, the main end-
358 products of butyrate-producing bacteria (19) have been shown to inhibit intestinal
359 inflammation and modulate immune responses (20), maintain barrier function (21), decrease
360 precancerous lesions due to DNA damage (22-24) and regulate DNA methylation (25).

361 Of interest, the observed dysbiosis was correlated with the histological and DNA
362 findings in the animals while the putative role of dysbiosis and inflammation in the initiation
363 of cancer in the colonic mucosa remained unclear. We used AOM as a co-factor together
364 with microbiota that could induce DNA alterations (22, 25). DNA alterations were more
365 numerous in the colonic mucosa than in splenic tissue, effects which were abolished in the
366 colon by PEG in CRC- μ +AOM recipients. Furthermore, transcripts of factors such as HES,
367 KLF4, and ELF3 involved in the *Wnt* and *Notch* pathways, were more abundant in CRC- μ
368 than in N- μ mice recipients, depending on higher *Bacteroides* and lesser *Coprococcus* in
369 mouse feces after human FMT (SI Appendix, Fig. S5). Thus, our findings suggest that
370 histological alterations in the colonic mucosa might be due to an imbalance in microbiota
371 composition with CRC- μ being associated with greater DNA damage and/or gene
372 methylation/demethylation changes in the colonic mucosa. Although there was globally a
373 greater number of unmethylated probes in the CRC- μ +AOM group than in the N- μ group,
374 few genes (i.e. *Wif1*) might be methylated after FMT. This is suggested by the observed
375 human CRC- μ induced DNA methylation/demethylation imbalance in mouse colonic
376 mucosa with an additive effect of AOM. These findings are consistent with previous

377 evidence of gene expression silencing by gene methylation in overall one third of human
378 tumor tissues (5) and significant associations between abundance of *Parvimonas micra* and
379 *Bacteroides fragilis* with highly methylated tumors (16). To evaluate this hypothesis, we
380 confirmed that *Parvimonas micra* species, was over abundant in the microbiota of those
381 patients presenting a higher methylation gene index in the blood first by analyzing 16sRNA
382 and then by using whole metagenome analysis for the confirmation at the species levels
383 (Table 3). Thus, one would suggest that genera such as *Parvimonas* and *Proteobacteria* may
384 use epigenetic pathways for adaptation to environmental factors (4, 26-30), with
385 methylation/demethylation as a pivotal mechanism (31-32).

386 In our cohorts, the *Prevotella* genus, a leading source of inter-individual gut
387 microbiota variation associated with long-term fiber intake (8), was more abundant in
388 individuals with CMI_≤2. Of interest, *Parvimonas* and *Parasutterella* were the bacteria most
389 closely associated with an animal-based diet and were related to a high fat intake (8), both of
390 which probably result in greater bile acid release and higher enteric deoxycholic acid
391 concentrations. These two bacteria co-exclude anti-inflammatory bacteria such as
392 *Faecalibacterium* and *Eubacterium* as seen currently and reported by us and others (10, 12-
393 15). Of interest, was also, *SFRP2* hypermethylation in tumor tissue and blood (SI Appendix,
394 Fig. S10A&C) which was associated with *Bilophila*, another proinflammatory genus (Tables
395 S3 and S10), and that co-excluded currently *Faecalibacterium* in the CRC microbiota.
396 Increased abundance of the *Bilophila* genus was associated with *SFRP2* gene promoter
397 demethylation after black raspberry supplementation (33) used as probiotics.

398 Our present results might be affected by various study limitations. Firstly, in our prospective
399 validation trial (NCT01270360), the blood CMI was determined at a single laboratory, and
400 bias may therefore have occurred. The blood CMI was performed on serum and was
401 negative in some CRC patients. Different results might have been obtained using plasma

402 instead of serum. Thus, methylation was further investigated in random samples by
403 performing a digital PCR test known to be more sensitive than routine qPCR as previously
404 reported (34). Even with dPCR test, sensitivity was higher in the symptomatic CRC patients
405 likely due to the more advanced CRC in symptomatic patients as compared to asymptomatic
406 CRC individuals. This would suggest a blood CMI positivity test in CRC patients with
407 symptoms was likely due to the presence of tumor cells in the bloodstream rather than to
408 systemic plasticity of peripheral blood cells such as mononuclear cells. A second limitation
409 of this study is that only three genes were incorporates in the blood CMI. Additional genes
410 such as *SFRP2* might enhance sensitivity of the test and might yield different bacterial
411 clustering patterns to those reported here. Furthermore, while viruses and CRC are still
412 subject to discussion, we cannot rule out the hypothesis that in CRC patients particular
413 phages modulate the gut microbiota of patients, inducing a dysbiotic profile compared to
414 healthy controls (35).

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

422

423 **Individuals & Materials & Methods**

424 *Recruitment of participants and collection of samples*

425 Patients referred to University hospitals for colonoscopy were enrolled in several
426 prospective cohorts. Effluents and tissues from participants were used for experimental,

427 proof-of-concept, and for a pilot study that included 266 individuals from a cohort named
428 CCR1 (SI Appendix, Table S6). A second cohort, named CCR2 (SI Appendix, Table S7)
429 was constituted for validation; this was composed of two sub-cohorts: one including only
430 symptomatic (named Valihybritest study) and the second including only asymptomatic
431 individuals (named Vatnimad) recruited from mass screening programs. All individuals
432 underwent colonoscopy due to symptoms or due to a positive fecal blood test (FOBT). Both
433 sub-cohort studies were registered on ClinicalTrials.gov (NCT01270360) and 1000
434 consecutive colonoscopies were to be enrolled, among them 500 individuals (Vatnimad)
435 without any digestive symptoms. The study protocol was approved by the ethics committee
436 of CPP Paris Est-Henri Mondor #10-006 on 2010). All participants signed an informed
437 consent.

438 Exclusion criteria for these cohorts were a history of colorectal surgery due to CRC,
439 familial adenomatous polyposis, Lynch syndrome, infection, and inflammatory bowel
440 disease and exposure to antibiotics during the 3 weeks preceding the colonoscopy.
441 Asymptomatic individuals had a FOBT (10) or a FIT before colonoscopy as enrolled in the
442 pilot or in the validation (Vatnimad) studies.

443

444 **Studies of microbiota from human participants**

445 *16S rRNA gene and whole metagenomic sequencing on stools*

446 Stool samples were collected in donors and patients during experimental and
447 validation studies, respectively; samples of stool were collected and stored within 4 hours
448 for DNA extraction using the GNOME[®] DNA Isolation Kit (MP Biomedicals, Santa Ana,
449 CA) as previously described (9-10, 12). After amplification by PCR of the V3-V4 region of
450 the 16S rRNA gene, sequencing was performed using a 250-bp paired-end sequencing
451 protocol on the Illumina MiSeq platform. Raw FASTQ files were demultiplexed, quality-

452 filtered using Trimmomatic (Sliding windows of 2 with a quality score of 20), and merged
453 using fastq-join from ea-utils (<https://expressionanalysis.github.io/ea-utils>). Taxonomic
454 assignments were performed using Qiime2 (no quality filtering; default parameters) (36)
455 with the SILVA-123 database; OTU were constructed using UCLUST (threshold of 97% of
456 similarity), Chimera Slayer for chimera removing and SILVA 16S rRNA database (version
457 123) for taxonomical assignment. The intergroup high similarity and intragroup low
458 similarity of microbiota were assessed by β -diversity, PCoA (generated by Qiime using
459 unweighted unfrac metrics). We subjected study populations to two Principle Coordinates
460 Analyses, independently of other data sets, and we investigated separation of CRC
461 microbiota from control microbiota in donors in the experimental study as well as in controls
462 versus CRC patients in the clinical trials. All microbiome statistical tests were produced
463 using the MetagenomeSeq packages or the Shaman Webserver that used the DESeq2
464 packages for differential expression analyses; therefore every statistical analysis for
465 microbiome have been corrected according to gender, age and BMI and adjusted for multiple
466 testing.

467 For a deep identification of bacteria species in the current study, DNA samples were
468 submitted to whole metagenomic analyses as previously described (10).

469

470 **Studies of methylation from human participants**

471 *DNA isolation and bisulfite (BS) conversion*

472 DNAs from colonic tissues (QIAamp DNA Mini Kit, Qiagen), blood and stool samples
473 (QIAamp DNA stool Mini Kit, Qiagen) were extracted using the ZR Serum DNA kit
474 (Ozyme, Montigny-le-Bretonneux, France) according to the manufacturer's protocol. The
475 DNA samples were then exposed to sodium bisulfite at 50°C in the dark for 16 hours (EZ
476 DNA Methylation kit, Zymo Research) to convert unmethylated cytosine nucleotides into

477 uracil nucleotides (subsequently converted to thymidine nucleotides during PCR cycling)
478 without changing the methylated cytosines as detailed elsewhere (10, 12).

479

480 ***DNA methylation using Illumina Golden Gate methylation bead arrays***

481 The GoldenGate Methylation Cancer Panel I (Illumina, San Diego, CA) was used to
482 probe 500 ng of each bisulfite-converted DNA sample of human (n=18, 9 CRC patients and
483 9 controls) including tissue (n=18), stool (n=18), and blood (n=18), the stool samples were
484 used for fecal microbiota transfer to germ free mice (SI Appendix, Table S11). Methylation
485 levels ranged from 0% to 100.0% were used for the calculation of the ratio of the methylated
486 signal intensity. The strategy for hierarchical clustering of gene candidates are further
487 described in Figure 3 and in SI Appendix, Fig. S8-S11. Briefly, comparisons across tissue,
488 stool, and blood samples identified genes with CpG loci methylation levels in the promotor
489 above the expected number. The CMI was computed by addition of the methylation values
490 of the three genes generated by the discovery study (characteristics of individuals in the pilot
491 study in SI Appendix, Table S12). Primers targeting all genes, (including albumin gene-
492 ALB, devoid of CpG sites and used as a housekeeping gene and for normalization of DNA
493 amounts), are reported elsewhere (SI Appendix, Table S13).

494

495 ***Quantitative methylation-specific PCR amplification and verification of the specificity***

496 Bisulfite-converted universal human methylated DNA standard (Zymo Research)
497 served as a calibrator and positive control and DNA-free distilled water as a negative
498 control. Quantitative, single-gene methylation-specific PCR (QS-MSP) and quantitative
499 multiplex methylation-specific PCR (QM-MSP) were applied. The relative methylation level
500 was determined using the $2^{-\Delta\Delta C_t}$ formula. Briefly, for each PCR run, a KAPA PROBE master

501 mix (Kapa Biosystems, Wilmington, MA) was prepared with pre-defined concentrations of
502 genes candidates.

503

504 ***Statistical analyses of blood CMI and FIT data in the validation cohort***

505 With the type I error set at 0.05 and assuming that CRC would be found in 8% of
506 asymptomatic and 25% of symptomatic individuals, to detect at least 60% of CRCs and 20%
507 of polyps with 90% specificity in average-risk (asymptomatic and aged ≥ 50 years) and high-
508 risk (history of polyps or sibling with CRC) individuals with 80% power, 1000 participants
509 (with at least 400 in each sub cohort) were needed. Characteristics of study populations were
510 described using number (%) for qualitative variables and mean \pm SD for quantitative
511 variables.

512 The main endpoint was identifying those individuals with advanced neoplasia
513 (invasive carcinoma or carcinoma *in situ* or specific carcinoma on any aspect in the rectum
514 or colon, roughly called CRC). Demographic, clinical, blood methylation and FIT data were
515 compared between patients with and without CRC using Pearson's chi-square test for binary
516 variables and Student's *t* test for continuous variables. Blood methylation data (CMI and
517 methylation of each of its three components, *Wif1*, *PENK*, and *NPY*) and FIT data were
518 handled as both binary and continuous variables. The following values were predefined to
519 classify tests as positive: >2 for the CMI and >150 ng/device for the FIT.

520 To determine whether CMI and FIT were associated with CRC, we built a multivariate
521 logistic regression model as described (37) adjusted for age, gender and BMI and the
522 adjusted odds ratios (ORs) and hazard ratios with their 95% confidence intervals (95% CIs)
523 were computed. All tests were two-tailed and *P* values <0.05 were deemed significant. The
524 statistical analysis software was Stata SE v15.0 (College Station, TX).

525

526 **Studies from experiments in mice**

527 Fresh stool samples were obtained from 9 females and 9 males in CCR1 cohort, for fecal
528 microbiota transfer (FMT) to germ-free mice. They were 9 with CRC and 9 with normal
529 colonoscopy (SI Appendix, Table S11).

530

531 *Fecal microbiota transfer (FMT) experiments*

532 FMT was performed in male C3H/HeN germ-free 8-week-old mice (Design of the
533 experimental study in SI Appendix, Fig. S1). Mice were maintained in gnotobiotic isolators,
534 *ad lib* for 1 week of acclimation to the laboratory conditions followed by fecal microbiota
535 transfer (FMT, day 0) then by 7 or 14 weeks of follow-up, after which the mice were
536 sacrificed. The fecal microbiota donors (SI Appendix, Table S11) were 9 consecutive
537 individuals with normal and 9 consecutive patients with CRC at colonoscopy from cohorts.
538 They were considered in the current study as normal microbiota (N- μ) and CRC microbiota
539 (CRC- μ) donors, were informed about the experimental study and accepted giving additional
540 stools, if needed, for animal experimentation during the study period. Fresh stools were
541 given by oral gavage, as follows: n=53 in 7-week study (CRC- μ transfer, n=30, and N- μ
542 transfer, n=23) and n=132 in 14-week study (CRC- μ transfer, n=66, and N- μ transfer, n=66).
543 In addition to the FMT, the mice were given intraperitoneal injections of either, the
544 carcinogen azoxymethane (AOM; Sigma; 8 mg/kg body weight once a week for three or 10
545 weeks), here chosen as a potential environmental exposure, or saline. In the 14-week study,
546 24 animals were given also polyethylene glycol (PEG3350) at a non-laxative dosage (1%) in
547 their drinking water to impede adherence of bacteria to the gut mucosa.

548 Associations between mice colonic tissue events, type of transferred microbiota (CRC- μ vs.
549 N- μ), chemical product given (AOM vs. saline), and bacterial composition in stools, were
550 assessed to evaluate potential associations by using Spearman's tests. The experimental

551 procedures were approved by the local ethics committee (Committee-reference number 45;
552 approval #12/076).

553 ***Cytokine and chemokine and cell signal measurements in mouse colonic mucosa in vivo***

554 cDNA synthesis from total RNA extracted from colon mucosa scrapings of mice, were
555 quantified using SYBR Green PCR Master Mix cDNA with the Light Cycler 480 System
556 (Roche Diagnostics) according to the manufacturer's instructions. The mRNA levels each
557 group (n=3) quantified and transcripts involved in cell renewal and those of involved in
558 inflammation (TNF α , MIP2, IL10, IL1 β , IL6, IL17 and IL17R) were analyzed. Three
559 housekeeping genes (GADPH, HRPT1, and TBP) were used. The comparative $\Delta\Delta$ CT-
560 method was used for relative mRNA quantification of target genes, normalized to GAPDH
561 and a relevant control equal to $2^{-\Delta\Delta$ CT. Relative levels of genes involved in cell renewal
562 (*Kfl4*, *Elf3*, *MATH1* and *Hes1*) or encoding proinflammatory cytokines and chemokines
563 (TNF α , MIP2, IL10, IL1 β , IL6, IL17, and IL17R) were analyzed using quantitative real-time
564 PCR (qRT-PCR) as previously described (38).

565

566 **Contribution:**

567 Iradj Sobhani: clinical trials, data collection, data analyses, funding, manuscript preparation
568 (writing and revising); corresponding author
569 Emma Bergsten: animal and experimental data, bacterial cultures and analysis, and
570 manuscript revising
571 Severine Couffin: animal and experimental data, histology, and manuscript revising
572 Aurelien Amiot: clinical data, monitoring, and manuscript revising
573 Biba Nebbad: microbiology, cultures, and manuscript revising
574 Caroline Barau: tissue and body fluid collection and quality assessment, manuscript revising
575 Nicola de'Angelis: surgery and post-surgical monitoring and clinical data, and manuscript
576 revising
577 Sylvie Rabot: animal experiments, data collection, biochemical assays, and manuscript
578 revising
579 Florence Canoui Poitrine: clinical data and statistical analysis, manuscript revising
580 Denis Mestivier: bioanalysis of data, bioinformatics analysis and manuscript reviewing
581 Thierry Pédrón: bacterial and cellular studies and manuscript revising
582 Khashayarsha Khazaie: inflammatory and immunology screening in mice and human tissues
583 and manuscript revising
584 Philippe Sansonetti: head of the laboratory, microbial function and manuscript revising

585

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609

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617

618

619 **Conflict of interest statement**

620

621 None of authors have any conflicts of interest to declare regarding this study; some of
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624

625

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818 **Figures legends**

819

820 **Figure 1. Histological patterns of murine colonic mucosa following FMT**

821 After the intestine was removed *en bloc* from caecum to anus, mucosa was carefully pinned
822 flat, without folds, to examine the totality of the colonic mucosa which were stained with
823 0.2% methylene blue (left slides of couple slides A to D) or HES (right slides of couple
824 slides A to D). Numbers of mice were as follows: n=53 in 7-week study (CRC- μ transfer,
825 n=30, and N- μ transfer, n=23) and n=132 in 14-week study (CRC- μ transfer, n=66, and N- μ
826 transfer, n=66). Bar scale: 50 μ m.

827 **A)** After FMT from healthy human controls (N- μ), no aberrant crypt foci (ACF) were visible
828 in the colonic mucosa (here after 14 weeks in left) and pattern of crypts was normal by HES
829 staining (right).

830 **B)** Elevated numbers of ACF were observed after FMT from patients with colorectal cancer
831 (CRC- μ) as compared to N- μ with multiple ACF (arrow in left slide) as verified by HES
832 (right slide).

833 **C)** ACF counts were higher in the animals given azoxymethane (AOM) as compared to N- μ .
834 Arrows indicate double ACF under bleu coloration (left slide) and illustration on HES slide
835 of colonic mucosa.

836 **D)** The combination of CRC- μ and AOM increased the ACF count with dysplasia in rare
837 cases (arrow).

838 **E)** No ACF no inflammatory cell infiltrate was visible in mice given N- μ under NaCl (HES)
839 although FISH staining showed density of bacteria trapped in the mucus layer (arrow, left
840 slide).

841 **F)** No ACF, no inflammatory nor injury were noticed when mice received polyethylene
842 glycol (PEG) in their drinking water as shown by H&S staining (HES) when FISH staining
843 shows clear decrease in density of bacteria trapped in the proximal mucus layer (arrow)

844 **G)** Representative pictures of KI67 staining after human FMT from patients with colorectal
845 cancer (CRC- μ) or from N- μ recipients.

846 **H)** Cell Proliferation assessed by Ki67 staining and ACF quantification 7 and 14 weeks after
847 FMT in mice in the intestinal mucosa were higher in CRC- μ than in N- μ recipients.

848 **I)** The number of ACF counts were enhanced depending on the length of mucosa examined
849 and the mice subgroups.

850 **J)** Comparative transcriptional levels of a set of inflammatory cytokines in the colonic
851 mucosa assessed by murine cytokine qPCR quantification showing a trend to higher IL1,
852 IL6, MIP2, IL17 and lower IFN γ , IL10 and IL23 in mice given CRC- μ alone (fold vs N- μ
853 given mice) with AOM boosting this effect that reached significance for IL6, TNF α , IL10 in
854 mice given CRC- μ +AOM (vs. N- μ +AOM).

855

856 **K)** Inflammatory cell infiltrate in the colonic mucosa as assessed by semi quantification on
857 HES stained slides (ten consecutive fields) under optic microscope magnification 20: a
858 pathologist blinded to animal groups used a semi-quantitative score to evaluate myeloid cell
859 infiltrate in the colonic mucosa as 0, 1, and 2 indicating absence, scarce, and numerous
860 inflammatory cells, respectively. The groups were compared by one-way ANOVA followed
861 by the Tukey-Kramer multiple comparisons post-hoc test. And no significant difference in
862 between mice groups was observed

863 * P <0.05; NS: not significant

864

865 **Figure 2. DNA changes in mice after human FMT**

866 Whole genome sequencing of total DNA extracted from colonic mucosa (n=12) and spleen
867 samples (n=6) were performed. **A.** All single-nucleotide polymorphisms (SNPs) in the colon
868 and spleen samples are compared to the reference mouse genome (GRCm38) and mutations
869 levels within gene segments are indicated. Mice given AOM showed the highest levels of
870 DNA alterations in exons or introns with a trend of additive effect of CRC- μ as compared to
871 N- μ . **B)** Distribution of animal subgroups according to the total gene mutations. DNA
872 changes in single-nucleotides with Pca scatter diagrams for colonic or spleen samples in the
873 groups of mice; the groups are identified by the type of human microbiota received (CRC- μ
874 or N- μ for CRC patients' and controls' stool, respectively) and type of treatment (AOM or
875 NaCl for azoxymethane or saline, respectively). **C)** Correlation circle of targeted gene
876 mutations in the colonic mucosa and spleen tissues according to Pca; vector length reflects
877 targeted gene mutation weight in the first two component analyses; targeted mutated genes
878 are indicated (those of Wnt pathway in red color). **D)** When mutations in all Wnt genes were
879 pooled together, rates of mutations were significantly higher in mice given AOM with an
880 additive effect of CRC- μ . The total number of mutations in *Wnt* pathway genes in both
881 colonic mucosa and spleen (Sp) was the highest in the animals given the CRC- μ and AOM

882 combination (see also Figure S6). The number of mutations was greater in colonic mucosa
883 (but not in spleen tissues) with AOM combined with CRC microbiota compared to AOM
884 combined with control microbiota. There was no significant effect in between colonic
885 mucosa due to CRC- μ alone as compared to N- μ alone. Col, colonic mucosa; Sp, spleen.

886 **E)** DNA epigenetic changes were investigated by using mEPIC array (39). The methylation
887 level of probes (n=63 987) were estimated after bisulfite modification of DNAs. Changes
888 based on the methylation of probes were investigated on DNAs from colon samples (n=16; 4
889 mice from each experimental group): the level of methylated probes was quantified as
890 reported (39) and ranged from 0 (not methylated) to 1 (fully methylated). DNAs were
891 classified as unmethylated if the methylation value was <0.2 and as hypermethylated if the
892 methylation value was >0.799 . Overall, mean and median values of all probes pooled
893 (n=63 987) in each group of mice showed lowest values in the group of CRC- μ + AOM
894 recipients and highest in control microbiota recipients.

895 **F)** Most of the probes were unmethylated in all animal groups; elevated numbers of both
896 hypomethylated and hypermethylated probes were observed in the mice given CRC- μ +
897 AOM.

898 **G)** Probes whose methylation level changed or remained unchanged under AOM in mice
899 given N- μ - or CRC- μ . Mice receiving CRC- μ had a greater number of genes with changed
900 methylation levels

901 **H)** Number of hypermethylated probes in each group showing highest level in mice given
902 CRC- μ and AOM combination compared to all other groups.

903 ** $P < 0.01$, * $P < 0.05$; NS: not significant

904

905

906 **Figure 3. Identification of hypermethylated genes related to fecal microbiota in human**

907 Overview on the strategy from experimental approach for the validation of gene methylated
908 targets in human based on microbiota donors (CRC patients or controls) in germ-free mice
909 experiments. Methylated genes in CRC-associated tissues and fluids were identified based
910 on their power for showing differences between normal colonoscopy individuals and CRC
911 patients. **A)** Human tissues and effluents were submitted to methylation gene array. Based on
912 significant differences of methylation values in CpG probes between controls (n=9) and
913 CRC patients (n=9) donors, genes were selected according to the promoter segments
914 hypermethylated in CRC patients. **B)** Bidimensional (right panel) and tridimensional (left

915 panel) distribution of genes regarding the difference in methylation values are indicated; in
916 red color are indicated 7 selected more discriminant genes regarding CRC patients and
917 controls; D: difference.

918

919 **Figure 4. Distribution of bacteria in fecal microbiota from patients with colorectal**
920 **cancer (CRC) and controls with normal colonoscopy findings**

921

922 Overall, 348 individuals from CCR2 cohort (173 asymptomatic individuals enrolled via a
923 mass CRC-screening program, and 165 patients from “Vatnimad” and “symptomatic” sub-
924 cohorts, respectively) enrolled. Invasive carcinoma, carcinoma in situ or specific carcinoma
925 on either flat mucosa or within a polyp in the rectum or colon were defined as CRC (n=177);
926 controls had no malignancy or significant polyp visible by full colonoscopy (n=171).

927 **A)** Pattern of microbiota clustering according to the diagnosis as assessed by Principal
928 Coordinate Analysis. The genus-level analysis based on distance matrix variances showed
929 significant differences between CRC patients and controls. Fecal DNA was subjected to
930 metagenomic sequencing of the conserved V3-V4 region of the 16S rRNA gene. The
931 amplicons were purified, quantified, and pooled then sequenced on an Illumina MiSeq
932 platform. For the analysis of 16S rRNA gene sequences, raw MiSeq FASTQ files were
933 demultiplexed, quality-filtered using Trimmomatic, and merged. Taxonomic assignments
934 were performed using Qiime2 (default parameters) with the SILVA-123 database. The
935 statistical analysis was done with MetagenomeSeq (36).

936 **B)** Pattern of microbiota clustering according to the blood methylation test as assessed by
937 Principal Coordinate Analysis. Analysis of variance using distance matrices on 789 OTUs
938 (metagenomeSeq_1.16.0) from 362 individuals (175 with normal colonoscopy findings and
939 187 with advanced neoplasia) demonstrated a significant difference between the groups with
940 positive and negative blood CMI values (≥ 2 and ≤ 2 , respectively).

941 **C)** Distribution of genera in fecal microbiota in the groups with positive and negative blood
942 CMI values (≥ 2 and ≤ 2 , respectively); Shaman c3bi platform; Institut Pasteur, Paris, France;
943 <http://shaman.c3bi.pasteur.fr/>). A maximum likelihood phylogenetic tree was tested (see
944 Methods). Brown-to-red colors indicate negative CMI results (≤ 2) and blue colors indicate
945 positive CMI results (> 2). Note that diversity was less in the group with a positive CMI
946 compared to the group with a negative CMI.

Table 1. Characteristics of participants in the validation CCR2 cohort^a

		CRC (n=187)	No CRC (n=812)	<i>P</i> value ^b
		Number (%) or mean ± SD		
Age, years (n=981)		63 [57-71]	60 [53-68]	<0.001
Gender (n=981)	Female	77 (41.2)	392 (48.8)	0.06
Body mass index, kg/m ²		25.31 [22.86-28.09]	25.31 [22.39-28.7]	0.96
History of GI cancer, (n=869)	Yes	7 (4.4)	31 (4.4)	0.97
History of non-GI cancer (n=868)	Yes	29 (18.4)	88 (12.4)	0.049
Family history of cancer (n=866)	Yes	19 (11.7)	114 (16.4)	0.13
Diabetes (n=980)	Yes	32 (17.1)	28 (16.3)	0.74
Blood methylation test				
Cumulative (<i>Wif1</i> + <i>PENK</i> + <i>NPY</i>) methylation index	>2	36 (20.3)	36 (4.6)	<0.001
<i>Wif1</i> + <i>PENK</i> + <i>NPY</i>	<i>Continuous</i>	0.16 [0.01-1.16]	0.12 [0.01-0.40]	<0.001
<i>Single gene</i>				
<i>Wif1</i> (n=956)	>2	20 (11.4)	21 (2.7)	<0.001
	<i>Continuous</i>	0.06 [0-0.49]	0.07 [0-0.24]	0.002
<i>PENK</i> (n=956)	>2	3 (1.7)	3 (0.4)	0.045
	<i>Continuous</i>	0 [0-0.04]	0 [0-0.02]	0.052
<i>NPY</i> (n=956)	>2	11 (2.3)	12 (1.5)	<0.001
	<i>Continuous</i>	0 [0-0.03]	0 [0-0]	0.03
CMI† in multivariate analysis Adjusted OR* [95% CI]	>2	4.92 [2.79-0.68]		0.005
FIT limited to the Vatnimad sub-cohort (n=468)	>150 ng**	45 (37.8)	20 (5.7)	<0.001
FIT limited to the Vatnimad sub-cohort (n=468)	<i>Continuous</i>	61.66 [1-379]	1 [0-9.67]	<0.001
FIT† in multivariate analysis Adjusted OR* [95% CI]				
Negative		reference		
Positive				

Not done	8.69 [4.66-16.21] 0.50[0.33-0.75]	<0.001 0.24

^a The cohort included 981 individuals from two 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. ^bStudent's *t* test and chi-square test for continuous and binary variables, respectively. *adjusted for age, gender and all variables listed in the table; †Positivity 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.

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

Comparisons between controls and CRC patients according to tumour staging and cohorts

Vatnimad (stage 0, I, II)			Symptomatic (stage III, IV CRC)		
Genus	Log ₂ fold change	Adjusted P values	Genus	Log ₂ fold change	Adjusted P values
<i>Ruminococcus</i>	-1.3	4.37E-02			
<i>Gemella</i>	-1.2	4.61E-02			
<i>Parvimonas</i>	-3.4	1.10E-09	<i>Parvimonas</i>	-2.0	1.16E-04
<i>Parasutterella</i>	-1.5	4.47E-02	<i>Parasutterella</i>	-1.4	1.72E-02
<i>Mogibacterium</i>	1.3	9.71E-03	<i>Mogibacterium</i>	1.2	5.41E-03
<i>Butyrivibrio</i>	1.0	2.18E-03			
<i>Lactonifactor</i>	1.0	2.35E-02	<i>Megasphaera</i>	1.3	1.10E-02
<i>Oscillospira</i>	0.8	4.93E-02	<i>Olsenella</i>	1.6	1.64E-05
<i>Howardella</i>	1.1	4.16E-02	<i>Howardella</i>	1.1	1.10E-02
<i>Abiotrophia</i>	0.9	2.31E-02	<i>Abiotrophia</i>	0.9	1.03E-02
<i>Eubacterium</i>	2.5	1.49E-07	<i>Eubacterium</i>	2.3	4.64E-08
<i>Acetitomaculum</i>	0.8	4.16E-02			
<i>Ezakiella</i>	1.1	2.76E-03	<i>Ezakiella</i>	0.9	1.03E-02
Comparison between individuals according to blood cumulative methylation index (CMI) values ≤ 2 (negative) versus >2 (positive)					
Genus	Log ₂ fold change	Adjusted P values	Genus	Log ₂ fold change	Adjusted P values
<i>Coprococcus</i>	1.1	4.717E-03	<i>Coprococcus</i>	1.26	4.0E-04
<i>Gemella</i>	-1.7	3.02E-02	<i>Dialister</i>	2.68	3.1E-04
<i>Parvimonas</i>	-2.3	4.10E-06	<i>Parvimonas</i>	-2.39	6.04E-06
<i>Peptostreptococcus</i>	-1.5	3.05E-5	<i>Peptostreptococcus</i>	-1.6	6.0E-04
<i>Oxalobacter</i>	-10.5	4.71E-04	<i>Fusobacterium</i>	-1.8	2.11E-03
<i>Acidamicococcus</i>	-1.9	3.05E-05	<i>Acidaminococcus</i>	-2.61	1.0E-04
			<i>Mitsuokella</i>	-2.2	5.24E-04
<i>Howardella</i>	1.9	3.06E-03	<i>Howardella</i>	1.1	1.10E-02
<i>Eubacterium</i>	2.5	1.49E-07	<i>Enterococcus</i>	2.7	5.9E-04
<i>Acetitomaculum</i>	0.8	4.16E-02			

<i>Shewanella</i>	-11.2	1.13E-3			
<i>Phenylobacterium</i>	11.4	3.71E-03			

*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.

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= logarithmic value; P values are given after adjustment on "age, gender and BMI" using Bonferroni-corrected Mann-Whitney U test.

Table 3. Species in the fecal microbiota in individuals with blood methylation test (negative versus positive)

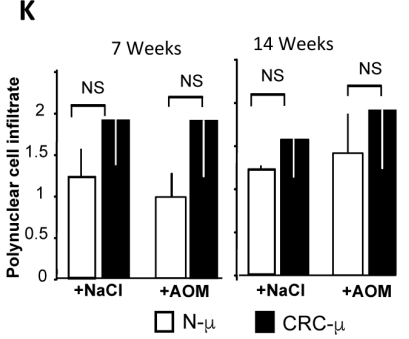
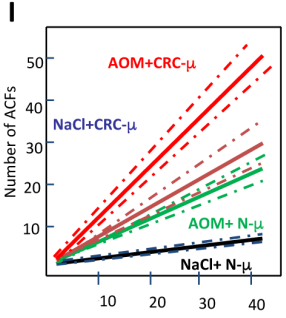
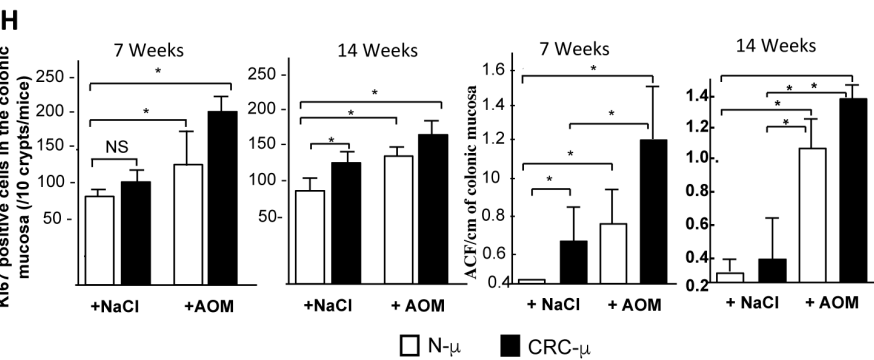
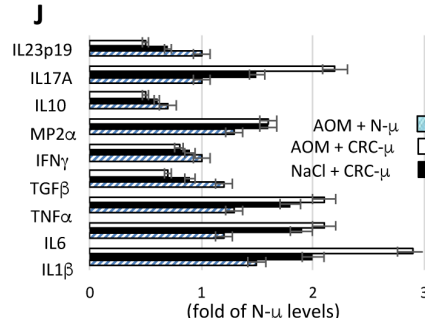
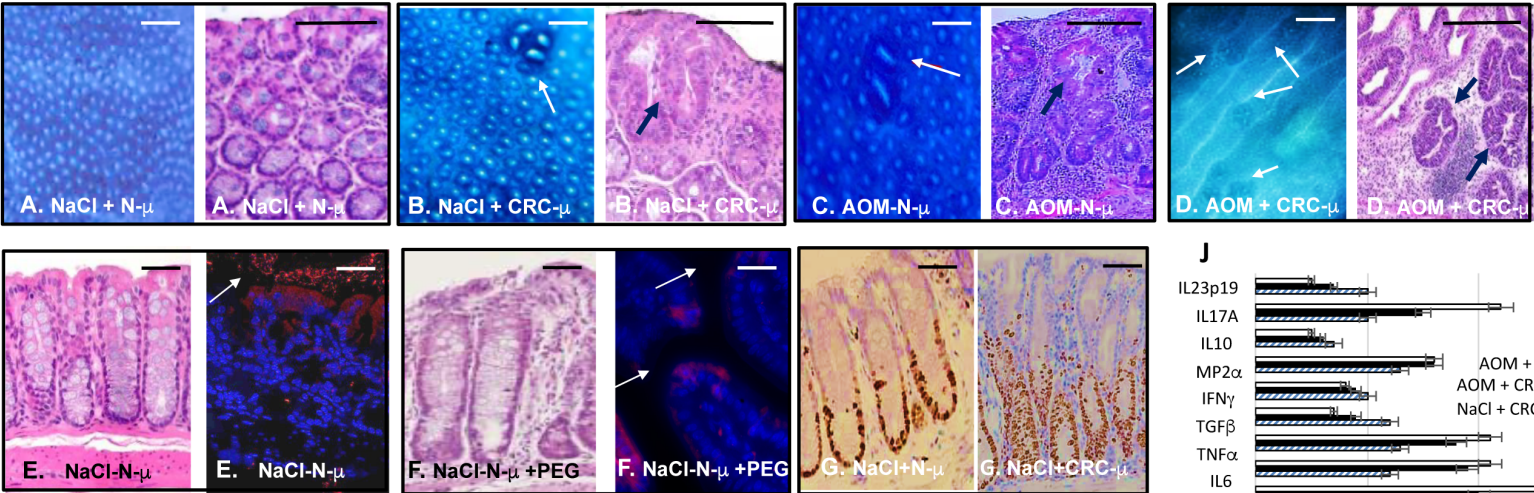
Species	Mean at baseline	Fold change	Log ₂ fold change	P value
Faecalibacterium prausnitzii [1574]	2480.5	2.2e+00	1.159	0.042
Ruminococcus sp. SR1/5 [1621]	2022	2.5e+00	1.354	0.004
Eubacterium hallii [1597]	1942.6	2.2e+00	1.14	0.045
Clostridium sp. L2-50 [1593]	1306.6	3.8e+00	1.945	0.004
Coprococcus comes [1616]	1306.5	2.1e+00	1.107	0.022
Dialister invisus [1259]	1023	6.2e+00	2.65	0.004
Coprococcus eutactus [1592]	941	2.9e+00	1.569	0.042
Bacteroides eggerthii [1097]	938	2.9e-01	-1.745	0.003
Ruminococcus obeum [1619]	714	2.3e+00	1.246	0.002
Clostridium bolteae [1598]	478	3.1e-01	-1.686	0.002
Bacteroides sp. D2 [1094]	367.5	3.8e-01	-1.384	0.042
Enterococcus faecalis [1363]	240	6.6e+00	2.723	0.042
Mitsuokella multacida [1653]	220	2.3e-01	-2.12	0.003
Parvimonas micra [1505]	211	2.1e-01	-2.22	0.003
Peptostreptococcus stomatis [1530]	110.5	2.0e-01	-2.26	0.002
Veillonella atypica [1260]	84.9	1.9e-01	-2.34	0.005
Streptococcus equinus [1381]	60.66	1.8e-01	-2.42	0.042
Gemella morbillorum	53	1.3e-01	-2.921	0.00004

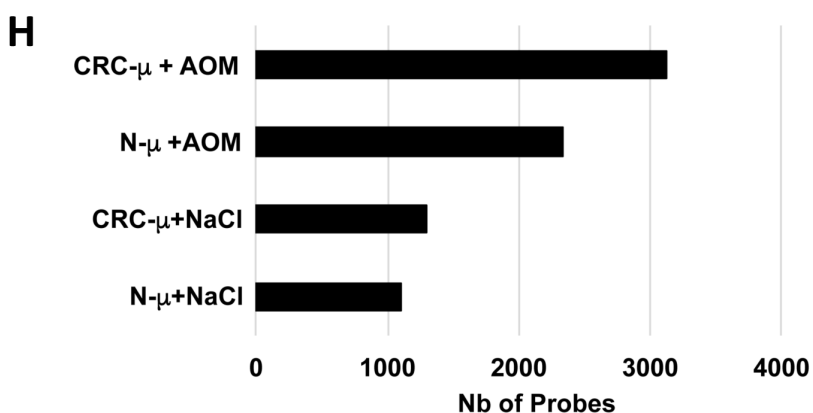
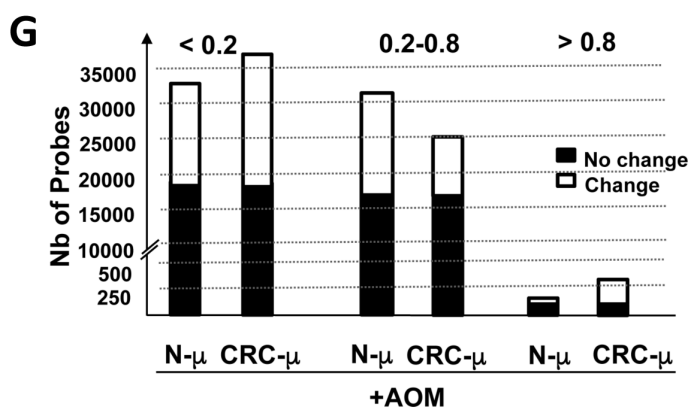
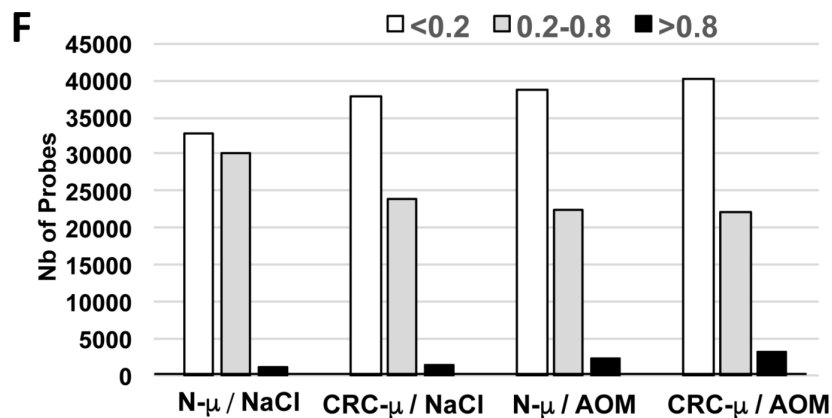
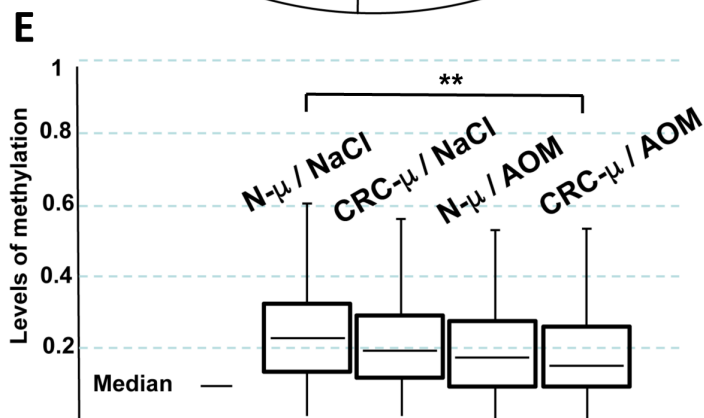
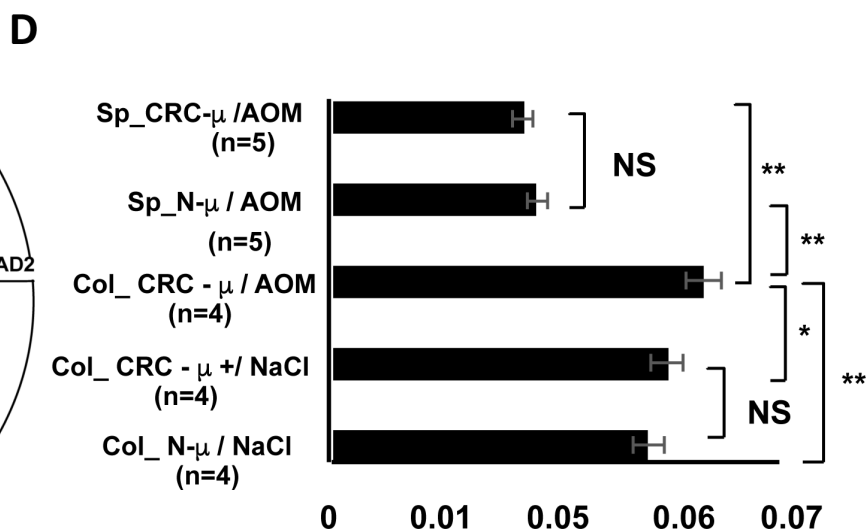
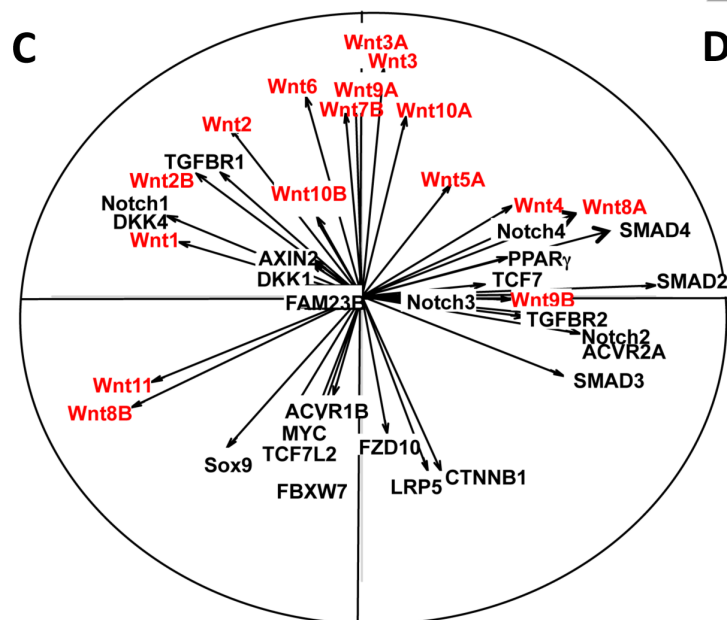
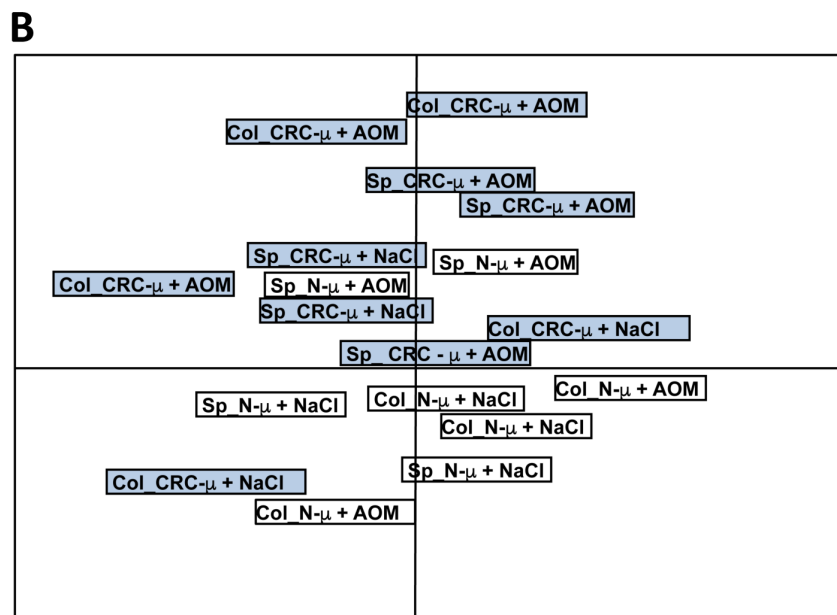
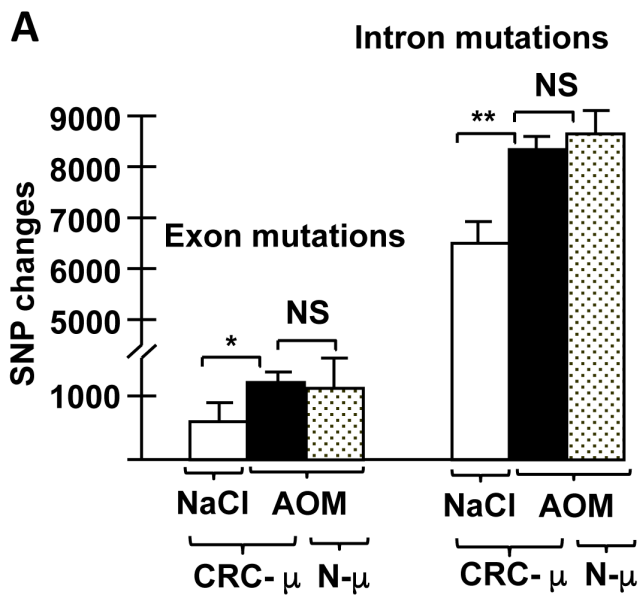
[1302]

Parvimonas sp. oral taxon 110 [1506]	35.6	2.7e-01	-1.86	0.006
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Parvimonas sp. oral taxon 393 [1507]	35	3.2e-01	-1.622	0.026
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Stool samples were submitted to whole metagenomic sequencing of fecal bacteria DNA (Controls, n=61 and 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). log₂=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 “-“ meaning the bacteria is higher in CRC patients’ microbiota and “+” meaning that the bacteria abundance was higher in controls.

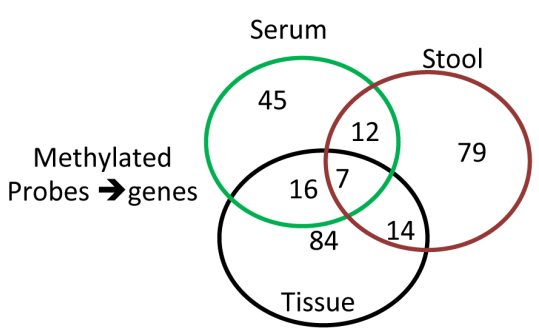




A Human Fecal Microbiota Transfer in mice → methylation/demethylation genes
 Back to the donors' tumours and effluents

→ using DNA methylation-array

1505 CpG probes → (statistical analysis CRC vs Control) → 195 CpG Probes highly methylated in CRC tissues or effluents



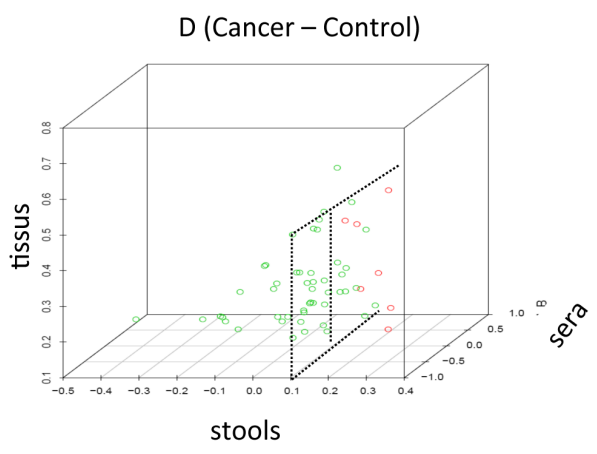
→ Selection for Gene candidates (see B)

- ALX4
- NPY
- PENK
- SEPT9
- SFRP1
- SFRP2
- SFRP3
- Wif1

→ Final selection for pilot and validation studies

- NPY
- PENK
- Wif1

B



D (Cancer - Control)

