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Cancer-associated fecal microbial markers in colorectal cancer detection

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Colorectal cancer (CRC) is the second most common cause of cancer death in the western world. An effective screening program leading to early detection of disease would severely reduce the mortality of CRC. Alterations in the gut microbiota have been linked to CRC, but the potential of microbial markers for use in CRC screening has been largely unstudied. We used a nested case–control study of 238 study subjects to explore the use of microbial markers for *clbA*+ bacteria harboring the *pks* pathogenicity island, *afa-C*+ diffusely adherent *Escherichia coli* harboring the *afa-1* operon, and *Fusobacterium nucleatum* in stool as potential screening markers for CRC. We found that individual markers for *clbA*+ bacteria and *F. nucleatum* were more abundant in stool of patients with CRC, and could predict cancer with a relatively high specificity (81.5% and 76.9%, respectively) and with a sensitivity of 56.4% and 69.2%, respectively. In a combined test of *clbA*+ bacteria and *F. nucleatum*, CRC was detected with a specificity of 63.1% and a sensitivity of 84.6%. Our findings support a potential value of microbial factors in stool as putative noninvasive biomarkers for CRC detection. We propose that microbial markers may represent an important future screening strategy for CRC, selecting patients with a "high-risk" microbial pattern to other further diagnostic procedures such as colonoscopy.

Tumor Markers and Signatures

Colorectal cancer (CRC) is the second most diagnosed cancer in women, and the third in men worldwide.[1,2] The mortality of patients with metastatic disease is high, indicating the necessity of a good and reliable screening method to detect the tumor at an early operable stage. Colonoscopy is currently the most reliable method for detection of early staged CRC, but it is uncomfortable for the patients, time consuming and costly. Recent studies have shown that changes in the intestinal microbiota are associated with CRC.[3–6] A noninvasive screening method, analyzing cancer-associated microbial alterations in stool, may have many benefits for both the health care system and the participating patients.

The gut microbiota plays many important roles in digestion, but has at the same time been implicated in diseases of the host. In the last years, accumulating evidence suggests that interactions between the mucosa and the microbiota are important, in both immunology and tumorigenesis (reviewed in Ref. 7). A number of studies have also provided mechanistic evidence that specific bacterial populations can change the milieu in the mucosa, promoting a proinflammatory response, and inducing double-stranded DNA breakage and mutations that can lead to tumor initiation and progression.[8–17]

Bacteria positive for *clbA* harbors the *pks* pathogenicity island and produces colibactin, a genotoxin capable of inducing double-stranded DNA breaks and cellular senescence, leading to increased production of growth factors that can stimulate tumor growth.[18,19] Colibactin-producing *Escherichia coli* (*E. coli*) and *afa-C*+ diffusely adherent *E. coli* (DAEC) have both been linked to human CRC.[20–22] *Escherichia coli* carrying *pks* have been found in 56–67% of human colorectal tumors compared to around 20% in controls.[20,22] DAEC carrying the afimbrial adhesin (*afa-1*) operon were shown by Prorok-Hamon *et al.* to be more common among *E. coli*

Key words: gut microbiota, *clbA*, *F. nucleatum*, stool, screening, colorectal cancer

Abbreviations: CRC: colorectal cancer; *E. coli*: *Escherichia coli*; DAEC: diffusely adherent *E. coli*; *F. nucleatum*: *Fusobacterium nucleatum*; FECSU: Faecal and Endoscopic Colorectal Study in Umeå; CAMA: cancer-associated microbial alterations

Additional Supporting Information may be found in the online version of this article.

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What's new?

Nobody looks forward to a colonoscopy, and now a pair of telltale bacteria could help people avoid them. Researchers know that microbial changes occur in colorectal cancer, and have hoped these microbial changes could provide less invasive screening tools to detect tumors. These authors conducted a nested case–control study investigating 3 bacterial markers in 238 patients. Two of the markers, *clbA*+ bacteria and *Fusobacterium nucleatum*, successfully predicted colorectal cancer with high sensitivity, particularly when tested together.

strains isolated from CRC patients compared to controls.[21] Compared to 17–36% of controls, 67–80% of colorectal tumors are found to be positive for *afaC*.[21,22] The *afa-1*+ DAEC have the ability to adhere to, and invade epithelial cells and likely play a role in epithelial-to-mesenchymal transition.[16]

Fusobacterium nucleatum (F. nucleatum) is part of the commensal flora of the gut and oral cavity, but has been linked to a number of pathological conditions, including periodontitis, appendicitis, inflammatory bowel disease (IBD) and CRC.[23–28] *F. nucleatum* has been found in higher levels in CRC, and adenomas, compared to adjacent normal tissue.[10,23–25] It is a highly adhesive bacterial species and has the ability to invade colonic epithelial cells.[13,29] Additionally, recent studies showed that, in human CRC, high amounts of *F. nucleatum* in tumor tissue is correlated to low infiltration of T lymphocytes and poor patient prognosis.[30,31]

In this study, we investigated the utility of microbial markers for *clbA*+ bacteria (*clbA*) and, *afa-1*+ DAEC (*afaC*), and *F. nucleatum*, in CRC detection, using 238 human stool samples from the FECSU (the Faecal and Endoscopic Colorectal Study in Umeå) cohort.

Material and Methods

Study cohort

The study is based on the Faecal and Endoscopic Colorectal Study in Umeå (FECSU) cohort of 1136 patients who went through colonoscopy at the University Hospital in Umeå, Sweden, between the years 2008–2013 (September 2008 to March 2013). Indications for colonoscopy were gastrointestinal symptoms of large bowel disease, visible bleeding and/or positive fecal hemoglobin (F-Hb) test. A total of 2660 patients were scheduled for colonoscopy during the time period (see flow chart in Fig. 1). Independent of underlying indications, 1997 patients were invited to participate in the study. Exclusion criteria were planned colonoscopy within 1 week, dementia and low-performance status, including mentally or physically disabled persons. Of the invited patients, 861 patients denied participation.

Patients were asked to leave stool samples before the precolonoscopy cleansing procedure started. Study information and tubes for stool sample collection were sent to the patients together with the invitation for the clinical colonoscopy examination. The colonoscopy was routinely performed at the endoscopy unit and the clinical findings were recorded. Biopsies were taken when clinically relevant. Lesions/findings were recorded by a pathologist, and the neoplastic lesions were further classified as low-grade dysplasia, high-grade dysplasia or adenocarcinoma according to the WHO classification of tumors of the digestive system.[32] If several lesions were present, the most severe lesion was recorded for the patient.

The study protocol was approved by the Regional Ethical Review Board in Umeå, Sweden (Dnr 08–184 M; Dnr 07–045 M). All individuals in this study have signed a written consent form.

Selection of study subjects

Cases with CRC or dysplasia were identified by reading patient records including the pathology reports. Patients selected for the study included all the 39 identified cases of CRC, all the 135 cases of low- and high-grade dysplasia and 66 controls. All study subjects included were adults with the youngest 34 years of age. As the number of cases with high-grade dysplasia was low (only ten cases), both low- and high-grade dysplasia were included in one group of dysplasia. Controls were selected from the group of FECSU patients where a biopsy was taken, but recorded with no neoplastic findings. Patients with IBD or findings of hyperplastic polyps were excluded from the controls. The controls were matched by age and gender to the CRC cases and a randomized subset of the dysplasia group.

Stool/tissue collection and storage

With the envelope of invitation for colonoscopy examination that were sent to the patients, three tubes for stool sample collection were enclosed, along with information about the collection procedure; one tube containing 5 ml of preservative buffer, RNA*later*® (Ambion®), one tube for fecal hemoglobin (F-Hb) analysis and a third tube for F-calprotectin (data not presented). Stool samples were collected by the participant prior to the preparation for the colonoscopy procedure. The samples were stored at room temperature for a maximum of 7 days before being processed at the lab facility. RNA*later*® is bacteriostatic (the bacteria remain intact but do not grow). The adequacy of using RNA*later*® as a preservative was validated by comparison of DNA yield and quality of samples stored in RNA*later*® in room temperature for 5 days, to that of immediately frozen samples (Supporting Information, Table S1). The samples containing RNA*later*® were centrifuged for 20 min at 2000 rpm, and excess fluid was removed before storage at

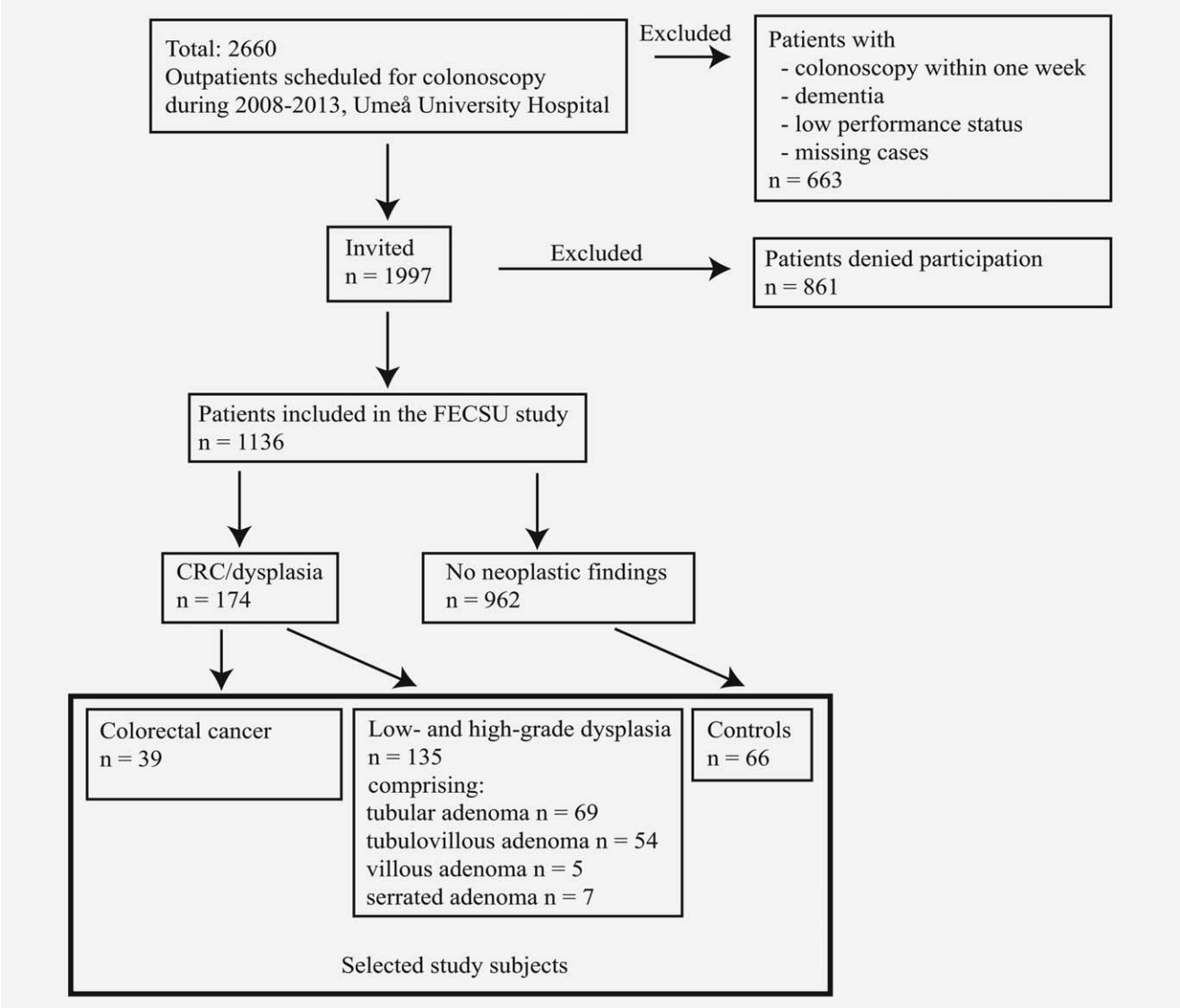

Figure 1. Flow chart describing the FECSU cohort and the selection of study subjects.

−80°C. F-Hb was analyzed manually using a fecal immunochemical test (FIT), Analyz F.O.B Test (ANL products AB), according to manufacturer's instructions.

DNA extraction

Stool DNA (sDNA) was prepared from approximately 0.2 g stool using QIAamp® DNA Stool Mini Kit (Qiagen) according to the manufacturer's instructions. Double stranded DNA-recovery was measured using Qubit® dsDNA BR Assay (Molecular probes) with the Qubit® 2.0 Fluorometer.

Detection of microbial markers in stool by real-time qPCR

Quantitative PCR assays targeting the *clbA* gene and the *afaC* gene of the *afa-1* operon were used to detect *pks*+ bacteria and Afa-1 adhesin-expressing DAEC, respectively. *Escherichia coli* Nissle 1917 was used as a positive control for *pks*. Real-time qPCR reactions were run in duplicates using the SYBR Green PCR kit on the 7900HT Fast Real-Time PCR System (Applied Biosystems). The following cycling conditions were used: for *clbA*, 5 min at 95°C, 40 cycles of 15 sec at 95°C, 30 sec at 50°C and 1 min at 72°C; for *afaC*, 10 min at 95°C, and 40 cycles of 15 sec at 95°C, and 15 sec at 65°C; for the *16S* rRNA gene, 5 min at 95°C, 40 cycles of 10 sec at 95°C, and 30 sec at 60°C. The quantification cycle (C_q) was calculated using the SDS 2.4 software (Applied Biosystems). The performance of the PCR assays was checked by analyses of replicates, serial dilutions, melting curves and separation on agarose gels. Samples not amplified with the appropriate amplicon length and not within 38 cycles were considered negative for the microbial factors.

F. nucleatum was assessed by real-time qPCR using the Microbial DNA qPCR Assay (Qiagen) containing a FAM-labeled probe specific for *F. nucleatum* 16S rRNA gene (GeneBank Acc. FJ471654.1) according to manufacturer's instructions.

F. nucleatum subsp. nucleatum Knorr (ATCC 25586 D-5) was used as positive control. The reactions were run on the Applied Biosystems 7900HT Fast Real-Time PCR System using the following cycling conditions; 95°C for 10 min, 40 cycles of 15 sec at 95°C, and 2 min at 60°C. Samples amplified within 38 cycles were considered to have template concentration positive for the specific sequence. The level of *F. nucleatum* in each sample was given as a relative quantification with the total microbial *16S* rRNA gene DNA in each sample as reference. The relative levels of *F. nucleatum* were calculated using $2^{-\Delta Cq}$, giving $\Delta C_q = C_q^{F.\ nucleatum} - C_q^{16S\ rRNA\ gene}$.

Two samples were excluded from qPCR analyses due to technical difficulties. The following primer sequences were used in this study: ***clbA***, *forward* 5′-ATGAGGATTGATATA TTAATTGGACA-3′ [33] and *reverse* 5′-GGTTTGCCATA TTTGCACGTAC-3′ product size 233 bp, ***afaC***; *forward* 5′-CGGCTTTTCTGCTGAACTGGCAGGC-3′[34] and *reverse* 5′-CCGCTCAGCACGTATGTATGAACTC-3′ product size 200 bp; ***16S* rRNA gene** *forward* 5′-CCATGAAGTCGGAA TCGCTAG-3′ and *reverse* 5′-GCTTGACGGGCGGTGT-3′ (*16S* rRNA Gene Universal Bacteria Control Primers from the NEBNext® Microbiome DNA Enrichment Kit (Biolabs)).

Statistical methods

Statistical analyses were performed using the IBM SPSS Statistics 23 (SPSS Inc.). χ^2 tests were used to compare categorical variables, unless expected frequencies were <5 when Fisher's exact test was used. The nonparametric Kruskal–Wallis *H* or Mann–Whitney *U* test was used to compare differences in continuous variables between groups. *p* values <0.05 were considered statistically significant. Area under the receiver operating characteristic (ROC) curve was calculated using the variable for *F. nucleatum* and cancer diagnosis, and the Youden's index was used to identify the cutoff for *F. nucleatum* levels that gave the most sensitive and specific assay to detect cancer. This cutoff was used in the *F. nucleatum* assay to identify stool samples as positive (with high levels of *F. nucleatum*) or negative (with low levels of *F. nucleatum*). In the CAMA test combining microbial markers or the test combining CAMA with F-Hb, a negative test result was given to stool samples negative for both markers and a positive test result given to stool samples with one or both markers positive. Sensitivity was defined as the percent of CRC or dysplasia cases with a positive test result. Specificity was defined as the percent of nested controls with a negative test result.

Results

Patient characteristics

The overall study cohort included 1136 patients invited to colonoscopy after presenting with symptoms from the large bowel. A flow chart describing the collection of the study cohort with inclusion/exclusion criteria can be found in Fig. 1. Among these, 39 patients were diagnosed with CRC and 135 patients with different degrees of dysplasia, and were selected for further studies. Also included in the study were 66 matched controls, who underwent colonoscopy but with no pathological findings. The clinical characteristics of the included study subjects are presented in Table 1. Most of the cancers were found in stage II (53.8%) and in left colon (43.6%).

Table 1. Clinical characteristics of study patients[1]

	Total	Control	Dysplasia	Cancer
	n = 238	*n* = 65	*n* = 134	*n* = 39
Age (%)				
34–59	33 (13.9)	6 (9.2)	23 (17.2)	4 (10.3)
60–69	94 (39.5)	22 (33.8)	60 (44.8)	12 (30.8)
70–80	85 (35.7)	26 (40.0)	41 (30.6)	18 (46.2)
>80	26 (10.9)	11 (16.9)	10 (7.5)	5 (12.8)
Gender (%)				
Female	103 (43.3)	30 (46.2)	54 (40.3)	19 (48.7)
Male	135 (56.7)	35 (53.8)	80 (59.7)	20 (51.3)
Location (%)	*n* = 173			
Right colon	49 (28.3)	n.a.	37 (27.6)	12 (30.8)
Left colon	76 (43.9)	n.a.	59 (44.0)	17 (43.6)
Rectum	48 (27.7)	n.a.	38 (28.4)	10 (25.6)
Stage (%)				
I		n.a.	n.a.	2 (5.1)
II		n.a.	n.a.	21 (53.8)
III		n.a.	n.a.	8 (20.5)
IV		n.a.	n.a.	7 (17.9)

[1]Shown are patients with complete data sets for microbial markers.
Abbreviation: n.a., not applicable.

Bacteria positive for *clbA* are more abundant in stool of patients diagnosed with CRC

A qPCR-assay targeting the *clbA* gene was used to detect colibactin-producing bacteria in DNA from stool of study patients. *clbA* was more often detected in stool samples of CRC patients compared to patients with dysplasia ($p = 0.004$) or controls ($p < 0.001$) (Fig. 2*a*). The difference in *clbA* detection frequency between patients with dysplasia and controls was of borderline significance ($p = 0.055$), but the stepwise increased frequency from controls, to dysplasia to cancers indicate that *clbA* may represent a useful marker for early changes leading to CRC. With a specificity of 81.5%, the *clbA* assay detected 56.4% of CRCs and 31.3% of dysplasias (Fig. 2*a* and Table 2). The clinical characteristics of cancer patients in relation to *clbA* can be found in Supporting Information, Table S2.

DAEC carrying *afa-1* were detected in DNA from stool using a qPCR assay targeting the *afaC* gene in the *afa-1* operon. Very few of the stool samples were positive for *afaC* (Fig. 2*b*). The stool samples originating from individuals diagnosed with CRC were slightly more frequently positive

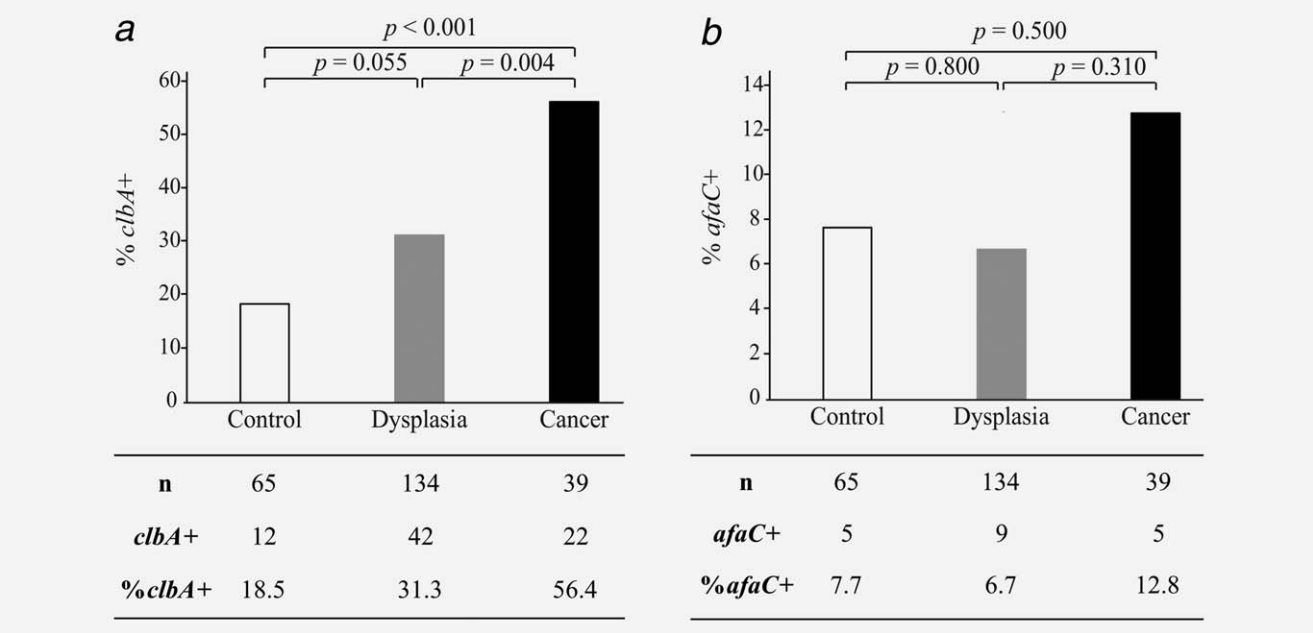

Figure 2. Bacteria carrying *clbA* are abundant in stool of CRC patients. Differences in absolute number (*n*) and percentage (%) of (a) *clbA*- and (b) *afaC*-positive stool samples between controls, and patients diagnosed with dysplasia or cancer are illustrated.

Table 2. Microbial alterations in stool of patients diagnosed with dysplasia or cancer

	Total	Control	Dysplasia	Cancer	*p* value
***clbA* (%)**	*n* = 238	*n* = 65	*n* = 134	*n* = 39	<0.001
Negative	162 (68.1)	53 (81.5)	92 (68.7)	17 (43.6)	
Positive	76 (31.9)	12 (18.5)	42 (31.3)	22 (56.4)	
***afaC* (%)**					0.46[1]
Negative	219 (92.0)	60 (92.3)	125 (93.3)	34 (87.2)	
Positive	19 (8.0)	5 (7.7)	9 (6.7)	5 (12.8)	
***F. nucleatum* (%)**					<0.001
Low	169 (71.0)	50 (76.9)	107 (79.8)	12 (30.8)	
High	69 (29.0)	15 (24.3)	27 (20.1)	27 (69.2)	
F-Hb (%)	*n* = 178	*n* = 41	*n* = 108	*n* = 29	<0.001
Negative	129 (72.5)	37 (90.2)	82 (75.9)	10 (34.5)	
Positive	49 (27.5)	4 (9.8)	26 (24.1)	19 (65.5)	

Unless otherwise indicated, χ^2 test was used for categorical variables.
[1]Fisher's exact test.
Abbreviation: F-Hb, immunochemical fecal hemoglobin test.

for *afaC* than samples from dysplasias or controls, but this was not statistically significant. With a specificity of 92.3%, the *afaC* assay detected only 12.8% of the cancers (Table 2). Notably, of the 5 *afaC*+ cancers, 4 were also *clbA*+, a pattern that was not found among the dysplasia or control groups (data not shown).

F. nucleatum is enriched in stool of patients with CRC

Quantitative PCR was applied to detect *F. nucleatum* in DNA from stool of study patients. *F. nucleatum* was found in stool of all patients, however, at varying levels (Fig. 3*a*). Patients diagnosed with CRC displayed significantly higher levels of *F. nucleatum* in stool compared to patients with dysplasia ($p < 0.001$) and controls ($p < 0.001$). No difference was found in *F. nucleatum* DNA levels between patients with dysplasia and controls. The clinical characteristics of cancer patients in relation to *F. nucleatum* can be found in Supporting Information, Table S2.

The area under the ROC curve was 0.737 for detection of CRC (Fig. 3*b*). A cutoff (0.00026) was selected that gave the

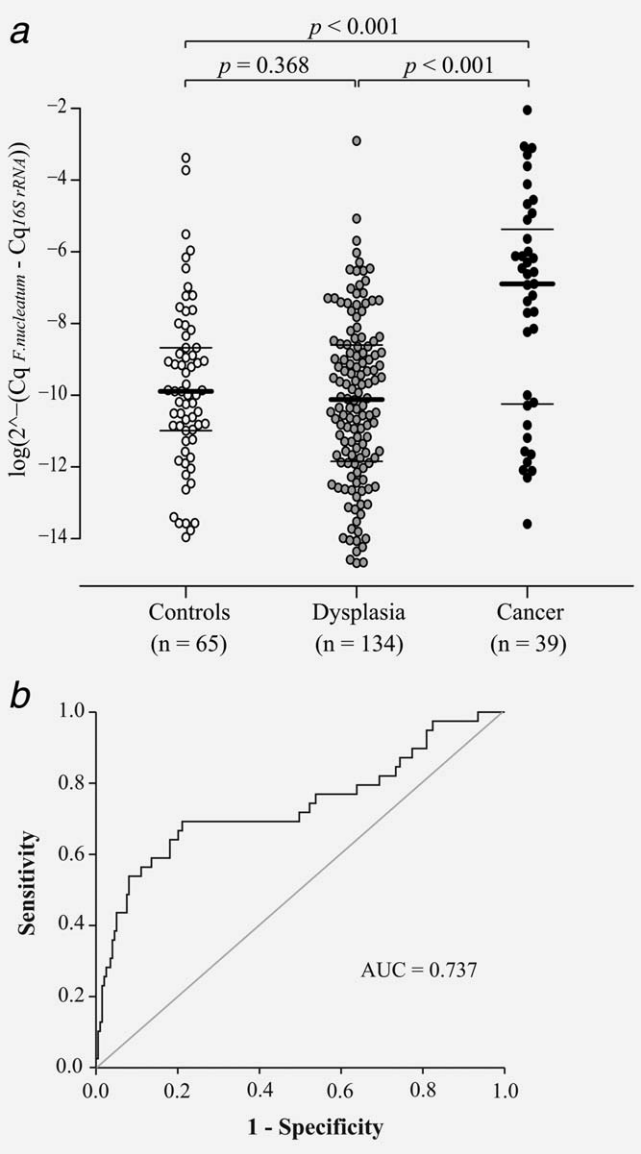

Figure 3. Increased levels of *F. nucleatum* are detected in stool of CRC patients. (*a*) A Beeswarm Boxplot is used to illustrate the relative levels of *F. nucleatum* in stool of control patients, and patients diagnosed with dysplasia or cancer. Horisontal lines indicate median (in bold) and quartiles. (*b*) An ROC curve displaying the specificity and the sensitivity for the *F. nucleatum* assay. The ROC curve was calculated using the variable for *F. nucleatum* and cancer/no cancer. The level of *F. nucleatum* in each sample is given as a relative quantification with the total microbial *16S* rRNA gene DNA in each sample as reference $2^{\wedge(-\Delta Cq)}$, $\Delta Cq = Cq^{F.\ nucleatum} - Cq16^{S\ rRNA\ gene}$).

most reliable analysis for detecting cancer in the study patients. With a specificity of 76.9%, the *F. nucleatum* assay detected 69.2% of CRCs and 20.1% of dysplasias (Table 2). At the selected cutoff, the *F. nucleatum* assay detected CRC with a higher sensitivity (69.2%) than *clbA* (56.4%) and the immunochemical F-Hb test currently used in the clinic (65.5%). However, the F-Hb test was more specific (90.2%) than *F. nucleatum* (76.9%), detecting <10% false positives (Table 2).

A test combining markers of microbial alterations in stool predicts CRC

To improve the CRC detection assay, the two microbial markers detecting *clbA* and *F. nucleatum* were combined in a single test here termed the cancer-associated microbial alterations (CAMA) test, where one or more positive markers predicts CRC. The *afaC* assay was excluded from the test since *afaC* was detected in very few cases and did not significantly differ between controls and cancer. At a specificity of 63.1%, the CAMA test detected CRC with a sensitivity of 84.6% (Table 3). Combining the CAMA test with the immunological F-Hb test, slightly increased sensitivity (89.7%) for detection of CRC, but at the same time specificity (61.0%) was slightly decreased.

DISCUSSION

In this nested case–control study, we explored the utility of using fecal microbial markers of microbial alterations in CRC detection. We found that individual markers for *clbA*+ bacteria and *F. nucleatum* were more highly abundant in stool of patients with CRC compared to controls, and could predict cancer with a sensitivity of 56.4% and 69.2%, respectively. The specificity of both assays was close to 80%. Combining the two markers into the CAMA test increased sensitivity to 84.6%, but with the drawback of reduced specificity. When combining CAMA and the immunochemical F-Hb test, the sensitivity was even higher 89.7%, but the specificity was slightly reduced. Our findings support the potential role of microbial factors in stool as putative noninvasive biomarkers for CRC detection.

We chose a nested case–control model, as it is generally more efficient than a case–control design with the same number of selected controls. However, one limitation of this study is that it is not randomized, as all patients selected for colonoscopy presented with symptoms from the large bowel. The controls, even though recorded without disease, may therefore not have represented a true healthy population. Another limitation is the lack of a validation cohort. The CAMA test was able to detect cancer with a high sensitivity, suggesting it to be a good test to detect cancer. In this study it was found to be more sensitive than the F-Hb test currently used in clinic. The relatively low specificity of the test would however result in around 35–40% of tested patients being diagnosed with a "high-risk" flora but without showing signs of dysplasia or neoplasia. This may cause psychological burden for the screening participants and will require continuous follow up by further tests and colonoscopy. It will be with great interest that we follow up on the currently healthy patients diagnosed with a "high risk" microbial pattern, to find out whether or not these patients later on will develop disease. Further studies and verifications in additional cohorts are required to understand the full potential of microbial markers in CRC progression and screening.

Table 3. A test combining microbial markers and F-Hb in CRC screening

	Total	Control	Dysplasia	Cancer	*p* value
CAMA (%)	*n* = 238	*n* = 65	*n* = 134	*n* = 39	<0.001
Negative	118 (49.6)	41 (63.1)	71 (53.0)	6 (15.4)	
Positive	120 (50.4)	24 (36.9)	63 (47.0)	33 (84.6)	
CAMA/F-Hb (%)	*n* = 178	*n* = 41	*n* = 108	*n* = 29	<0.001[1]
Negative	70 (39.3)	25 (61.0)	42 (38.9)	3 (10.3)	
Positive	108 (60.7)	16 (39.0)	66 (61.1)	26 (89.7)	

[1]A positive score was given to stool samples positive for one or both of *clbA* and *F. nucleatum*. Unless otherwise indicated, χ^2 test was used for categorical variables.
[2]Fisher's exact test.
Abbreviation: CAMA, cancer-associated microbial alterations.

Advantages of the microbial marker test is that it represents a cost-efficient, straight forward, and noninvasive procedure. It is likely that a higher number of patients would agree to a screening procedure leaving stool samples compared to screening by colonoscopy. The stool samples were collected prior to the precolonoscopy cleansing procedure and stored in a chemical stabilizer with bacteriostatic activity, which according to our evaluation preserves microbial DNA quality. The test might, however, be sensitive to antibiotic treatment and stool sampling, as it is a single test, randomly sampled by the patient from a small amount of stool. Data on ongoing antibiotic treatment has been collected for all patients in the FECSU cohort, but no patients included in this study were registered with ongoing antibiotic treatment. However, we cannot exclude long-term changes in the composition of the microbiota associated with previous antibiotic treatment.

F-Hb tests are currently the most commonly used noninvasive screening method in CRC and are generally found to be more specific than our CAMA test.[35] A major advantage of microbial markers in relation to F-Hb tests is that they could detect also nonbleeding lesions. Microbial markers such as for *F. nucleatum* have also been suggested to be useful for detecting serrated polyps, not fully efficiently detected by F-Hb tests.[36,37] *F. nucleatum* levels in stool have previously been assessed as a noninvasive biomarker in CRC. In a study by Kostic *et al.*, a significant stepwise increase in *F. nucleatum* levels was found from controls to adenomas to carcinomas, suggesting the potential of *F. nucleatum* as a marker for detection of early changes.[10] In another study by Flanagan *et al.*, *F. nucleatum* was found, like in our study, to be more abundant in stool of carcinomas but with no significant difference between adenomas (or dysplasias) and controls.[38] In our study, *clbA* turned out to be the most promising marker for early detection, as the prevalence increased already with dysplastic lesions. Further studies of microbial factors as early detection markers are required to elucidate these differences.

As recently described in the bacterial driver-passenger model, some bacteria are likely procarcinogenic and involved in CRC development while others defined as passengers may be involved in later stages of tumor progression.[39] Colibactin-producing *E. coli* can increase the mutation rate of infected cells,[9] and could therefore be an example of a bacterial driver. On the other hand, a change in the microbiota could instead be a consequence of epithelial changes following CRC progression. *F. nucleatum* has been shown to bind to epithelial cells through a Fap2/Gal-GalNAc interaction, where Gal-GalNAc is overexpressed in CRC cells compared to non-neoplastic epithelial cells.[40] These findings suggest that *F. nucleatum* may be a bacterial passenger. Further studies, including the evaluation of bacterial markers in tumor tissue, are needed to elucidate the roles of microbial shifts in CRC development and progression.

Models of microbial alterations combining different "high-risk" bacteria may improve the specificity of diagnostic tests for CRC. A few metagenomics studies have addressed variations of the microbiome in stool of patients with colorectal adenomas or carcinomas for potential use as noninvasive screening markers for CRC.[41–44] In a study by Yu *et al.*, compositional differences for several bacterial species were identified in patients with CRC compared to controls. Using qPCR, the specific markers buturyl-CoA dehydrogenase (*F. nucleatum*) and *rpoB* (*Parvimonas micra*) were further found to be highly enriched in stool of patients with early stages of CRC.[42] In a study by Zackular *et al.*, they identified a panel of microbial markers that was differentially expressed in controls, adenomas and carcinomas. These changes in the gut microbiome could significantly complement the ability of clinical characteristics, and the gFOBT test to identify the different patient diagnoses.[44] Furthermore, Wong *et al.* very recently showed that quantitation of fecal *F. nucleatum* improved and had a complementary value added to the FIT test.[45] Therefore, combining different microbial markers of "high-risk" flora with F-Hb tests, clinical characteristics and tumor-specific DNA, RNA or protein biomarkers in stool may be a putative screening strategy in the future to more accurately identify patients in early stages of disease progression.

In conclusion, we suggest that analyses of markers of microbial alterations in stool may be putative noninvasive diagnostic markers for CRC. We suggest that detection of a "high-risk" microbial pattern in stool may identify patients

with increased risk of developing CRC. Future studies combining different microbial markers and as well as other biomarkers in a true population-based setup may lead to important advances in CRC screening. Further studies are also needed to address the role of the microbiota in cause or consequence of tumor progression. These studies may lead to important understandings of the role of the microflora in progression of CRC and the identification of important microbial markers for detection of early disease.

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CONTRIBUTORS

Study concept and design: VE, MLW, JR, OA, PK, RP; acquisition of data: ALB, VE, CZ; data analyses: ALB, VE, SE, PL; drafting of the manuscript: ALB, VE, CZ, MLW, SE, RP. Critical revision of the manuscript for important intellectual content: PL, PK, OA, JR. All authors approved the final version of the manuscript.

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