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Fusobacterium and colorectal cancer: causal factor or passenger? Results from a large colorectal cancer screening study

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Abstract

Colorectal cancer is a leading cause of morbidity and mortality worldwide in both men and women. The gut microbiome is increasingly recognized as having an important role in human health and disease. Fusobacterium has been identified in former studies as a leading gut bacterium associated with colorectal cancer, but it is still not clear if it plays an oncogenic role. In the current study, fecal samples were collected prior to bowel preparation from participants of screening colonoscopy in the German BliTz study. Using 16S rRNA gene analysis, we examined the presence and relative abundance of Fusobacterium in fecal samples from 500 participants, including 46, 113, 110 and 231 individuals with colorectal cancer, advanced adenomas, non-advanced adenomas and without any neoplasms, respectively. We found that the abundance of Fusobacterium in feces was strongly associated with the presence of colorectal cancer (P-value < 0.0001). This was confirmed by PCR at the species level for Fusobacterium nucleatum. However, no association was seen with the presence of advanced adenomas (P-value = 0.80) or non-advanced adenomas (P-value = 0.80), nor were there any associations observed with dietary or lifestyle habits. Although a causal role cannot be ruled out, our observations, based on fecal microbiome, support the hypothesis that Fusobacterium is a passenger that multiplies in the more favorable conditions caused by the malignant tumor rather than a causal factor in colorectal cancer development.

Introduction

With estimated numbers of 1360000 new cases and 694000 deaths in 2012, colorectal cancer (CRC) is a leading cause of morbidity and mortality worldwide. It is the third most common cancer in men, after lung and prostate cancer, and the second most common cancer in women after breast cancer. Almost 55% of CRC cases occur in more developed regions, and the highest mortality rates are observed in central and eastern Europe (1). Hereditary forms, such as familial adenomatous polyposis and hereditary non-polyposis colon cancer, account for <5% of CRCs, whereas the majority of the cases are sporadic and thought to be related to lifestyle and environmental exposures (2,3). Recently, increased attention has been devoted to a potential role of the gut microbiome in colorectal carcinogenesis (4–7). Several studies reported significant differences in the
abundance of specific bacteria in the gut microbiome of patients with CRC compared with healthy controls.

One common bacterium, found to be significantly enriched in the gut microbiome of people diagnosed with CRC compared with controls, was *Fusobacterium* (8–12). Members of the genus *Fusobacterium* are a group of obligate anaerobic gram-negative bacteria belonging to the phylum *Fusobacteria* (13) and are part of the normal microbiota of the human oral cavity, upper respiratory tract and intestinal tract. Though most of the members of the *Fusobacterium* genus are normal commensals, the genus also comprises some pathogenic species among which are *Fusobacterium nucleatum*, *Fusobacterium necrophorum* and *Fusobacterium ulcerae* (14).

Some studies showed that species and subspecies of this genus, especially *F. nucleatum*, are enriched in either human colorectal adenoma or carcinoma tissue compared with adjacent healthy tissue (9,15–17), whereas others observed enrichment of *Fusobacterium* and *F. nucleatum* in fecal samples of people diagnosed with CRC or adenomas compared with healthy controls (8–11,18–20). Nevertheless, it is still uncertain if *Fusobacterium* is an oncogenic agent contributing to the development of CRC or an opportunistic bacterium, flourishing under the different conditions in the gut caused by the colorectal tumor.

The aim of this study was to examine the prevalence and abundance of *Fusobacterium* in stool samples collected from participants diagnosed at various stages of colorectal carcinogenesis at screening colonoscopy, i.e. those carrying non-advanced adenoma, advanced adenoma or CRC compared with individuals free of neoplasms, in order to provide clues as to a possible role of this bacterium in the development of CRC. Furthermore, as recent studies have shown that diet plays a key role in altering the composition of the gut microbial community (21,22), we also examined possible associations of *Fusobacterium* with sociodemographic, nutritional and lifestyle factors. Last but not least, we assessed the potential of *Fusobacterium* for early detection of CRC.

**Materials and methods**

**Study population and sample collection**

Participants were selected from the BliTz study. Details of the study design have been reported elsewhere (23–26). Briefly, BliTz is an ongoing prospective screening study among participants of screening colonoscopy in Germany that was initiated in 2005 with the primary aim of developing novel non-invasive tests for CRC screening. It is conducted in cooperation with 20 gastrointestinal practices in southern Germany. Pre-colonoscopy blood and stool samples, questionnaire data, colonoscopy and histology of the University of Heidelberg, Germany. Written informed consent was obtained from all participants with 20 gastrointestinal practices in southern Germany. Pre-colonoscopy blood and stool samples, questionnaire data, colonoscopy and histology

**Fecal sample collection and preparation**

Participants were given stool collection devices at a pre-colonoscopy practice visit. They were asked to fill small containers with fresh stool prior to their next practice visit, immediately freeze them at their home and bring them to the practice visit. At the practice, the stool-filled containers were immediately stored at −15 to −40°C, shipped on dry ice to a central laboratory and stored again at −70°C until further processing. The median time between stool collection date as reported by the participants and the arrival at the central laboratory was 7 days.

**Bacterial analysis of fecal samples and processing of sequencing data**

A small aliquot of stool was cut from each frozen stool sample and sent on dry ice to the laboratory for conducting microbiome measurements. DNA extraction and amplification of the V1–V2 region of the 16S rRNA gene platform was done as described previously (27) and obtained amplificates were sequenced on Illumina MiSeq in paired-end mode (2 × 250). The V1–V2 region was chosen as it provides the highest phylogenetic resolution ensuring most robust sequence classifications. Paired-end raw reads were then merged, quality filtered according to Cole et al. (28) and additionally filtered for a length of >250 bp. This yielded a mean of 41329 ± 12690 SD sequences per sample (lowest: 14458 sequences) that were subsequently analyzed using a ‘taxonomy supervised’ approach, where all sequences were directly assigned a taxonomic affiliation using Ribosomal Database Project (RDP) naïve Bayesian classifier applying a confidence cutoff of 80% (29). This method provides reliable information for taxonomic composition of a community at the genus level and enables quantitative analysis also for very low abundance taxa such as *Fusobacterium* spp. that might be filtered out by common operational taxonomic unit (OTU)-based approaches.

To get insights beyond the genus level, all reads classified as *Fusobacterium* spp. were aligned in MOTHUR (Gotway algorithm on SILVA reference database) and subjected to pre clustering (diff. = 2 bp). This procedure yielded tight clusters with sequences showing >98% identity to the respective representative sequence of each cluster. A neighboring tree was constructed based on representative sequences obtained after pre clustering together with reference sequences and assigned species/subspecies affiliations (for the reference tree and details on procedures, see Supplementary Figure S2, available at Carcinogenesis Online). Singletones (5.4% of all *Fusobacterial* sequences) were omitted in this analysis. Raw sequences were submitted to the European Nucleotide Archive (ENA) under accession no. PRJEB20036.

In order to properly address differences in library size and heteroscedasticity in the microbiome sequencing data without losing power (30), we followed the methodology formerly used in similar studies (8,9) and normalized the *Fusobacterium* abundance using percentage normalization to create relative abundance (RA) data.

**Fusobacterium nucleatum multiplex PCR**

Primers and probes for *F. nucleatum* multiplex PCR were specifically designed to detect the presence of all four completely sequenced *Fusobacteium* subspecies including *animalis*, *polymorphum*, *vincentii* and *nucleatum* by targeting acyl-coA-dehydrogenase (CoA, NCBI accession nos CP012713.1, CP013121.1, CP003700.1, CP012717.1) and murein hydrolase exporter (Fn1592, NCBI accession nos CP012713.1, CP013121.1, CP003700.1, CP012717.1) genes (Supplementary Table 1, available at Carcinogenesis Online). However, the PCR was not designed to distinguish between the subspecies. For internal DNA quality control, human DNA polymerase (polA) was co-detected.

Multiplex PCR and subsequent hybridization of PCR products was performed as described elsewhere (31). Briefly, multiplex PCR was performed in a final reaction volume of 25 µl comprising 1x Multiplex PCR Kit buffer (Qiagen, Hilden, Germany), containing 3 mM MgCl2, dNTP mix,
0.5× Q-solution and HotStartTaq DNA polymerase, 0.15–0.6 µM of each primer and 86 ng of purified DNA. A 15 min enzyme activation step at 95°C was followed by 40 cycles of amplification in a Mastercycler (Eppendorf, Hamburg, Germany). Each cycle included a denaturation step at 94°C for 30 s, an annealing step at 61°C for 90 s, and an extension step at 72°C for 60 s. The final extension step was prolonged for further 10 min and reactions were kept at 4°C.

The detection of amplicons was performed via hybridization reaction, adding 10 µl of PCR products to a mixture of SeroMap-beads coupled to F. nucleatum CoA and Fn1532 specific oligonucleotide probes. Next, heat denaturation, hybridization under stringent conditions and incubation with streptavidin-R-phycoerythrin (Molecular Probes, Leiden), followed by Luminex read-out, were performed to obtain median fluorescence intensity (MFI) values per target for each specimen.

For each probe, MFI values in reactions with no PCR product added to the hybridization mixture were considered as background values. Net MFI values were computed by subtraction of 1.2 times the median background value plus 5 MFI. All samples were analyzed in duplicates. Samples were defined as positive if the net MFI values in both duplicates were >5 MFI, and one fecal sample was scored positive if at least one of the two F. nucleatum genes was positive. If neither a F. nucleatum gene nor polA was above their respective cutoff in both duplicates, the sample was defined as ‘invalid’ and excluded from further analyses.

Statistical analysis

Characteristics of participants were tabulated and stratified by the four study groups that were defined according to the most advanced finding at colonoscopy (CRC, advanced adenoma, non-advanced adenoma or no neoplasm). F. nucleatum was studied in two ways: (i) presence/absence of F. nucleatum counts (binary) and (ii) RA (expressed as percentage). The groups were compared both across four groups and the CRC or adenoma groups versus the control group separately. We also assessed the association of F. nucleatum RA with CRC stage within the CRC group. The associations between F. nucleatum RA and sociodemographic, nutritional and lifestyle factors were assessed for all participants as well as for the CRC and control groups.

Finally, the association of F. nucleatum with presence of CRC was analyzed using logistic regression modeling, and its predictive ability was estimated using the area under the receiver-operating characteristic curve (AUC). All statistical tests (Fisher’s exact test for binary variables and Kruskal–Wallis rank sum test for numerical and ordinal characteristics)
Results

Fusobacterium genus and CRC

Study participants' characteristics are displayed in Table 1. The genus Fusobacterium was found in stool samples of 27.2% of study participants. Fusobacterium was found to be significantly more common in the CRC group (54.3%) than in all other groups (23.6–25.1%) (Kruskal–Wallis rank sum test, P < 0.001, Table I and Figure 2a). The stage distribution of CRC cases in our sequencing data was: stage I n = 18, stage II n = 6, Stage III n = 19 and stage IV n = 3. Within the CRC group, Fusobacterium RA was positively associated with cancer stage (Kruskal–Wallis rank sum test, P-value = 0.049) (Figure 2b). A Dunn’s post hoc test showed significant differences between stage I and stage II (unadjusted P-value = 0.012) and stage I and stage III (unadjusted P-value = 0.042). No statistically significant differences in Fusobacterium RA were found between advanced adenoma (n = 113) and control groups (n = 231) (P-value = 0.802) or non-advanced adenoma (n = 110) and control groups (P-value = 0.803).

The sequencing of regions V1–V2 enabled us to look at Fusobacterium beyond the genus level, applying a two-step phylogenetic approach. First, all obtained sequences that clustered in the main clade constituting all F. nucleatum reference strains, Fusobacterium periodonticum and two strains isolated from animals were analyzed together as ‘Fusobacterium clade’. This clade contained 70.1% of all Fusobacterial reads (excluding singletons), that were present in 23% of participants, where they found to be significantly more common in CRC participants (51%) than in healthy controls (20%) (Fisher’s exact test, P-value < 0.001), whereas no significant differences between carriers of adenomas or advanced adenomas and healthy controls were observed. Within the CRC group, RA of this clade was not associated with cancer stage (P-value = 0.160). In the second step, sequencing results constituting ‘Fusobacterium clade’ were assigned a species/subspecies based on their position on the tree (88.8% of sequences).

Fusobacterium simiae, F. periodonticum, F. nucleatum, F. nucleatum ssp. animalis, F. nucleatum ssp. vincentii and F. nucleatum ssp. polymorphum were present in 1.3, 7.8, 0.9, 11.7, 7.8 and 5.6%, of healthy participants, respectively. Among participants with CRC, these taxa were present in 2.2, 23.9, 4.3, 19.6, 15.2 and 13.0% of cases, respectively. A statistically significant difference in association between CRC patients and controls was only found for the species F. periodonticum (P-value = 0.003).

Fusobacterium nucleatum and CRC

To further validate the sequencing results, we conducted PCR for F. nucleatum. The PCR analysis included 430 participants of whom 44 were diagnosed with CRC, 94 participants with advanced adenoma, 99 participants with non-advanced adenoma and 193 healthy controls. Fusobacterium nucleatum was, in accordance with the sequencing results, much more commonly found in the CRC group (45%) compared with all other groups (advanced adenomas: 28%; non-advanced adenomas: 24% and controls: 30%; P-value = 0.022). In accordance with the sequencing results, no statistically significant difference was detected between participants with advanced adenoma (N = 94) and controls (N = 193) in the prevalence of F. nucleatum (P-value = 0.783).

Fusobacterium, nutrition and health behavior

We analyzed the relationship between Fusobacterium RA at genus level and dietary exposures, including consumption of processed meat products, red meat, meat in general, the frequency of vegetable consumption, smoking, drinking alcohol in general and beer in particular and the consumption of whole grains. Statistical analyses were conducted within the CRC group, within the advanced adenoma group, within the control group alone and within the entire study population. No

Table 1. Study population characteristics according to most advanced finding of colonoscopy

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>All (N = 500)</th>
<th>Colorectal cancer</th>
<th>Advanced adenoma</th>
<th>Non-advanced adenoma</th>
<th>Control (N = 231)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>50–86</td>
<td>55–81</td>
<td>50–86</td>
<td>50–79</td>
<td>50–80</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Mean</td>
<td>63.2</td>
<td>66.9</td>
<td>64.8</td>
<td>62.4</td>
<td>62.1</td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Men</td>
<td>280 (56%)</td>
<td>31 (67.4%)</td>
<td>71 (62.8%)</td>
<td>68 (61.8%)</td>
<td>110 (47.6%)</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td>Women</td>
<td>220 (44%)</td>
<td>15 (32.6%)</td>
<td>42 (37.2%)</td>
<td>42 (38.2%)</td>
<td>121 (52.4%)</td>
<td></td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>26.8</td>
<td>28.3</td>
<td>26.2</td>
<td>26.9</td>
<td>26.8</td>
<td>0.497**</td>
</tr>
<tr>
<td>IQR</td>
<td>24.3–30.0</td>
<td>25.0–30.4</td>
<td>24.3–28.8</td>
<td>24.8–29.8</td>
<td>24.5–30.0</td>
<td></td>
</tr>
<tr>
<td>Family history of CRC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>471 (94.2%)</td>
<td>43 (93.5%)</td>
<td>105 (92.9%)</td>
<td>104 (94.5%)</td>
<td>219 (94.8%)</td>
<td>0.864**</td>
</tr>
<tr>
<td>No</td>
<td>29 (5.8%)</td>
<td>3 (6.5%)</td>
<td>8 (7.1%)</td>
<td>5 (5.5%)</td>
<td>12 (5.2%)</td>
<td></td>
</tr>
<tr>
<td>Smoking status</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Current</td>
<td>59 (11.8%)</td>
<td>3 (6.5%)</td>
<td>17 (15.0%)</td>
<td>17 (15.5%)</td>
<td>22 (9.6%)</td>
<td>0.204**</td>
</tr>
<tr>
<td>Never</td>
<td>208 (41.6%)</td>
<td>25 (54.3%)</td>
<td>47 (40.0%)</td>
<td>44 (40%)</td>
<td>92 (40%)</td>
<td></td>
</tr>
<tr>
<td>Alcohol (on how many days per week)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>105 (22.8%)</td>
<td>8 (19.5%)</td>
<td>20 (18.5%)</td>
<td>26 (26.3%)</td>
<td>51 (23.9%)</td>
<td>0.369**</td>
</tr>
<tr>
<td>1 day</td>
<td>94 (20.4%)</td>
<td>5 (12.2%)</td>
<td>23 (21.3%)</td>
<td>17 (17.2%)</td>
<td>49 (23.0%)</td>
<td></td>
</tr>
<tr>
<td>&gt;1 day/week</td>
<td>26 (23%)</td>
<td>29 (68.3%)</td>
<td>65 (60.2%)</td>
<td>56 (56.5%)</td>
<td>113 (53.1%)</td>
<td></td>
</tr>
<tr>
<td>Fusobacterium presence</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>136 (27.2%)</td>
<td>25 (54.3%)</td>
<td>27 (23.9%)</td>
<td>26 (23.6%)</td>
<td>58 (25.1%)</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td>No</td>
<td>364 (72.8%)</td>
<td>21 (45.7%)</td>
<td>86 (76.1%)</td>
<td>84 (76.4%)</td>
<td>173 (74.9%)</td>
<td></td>
</tr>
</tbody>
</table>

BMI, body mass index; IQR, interquartile range.
*Kruskal–Wallis rank sum test.
**Fisher exact test.

Note: P-values below a significance level of 0.05 were considered statistically significant. We used R version 3.2.3 (2015-12-10) for all statistical analyses (32).
statistically significant associations were found between dietary and lifestyle exposures and Fusobacterium genus RA, except for a positive association between higher body mass index and Fusobacterium RA in the control group alone and an inverse association between vegetable consumption and Fusobacterium in the advanced adenoma group (Table 2).

**Colorectal cancer detection models with Fusobacterium**

Figure 3 shows the results of a predictive model to distinguish CRC cases (n = 46) from the rest of the study population (n = 454). A model including only Fusobacterium had an AUC of 0.676, whereas a model including only age and sex had an AUC of 0.677. The combined model yielded an improved AUC of 0.715, though the difference between the combined model and the one based only on age and sex had only borderline statistical significance (P-value = 0.052). When comparing CRC cases (n = 46) to healthy controls (n = 231), the AUC of the model including only Fusobacterium was 0.675. The combined model had an AUC of 0.720 compared with 0.580 for F.nucleatum and AUC of 0.714 for age and sex alone. On the other hand, we did not observe Fusobacterium genus improving the ability of the age and sex based model to discern between adenomas, either advanced or non-advanced and healthy controls (Supplementary Figure S1, available at Carcinogenesis Online).

**Discussion**

In this study, we conducted a 16S rRNA gene analysis in fecal samples of 500 participants of screening colonoscopy, including 46 participants diagnosed with CRC, 113 with advanced adenoma, 110 with non-advanced adenoma and 231 controls free of neoplasms, all recruited in a true population-based CRC screening setting. Fusobacterium genus abundance in feces was found to be strongly associated with presence of CRC, but not with presence of advanced or non-advanced adenoma. Within the CRC cases, abundance of Fusobacterium was positively associated with more advanced cancer stages (though numbers in some groups were very small). When used in a predictive model for CRC diagnosis, Fusobacterium slightly improved performance of the model based solely on age and sex. To validate our sequencing results, we have also conducted multiplex PCR for F.nucleatum on a large subset of our study. PCR results were similar to sequencing results at the genus level.

Species belonging to the Fusobacterium genus are commensal anaerobes that are commonly found in the human oral...
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Some species belonging to the *Fusobacterium* genus have also been found to be associated with occurrence of CRC. It is, however, still under debate whether *Fusobacterium* is a passenger taking advantage of the conditions caused by the tumor or if the bacterium has a causative role in CRC development.

A potential role of *Fusobacterium* as a carcinogenic agent is supported by several observations. Nosho et al. (11,33) found an association between highly enriched *F.nucleatum* in colorectal carcinoma tissue and microsatellite instability high status (MSI high) (34). According to that study, *F.nucleatum* increases the production of reactive oxygen species and inflammatory cytokines in CRC, leading to reduced enzymatic activity of mismatch repair proteins and MSI. Rubinstein et al. (35) indicate that *F.nucleatum* adheres to and invades endothelial and epithelial cells, through its FadA adhesin. This is accompanied by increased expression of transcription factors, oncogenes, Wnt and inflammatory genes as well as growth stimulation of CRC cells.

Kostic et al. (9) found that the introduction of human *F.nucleatum* accelerated the onset of colonic tumors in mice. They reported that *Fusobacterium* selectively expanded myeloid-derived immune cells that are key components in neoplastic progression and was also associated with a human CRC gene expression signature.

On the other hand, other studies, including the current study, found an association of *F.nucleatum* in feces with colorectal neoplasms exclusively for CRC, but not for colorectal adenomas, the precursor of most CRCs (8,10). This finding, along with the positive association of *Fusobacterium* genus with more advanced tumor stage, supports the assumption that *Fusobacterium* is a passenger bacterium or that it only promotes cancer progression in further advanced colorectal lesions rather than playing a causative role at the onset of carcinogenesis.

It would, however, be conceivable that even in the case that *Fusobacterium* does not play a causal role in the initiation and early development of CRC it could still be useful as a screening tool.

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**Table 2. Association of nutritional and lifestyle factors with *Fusobacterium* RA**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Healthy controls</th>
<th>Advanced adenoma</th>
<th>Colorectal cancer</th>
<th>Total study population</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>N</em> = 231</td>
<td><em>N</em> = 113</td>
<td><em>N</em> = 46</td>
<td><em>N</em> = 500</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMI (kg/m²) (&lt;25/&lt;25 overweight)</td>
<td>0.423†</td>
<td>0.736</td>
<td>0.232</td>
<td>0.241</td>
</tr>
<tr>
<td>Age (years) (&lt;65/&gt;65 elderly)</td>
<td>0.043†</td>
<td>0.403</td>
<td>0.590</td>
<td>0.824</td>
</tr>
<tr>
<td>Processed meat (none/conce a week/few times a week/&gt;once per day)</td>
<td>0.606†</td>
<td>0.431</td>
<td>0.402</td>
<td>0.665</td>
</tr>
<tr>
<td>Red meat (none/conce a week/few times a week/&gt;once per day)</td>
<td>0.807†</td>
<td>0.929</td>
<td>0.600</td>
<td>0.482</td>
</tr>
<tr>
<td>Any meat (none/conce a week/few times a week/&gt;once per day)</td>
<td>0.262†</td>
<td>0.554</td>
<td>0.657</td>
<td>0.696</td>
</tr>
<tr>
<td>Vegetables (none/conce a week/few times a week/&gt;once per day)</td>
<td>0.802†</td>
<td>0.520</td>
<td>0.559</td>
<td>0.764</td>
</tr>
<tr>
<td>Whole grains (none/conce a week/few times a week/&gt;once per day)</td>
<td>0.726†</td>
<td>0.032</td>
<td>0.114</td>
<td>0.451</td>
</tr>
<tr>
<td>Alcohol (none/conce a week/&gt;1 day/week)</td>
<td>0.823†</td>
<td>0.801</td>
<td>0.082</td>
<td>0.808</td>
</tr>
<tr>
<td>Smoking (never/past/current)</td>
<td>0.597†</td>
<td>0.670</td>
<td>0.440</td>
<td>0.573</td>
</tr>
</tbody>
</table>

BMI, body mass index.
†Kruskal–Wallis rank sum test and asymptotic.
†Wilcoxon–Mann–Whitney test.

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**Figure 3.** Predictive models including *Fusobacterium*. (a) Colorectal cancer cases (*n* = 46) compared with all the others (*n* = 454). (b) Colorectal cancer cases (*n* = 46) compared with healthy controls (*n* = 231).
tool if its presence helped to reliably detect early stage cancers. To date, there are at least two studies that used Fusobacterium in classification algorithms for discrimination between CRC patients and healthy controls that were validated in independent samples (8,11). Zeller et al. (8) have created a metagenomics classifier for non-invasive early detection of CRC based on a combination of marker species found in fecal samples. The classifier model consisted of 22 species, which included two Fusobacterium subspecies: F.nucleatum subsp. vincenii and F.nucleatum subsp. animalis. This model alone had an AUC of 0.84 and an AUC of 0.87 when it was combined with guaiac fecal occult blood test (Hemoccult gFOBT) for diagnosing CRC in the study population.

Another model for the early detection of CRC was suggested by Yu et al. (11) and included several gene markers, one of which was a gene from F.nucleatum. The researchers found that the combined qPCR measurements of just 2 OTUs, butyryl-CoA dehydrogenase from F.nucleatum and rpob from Parvimonas micra, accurately classified CRC samples in a Chinese population of 47 CRC cases and 109 healthy controls with an improved AUC of 0.84. Prevalence and relative abundance of these two markers were higher in stage II and III than in stage I, which is in line with our results with regards to stage-specific RA of Fusobacterium. However, in the interpretation of the apparently higher AUC achieved in these studies compared with ours it has to be kept in mind that both previous studies included clinically selected CRC cases rather than CRC cases detected by screening. The findings of these studies may not necessarily hold in a true screening setting as clinical diagnoses are typically made at more advanced stages and after diagnostic colonoscopies, which may alter the gut microbiome. Furthermore, even those apparently high AUCs are lower than AUCs that can be achieved with fecal immunochemical tests for hemoglobin (36).

The low prevalence of Fusobacterium in fecal samples from participants with advanced adenoma and stage I cancer may further limit the use of species or subspecies from the Fusobacterium genus as biomarkers for the early detection of CRC in population-based screening. However, it should be elucidated whether and to what extent Fusobacterium may still be a possible target for therapeutic intervention that may improve the cancer outcome, as is the case in other diseases such as adverse pregnancy outcomes and rheumatoid arthritis (37).

A particular strength of this study was its conduction in a true screening population, i.e. the target population in which to assess potential use of Fusobacterium as a tool for early diagnosis of CRC or for risk stratification in CRC screening. Furthermore, parallel evaluation of Fusobacterium prevalence and abundance among people with CRC, advanced adenoma, non-advanced adenoma and healthy participants, enabled evaluation of a potential role of Fusobacterium in early stages of colorectal carcinogenesis. In particular, the study included a relatively large sample of 500 participants, among whom there were 46 and 113 participants with screening colonoscopy detected CRC and advanced adenoma, respectively. All stool samples for the study were collected before preparation for colonoscopy, a process that may affect the composition of the gut microbiome (38–40). The samples were collected at home and transported by the study participants to their gastroenterologists, thus representing the real environmental conditions of fecal sample collection in population-based screening and their influences, if any, on fecal Fusobacterium levels.

One limitation of the study is that we assessed Fusobacterium solely in stool samples, which only partially reflect the mucosal microbiota in CRC (41). Flanagan et al. (42) showed that although in their study F.nucleatum was more abundant in stool samples from CRC patients compared with individuals with adenoma or controls, the levels of Fusobacterium in the stool did not correlate with the levels of Fusobacterium found in cancer or adenoma tissue from the same individuals. Gevers et al. found that differences in the microbiome, including in Fusobacterium levels, could only be observed in tissue samples but not in stool samples collected at the time of diagnosis (43). Another limitation of the study is that information regarding antibiotic use by the participants in the period immediately before giving the stool samples, a potentially important confounder in microbiome studies, was not available so could not be controlled for in the analyses. However, recent or current antibiotics use is probably to be rare among healthy participants of a screening program, suggesting that the potential for confounding due to antibiotics use would have been very small.

In conclusion, although we found the prevalence of Fusobacterium to be higher in fecal samples of participants with CRC than in fecal samples of other participants, our results rather support the hypothesis that Fusobacterium is a passenger that multiplies in the more favorable conditions caused by the malignant tumor than the hypothesis that Fusobacterium is a causal factor in cancer development, even though a causative role in the progression of the tumor cannot, of course, be ruled out. Our findings also do not support a major role for the use of Fusobacterium alone as a tool in population-based screening programs, but may be combined with other microbiome biomarkers and clinical tests as has been demonstrated in previous studies. Further research should address whether Fusobacterium may still be a possible target for therapeutic intervention that may improve the cancer outcome.

**Supplementary material**

Supplementary data are available at Carcinogenesis online.

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**References**


