



Detection of *Fusobacterium nucleatum* in stool and colonic tissues from Norwegian colorectal cancer patients

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Abstract

Norway has one of the world's highest incidences of colorectal cancer (CRC). Accumulating research suggests that the intestinal microbiota may have an important role in initiation and progression of colorectal cancer. In order to evaluate microbiome-based biomarkers for non-invasive detection of CRC, the levels of *Fusobacterium nucleatum* and selected *Escherichia coli* toxin genes in stool and mucosa from a small cohort of Norwegian patients were investigated. The study cohort included 72 patients scheduled for colonoscopy. The patients were divided into three groups upon their examinations: cancer, polyp, and control groups. Levels of *F. nucleatum* in stool samples were significantly higher in the cancer group compared with the control group and the polyp group. High levels of *F. nucleatum* in stool reflected detection of *F. nucleatum* in the tumor tissues of colorectal cancer patients. However, no difference in the levels of *E. coli* toxin genes in neither stool nor biopsy samples between the patient groups was observed. This study suggests that a quantitative PCR assay targeting *F. nucleatum* in stool samples has the potential to be included in a larger panel of biomarkers for non-invasive testing for colorectal cancer.

Keywords CRC · *Fusobacterium nucleatum* · Early detection · Non-invasive · Biomarker · qPCR

Introduction

Despite developments in diagnostics and treatment, colorectal cancer (CRC) remains the second most common cause of cancer mortality in the western countries. Norway has one of the world's highest incidences of colon cancer, with 4332 new cases reported in 2017 (Cancer Registry of Norway: www.kreftregisteret.no). Early detection results in significantly improved outcomes for CRC patients. When diagnosis is set at stage I, patients have a 90–95% survival rate with surgical

intervention while at stage IV, the survival rate is reduced to just 5–10% (<https://seer.cancer.gov/statistics/>). Currently, sigmoidoscopy and colonoscopy are used for diagnostics of CRC in Norway. These are invasive methods that require extensive resources and therefore high costs. Alternatively, non-invasive methods such as immunochemical tests for hidden (occult) blood in stool samples (iFOBT) are also in use. The advantages with using these tests lie in the easily accessible sample material and low analytical costs in the laboratory. The drawbacks are, however, poor diagnostic sensitivity and specificity [1]. Therefore, adding new non-invasive biological markers to increase the overall sensitivity and specificity is highly attractive.

Colorectal cancer development is a multifactorial process involving an accumulation of mutations in the genes such as APC, KRAS, BRAF, and TP53, resulting in uncontrolled cell growth and tumor formation. The triggers for initiation of colorectal cancer are not fully characterized, but the risk factors are associated with high intake of animal fat, red meat, processed food, and low consumption of fiber-rich food [2]. We are becoming increasingly aware that the intestinal microbiota may be involved in the initiation and facilitation of CRC [3, 4]. Several models for bacterial involvement in initiation

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and development of CRC have been presented (reviewed in [5]). The mechanisms include DNA damage from bacterial virulence factors such as genotoxins, activation of inflammatory and oncogenic signaling pathways, and production of tumor-promoting metabolites such as secondary bile acids. A number of microbes have been proposed as candidates for CRC initiation, in particular, *Fusobacterium nucleatum* and *Escherichia coli*. An expert review from 2016 summarizes the evidence supporting oncogenic roles of *F. nucleatum* and *E. coli* [6]. The authors present supporting evidence from molecular studies illustrating a strong correlation between *F. nucleatum* and carcinogenesis. *F. nucleatum* may directly contribute to carcinogenesis through attachment of its FadA adhesion protein to the extracellular domain of E-cadherin on the surface of intestinal epithelial cells. This may result in E-cadherin/ β -catenin activation via the WNT signaling pathway [7–11]. β -Catenin accumulates in the cytosol, translocates into the nucleus, and activates its target genes such as NK- κ B and the proto-oncogenes C-MYC and C-JUN [7]. The contributory rather than consequential role of *F. nucleatum* in tumor formation is supported by studies using models with preexisting mutations in the tumor suppressor gene APC. “First-hit” APC mutations appear to be sufficient for *F. nucleatum* to exert its inflammatory and oncogenic effects [12]. The present hypothesis is therefore that *F. nucleatum* emerges early in cancer development and is a risk factor for progression from adenoma to cancer [13]. Furthermore, invasive *F. nucleatum* can also be found in liver metastases, suggesting a persistent association with tumor cells during various stages of colorectal cancer development [14].

Despite the accumulating evidence correlating *F. nucleatum* to colorectal cancer, this has only been shown for a subgroup of colorectal cancer patients. Given the large interindividual differences in intestinal microbiota, it remains unlikely that bacterially induced oncogenic progression is driven by the same bacterial species in all cancer patients. *E. coli* have repeatedly been associated with colorectal cancer tumor sites, and evidence for a contributory role of *E. coli* toxins in colorectal cancer is emerging [15, 16]. The virulence factor colibactin, part of the polyketide synthase (*pks*) island, has been most widely studied. It is suggested to promote tumor progression by miRNA silencing of p53 and hence cellular senescence and hepatocyte growth factor secretion [5]. Other *Escherichia* virulence factors studied in relation to CRC are cytolethal distending toxin (*cdt*), toxin coregulated pilus synthesis outer membrane protein C (*tcpC*), and arginine succinyltransferase (*astA*) [17, 18].

The majority of studies have investigated *F. nucleatum* and *E. coli* toxins in mucosal samples, from tumor and adjacent tissues [15, 19–21]. Recent studies also illustrate that *F. nucleatum* can often be detected in stool samples from CRC patients [22–24]. The presence of *F. nucleatum* and *E. coli* toxin genes has not previously been studied in samples

from Norwegian cancer patients, and their use as marker genes for CRC in the Norwegian population has therefore not been evaluated. The aims of the present study were to estimate the levels of *F. nucleatum* and selected *E. coli* toxin genes in stool and mucosa samples from Norwegian colorectal cancer patients and to evaluate the use of *F. nucleatum* and *E. coli* quantitative PCRs in a panel of non-invasive biomarkers for detection of colorectal cancer in its early stages.

Materials and methods

Study population and samples

Patients with scheduled colonoscopy at Akershus University Hospital (Ahus) from 2014 to 2017 were included in the study ($n = 72$). The indications for colonoscopy were gastrointestinal bleeding, weight loss, changes in bowel movements, or detection of polyps/tumors on CT colonography. Prior to the procedure, patients were informed that additional samples would be taken, given their consent, and informed of their rights to withdraw from the study at any time. Written informed consent was obtained from all included participants. The regional committee for medical and health-related research ethics (REK 2012/1944) and the data protection manager at Ahus have approved the study.

The 72 patients were divided into three groups based on findings during colonoscopy: one group with colorectal cancer ($n = 25$), one group with adenomatous polyps ($n = 25$), and one control group without pathological findings ($n = 22$). Median age was 70, 69, and 57 for the cancer, polyp, and control groups, respectively. The proportions of females were 28%, 56%, and 41%, respectively, in the three different groups. Each patient collected a stool sample in RNeasy RNA Stabilization buffer (Qiagen, Hilden, Germany) prior to their bowel preparation or 1 week after colonoscopy. When samples arrived at the laboratory, they were homogenized and stored at $-80\text{ }^{\circ}\text{C}$. During colonoscopy, biopsies (2×2 mm on average) were collected from different positions in the colon: position 1, colon ascendens; position 2, from the polyp or cancerous tissue; position 3, adjacent healthy tissue; and position 4, colon sigmoideum. Immediately after collection, the biopsies were fixed in Allprotect Tissue Reagent (Qiagen, Hilden, Germany) and stored according to the manufacturer’s recommendations.

Classification of the tumors

All tumors were examined by the Dept. of Pathology and classified into stages based on the UICC TNM Classification system. In this anatomically based system, the category T

describes the primary tumor size and/or extent, the category N describes the regional lymph node involvement, and the category M describes the presence of distant metastatic spread. According to the National Health Department in Norway, the TNM should be given with the prefix p, indicating assessment done by a pathologist (Table 1).

DNA extraction from stool and biopsies

DNA was extracted from all fecal samples with PSP® Spin Stool DNA Kit (Strattec Molecular GmbH, Berlin, Germany) based on previous results for optimal DNA output and bacterial diversity [25]. Biopsies were extracted with AllPrep DNA/RNA Mini Kit (Qiagen, Hilden, Germany) according to the procedure described by Moen et al. [26]. NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) was used for measurements of concentration and purity of the extracted DNA. DNA from biopsy and stool samples were

diluted 10- and 100-fold, respectively, in PCR-grade water to facilitate downstream analysis.

Quantitative PCR (qPCR)

Fusobacterium nucleatum-specific qPCR

The PCR reactions were performed with primers for *F. nucleatum* designed by Flanagan et al. [13] but with a slightly modified reverse sequence (Table 2). The primers amplify a portion of the gene encoding the antitermination factor NusG from *F. nucleatum*. The quantitative PCR (qPCR) reactions were performed on the Applied Biosystems 7900 Real-Time PCR System (Thermo Fisher Scientific) using SYBR® Select Master Mix (Thermo Fisher Scientific) in 20 µl reactions. The cycling conditions were as follows: 50 °C for 2 min (uracil N-glycosylase activation), followed by one hold at 96 °C for 2 min (activation of *Taq*

Table 1 pTNM classification of the tumors from CRC patients

| Patient | Patient | | | Tumor | | | Classification | | |
|---------|-----------|-----|-----------------------|-----------------|------------------|------------|----------------|---|---|
| | Sample ID | Age | Sex | Location | Size | Morphology | T | N | M |
| C01 | 69 | M | Sigmoid colon | 20 mm | Adenocarcinoma | 2 | 0 | 0 | |
| C02 | 74 | M | Sigmoid colon | 15 × 10 × 5 mm | Haggitt: level 2 | | | | |
| C03 | 57 | F | Cecum | 45 mm | Adenocarcinoma | 2 | 0 | 0 | |
| C04 | 53 | F | Cecum | 60 mm | Adenocarcinoma | 3 | 2 | 0 | |
| C05 | 70 | M | Rectosigmoid junction | 130 mm | Adenocarcinoma | 3 | 2 | 0 | |
| C06 | 80 | F | Transverse colon | 50 mm | Adenocarcinoma | 3 | 1 | 0 | |
| C07 | 69 | M | Rectum | 20 mm | Adenocarcinoma | 2 | 0 | 0 | |
| C08 | 71 | M | Sigmoid colon | 35 mm | Adenocarcinoma | 2 | 0 | 0 | |
| C09 | 67 | M | Ascending colon | 60 mm | Adenocarcinoma | 3 | 1 | 0 | |
| C10 | 72 | M | Sigmoid colon | 28 mm | Adenocarcinoma | 2 | 0 | 0 | |
| C11 | 80 | F | Ascending colon | 35 mm | Adenocarcinoma | 3 | 0 | 0 | |
| C12 | 75 | M | Transverse colon | 28 mm | Adenocarcinoma | 1 | 0 | 0 | |
| C13 | 68 | M | Sigmoid colon | 60 mm | Adenocarcinoma | 3 | 0 | 1 | |
| C14 | 68 | M | Cecum | 18 mm | Adenocarcinoma | 2 | 0 | 0 | |
| C15 | 63 | M | Rectosigmoid junction | 35 mm | Adenocarcinoma | 3 | 0 | 0 | |
| C16 | 84 | M | Cecum | 70 mm | Adenocarcinoma | 4 | 2 | 1 | |
| C17 | 76 | M | Ascending colon | 23 mm | Adenocarcinoma | 3 | 2 | 0 | |
| C18 | 65 | M | Sigmoideum | 20 mm | Adenocarcinoma | 1 | 0 | 0 | |
| C19 | 70 | F | Cecum | 38 mm | Adenocarcinoma | 2 | 0 | 0 | |
| C20 | 55 | F | Cecum | 70 mm | Adenocarcinoma | 4 | 1 | 1 | |
| C21 | 45 | M | Sigmoid colon | 55 mm | Adenocarcinoma | 4 | 1 | 1 | |
| C22 | 66 | M | Sigmoid colon | 40 mm | Adenocarcinoma | 3 | 0 | 0 | |
| C23 | 82 | M | Rectum | 40 mm | Adenocarcinoma | 2 | 0 | 0 | |
| C24 | 81 | M | Rectum | 25 mm | Adenocarcinoma | 2 | 0 | 0 | |
| C25 | 73 | F | Sigmoid colon | 42 × 46 × 56 mm | Adenocarcinoma | 4b | 1b | 0 | |

Table 2 Primers and probes used in the study

| | Primer sequence 5'-3' | Primer conc. | Amplicon size | Ta | PCR efficiency/LOD | Reference |
|------------------------|----------------------------------|--------------|---------------|-------|--------------------|-----------|
| <i>F. nucleatum</i> -F | CAACCATTACTTTAACTCTACCATGTTCA | 400 nM | 105 bp | 57 °C | 98.6%/3 fg DNA/μl | [13] |
| <i>F. nucleatum</i> -R | TACTGAGGGAGATTATGTA AAAATC | | | | | |
| <i>Pks</i> -F | GCGCATCTCAAGAGTAAATA | 500 nM | 280 bp | 60 °C | 94%/0.6 pg DNA/μl | [17] |
| <i>Pks</i> -R | GCGCTCTATGCTCATCAACC | | | | | |
| <i>tcpC</i> -F | TCGGCGATAGCTTAAGGAGA | 500 nM | 216 bp | 56 °C | 89%/0.4 pg DNA/μl | [18] |
| <i>tcpC</i> -R | CCGCCAAATAATGGCTGTAT | | | | | |
| <i>CNF1</i> -F | AGCGTGCAATCTATCCGTATTT | 500 nM | 173 bp | 56 °C | 110%/0.6 pg DNA/μl | [18] |
| <i>CNF1</i> -R | TGGAATTTCCCCAGTATAGGTG | | | | | |
| <i>astA</i> -F | TGCCATCAACACAGTATATCCG | 500 nM | 102 bp | 63 °C | 103%/0.4 pg DNA/μl | [27] |
| <i>astA</i> -R | ACGGCTTTGTAGTCCTTCCAT | | | | | |
| 16S rRNA-F | ACTCTACGGGAGGCAGCAGT | 400 nM | 198 bp | 57 °C | 98.6%/1 fg DNA/μl | [28] |
| 16S rRNA-R | CCTAGCTATTACCGCGGCTGCT | | | | | |
| β-globin-F | ACACAACGTGTTCTACTAGCAACCTC | 375 nM | 111 bp | 60 °C | 95%/0.1 ng DNA/μl | [29] |
| β-globin-R | CCAACCTCATCCACGTTACCT | | | | | |
| β-globin-p | TGCACCTGACTCCTGAGGAGAAGTCT GC | | | | | |
| 341-F 16S rRNA seq-F | CCTACGGGNGGCWGCAG | – | 464 bp | – | | [30] |
| 805-R 16S rRNA seq-R | GACTACHVGGGTATCTAATCC | | | | | |

Ta annealing temperature, LOD limit of detection

DNA polymerase), and 40 cycles at 95 °C/15 s, 57 °C/15 s, and 72 °C/20 s.

Escherichia coli toxin-specific qPCR

Four qPCR reactions targeting four different *E. coli* toxin genes (*pks*, *CNF1*, *tcpC*, and *astA*) were performed using cycling conditions as previously described [17, 18, 27] (Table 2). The PCR reactions were performed with SYBR® Select Master Mix (Thermo Fisher Scientific) in 20 μl reactions.

Reference gene qPCR

Total bacterial DNA determined by 16S rRNA qPCR was used to normalize the target genes in stool samples. The PCR master mix and cycling conditions were as described for *F. nucleatum* [28]. A qPCR targeting the human β-globin gene was used for target gene normalization in biopsies [29] (Table 2).

qPCR evaluations

PCR efficiency and limit of detection (LOD) were determined for all qPCR assays using 10-fold dilution series of genomic DNA from control strains of *F. nucleatum*, extraintestinal *E. coli* (ExPEC), enteroaggregative *E. coli* (EAEC), and enterohemorrhagic *E. coli* (EHEC) (taxonomy confirmed by 16S rRNA gene sequence and/or characterized at the Norwegian Institute of Public Health). Specificity of the

PCR primers was determined using a panel of genomic DNA isolated from stool-associated bacterial species ($n = 20$).

Statistical analysis of qPCR data

Stool samples

Stool samples from the cancer group ($n = 23$), polyp group ($n = 25$), and control group ($n = 22$) were analyzed for quantities of *F. nucleatum* and the presence of four *E. coli* toxin genes using qPCR as described above. All qPCR reactions were performed in duplicate. Relative quantities of *F. nucleatum* and *E. coli* toxin genes in each stool sample were determined with $2^{-\Delta Ct}$ using the 16S rRNA gene as a reference gene: $\Delta Ct = Ct_{Fn/E.coli} - Ct_{16S\ rRNA}$ [13]. The fold difference in *F. nucleatum* or *E. coli* toxin gene levels between the patient groups were calculated by the $2^{-\Delta\Delta Ct}$ method. For statistical comparison of means between independent groups, one-way ANOVAs were performed to test differences in both *F. nucleatum* and *E. coli* toxin levels between the cancer, polyp, and control groups. Significance was set at $\alpha = 0.05$; a comparison of means was performed using the post hoc Tukey multiple comparison test. A test of normality was carried out using the Shapiro-Wilk test and also Levene's test for homogeneity of variance. Due to the non-Gaussian nature of the qPCR data distribution, non-parametric testing was deemed appropriate; consequently, the one-way ANOVA and the Tukey post hoc test were replaced with the Kruskal-Wallis rank sum test and the pairwise Wilcoxon rank sum test,

respectively. Statistical comparisons between groups were performed in the open-source software R (version 3.3.2 (2016-10-31)).

Biopsy samples

Biopsies from 21 cancer patients, 11 polyp patients, and 11 control patients were analyzed for quantities of *F. nucleatum* and the presence of four *E. coli* toxin genes using qPCR as described above. Levels of *F. nucleatum* and *E. coli* toxin genes were related to human β -globin: $\Delta Ct = Ct_{Fn/E.coli} - Ct_{\beta\text{-globin}}$. Repeated measures ANOVAs were performed to test differences in both *F. nucleatum* and *E. coli* toxin levels between the colon ascendens, cancerous tissue, adjacent healthy tissue, and colon sigmoideum biopsies in colon cancer patients. Post hoc Tukey multiple comparisons of means were again implemented to identify which pair(s) of biopsy position means differed significantly. We used the Shapiro-Wilk normality test and Mauchly's test of sphericity to formally test these assumptions. Non-parametric testing was performed when appropriate, replacing the repeated measures ANOVA and the Tukey post hoc test with the Friedman rank sum test and the pairwise Friedman-Nemenyi multiple comparison test, respectively. All significance levels were set at $\alpha = 0.05$.

16S rRNA massive parallel sequencing

For verification of qPCR results, stool samples from the cancer and control groups ($n = 27$), as well as two negative processing controls (included in the whole sample processing procedure from DNA extraction), were sent for 16S rRNA massive parallel sequencing by a commercial laboratory, Omega Bioservices (Atlanta, GA, USA). 12.5 ng DNA was used as input and the V3-V4 region of the bacterial 16S rRNA gene sequences was amplified using the primer pair 341F-805R (Table 2) and KAPA HiFi HotStart ReadyMix (Kapa Biosystems, Wilmington, MA). PCR products were purified with Mag-Bind RxnPure Plus magnetic beads (Omega Biotek, Norcross, GA) before performing a second index PCR amplification with the same master mix. Libraries (~600 bp) were normalized, pooled, and sequenced (2×300 bp paired-end read setting) using the MiSeq platform (Illumina, San Diego, CA, USA).

Sequences were preprocessed, quality filtered, and analyzed using QIIME2 1.9. Further analysis was performed in the Illumina BaseSpace 16S metagenomics application. The read classifier was Illumina-modified RDP, and taxonomy was assigned using the Illumina-curated version of the May 2013 release of the Greengenes Consortium Database 13.5 (performed by Omega Bioservices). Taxonomic IDs with only one aligned sequence read were discarded. The microbial classifications were compared at different taxonomic levels (genus and species) between the groups.

Results

Quantitative PCR results

Levels of *Fusobacterium nucleatum* in stool samples are associated with colorectal cancer and reflect the tumor environment

Significantly higher levels of *Fusobacterium nucleatum* were observed in stool samples from the cancer group compared with the polyp group (Tukey $p = 0.00074$ and Wilcoxon $p = 0.0028$) and the cancer group compared with the control group (Tukey $p = 0.00014$ and Wilcoxon $p = 0.0073$) using qPCR (Fig. 1). Comparison of *F. nucleatum* levels between the cancer and control groups with the $2^{-\Delta\Delta Ct}$ method illustrated a fold difference of 66, in favor of the cancer group. The difference between the cancer and polyp groups was 97-fold. No significant difference was detected between the polyp and control groups (Tukey $p = 0.928$ and Wilcoxon $p = 0.495$).

Figure 2 illustrates that 35% of cancer patients and none of the control or polyp patients in this study would be identified with a ΔCt ($Ct^{Fn} - Ct^{16S\ rRNA}$) cutoff of 12. High levels of *F. nucleatum* in stool samples correlated with detection of *F. nucleatum* in tumor tissue. In 69% (9/13) of cancer patients that tested positive for *F. nucleatum* in their cancerous tumor biopsies, high levels were identified in stool.

Fusobacterium nucleatum is associated with tumor tissues

The relative abundance of *F. nucleatum* in different positions along the colon was determined using qPCR. Irrespective of sample position, *F. nucleatum* was detected in samples from 60%, 18%, and 18% of patients in the cancer, polyp, and control groups, respectively. Further analyses of samples from the cancer group ($n = 21$) illustrated that *F. nucleatum* was detected in 52% ($n = 13$) of samples from tumor tissue, 24% ($n = 6$) of healthy tissue sample from the ascending colon, 36% ($n = 9$) of healthy tissue samples adjacent to the tumor, and 36% ($n = 9$) of healthy tissue samples from the colon sigmoideum. Comparison of *F. nucleatum* relative quantities suggested higher levels of *F. nucleatum* in the tumor tissue compared with adjacent healthy tissue (6.3-fold), colon ascendens (13-fold), and colon sigmoideum (11-fold) (Fig. 3). The non-parametric Friedman test showed significant differences only between cancerous tissue and colon ascendens ($p = 0.036$).

Association between tumor position, tumor stage, and *Fusobacterium nucleatum*

Tumors located in the proximal part of the colon were more frequently associated with *F. nucleatum*. *F. nucleatum* was detected in 72% (8/11) of tumors in the proximal part of the

Fig. 1 Relative quantities of *F. nucleatum* in stool samples. qPCR data illustrate higher quantities of *F. nucleatum* in the cancer group (C) relative to the control group (K) and the polyp group (P). The boxplot shows the median and interquartile range of the relative *F. nucleatum* quantifications

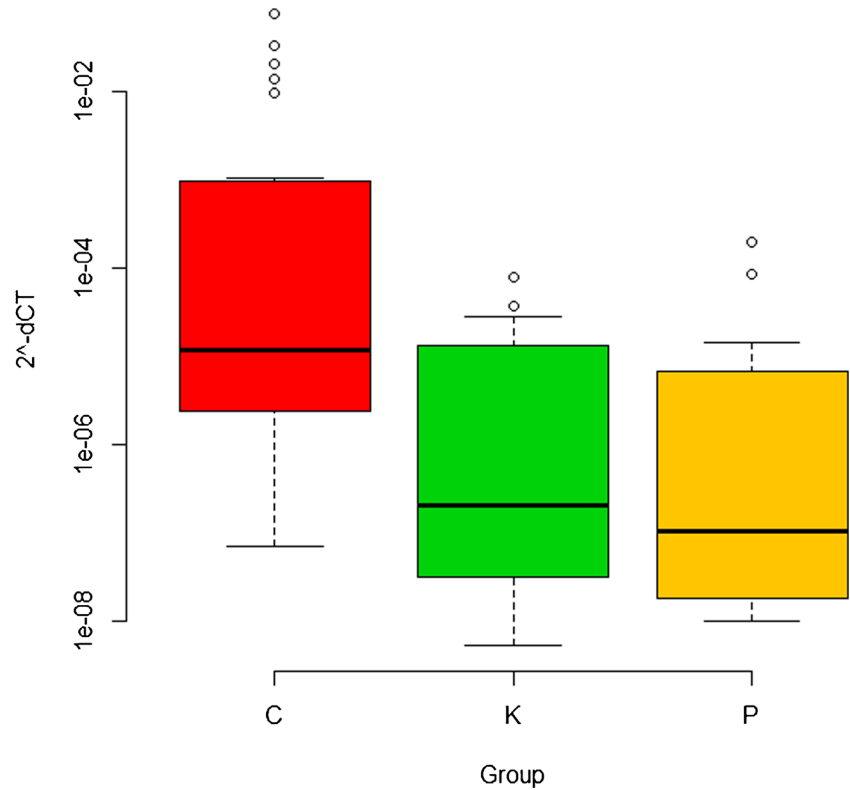


Fig. 2 The figure illustrates that 35% of stool samples from cancer patients and none of the stool samples from polyp or control patients were detected with ΔCt ($Ct^{F.nucleatum} - Ct^{16S rRNA}$) values below 12. These samples were all from cancer patients in early stages and represented 69% of cancer patients with *F. nucleatum* in their superfluous tumor biopsies. Cancer patients with distant metastatic spread had higher ΔCt values or *F. nucleatum* was undetected in stool

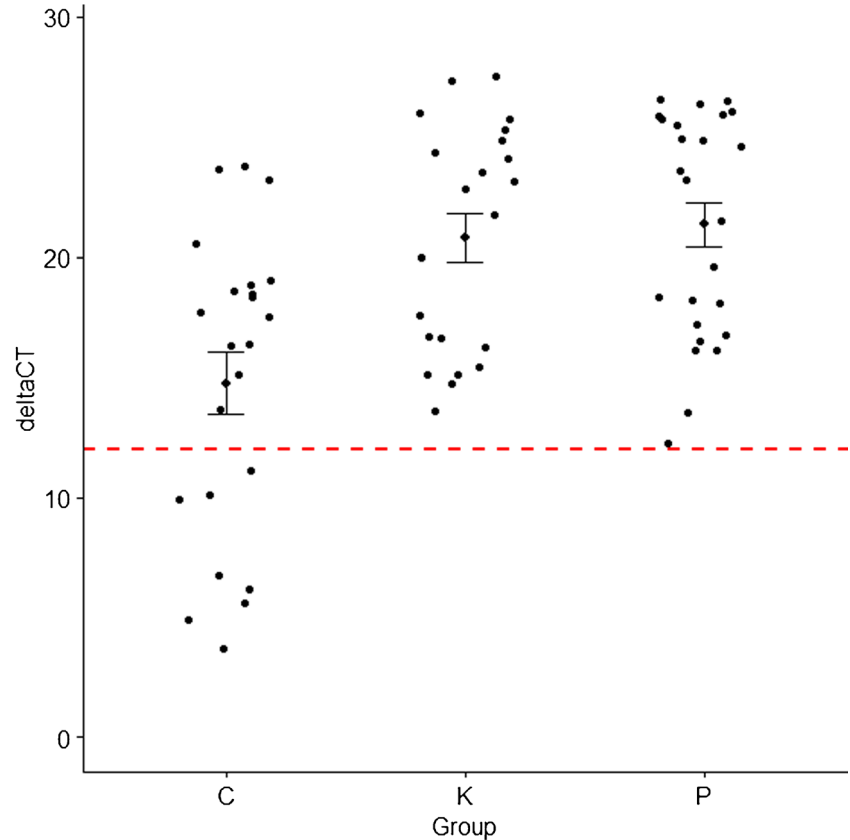
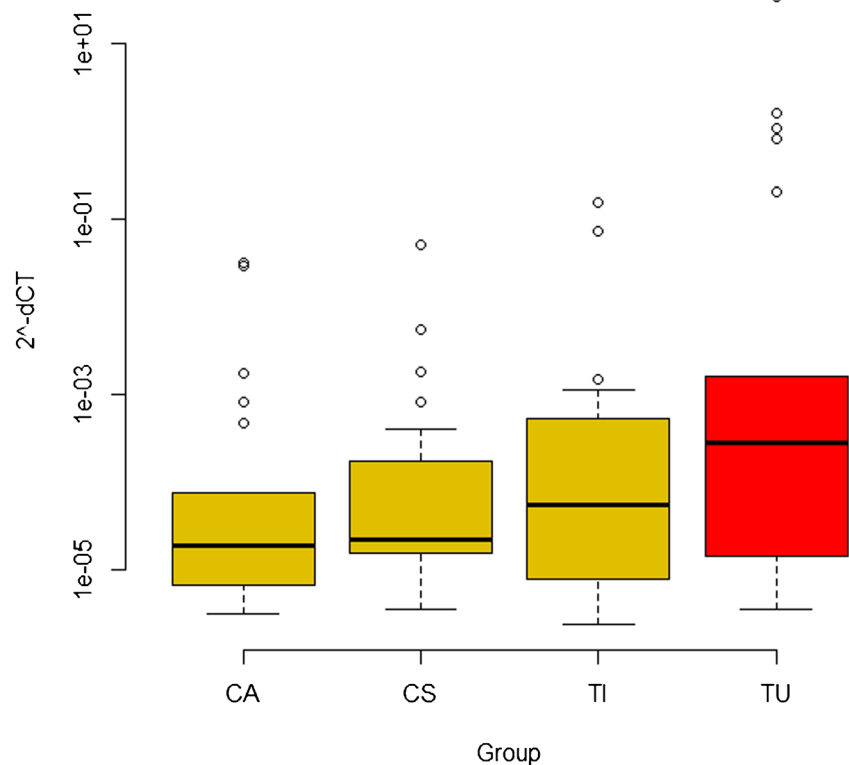


Fig. 3 Relative quantities of *F. nucleatum* in different positions of the colon in cancer patients. Statistical analysis (Friedman test) showed significant differences only between cancerous tissue (TU) and colon ascendens (CA) ($p = 0.036$). The boxplot shows the median and interquartile range of the relative *F. nucleatum* quantifications. CS *colon sigmoideum*, TI *healthy tissue adjacent to the tumor*



colon, compared with 35% (5/14) of tumors in the distal part of the colon and rectum. A trend of wider distribution of *F. nucleatum* in the colorectum, illustrated by detection in multiple positions, was observed when the tumor was located in the proximal part of the colon. Seven of the 13 tumors with *F. nucleatum* were positive also in adjacent tissues, notably six of these seven tumors were located in the proximal part of the colon (Fig. 4). The qPCR data did not reveal any differences in quantities of *F. nucleatum* in tumors located in the proximal versus distal part of the colon, nor did they detect any differences in *F. nucleatum* quantities between different TNM classifications. These numbers are too small for statistical comparison.

Levels of *E. coli* toxin genes in stool samples and colonic mucosa are not associated with colorectal cancer

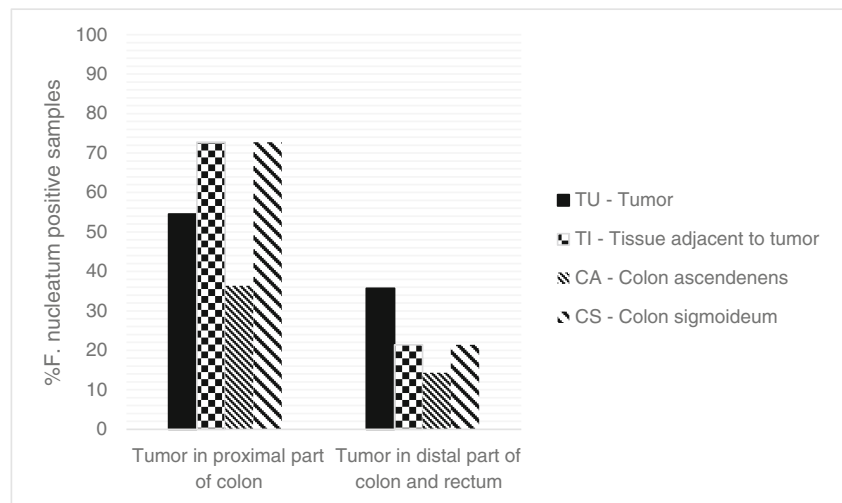
No statistical significant differences in *E. coli* toxin gene levels in stool were observed between the patient groups (one-way ANOVA $p = 0.61$, Tukey $p = 0.41$, and Wilcoxon $p = 0.59$). Irrespective of sample position, *E. coli* toxin genes were detected in biopsies from 52%, 27%, and 45% of patients in the cancer, polyp, and control groups, respectively. Further analyses of samples from the cancer group did not result in any significant differences between the different positions (repeated measures ANOVA $p = 0.36$). The *E. coli* toxin biopsy data met the assumptions of both normality and sphericity.

The same toxin gene was detected in two or more positions of the colon in 90% of the patients with *E. coli* toxins in stool and in three or four positions in 70% of the patients with *E. coli* toxins in stool, illustrating that the toxin-producing *E. coli* were distributed in the entire colorectum. The results from biopsies correlated with results from stool samples. In 95% of patients that tested positive for an *E. coli* toxin gene in a biopsy, the same gene was also detected in the stool sample.

Evaluation of qPCR results by massive parallel sequencing of the bacterial 16S rRNA gene

Illumina 300-bp paired-end sequencing generated, after quality filtering and processing, between 109,348 to 196,219 sequence reads per sample. Between 80 and 95% of the sequences were taxonomically assigned to genus level. The Shannon diversity indexes ranged from 2.273 to 2.296 and between 346 and 535 species were identified per sample. Results from 16S rRNA sequencing confirmed the results from *F. nucleatum* qPCR. Sequence data on species and genus level illustrated higher relative abundance of *F. nucleatum* and *Fusobacterium* spp. in the cancer group compared with the control group (Fig. 5). 16S rRNA sequencing data could not be used to verify the presence or quantities of *E. coli* toxin genes, but the data illustrated similar quantities of *Escherichia* sp. between the cancer and control groups (Fig. 5).

Fig. 4 Relative numbers of *F. nucleatum*-positive biopsies. *F. nucleatum* was detected more frequently in all tissues when the tumor was located in the proximal part of the colon compared with the distal part of the colon and rectum



Discussion

The aims of the present study were to determine the levels of *F. nucleatum* and selected *E. coli* toxin genes in stool and mucosa samples from Norwegian colon cancer patients and to evaluate the use of *F. nucleatum* and *E. coli* quantitative PCRs as potential microbiome-based biomarkers for detection of colorectal cancer in its early stages.

We identified significantly higher levels of *F. nucleatum* and *Fusobacterium* spp. in stool samples from the cancer group compared with the control group and the polyp group. Furthermore, *F. nucleatum* was more frequently detected in biopsies from the cancer patients. This is in line with previous studies from the USA, Canada, China, and Japan [22–24, 31], and it illustrates that *F. nucleatum* is a microbe associated with a subgroup of colorectal cancer patients also in Norway.

A number of previous studies have dismissed stool as sample material since this is not reflective of the microenvironment in colonic mucosa. A study from Mira-Pascual et al. illustrated more similar microbial communities in stool samples between control, adenoma, and CRC patients than within biopsy samples from the same patient [32]. Although stool samples do not represent the same microbiota as tumor tissues in neither richness nor diversity, there is increasing evidence that important microbial signatures associated with CRC are

reflected in stool [13, 22–24]. To identify cancer patients with high levels of *F. nucleatum* in stool, a $\Delta Ct (Ct^{Fn} - Ct^{16S\ rRNA})$ cutoff of 12 was set. Using this approach, high levels of *F. nucleatum* in stools were detected in 35% of cancer patients in the present study. These patients were diagnosed in early cancer stages. Three patients with distant metastatic spread had lower levels of *F. nucleatum* in stool (Fig. 2). No difference in *F. nucleatum* abundance was identified in stool samples between the polyp group and the control group, and *F. nucleatum* was identified in the mucosa from only one patient from the polyp group; hence, we find no evidence for *F. nucleatum* correlation with polyp development. High levels of *F. nucleatum* in stool may therefore have potential as a non-invasive biomarker for detection of colorectal cancer in its early stages. However, our results indicated that a quantitative PCR targeting fecal amounts of *F. nucleatum* was capable to identify just a subset of colorectal cancer patients, and therefore it can be used in combination with other biomarkers. The subset of *F. nucleatum*-high patients may represent a distinct CRC group and will be the focus of further research.

Colorectal cancer is typically classified into rectal, distal colon, and proximal colon cancers, which are known to have different clinical, pathological, and epidemiological features [33]. Several studies have shown that *F. nucleatum* is frequently associated with CpG island methylator phenotype–

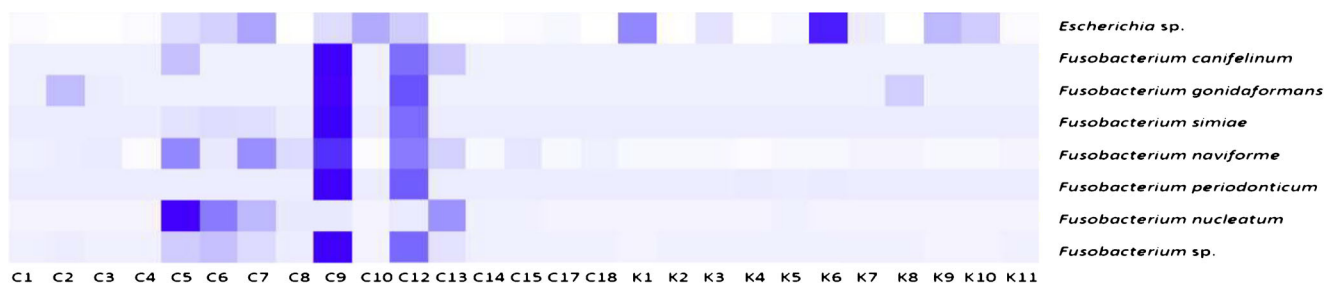


Fig. 5 Heat map from massive parallel sequencing of the 16S rRNA gene in stool samples. Higher relative abundance of *Fusobacterium* spp. was detected in cancer patients (C) compared with controls (K). Dark blue color reflects high relative number of reads within the same sample

high lesions, which typically occur in the proximal colon (reviewed in [34]). Results from the present study suggested that *F. nucleatum* was detected more frequently and in several positions of the colon when the tumor was located in the proximal part of the colon compared with the distal part and rectum. Comparison of *F. nucleatum* quantities in tumors versus the other sample sites indicated, but did not confirm statistically, a higher *F. nucleatum* abundance in the tumor site compared with the other sample positions. Due to the bias of more frequent detection of *F. nucleatum* in the proximal part of the colon, the comparisons should be performed separately for groups with different tumor locations. This will be the focus in ongoing studies in our group. Our results are in line with Mima et al. who illustrated that the proportion of *F. nucleatum*-high colorectal cancers gradually increased from the rectum to the cecum [35]. The authors argue that pathogenic influence of the microbiota on neoplastic and immune cells varies along the proximal to distal axis of the colorectum and challenges the prevailing two-colon (proximal vs. distal) dichotomy paradigm [35].

Although *E. coli* has been suggested to play a role in the pathogenesis of colorectal cancer, the precise role of this diverse species and its toxin genes are not clearly defined. This study found no significant difference in the number or the relative quantities of *E. coli* toxin genes in stool or biopsy samples between the patient groups, neither did we identify differences between tumor tissue and healthy tissue in CRC patients, as previously suggested [15, 36]. Dutilh et al. found that enterobacterial toxins were among the most highly expressed in CRC tissues while *Enterobacteriaceae* were not among the most abundant species [37]. This suggests that extended metagenomic and metatranscriptomic studies are needed to identify the exact role of *Enterobacteriaceae* and toxin contribution to colorectal cancer.

To our knowledge, this is the first study to evaluate the levels of *F. nucleatum* and *E. coli* toxins in Norwegian patients. In the present study, we have included two different control groups, one group with adenomatous polyps and one control group without any pathological findings, although scheduled for colonoscopy based on symptoms. These are patients that need to be differentiated from colorectal cancer patients and are therefore considered representative controls. A healthy control group without any symptoms would possibly reflect larger differences between the groups but would leave a gap of knowledge relating to patients suffering from similar symptoms as colorectal cancer patients.

There are some limitations of the present study. The number of patients included in each group ($n = 22\text{--}25$) is low, and the approach therefore needs to be tested on a larger sample material. Furthermore, *F. nucleatum* is a heterogeneous species with five proposed subspecies: *ss animalis*, *ss fusiforme*, *ss nucleatum*, *ss polymorphum*, and *ss vincentii*. The primers used to detect *F. nucleatum* do not differentiate between the

five subspecies, which could have different contributory effects to colorectal cancer. There could also potentially be cross-reactions between the different species of the *Fusobacterium* genus.

Conclusion

Approximately 35% of the cancer patients and none of the control patients in the present study were identified as *F. nucleatum*-high using qPCR on stool samples with a ΔCt cutoff of 12. *F. nucleatum* qPCR could potentially be included in a larger panel of non-invasive stool biomarkers for detection of colorectal cancer, but further studies are necessary to confirm this hypothesis.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical considerations The regional committee for medical and health-related research ethics (REK 2012/1944) and the data protection manager at Ahus have approved the study. Written informed consent was obtained from all participants.

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