Detection of *Fusobacterium nucleatum* DNA in primary care patient stool samples does not predict progression of colorectal neoplasia

Alan Aitchison¹, John F. Pearson², Rachel V. Purcell¹, Frank A. Frizelle¹, Jacqueline I. Keenan¹*¹

¹ Department of Surgery, University of Otago Christchurch, Christchurch, New Zealand, ² Biostatistics and Computational Biology Unit, University of Otago Christchurch, Christchurch, New Zealand

* jacqui.keenan@otago.ac.nz

Abstract

**Background**

Carriage of certain bacterial species may represent potential biomarkers of colorectal cancer (CRC). Prominent among these is *Fusobacterium nucleatum*. We explored the association of *F. nucleatum* DNA in stool samples with the presence of colonic neoplastic lesions in a cohort of primary care patients, and compared our findings with those from an unrelated cohort of colonoscopy patients followed clinically over time.

**Methods**

Carriage rates of *F. nucleatum* in stool samples were assessed in 185 patients referred for a faecal immunochemical test (FIT) by their general practitioners (GPs). Comparisons were made with stool samples from 57 patients diagnosed with CRC and 57 age-matched healthy controls, and with tissue samples taken at colonoscopy from 150 patients with a decade of subsequent clinical follow-up.

**Findings**

*F. nucleatum* DNA was found at a high rate (47.0%) in stool samples from primary care patients, and more often in stool samples from CRC patients (47.4%) than in healthy controls (7.0%), (P = 7.66E-7). No association was found between carriage of *F. nucleatum* and FIT positivity (P = 0.588). While evidence of stool-associated *F. nucleatum* DNA was significantly more likely to indicate a lesion in those primary care patients progressed to colonoscopy (P = 0.023), this finding did not extend to the progression of neoplastic lesions in the 150 patients with a decade of follow up.

**Conclusion**

The finding of *F. nucleatum* DNA at similar rates in stool samples from patients diagnosed with CRC and in primary care patients with pre-cancerous lesions supports growing awareness that the presence of these bacteria may be a biomarker for increased risk of disease.
However, molecular evidence of *F. nucleatum* did not predict progression of colonic lesions, which may lessen the utility of this bacterium as a biomarker for increased risk of disease.

**Introduction**

Colorectal cancer (CRC) is a considerable health burden globally, being the second most diagnosed cancer in women and third in men [1]. CRC is usually surgically curable in the early stages (I and II) of the disease, with a 5-year relative survival of about 90% [2]. The key to a good prognosis is early diagnosis. This remains an issue however, not least because patients with early stage disease have no symptoms or present with non-specific symptoms in primary care that may require multiple visits before referral for investigation. Accordingly, development of accurate screening tools could considerably reduce the burden of CRC.

The adenoma-carcinoma sequence is well described and, as such, the adenoma provides a target for screening for precancerous lesions. Colonoscopy still provides the most reliable method for screening for adenomas however this relatively expensive, resource-intensive and invasive procedure and as such is not a good population screening tool. Accordingly, biomarkers of early-stage disease that identify at-risk individuals, who would benefit from clinical investigation, are needed. The most widely used non-invasive screening test is the faecal immunochemical test (FIT), which is based on the detection of faecal haemoglobin (f-Hb) in stool samples. While this test has utility as a population-based screening tool, it has limited ability to detect small adenomas (< 1 cm in size) in a primary care setting [3]. While stool-based DNA testing may be more sensitive than FIT with regard to detection of larger lesions, it also lacks the sensitivity to detect small adenomas [4]. Accordingly, other biomarkers of early-stage disease are needed.

There is growing evidence of an association of gut bacteria with CRC. Globally, changes in the composition of gut microbiota (dysbiosis) have been described [5, 6], while an increasing number of studies find that certain species of gut microbiota carrying a range of virulence factors are more prevalent in individuals with CRC when compared to age-matched healthy controls. These bacterial species include enterotoxigenic strains of *Bacteroides fragilis* (ETBF) [7], and strains of *E. coli* that carry the *pkS* gene cluster encoding the synthesis of the colibactin genotoxin [8]. Long-term colonic carriage of ETBF is associated with significant risk of developing low-grade colonic dysplastic lesions [7] and colonic carriage of *pkS+ E. coli* is reportedly increased in colon cancer [9]. Moreover, animal modelling has been used to illustrate how the *B. fragilis* toxin and *E. coli* colibactin toxin may work together to promote colon cancer [10]. *Fusobacterium nucleatum*, a common member of the oral microflora, has also been associated with CRC [11–13]. While it has been argued that *F. nucleatum* may be an opportunistic pathogen in CRC [14], other evidence supports active involvement of these bacteria in colonic oncogenesis by recruitment of tumour-infiltrating myeloid cells [15] and/or by activation of the Wnt signalling pathway via interaction of *F. nucleatum* FadA protein with E-cadherin [16].

Here we investigate the prevalence of *F. nucleatum* carriage in a cohort of 185 patients presenting in primary care with bowel symptoms, and correlate the presence of this potential driver of colorectal carcinogenesis with the presence of f-Hb in the same stool samples [3] and, where possible, clinical follow up.

**Methods**

A total of four cohorts were investigated in this study, as detailed below. These included stool samples from patients presenting in primary care, unrelated stool samples from age-matched
CRC patients and self-reporting healthy community controls, and mucosal biopsies from unrelated patients referred for colonoscopy (Fig 1).

Primary care patient cohort

One hundred and eight-five patients presenting to their general practitioners (between 2014 and 2017) with bowel problems (hereafter referred to as primary-care patients) and subsequently referred for FIT (to detect the presence of f-Hb) gave written informed consent for their stool samples to also be screened for bacterial biomarkers, and for possible clinical follow-up. This study was approved by the University of Otago Human Ethics Committee (H14/019). Samples were stored at -80˚C until DNA extraction.

Unrelated stool sample cohorts

For comparison, stool samples from two age-matched cohorts were also investigated for molecular evidence of *F. nucleatum*. These included samples from 57 individuals, diagnosed with CRC between 2012 and 2014, using standard endoscopic, histological or radiological criteria, who provided a stool sample prior to surgery. Patients found to have had pre-operative chemo-radiation therapy were not included in the study. Additionally, 57 stool samples from a cohort of 125 collected from healthy volunteers who self-reported no evidence of bowel problems at the time of sampling (healthy controls) were age-matched to the CRC patients. All gave written informed consent for their stool samples to be screened for bacterial biomarkers. The CRC patient and healthy control cohorts were initially collected to determine carriage...
rates of ETBF in the cohorts [17] (Southern Health and Disability Ethics Committee (URA/12/02/005/AM03). The three stool sample cohorts (primary care, CRC and healthy controls) are described in S1 Table in S2 File. Stool samples were stored at -80°C until DNA extraction.

**Colonic biopsy cohort**

For further comparison, mucosal biopsies collected from 150 patients referred for colonoscopy at our institution between February 2003 and August 2005 were also investigated for molecular evidence of *F. nucleatum*. Patients provided written informed consent for tissue to be collected from up to four different sites in the colon: A, terminal ileum; B, caecum; C, transverse colon; D, recto-sigmoid colon, and for monitoring of clinical follow-up over time (Upper South A Regional Ethics Committee CTY/02/08/132). Details of this cohort are described in S2 Table in S2 File. The samples taken for analysis were macroscopically normal, i.e. no overtly dysplastic, polypoid or cancerous tissue samples were used. Patients had not had previous colonic resections. The samples were frozen in liquid nitrogen and transferred to -80°C until DNA extraction.

Follow-up data was available for 134 patients up to June 2015 (10 to 12 years). Sixteen patients were lost to follow-up during this period, including four who died of CRC, 11 who died of other causes, and one patient who moved to a different country. Clinical data available for the remaining patients at the time of sampling and during this follow-up period included development of CRC, number and type of polyps, presence and type of dysplasia, and side (left or right) of colonic disease, diagnosed from this or subsequent colonoscopies; 75 patients had one or more subsequent colonoscopy.

**Sample preparation**

DNA was extracted directly from 100 mg aliquots of individual stool samples using a commercially available kit (Dynabeads DNA DIRECT™ Universal extraction kit, Life Technologie AS, Oslo, Norway). This extract was diluted 100-fold to reduce any inhibitory factors. A different kit (High-Pure PCR Template Preparation Kit, Roche, Nonnenwald, Germany) was used, as per the manufacturer’s instructions, to extract DNA from the biopsies. Purified DNA was quantified using the NanoDrop 2000c spectrophotometer (Thermo Scientific, Asheville, NC, USA), with 260/280 absorbance ratios being between 1.7 and 2.1 for all extracted samples. All extracts were stored at -20°C.

**Stool sample PCR**

SYBR-Green chemistry was used to detect evidence of *F. nucleatum* in the stool samples, as described previously [17]. Briefly, 25–35 ng of DNA was used along with 0.5 μM of each primer, 5 μl PerfeCTa SYBR Green Fastmix (Quantabio, Beverly, MA, USA) and 1.5 μl H₂O in a 10μl reaction run on a LightCycler 480 thermocycler (Roche Diagnostics, Indianapolis, IN, USA) for one 5 min cycle of 95°C followed by 45 cycles of 95°C for 10 sec, 57°C for 10 sec and 70°C for 15 sec. Primer details for the *F. nucleatum* nusG are shown in S3 Table in S2 File. *Fusobacterium nucleatum subsp. nucleatum* (ATCC 25586) was used as a reference strain and a standard curve was made using extracted DNA from this reference strain according to the method of Dolezel *et al* [18]. *F. nucleatum* has a genome size of 2.4Mb and a single *F. nucleatum* genome weighs 2.43 fg (2.4 Mb/987 Mb [1pg of double-stranded DNA] = 0.00243 pg). Therefore, 1 ng of *F. nucleatum* DNA contains approximately 411,523 copies of the genome (1000pg/0.00243 pg). Samples were considered positive if two of the three replicates amplified *F. nucleatum* DNA.
Incidence of *F. nucleatum* in colonic biopsies analysed by qPCR

TaqMan probes (S4 Table in S2 File) designed to detect *F. nucleatum* nusG gene and a reference gene, *PGT* [12], were used to screen genomic DNA isolated from the colonic biopsies. Each reaction consisted of 25–35 ng of genomic DNA, 5 μl of TaqMan Fast Advanced Master Mix (Applied Biosystems), and 0.5 μl TaqMan primer/probe (Thermo Fisher) in a 10 μl reaction. A LightCycler480 thermocycler was used, and thermal cycling conditions were as follows: 1 cycle of 95˚C for 10 mins, followed by 50 cycles of 95˚C for 10 secs and 60˚C for 30 secs. All reactions were performed in triplicate. DNA extracted from *F. nucleatum* subsp. *nucleatum* (ATCC 25586) was used as a positive control.

Faecal Immunochemical Test (FIT)

A qualitative (one-step membrane cassette) immunoassay was used for detecting f-Hb in each stool sample (Ngaio Diagnostics Ltd, Nelson, New Zealand). This assay detects human haemoglobin above 50 μg of f-Hb per gm of faeces, and is shown to be specific for human haemoglobin.

Statistical analysis

For stool samples, counts of positive samples were compared between 3 cohorts, healthy controls, primary care and CRC patients using Fisher exact tests. Counts of positive samples between control and both patient cohorts (CRC and primary care) were compared with odds ratios and 95% Wald confidence intervals using Fisher exact P-values. For comparisons with zero counts, the Haldane Anscombe correction was applied prior to calculating odds ratios and confidence intervals. The association of biopsy positivity with location and outcome was assessed using generalized mixed effects logistic regression including fixed effects for age and sex and a random effect for subject, for details see Purcell *et al.* [7]. Abundance was compared by by Welch’s one-way test for differences in means assuming unequal variances on log transformed values followed by posthoc t-tests with P values corrected for multiple comparisons by the Bonferroni method. All tests were 2-sided and considered statistically significant at P<0.05. Analysis was performed in R 4.0.5, using the epiR package.

Results

Stool sample analysis

Across cohorts, *F. nucleatum* DNA was detected more often in the stools of patients than in healthy controls (Fig 2, Table 1). PCR detected evidence of *F. nucleatum* DNA in 87/185 of the primary care-patient samples and 27/57 cancer patient samples, significantly greater than in 4/57 of the age-matched healthy controls (P = 3.02x10^-9, 7.66x10^-7 respectively).

There was evidence for differences in the abundance of *F. nucleatum* across the 3 cohorts (F(2,12.6) = 25.7, P = 0.00004), with both primary care and CRC cohorts showing significantly more abundant *F. nucleatum* than healthy controls, P = 0.0008 and 0.0023 respectively, while the difference detected between primary care and CRC cohorts was not statistically significant, P = 0.341, where P values have been corrected for multiple comparisons.

Clinical investigation

Within the primary care cohort, 82 of the 185 patients subsequently progressed to further investigation that included colonoscopy. Thirty-two of these patients (39%) were found to have evidence of lesions that included CRC (n = 2) and polyps (n = 33) (Table 2). Seven patients presented with lesions greater than 1 cm in size, including the two patients identified
Fig 2. Abundance of *F. nucleatum* in stool samples. *F. nucleatum* abundance is significantly higher in primary care and CRC stool samples compared to healthy controls (P = 0.0008 and P = 0.0023, respectively) but is not significant between primary care and CRC cohorts, P = 0.341. Values for individual samples are shown in S1 File.

https://doi.org/10.1371/journal.pone.0269541.g002
with CRC, four patients with tubular adenomas (all reported as having low-grade dysplasia), and one patient found to have multiple sessile serrated adenomas. The other 25 patients had lesions of less than 1 cm while the remaining 50 patients were reported as having a normal colonoscopy. Patients with diverticulosis were considered normal if no evidence of lesions was found.

Twenty-one of the 40 patients in primary care with evidence of \textit{F. nucleatum} DNA in their stool samples and who progressed to colonoscopy were found to have evidence of lesions. The majority of these patients (n = 19) presented with polyps that were less 1 cm in size. Polyps included tubular adenomas, tubulovillous adenomas and serrated polyps (including hyperplastic polyps and sessile serrated adenomas). These were reported in 12, 1 and 11 patients, respectively, reflecting histological evidence of more than one polyp subtype in several patients. Ten of these patients were found to have polyps with evidence of low-grade dysplasia. The two patients with lesions greater than 1 cm were found to have multiple sessile serrated adenomas and CRC, respectively. Eleven patients with no evidence of \textit{F. nucleatum} DNA in their stool samples were also progressed to colonoscopy (Table 2). While the types of clinical lesions found in the two groups were similar, the presence of \textit{F. nucleatum} DNA in the stool was associated with a significantly greater risk of having any lesion found (OR = 3.11, 95% CI [1.23, 7.87], P = 0.023).

We have previously reported FIT positivity (>50 \textmu g of Hb per gm of faeces) in 29 of the 185 samples collected from patients in the primary care cohort [3]. Across this cohort, 15 of the 29 patients who had evidence of faecal haemoglobin in their stool sample also had evidence of \textit{F. nucleatum} DNA (52%). There was no association of \textit{F. nucleatum} positivity with regard to f-Hb status (P = 0.588).

To further investigate the proposed link between colonic carriage of \textit{F. nucleatum} and lesions in the colon we looked retrospectively for molecular evidence of \textit{F. nucleatum} in biopsies collected from up to four colonic sites from 150 patients undergoing colonoscopy. The age of these patients at the time of the procedure ranged from 19–88 years (mean = 55 years) and there were 100 females and 50 males (S2 Table in S2 File). Previous medical history, along with follow-up medical reports and subsequent colonoscopies were used to generate clinical characteristics for the cohort. Eleven patients had previously diagnosed CRC with an additional nine

Table 1. \textit{F. nucleatum} positivity relative to a healthy control cohort.

| Cohort     | Positive (%) | OR [95% CI] | P     |
|------------|--------------|-------------|-------|
| Controls   | 57           | 7.0         |       |
| GP Patients| 185          | (47.0)      | 11.76 [4.09,33.83] | 3.02E-9 |
| CRC Patients| 57          | (47.4)      | 11.93 [3.81,37.35] | 7.66E-7 |

Count of positive samples in patient cohorts compared to controls by Odds Ratios (OR) with 95% Confidence Intervals (CI) and P values.

https://doi.org/10.1371/journal.pone.0269541.t001

Table 2. Association of stool-associated \textit{F. nucleatum} DNA with clinical lesions in the primary care cohort progressed for clinical investigation.

| Diagnosis | Any lesion at colonoscopy (n = 32) | \textit{F. nucleatum} positive (n = 21) | \textit{F. nucleatum} negative (n = 11) |
|-----------|-------------------------------------|----------------------------------------|---------------------------------------|
| CRC       | 2                                   | 1                                      | 1                                     |
| SP        | 14                                  | 11                                     | 3                                     |
| TA        | 15                                  | 12                                     | 3                                     |
| TVA       | 4                                   | 1                                      | 3                                     |

CRC, colorectal cancer; SP, serrated polyp; TA, tubular adenoma; TVA, tubulovillous adenoma

https://doi.org/10.1371/journal.pone.0269541.t002
diagnosed at the time of colonoscopy or during the follow-up period, giving a total of 15% of patients with CRC. Sixty-six patients were diagnosed with having polyps, 23 of these reported as having more than one type of polyp present. As above, the types of polyps described were tubular adenomas, tubulovillous adenomas and serrated polyps, and were reported in 35, 16 and 40 patients, respectively. Low-grade dysplasia was reported in 19/150 patients and high-grade dysplasia in 9/150. A total of 77 patients were reported to have at least one colonic neoplastic lesion (dysplasia, polyps, adenomas, or CRC), and this was reported to be right sided (ascending) in 19/77, left sided (descending) in 45/77, and in both sides in 13 patients. The remaining 73 patients were not diagnosed with any of the lesions being investigated in this study (S2 Table in S2 File).

**Concordance of F. nucleatum in colonoscopy mucosal biopsies**

The presence of *F. nucleatum* was confirmed in colonoscopy samples from 88/150 patients (58.7%) following qPCR on DNA samples from up to four colonic sites. In 56 of these 88 patients each sampling site was positive for *F. nucleatum* DNA. The remaining 32 patients had at least one colonic site at which *F. nucleatum* DNA was undetectable. Sixty-two patients were negative for *F. nucleatum* DNA at all sites tested. Hence 118 out of 150 of patients had samples that were either *F. nucleatum* positive or negative at all sites, a raw concordance of 79%.

**Association of F. nucleatum with clinicopathological characteristics**

Univariate logistic regression analysis was used to determine associations between *F. nucleatum* positivity and clinicopathological parameters of the colonic biopsy cohort (Table 3). No significant associations were seen between *F. nucleatum* positivity and the presence of CRC, serrated polyps, high-grade dysplasia, low grade dysplasia, tubular adenomas or tubulovillous adenoma (P-values > 0.05). Analysis of the association of *F. nucleatum* positivity showed that the presence of *F. nucleatum* did not differ significantly by colonic site (Table 3). For comparison, we previously reported ETBF to be significantly more likely to be present in more distal samples (Table 3). Recto-sigmoid samples were more likely to be positive than transverse colon in ETBF-colonised individuals, and transverse colon more likely to be positive than

| Table 3. Association of *F. nucleatum* and ETBF positivity with clinical lesions in the colonic biopsy cohort, adjusted for age and gender. |
|---------------------------------|---------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                                 | *F. nucleatum* (this study)     |                  | ETBF            |                  |                  |                  |                  |
|                                 | OR     | 95% CI | P value | OR     | 95% CI | P value | OR     | 95% CI | P value |
| **Location**                    |        |        |         |        |        |         |        |        |         |
| Site                            |        |        |         |        |        |         |        |        |         |
| Recto-sigmoid                   | 1      | [0.298]| 0.298   | 1      | [0.298]| 0.0010*|        |        |         |
| Transverse                      | 1.86   | [0.84,12]| 0.69 | 0.21,2.26]| 0.152 | 2.79 | 0.30,2.33]| 0.738 |         |
| Caecum                          | 1.47   | [0.65,3.30]| 0.09 | [0.02,0.41]| 0.005*| 4.51 | [1.53,16.58]| 0.007*|         |
| **Diagnosis**                   |        |        |         |        |        |         |        |        |         |
| CRC                             | 0.766  | [0.88,8.07]| 0.087 | 0.30,2.33]| 0.007*| 0.84 | [1.31,6.16]| 0.005*|         |
| SP                              | 1.74   | [0.82,3.85]| 0.152 | 1.11,5.58]| 0.294 | 2.79 | [1.31,6.16]| 0.007*|         |
| LGD                             | 1.02   | [0.38,2.82]| 0.958 | 4.51 | [1.53,16.58]| 0.005*| 4.18 | [1.98,9.77]| 0.005*|         |
| HGD                             | 0.97   | [0.24,4.17]| 0.966 | 1.98 | [0.49,9.77]| 0.347 | 1.98 | [0.49,9.77]| 0.347 |         |
| TA                              | 1.13   | [0.52,2.51]| 0.766 | 2.43 | [1.11,5.58]| 0.027 | 1.76 | [0.61,5.47]| 0.294 |         |
| TVA                             | 0.53   | [0.18,1.53]| 0.241 | 1.76 | [0.61,5.47]| 0.294 | 1.76 | [0.61,5.47]| 0.294 |         |

CRC, colorectal cancer; SP, serrated polyp; LGD, low-grade dysplasia; HGD, high-grade dysplasia; TA, tubular adenoma; TVA, tubulovillous adenoma; ETBF, enterotoxigenic *Bacteroides fragilis*. *significant after adjustment for multiple comparisons.

https://doi.org/10.1371/journal.pone.0269541.t003
caecal samples (P = 0.001). There was no evidence that *F. nucleatum* was associated with either presence or location of colonic disease. Likewise, no association was found between *F. nucleatum* positivity and patient age (P = 0.19) or gender (P = 0.90).

**Discussion**

This study found that the proportion of primary care patients with evidence of *F. nucleatum* DNA in their stool samples was similar to that found in CRC patients, and significantly higher than the carriage rate detected in the self-reporting healthy controls. While a number of studies have to date also reported an increased presence of *F. nucleatum* DNA in stool samples from patients presenting with adenomas and CRCs [12, 15, 19], this is the first study to extend this finding to patients presenting in primary care with bowel symptoms. We found molecular evidence of *F. nucleatum* in approximately 50% of stool samples from these individuals. This is in marked contrast to samples from age-matched self-reporting healthy individuals, where only 7% were found to be positive. Collectively, these findings reinforce the growing awareness that screening for *F. nucleatum* DNA may offer the opportunity for early identification of patients who would potentially benefit from clinical investigation and/or ongoing surveillance [20].

Bacteria in the mucosa of the colon are not necessarily well represented in stool, as exemplified by reportedly higher rates of ETBF carriage detected in the colonic mucosa in CRC patients [21] than in patient stool samples [17, 22]. Our finding however of a highly significant difference in the carriage rate of *F. nucleatum* DNA in CRC patient stool samples when compared to stool samples from healthy controls confirms other reports that suggest these bacteria are well represented in the lumen of the gut [12, 15, 19], in addition to being present in tissue samples as shown here and by others [11, 12, 15, 23]. Moreover, the observation that the relative abundance of *F. nucleatum* DNA in the in the primary care patients was notably higher than that detected in the stool samples from CRC patients reinforces the idea that ongoing surveillance of patients with molecular evidence of *F. nucleatum* in stool samples may be warranted.

Current thinking is that Fusobacteria may contribute to tumorigenesis via an inflammatory-mediated mechanism [15], a theory enhanced by the finding that Fusobacterium strains taken from inflamed tissue of IBD patients exhibited increased invasive potential compared to strains found in non-inflamed tissue [24]. More recently, studies suggest that *F. nucleatum* may promote CRC development by suppressing aspects of cell-mediated host immunity, and that the notable association between high-level colonisation by these bacteria and MSI-H tumours [23, 25] may relate to a reported association between the abundance of *F. nucleatum* in mucosal biopsies and a finding of serrated polyps at colonoscopy [26]. These studies do not, however, confirm a role for these bacteria in initiating as opposed to driving the serrated polyp pathway (or indeed colorectal carcinogenesis). Thus, while patients presenting in primary care with bowel symptoms were found to be significantly more likely to have molecular evidence of *F. nucleatum* in their stool samples when compared to healthy controls, these findings do not address cause or effect [27].

The premise that molecular screening for faecal microbiota such as *F. nucleatum* may complement FIT in identifying at risk individuals and/or at-risk populations who would benefit from clinical investigation has been explored [19, 28, 29]. Our study failed to find a significant association between molecular evidence of *F. nucleatum* and detection of f-Hb in the same stool samples. This is in contrast to the study by Wong *et al.* that showed quantitation of faecal *F. nucleatum* improved the diagnostic performance of the FIT with regard to detecting advanced adenomas [19]. This may, in part, reflect the cut off value of the FIT assay, which was notably lower in the Wong study than the threshold of the assay used here (20 μg and
similar to those reported by Grobbee and more recently screened for the presence of ETBF [7]. Clinical data was available for 134 of 150 patients between 2003 and 2005 originally to look for evidence of pathological findings at colonoscopy.

While the small numbers in our study preclude more meaningful analysis, the findings are consistent with those of previous studies. We did however find that molecular evidence of *F. nucleatum* in stool was associated with a significantly higher risk of having any lesion found. To investigate this further, we looked for evidence of pathological findings at colonoscopy. Of the 32 patients found to have lesions, most were small polyps (<1 cm) and there was no association of molecular evidence of *F. nucleatum* with serrated lesions compared to other neoplasia or a normal colon.

We did however find that molecular evidence of *F. nucleatum* in stool was associated with a significantly higher risk of having any lesion found. To investigate this further, we looked for the presence of *F. nucleatum* DNA in an unrelated cohort of colonic biopsies collected from 150 patients between 2003 and 2005 originally to look for evidence of *Helicobacter* spp. [34] and more recently screened for the presence of ETBF [7]. Clinical data was available for 134 of these patients, both at the time of sampling and during the 10–12 year follow-up period, and included development of CRC, the number and type of polyps, and the presence and type of dysplasia [7]. We found no significant association between clinico-pathological features and colonisation with *F. nucleatum*. Interestingly, Zakular et al. [35] report a similar finding using a 16S rRNA gene sequencing approach. Specifically, the presence of *F. nucleatum* was not significantly associated with the development of serrated polyps in our study. This finding was unexpected, given recent studies that find colonic carriage of *F. nucleatum* is associated with MSI-H [23, 36] or CIMP+ [26, 36] subtypes of CRC that are considered to develop through the serrated neoplasia pathway [37]. There was also no evidence that the presence of *F. nucleatum* was more likely to be detected in the proximal as opposed to the distal bowel, as reported elsewhere [26, 38]. It is possible that these differences may, in part reflect our reporting of the presence as opposed to the relative abundance of *F. nucleatum* in these samples and we acknowledge this as a potential limitation of our findings.

Levels of the *F. nucleatum* nusG gene and a reference control, prostaglandin transporter (PGT), were simultaneously measured in this study using a probe that detects *F. nucleatum* subsp. *nucleatum* [7, 11]. It was noted however that the *F. nucleatum* nusG primers used in the probe have differing sequence similarities to each of the four *F. nucleatum* subspecies, only two of which (nucleatum and animalis) are considered disease-associated [39]. While the forward primer had 100% identity across the entire length of the primer with all four subspecies, the reverse primer did not have any homology with the *animalis* subspecies (strain ChDC F332). Each of the remaining subspecies, *F. nucleatum* subsp. *nucleatum* (strain ATCC 25586), *F. nucleatum* subsp. *polymorphum* (strain ChDC F306) and *F. nucleatum* subsp. *vincentii* (strain KCOM 2931) contained three mismatches within the 5’ half of the reverse primer. Accordingly, while minor enough not to affect detection of *F. nucleatum* per se, it is likely that the use of this reverse primer may sufficiently affect the dynamics of the PCR to preclude assessment of relative abundance between samples. Whereas one study to date reports *F. nucleatum* subsp. *animalis* is the predominant *F. nucleatum* subspecies in CRC specimens [40], this is an area that warrants further investigation.
A potential limitation of our study might be seen as our focus on a single bacterial candidate as a novel biomarker for non-invasive diagnosis of CRC [41]. Our goal, however, in screening for a potential bacterial biomarker (alone, and in combination with FIT) was to identify symptomatic patients presenting in primary care who should be progressed for clinical investigation [42]. Accordingly, our primary care cohort did not include asymptomatic individuals [43]. Other potential limitations of this study include statistical power which was adequate for the primary comparison but limited in sub-group analysis by the low incidence of \( F. \) nucleatum in control samples and biases in presentation and referral criteria, for example by ethnicity which is better described elsewhere [44]. Additionally, the use of samples collected across different calendar times and from different cohorts with different selection criteria might also be considered a limitation of our study, although the findings suggest otherwise.

In summary, in addition to cancer and healthy control cohorts, this study uniquely investigates the carriage of \( F. \) nucleatum in a primary care cohort referred for FIT. Our results suggest that while \( F. \) nucleatum is detected at higher rates in the stool samples of individuals presenting in primary care with bowel problems than in healthy individuals, it may not necessarily by itself be a biomarker of lesions that have the potential to drive colon carcinogenesis.

Supporting information

S1 File. Abundance of \( F. \) nucleatum in patient cohorts. (XLSX)

S2 File. (DOCX)

Author Contributions

Conceptualization: Alan Aitchison, Frank A. Frizelle, Jacqueline I. Keenan.

Data curation: Alan Aitchison, Rachel V. Purcell, Jacqueline I. Keenan.

Formal analysis: John F. Pearson.

Funding acquisition: Frank A. Frizelle, Jacqueline I. Keenan.

Investigation: Alan Aitchison, Jacqueline I. Keenan.

Methodology: Alan Aitchison, Jacqueline I. Keenan.

Writing – original draft: Alan Aitchison, John F. Pearson, Jacqueline I. Keenan.

Writing – review & editing: Alan Aitchison, John F. Pearson, Rachel V. Purcell, Frank A. Frizelle, Jacqueline I. Keenan.

References

1. Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, et al. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. International journal of cancer Journal international du cancer. 2015; 136(5):E359–86. https://doi.org/10.1002/ijc.29210 PMID: 25220842
2. Buchwald P, Hall C, Davidson C, Dixon L, Dobbs B, Robinson B, et al. Improved survival for rectal cancer compared to colon cancer: the four cohort study. ANZ journal of surgery. 2018; 88(3):E114–E7. https://doi.org/10.1111/ans.13730 PMID: 27618786
3. Keenan J, Aitchison A, Leaman J, Pearson J, Frizelle F. Faecal biomarkers do not always identify pre-cancerous lesions in patients who present in primary care with bowel symptoms. The New Zealand medical journal. 2019; 132(1501):48–56. PMID: 31465327
4. Ahlquist DA, Zou H, Domanico M, Mahoney DW, Yab TC, Taylor WR, et al. Next-generation stool DNA test accurately detects colorectal cancer and large adenomas. Gastroenterology. 2012; 142(2):248–56; quiz e25–6. https://doi.org/10.1053/j.gastro.2011.10.031 PMID: 22062357

5. Sears CL, Garrett WS. Microbes, microbiota, and colon cancer. Cell host & microbe. 2014; 15(3):317–28.

6. Gagniere J, Raisch J, Veziant J, Barnich N, Bonnet R, Buc E, et al. Gut microbiota imbalance and colorectal cancer. World journal of gastroenterology: WJG. 2016; 22(2):501–18. https://doi.org/10.3748/wjg.v22.i2.501 PMID: 26811603

7. Purcell RV, Pearson J, Alitchson A, Dixon L, Frizelle FA, Keenan JI. Colonization with enterotoxigenic Bacteroides fragilis is associated with early-stage colorectal neoplasia. PLoS one. 2017; 12(2): e0171602. https://doi.org/10.1371/journal.pone.0171602 PMID: 28151975

8. Nougayrede JP, Homburg S, Taieb F, Boury M, Bruszkiwicz E, Gottschalk G, et al. Escherichia coli induces DNA double-strand breaks in eukaryotic cells. Science. 2006; 313(5788):848–51. https://doi.org/10.1126/science.1127059 PMID: 16902142

9. Flanagan L, Schmid J, Ebert M, Soucek P, Kunicka T, Liska V, et al. Fusobacterium nucleatum infection is prevalent in human colorectal carcinoma. Genome research. 2012; 22(2):299–306. https://doi.org/10.1101/gr.126516.111 PMID: 22009990

10. Bacteroides fragilis enterotoxin in the aetiology of colorectal cancer. Clinical microbiology and infection: the official journal of the European Society of Clinical Microbiology and Infectious Diseases: official publication of the European Society of Clinical Microbiology. 2006; 12(6):663–70. https://doi.org/10.1111/j.1469-0691.2006.01494.x PMID: 16842574

11. Dejea CM, Fathi P, Craig JM, Boleij A, Taddese R, Geis AL, et al. Patients with familial adenomatous polyposis harbor colonic biofilms containing tumorigenic bacteria. Science. 2018; 359(6375):592–7. https://doi.org/10.1126/science.aar3468 PMID: 29420293

12. Castellarin M, Warren RL, Freeman JD, Dreolini L, Krzywinski M, Strauss J, et al. Fusobacterium nucleatum infection is prevalent in human colorectal carcinoma. Genome research. 2012; 22(2):292–8. https://doi.org/10.1101/gr.126573.111 PMID: 22009989

13. Tjalsma H, Boleij A, Marchesi JR, Dutilh BE. A bacterial driver-passenger model for colorectal cancer: beyond the usual suspects. Nature reviews Microbiology. 2012; 10(8):575–82. https://doi.org/10.1038/nrmicro2819 PMID: 22728587

14. Kostic AD, Gevers D, Pedamallu CS, Michaud M, Duke F, Earl AM, et al. Genomic analysis identifies association of Fusobacterium with colorectal carcinoma. Genome research. 2012; 22(2):292–8. https://doi.org/10.1101/gr.126573.111 PMID: 22009990

15. Castellarin M, Warren RL, Freeman JD, Dreolini L, Krzywinski M, Strauss J, et al. Fusobacterium nucleatum infection is prevalent in human colorectal carcinoma. Genome research. 2012; 22(2):292–8. https://doi.org/10.1101/gr.126573.111 PMID: 22009989

16. Rubinstein MR, Wang X, Liu W, Hao Y, Cai G, Han YW. Fusobacterium nucleatum promotes colorectal carcinogenesis by modulating E-cadherin/beta-catenin signaling via its FadA adhesin. Cell host & microbe. 2013; 14(2):195–206. https://doi.org/10.1016/j.chom.2013.07.012 PMID: 23954158

17. Keenan JI, Alitchson A, Purcell RV, Greenlees R, Pearson JF, Frizelle FA. Screening for enterotoxigenic Bacteroides fragilis in stool samples. Anaerobe. 2016; 40:50–3. https://doi.org/10.1016/j.anaerobe.2016.05.004 PMID: 27166180

18. Dolezel J, Bartos J. Plant DNA flow cytometry and estimation of nuclear genome size. Ann Bot. 2005; 95(1):99–110. https://doi.org/10.1093/aob/mci005 PMID: 15596459

19. Wong SH, Kwong TNY, Choy TC, Luk AKC, Dai RZW, Nakatsu G, et al. Quantitation of faecal Fusobacterium improves faecal immunochemical test in detecting advanced colorectal neoplasia. Gut. 2017; 66(8):1441–8. https://doi.org/10.1136/gutjnl-2016-312786 PMID: 27797940

20. Gethings-Behncke C, Coleman HG, Jordaio HWT, Longley DB, Crawford N, Murray LJ, et al. Fusobacterium nucleatum in the Colorectum and Its Association with Cancer Risk and Survival: A Systematic Review and Meta-analysis. Cancer epidemiology, biomarkers & prevention: a publication of the American Cancer Society for Cancer Research, cosponsored by the American Society of Preventive Oncology. 2020; 29(3):539–48. https://doi.org/10.1158/1055-9965.EPI-18-1295 PMID: 31915144

21. Boleij A, Hechenbleikner EM, Goodwin AC, Badani R, Stein EM, Lazarev MG, et al. The Bacteroides fragilis Toxin Gene Is Prevalent in the Colon Mucosa of Colorectal Cancer Patients. Clinical infectious diseases: an official publication of the Infectious Diseases Society of America. 2015; 60(2):208–15.

22. Toprak NU, Yagci A, Guluoagli BM, Akin ML, Demirkalem P, Celenk T, et al. A possible role of Bacteroides fragilis enterotoxin in the aetiology of colorectal cancer. Clinical microbiology and infection: the official publication of the European Society and Infectious Diseases. 2006; 12(8):782–6. https://doi.org/10.1111/j.1469-0691.2006.01494.x PMID: 16842574
23. Viljoen KS, Dakshinamurthy A, Goldberg P, Blackburn JM. Quantitative profiling of colorectal cancer-associated bacteria reveals associations between fusobacterium spp., enterotoxigenic Bacteroides fragilis (ETBF) and clinicopathological features of colorectal cancer. PloS one. 2015; 10(3):e0119462. https://doi.org/10.1371/journal.pone.0119462 PMID: 25751261

24. Strauss J, Kaplan GG, Beck PL, Rioux K, Panaccone R, Deviney R, et al. Invasive potential of gut mucosa-derived Fusobacterium nucleatum positively correlates with IBD status of the host. Inflammatory bowel diseases. 2011; 17(9):1971–8. https://doi.org/10.1002/ibd.21606 PMID: 21830275

25. Hamada T, Zhang X, Mima K, Bullman S, Sukawa Y, Nowak JA, et al. Fusobacterium nucleatum in Colorectal Cancer Relates to Immune Response Differentially by Tumor Microsatellite Instability Status. Cancer Immunol Res. 2018; 6(11):1327–36. https://doi.org/10.1158/2326-6066.CIR-18-0174 PMID: 30228205

26. Ito M, Kanno S, Nosho K, Sukawa Y, Mitsuhashi K, Kurihara H, et al. Association of Fusobacterium nucleatum with clinical and molecular features in colorectal serrated pathway. International journal of cancer Journal international du cancer. 2015; 137(6):1258–68. https://doi.org/10.1002/ijc.29488 PMID: 25703934

27. Brennan CA, Garrett WS. Fusobacterium nucleatum—symbiont, opportunist and oncobacterium. Nature reviews Microbiology. 2019; 17(3):156–66. https://doi.org/10.1038/s41579-018-0129-6 PMID: 30546113

28. Baxter NT, Ruffin MT, Rogers MA, Schloss PD. Microbiota-based model improves the sensitivity of fecal immunochemical test for detecting colonic lesions. Genome Med. 2016; 8(1):37. https://doi.org/10.1186/s13073-016-0290-3 PMID: 27056827

29. Grobbee EJ, Lam SY, Fuhler GM, Blakaj B, Konstantinov SR, Bruno MJ, et al. First steps towards combining faecal immunochemical testing with the gut microbiome in colorectal cancer screening. United European Gastroenterol J. 2020; 8(3):293–302. https://doi.org/10.1177/2050640619890732 PMID: 32213018

30. D’Souza N, Georgiou Delisle T, Chen M, Benton S, Abulafi M, Group NFS. Faecal immunochemical test is superior to symptoms in predicting pathology in patients with suspected colorectal cancer symptoms referred on a 2WW pathway: a diagnostic accuracy study. Gut. 2021; 70(6):1130–8. https://doi.org/10.1136/gutjnl-2020-321956 PMID: 33087488

31. Fraser CG. Interpretation of faecal haemoglobin concentration data in colorectal cancer screening and in assessment of symptomatic patients. Journal of Laboratory and Precision Medicine. 2017; 2(96).

32. Robertson DJ, Imperial TF. Stool Testing for Colorectal Cancer Screening. Gastroenterology. 2015; 149(5):1286–93. https://doi.org/10.1053/j.gastro.2015.05.045 PMID: 26033932

33. van Doorn SC, Stegeman I, Stroobants AK, Mundt MW, de Wijkerslooth TR, Fockens P, et al. Fecal immunochemical testing results and characteristics of colonic lesions. Endoscopy. 2015; 47(11):1011–7. https://doi.org/10.1055/s-0034-1392412 PMID: 26126163

34. Keenan JI, Beaugie CR, Jasmann B, Potter HC, Collett JA, Frizelle FA. Helicobacter species in the human colon. Colorectal disease: the official journal of the Association of Coloproctology of Great Britain and Ireland. 2010; 12(1):48–53. https://doi.org/10.1111/j.1463-1318.2008.01672.x PMID: 20050183

35. Zackular JP, Baxter NT, Chen GY, Schloss PD. Manipulation of the Gut Microbiota Reveals Role in Colon Tumorigenesis. mSphere. 2016; 1(1):e00001–15. https://doi.org/10.1128/mSphere.00001-15 PMID: 27303681

36. Tahara T, Yamamoto E, Suzuki H, Maruyama R, Chung W, Garriga J, et al. Fusobacterium in colonic flora and molecular features of colorectal carcinoma. Cancer research. 2014; 74(5):1311–8. https://doi.org/10.1158/0008-5472.CAN-13-1865 PMID: 24385213

37. Rosty C, Hewett DG, Brown IS, Leggett BA, Whitehall VL. Serrated polyps of the large intestine: current understanding of diagnosis, pathogenesis, and clinical management. Journal of gastroenterology. 2013; 48(3):287–302. https://doi.org/10.1055/s-0035-1392412 PMID: 23208018

38. Mima K, Cao Y, Chan AT, Qian ZR, Nowak JA, Masugi Y, et al. Fusobacterium nucleatum in Colorectal Cancer Tissue According to Tumor Location. Clinical and translational gastroenterology. 2016; 7(11):e200. https://doi.org/10.1038/ctg.2016.53 PMID: 27811909

39. Kook JK, Park SN, Lim YK, Cho E, Jo E, Roh H, et al. Genome-Based Reclassification of Fusobacterium nucleatum Subspecies at the Species Level. Curr Microbiol. 2017; 74(10):1137–47. https://doi.org/10.1007/s00284-017-1296-9 PMID: 28687946

40. Ye X, Wang R, Bhattacharya R, Bouthes DR, Fan F, Xia L, et al. Fusobacterium Nucleatum Subspecies Animalis Influences Proinflammatory Cytokine Expression and Monocyte Activation in Human Colorectal Tumors. Cancer prevention research. 2017; 10(7):398–409. https://doi.org/10.1158/1940-6207.CAPR-16-0178 PMID: 28483840
41. Liang Q, Chiu J, Chen Y, Huang Y, Higashimori A, Fang J, et al. Fecal Bacteria Act as Novel Biomarkers for Noninvasive Diagnosis of Colorectal Cancer. Clinical cancer research: an official journal of the American Association for Cancer Research. 2017; 23(8):2061–70.

42. Keenan JI, Frizelle FA. Biomarkers to Detect Early-Stage Colorectal Cancer. Biomedicines. 2022; 10(2). https://doi.org/10.3390/biomedicines10020255 PMID: 35203465

43. Liang JQ, Wong SH, Szeto CH, Chu ES, Lau HC, Chen Y, et al. Fecal microbial DNA markers serve for screening colorectal neoplasm in asymptomatic subjects. Journal of gastroenterology and hepatology. 2021; 36(4):1035–43. https://doi.org/10.1111/jgh.15171 PMID: 32633422

44. Blackmore T, Norman K, Kidd J, Cassim S, Chepulis L, Keenan R, et al. Barriers and facilitators to colorectal cancer diagnosis in New Zealand: a qualitative study. BMC Fam Pract. 2020; 21(1):206. https://doi.org/10.1186/s12875-020-01276-w PMID: 33003999