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Detection of *Fusobacterium* spp in colorectal tissue samples using reverse transcription polymerase chain reaction with minor groove binder probes: an exploratory research

Cláudio J.N. Silva[a,]*, Yuliana O. Eremina[b,c,d], Susana Rodrigues[e], Rosa Coelho[e], Rosa Ramalho[e], Otília Lopes[f], Fátima Carneiro[a,b,g], Joana Sobrinho Simões[c]

Abstract

An unhealthy microbiome is intimately correlated with several disease states, including colorectal cancer, wherein bacteria might be the key to neoplastic initiation and progression. Recent studies revealed an enrichment of *Fusobacterium* in colorectal tumor tissues relative to surrounding normal mucosa.

Given the available evidence, we conducted an exploratory study quantifying the relative expression of *Fusobacterium* spp in 28 tissue samples from patients treated at Centro Hospitalar de São João belonging to 4 different groups: adenomas, paired normal tissue from patients with adenomas, carcinomas, and paired normal tissue from patients with colorectal carcinomas. To increase reverse transcription polymerase chain reaction quantification sensitivity, minor groove binders fluorescent probes were used, having in mind its implementation into routine clinical practice. Differences of *Fusobacterium* spp relative abundance between paired neoplastic lesions/normal tissue were examined by Wilcoxon signed-rank test and for all the other 2-group comparisons the Mann-Whitney *U* test was used.

Most of the adenomas studied belonged to clinical specimens showing either tubular or villous low-grade dysplasia and an enrichment of *Fusobacterium* relative to paired normal tissue was not found ($P=.180$). In the carcinoma group, 57% of samples displayed a positive status for this *bacterium* with the highest burden of detectable *Fusobacterium* belonging to a specimen with positive regional lymph node metastasis.

This is the first Portuguese study confirming a trend toward an overabundance of *Fusobacterium* in colorectal carcinomas compared to adenomas and paired samples of normal-looking mucosa, in keeping with the role of this *bacterium* in colorectal carcinogenesis. Further studies are needed to elucidate the relevance of *Fusobacterium* detection for the prevention and treatment of colorectal cancer.

Abbreviations: *APC* = adenomatous polyposis coli, CHSJ = Centro Hospitalar de São João, CIMP = CpG Island Methylator Phenotype, *COX-2* = cyclooxygenase 2, CRC = colorectal cancer, Eu = *Eubacterium*, Fn = *Fusobacterium nucleatum*, IBD = inflammatory bowel disease, IL = interleukin, *LEF* = lymphoid enhanced factor, MGB = minor groove binders, *MLH1* = mutL homolog 1, MSI = microsatellite instability, NF-κB = nuclear factor kappa-light-chain enhancer of activated B cells, NSAIDs = nonsteroidal anti-inflammatory drugs, PCR = polymerase chain reaction, *PTSG2* = prostaglandin-endoperoxide synthase 2, SSA = sessile serrated adenoma, *TCF* = T-cell factor, TNF = tumour necrosis factor.

Keywords: bacterial infection, colorectal cancer, *Fusobacterium* spp, microbiome, minor groove binder probes

CJNS and YOE contributed equally to the study.

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[a] Faculdade de Medicina da Universidade do Porto, [b] Instituto de Patologia e Imunologia Molecular da Universidade do Porto (IPATIMUP), [c] Serviço de Patologia Clínica, Centro Hospitalar de São João E.P.E., [d] Instituto de Saúde Pública do Porto (EPIUnit - ISPUP), [e] Serviço de Gastrenterologia, Centro Hospitalar de São João, E.P.E., [f] Faculdade de Medicina Dentária da Universidade do Porto, [g] Serviço de Anatomia Patológica, Centro Hospitalar de São João, E.P.E., Porto, Portugal.

[*] Corresponding author. Centro Hospitalar de Sao Joao EPE, 4200-319 Porto, Paranhos, Portugal. E-mail address: claudio.j.n.silva@gmail.com (Cláudio J.N. Silva).

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Introduction

In the gut, microbiota is made up of more than 10^{14} organisms (with *Bacteroidetes* and *Firmicutes* as the most prevalent phyla).[1] The symbiotic, mutualistic relationship between the human host and its microbiota has developed through a beneficial reciprocal adaptation, that is, co-evolution. The gut, for instance, provides a microenvironment for the bacteria stable in temperature and rich in nutrients, whereas the signals from the latter are necessary for the intestinal physiology.[2–5]

The important role played by the host microbiome in health raised the question of what the consequences are in a state of dysbiosis: what happens when our friends become foes? It strikes us as no surprise that an unhealthy microbiome is intimately correlated with several disease states, including inflammatory bowel disease, colorectal cancer (CRC), cardiovascular disease, diabetes mellitus, obesity, and multiple organ failure.[6,7] The goal of recent studies has focused on whether the dysbiosis found in a diseased state is one of its causes or a mere consequence. The intensive research about *Fusobacterium* spp role on CRC, in particular, illustrates that bacteria are not an epiphenomenon in disease but may actually be involved in cancer initiation and progression.[8,11]

Worldwide, colorectal carcinoma is the fourth main cause of cancer death, being responsible for approximately 694,000 deaths per year (World Health Organization, 2015).[12] Whole-genome sequence analysis of the CRC microbiome in 2012 showed an enrichment of *Fusobacterium* spp, *F mortiferum*, *F necrophorum*, and especially *F nucleatum* in tissues of CRC.[13] Another phyla, of which *Escherichia coli* takes part, also stood out in patients with CRC.[14] In regard to *Fusobacterium* species, subsequent studies comparing carcinoma and adenoma vs paired normal tissue by quantitative polymerase chain reaction (PCR) and 16s rDNA sequence analysis identified an overabundance of these bacteria in tumor tissues relative to surrounding normal mucosa or tissues from healthy controls.[7,15,16] The bacteria are also enriched in stool samples from patients with adenoma/carcinoma compared to healthy subjects.[10]

It may come as a bolt from the blue the fact that *Fusobacterium* is actually an opportunistic commensal, anaerobic *bacterium* that ordinarily colonizes our oral cavity, most notably the subgingival plaque and saliva.[17] This gram negative microorganism has not only been implicated in mouth disease, that is, periodontitis,[18] but also in extraoral conditions such as jugular thrombophlebitis, appendicitis, inflammatory bowel disease, and intrauterine infections with consequent neonatal sepsis.[15,19,20]

Recent speculation concerning the role of *F nucleatum* (Fn) in colorectal carcinogenesis sought to answer if Fn was a mere bystander in the tissues/stool of diseased individuals or (batten down the hatches!) if it played a causal role in colorectal carcinogenesis.[21] Functional studies on $Apc^{Min/+}$ mice support the notion that Fn may be involved in cancer initiation and progression: it stimulates proliferation only in CRC cell lines, but not in non-neoplastic cell lines. In fact, early initiating somatic mutations (such as adenomatous polyposis coli (*APC*) or *β-catenin* mutations present in adenomas) precede the enrichment in this bacterium, so that the latter is present early in colonic carcinogenesis. These somatic mutations are responsible for epithelial barrier defects (ie, loss of tight junctions, cell to cell contacts, and epithelial polarity) allowing both *Fusobacterium* and other bacteria to take part in the tumoral niche.[10,22,23]

Fusobacterium also promotes tumor growth through a nonimmune oncogenic response pathway involving a virulence factor, FadA adhesin, engaged in its strong adhesive and invasive abilities for epithelial cells.[14,24] This adhesion, however, did not stimulate the growth of noncancerous cell lines, suggesting that Fn promotes carcinogenesis only after an early mutation occurs.[9]

Given the available evidence, we aimed to conduct an exploratory research comparing the relative expression of *Fusobacterium* spp in colorectal tissue samples from patients belonging to 4 different groups: adenomas, paired normal tissue from patients with adenomas, carcinomas, and paired normal tissue from patients with carcinomas. This is the first study using reverse transcription PCR (RT-PCR) with minor groove binder (MGB) probes as the technique to determine the *Fusobacterium* spp abundance in colorectal tumor vs paired normal tissue, having in mind its usage for the detection of this *bacterium* in routine clinical practice.

Materials and methods

Biopsy sample collection

The study was approved by the ethics committee of the institution in which it was conducted. It is in compliance with Helsinki declaration.

A total of 14 patients were enrolled in this study. The first group of samples was drawn from 7 inclusion-criteria eligible consenting subjects who underwent colonoscopy screening and polyp resection from January to May, July to August, and October to December, 2015. As not to compromise standard pathologic processing and analysis, only a small sample of the tumor specimen being submitted to the Pathology Department was used by the members of this study. Based on the histologic features of the specimen being sent to the Pathology Department, all patients were classified as having adenomas. A paired sample of normal appearing colon mucosa at colonoscopy (1 cm from the macroscopic lesion) was also obtained from the same subjects for further comparison of Fn-relative abundance between paired normal/adenoma tissue. The second set of human samples consisted of 7 paired adenocarcinoma tissues and normal colon mucosa frozen in liquid nitrogen and retrieved from the institution tumor bank. All adenocarcinoma fragments belonged to subjects who underwent surgery in 2015 and did not meet the exclusion criteria based on clinical records. Exclusion criteria included antibiotic therapy within 8 weeks before colonoscopy or surgery, age <50 years, patients with previous colon adenocarcinoma/adenoma, a known synchronous cancer or other cancer diagnosis within the previous 5 years (to exclude possible cases of familiar CRC syndromes), or inflammatory bowel disease. Polyethylene glycol was the favored agent used for bowel cleansing (to minimize the effects of bowel preparation upon colorectal bacteria) and a written informed consent was obtained from all eligible subjects participating in the study. All the biopsy samples were stored at -80°C.

DNA extraction from biopsy samples

Biopsies were lysed with proteinase K at 56°C and automatically processed for DNA extraction with silica spin columns (QIAcube, QIAGEN) following manufacturer's recommendations (*QIAamp DNA Mini Kit*). An eluate volume of 150 μL was obtained and frozen at -20°C until tested. *QIAamp DNA Mini Kit* is useful for the isolation of genomic, mitochondrial, bacterial, parasite, or viral DNA with rapid purification of high-quality, ready-to-use DNA, providing both consistent high yields and complete removal of contaminants and inhibitors.

Bacterial strains and generation of standard curves

Lyophilized *F nucleatum* (ATCC 10953) was used as a positive control for *Fusobacterium* detection. After hydration, the *bacterium* was cultivated on chopped meat broth media under anaerobic conditions and strain identification was verified with VITEK MS automated microbial identification system with matrix-assisted laser desorption/ionization-time of flight. For total bacterial quantification, a well characterized *E coli* clinical strain was selected.

Known McFarland equivalence standards were used as a reference to adjust bacterial suspensions' turbidity to the expected cell count, that is, McFarland standard 0.5 for an approximate 1.5×10^8 *Fusobacterium* cell count/mL and 2.0 for an approximate 6.0×10^8 *E coli* cells/mL. Genomic bacterial DNA was then extracted and, 10-fold serially diluted and tested for *Fusobacterium* and total bacteria in duplex real-time PCR. Two standard curves were generated by Rotor-gene 3000 software for total and specific quantitative bacterial DNA detection. An inferior detection limit of 15 copies/μL for both

Table 1

Characteristics of molecular probes used in this study as previously described.[26]

Group	Positions	Sequences	Dye
All bacteria	321–337	ACTGAGACACGGTCCA	VIC
Fusobacterium	746–763	CTTTAGCGTCAGTATCT	FAM

FAM = 6-carboxyfluorescein; VIC = 6-carboxyrhodamine.

total bacteria and *Fusobacterium* was established. The C_T values at different dilution points were calculated.

Real-time PCR

The sample DNA content, quality, and the presence of PCR inhibitor substances were verified using a parallel real-time PCR reaction and melting curve analysis with primers targeting human *β-globin* gene, as described elsewhere,[25] and SYBR Green stain (*QuantiTect SYBR Green PCR kit*, QIAGEN). All real-time PCR experiments were carried out using the same thermal cycler (Rotor-Gene RG-3000 from Corbett Research). It was selected a real-time PCR protocol using MGB fluorescent probes as previously described[26] with some minor modifications. Molecular characteristics of the fluorescently labeled probes used in this study (Applied Biosystems) are further detailed in Table 1. To ensure the specificity of the real-time PCR assay, the probes were tested with human and viral DNA and no crossreaction was detectable.

A final reaction of 20 μL included HotMaster Taq buffer with 25 mM of Mg^{2+} (10×), 1.12 U of HotMaster Taq DNA polymerase (5 PRIME), 200 μM of dNTP Mix (PROMEGA), 0.4 μM of each primer (EU 16S and Fuso spp—Table 2), 0.2 μM of each probe, molecular biology grade water, and 5 μL of template DNA. The 16S rDNA sequence was amplified for quantitative PCR with a hold of 95°C for 3 minutes to activate Taq polymerase, followed by 50 cycles of 95°C for 15 seconds, 60°C for 1 minute, and 72°C for 20 seconds. The universal bacteria MGB probe was labeled with 6-carboxyrhodamine (VIC) and the *Fusobacterium*-specific probe labeled at the 5'end with 6-carboxyfluorescein (FAM) dye.

The primers utilized allowed the quantification of the total number of bacteria and *Fusobacterium* spp in the same reaction. In each reaction, to quantify the total number of bacteria, 1 negative and 2 positive controls (2 samples with known concentration of *E coli* and *Fusobacterium* DNA copies) were used, whereas in the determination of the *Fusobacterium* spp abundance, 1 positive (sample with known concentration of *Fusobacterium* DNA copies) and 2 negative controls (no DNA and a sample with known concentration of *E coli* DNA copies) were employed.

All biologic samples sent for the PCR assay were codified using numbers as to ensure the experiments were carried out blindly.

Table 2

Sequences of the forward and reverse polymerase chain reaction primers used in this study (from TIB MOLBIOL Syntheselabor GmbH)

	Forward (Fwd)	Reverse (Rev)
EU 16S	5′-ggTgAATACgTTCCCgg	5′-TACggCTACCTTgTTACgACTT
Fuso spp	5′-ggATTTATTgggCgTAAAgC	5′-ggCATTCCTACAAATATCTACgAA

Statistical analysis

The differences of *Fusobacterium* spp abundance relative to the total number of bacteria (% Fn/Eu) between paired adenoma vs normal tissue from patients with adenomatous lesions and paired carcinoma vs normal tissue from patients with colorectal carcinomas were examined by Wilcoxon signed-rank test, a nonparametric test used to determine whether there is a difference in the median scores of a dependent continuous variable between 2 related categorical groups. For all the other 2-group comparisons, we performed a nonparametric Mann-Whitney *U* test, to determine whether there are differences between 2 categorical independent groups on a continuous dependent variable, for non-normally distributed data. A P value <0.05 was considered statistically significant. All tests were performed using SPSS software.

Results

We examined *Fusobacterium* spp abundance relative to the total number of bacteria (% Fn/Eu) in 28 samples from the following 4 groups: (a) adenomas (n=7), (b) paired normal tissues from patients with adenomatous lesions (n=7), (c) carcinomas (n=7), and (d) paired normal tissue from patients with colorectal carcinomas (n=7). From all the fragments sent for PCR experiments, *Fusobacterium* spp positivity was detected in 8 of them (Table 3) and in 5 samples the numbers of total bacterial DNA remained below the detection limit (15 copies/μL) of the respective PCR assays. Therefore, we tested all the samples for the possible presence of PCR inhibitors interfering with the activity of reaction components using a parallel real-time PCR reaction with primers targeting human *β-globin* gene. Having obtained human *β-globin* gene amplification in all these samples, we found no inhibition and confirmed the efficiency of the DNA extraction step. We have also repeated the PCR assays in randomly selected samples to test and verify our results and found that those originally negative for *Fusobacterium* spp continued to have nondetectable *Fusobacterium* DNA copies and those with a former positive status for this *bacterium* maintained detectable levels with roughly the same load of *Fusobacterium*/total bacteria relative to previous experiments.

The 5 samples whose total bacterial DNA remained undetected (<15 copies/μL) may not be truly zero values but are due to technical limitations of the PCR assay (see Discussion for details). As we cannot be certain about *Fusobacterium* relative abundance in these samples, they were excluded from data analysis. Those in which *Fusobacterium* (but not total bacterial) DNA was inferior to the respective detection limit were included in statistical analysis as having a *Fusobacterium* spp abundance relative to total number of bacteria (% Fn/Eu) of 0.

As shown in Table 3, we found that *Fusobacterium* spp DNA was detected by real-time PCR with MGB probes in only 1 adenoma fragment, although at a high load (26.5% relative to total number of bacteria). In fact, there was no enrichment of this *bacterium* in adenomatous lesions relative to paired normal tissue from the same patient (P=.180, using Wilcoxon signed-rank test). Importantly, the histopathological review of our clinical specimens showed that all adenoma cases displayed low-grade dysplasia and tubular (n=4) or villous (n=2) architecture. The sample with detectable *Fusobacterium* spp DNA levels belonged to a specimen with the features of sessile serrated adenoma (SSA).

The carcinoma group was the one attaining the highest numbers of detectable *Fusobacterium* copies, wherein 57% of samples showed a positive status for this *bacterium* (Fig. 1) and to

Table 3

***Fusobacterium* spp abundance relative to total number of bacteria (% Fn/Eu) in samples used in the polymerase chain reaction assay**

Adenoma/carcinoma sample		Age	Sex	Fn/Eu (%)	Histopathological features	Paired normal tissue	Fn/Eu (%) in paired normal tissue
Adenomas	1	61	M	26.5	Sessile lesion. Serrated adenoma. Low-grade dysplasia	Yes	NA*
	2	75	M	0*	Pedunculated polyp. Tubular adenoma. Low-grade dysplasia	Yes	1.2
	3	82	F	0	Pedunculated polyp. Tubular adenoma. Low-grade dysplasia	Yes	0
	4	72	F	0	Sessile lesion. Villous adenoma. Low-grade dysplasia	Yes	0.2
	5	52	M	0	Pedunculated polyp. Tubular adenoma. Low-grade dysplasia	Yes	0
	6	64	F	0	Sessile lesion. Villous adenoma. Low-grade dysplasia	Yes	0
	7	76	M	NA	Pedunculated polyp. Tubular adenoma. Low-grade dysplasia	Yes	0
Carcinomas	8	66	M	0.1	Adenocarcinoma NOS, moderately differentiated, pT4N0R0	Yes	0
	9	66	M	54.3	Adenocarcinoma NOS, moderately differentiated, pT3N1bR0	Yes	0
	10	82	F	18.1	Adenocarcinoma NOS, poorly differentiated, pT3N0R0	Yes	0
	11	80	M	0.0002	Adenocarcinoma NOS, moderately differentiated, pT1N0R0	Yes	0
	12	52	M	NA	Adenocarcinoma NOS, moderately differentiated, pT3N1aR0	Yes	0
	13	61	M	NA	Adenocarcinoma NOS, moderately differentiated, pT3N0R0	Yes	0
	14	85	M	NA	Adenocarcinoma NOS, moderately differentiated, pT3N1bR0	Yes	0.1

* NA = not applicable, meaning that the numbers of total bacterial DNA remained < 15 copies/μL [inferior detection limit of the polymerase chain reaction (PCR) assay for total bacteria], 0 – numbers of *Fusobacterium* DNA < 15 copies/μL (inferior detection limit of the PCR assay for *Fusobacterium* spp).

F = female, M = male, N = spread to regional lymph nodes, NOS = not otherwise specified, p = stage given by pathologic examination of a surgical specimen, R = resection boundaries of the surgical specimen, T = size or direct extent of the primary tumor.

which belonged the fragment with the greatest burden of *Fusobacterium* spp (54.3% Fn/Eu). Colorectal carcinoma specimens were classified as adenocarcinoma not otherwise specified and were moderately (n = 6) or poorly differentiated (n = 1). Three out of 7 cancer specimens had positive regional lymph node metastasis with the aforementioned sample reaching the highest abundance of *Fusobacterium* spp belonging to 1 of these 3 cases (Table 3).

In this exploratory research, although there is a trend toward overabundance of *Fusobacterium* in colorectal carcinomas

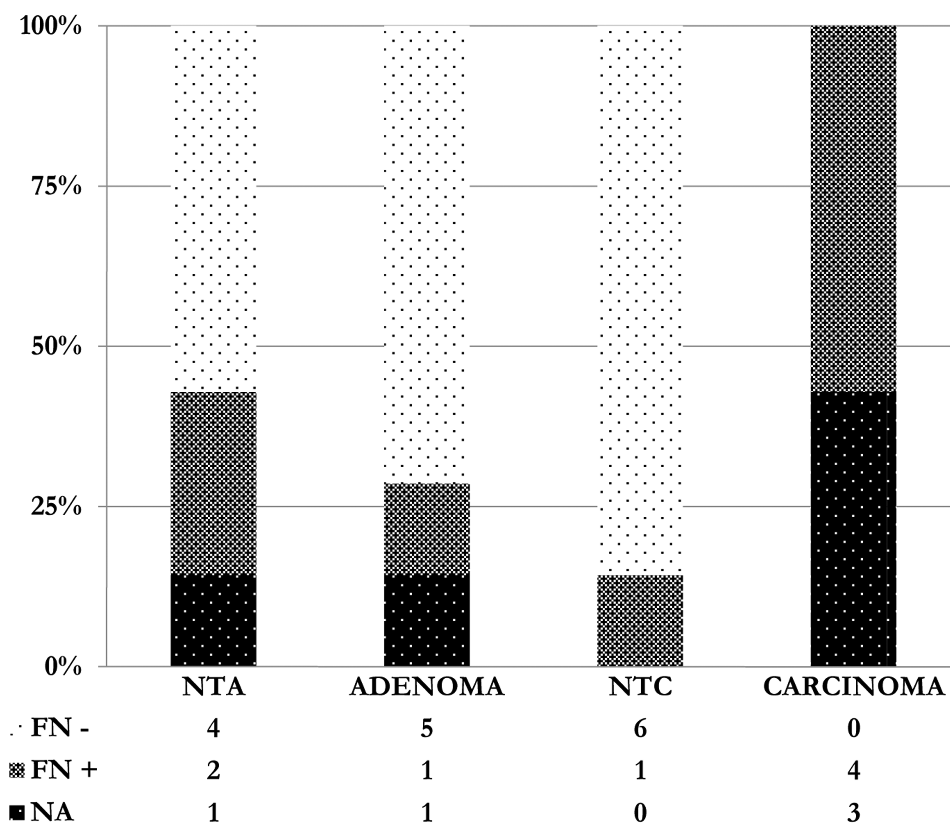

Figure 1. *Fusobacterium* spp detection in each group. Percentage and absolute number of patients in each group of colorectal samples with detectable *Fusobacterium* (FN +), without detectable *Fusobacterium* –<15 copies/μL (FN–) and in which total bacterial DNA remained <15 copies/μL (NA). NTA = paired normal tissue from patients with adenoma, NTC = paired normal tissue from patients with carcinoma.

compared to paired normal tissue ($P=.068$, using Wilcoxon signed-rank test), adenomas ($P=.067$, using nonparametric Mann-Whitney U test), and normal tissue from patients with adenomas ($P=.114$, using nonparametric Mann-Whitney U test), our results are not statistically significant due to the low sample size. We have not found any statistically significant difference in the relative expression of *Fusobacterium* spp between adenomas and normal tissue from patients with carcinomas or between normal tissue collected from patients with carcinomas and normal tissue from patients with adenomas ($P=.945$ and .534, respectively).

Discussion

The debate of whether *Fusobacterium* plays a causal role early in colorectal carcinogenesis is an ever ongoing question and, as mentioned previously, functional studies support the notion that this *bacterium* may be involved in cancer initiation and progression stimulating the proliferation of CRC cell lines harboring early initiating somatic mutations, such as the *APC* mutations present in adenomas. These are responsible for epithelial barrier defects that enable an enrichment of *Fusobacterium* early in the colonic carcinogenesis pathway (ie, adenomatous lesions). In fact, the available evidence[7,15,16] demonstrates an overabundance of these bacteria in both adenomas and carcinomas relative to paired surrounding normal mucosa.

Despite this, in our exploratory research using real-time PCR with MGB probes, we did not find a statistically significant enrichment (or even a trend toward an increase) of this *bacterium* in adenomatous lesions relative to paired normal tissue from the same patient. However, a previous European study,[27] which quantified *F nucleatum* levels in adenomas (grouped as tubular, tubulovillous, and low- or high-grade adenomas), colorectal carcinomas, and matched normal tissue, demonstrated *Fusobacterium* levels to be identical between tubular/tubulovillous adenomas and paired normal tissue but significantly higher in adenomas with a high-grade dysplasia. Remarkably, the pathologic analysis of our clinical specimens revealed that all our adenomas were composed of cells with low-grade dysplasia and if we assume an Fn enrichment with increasing stages of adenomatous dysplasia, then our data are in accordance with the study named earlier.

Interestingly, we detected *Fusobacterium* spp DNA (26.5%) in 1 fragment belonging to a clinical specimen whose histopathologic review showed features of a SSA. A study aiming to compare Fn expression and molecular characteristics of colorectal carcinoma[19] demonstrated high Fn levels to correlate with specific epigenetic profiles in CRCs: high-level CpG Island Methylator Phenotype (CIMP), microsatellite instability and mutL homolog 1 (*MLH1*) mismatch repair gene silencing. Another study analyzed the association between this *bacterium* and the molecular features of the colorectal serrated adenomas[28] and showed resemblances between SSAs (also harboring *BRAF* mutations and epigenetic silencing of the *MLH1* gene) and colorectal carcinomas carrying a high-level CIMP, thereby postulating SSAs to be their premalignant lesions. In the same study, despite being inconsistently detected in premalignant lesion (24%–35%), Fn was strongly associated with those carrying a high level CIMP status independent of their histopathological category. Is there a bridge linking *Fusobacterium* expression, SSA pathway, and CIMP-high colorectal carcinomas? Is our finding of a SSA fragment with *Fusobacterium* spp a luck of the draw or is there more to it than meets the eye?

In our case series, the highest levels of *Fusobacterium* spp DNA were found in fragments belonging to carcinoma specimens, in agreement with the available evidence. Remarkably, the fragment with the highest burden of detectable *Fusobacterium* (54.3%) belonged to a clinical specimen whose histology revealed positive regional lymph node metastasis, a finding that might not be just a sheer coincidence as tumors with Fn overabundance relative to paired normal tissue were previously shown to have an association with nodal metastasis.[15]

In this exploratory study, we have used real-time PCR with MGB probes for the detection of *Fusobacterium* spp having in mind its implementation into routine clinical practice. This is the first study using this method to determine the *Fusobacterium* spp load in both colorectal adenomas or carcinomas and paired normal tissue, making it possible to measure total and specific bacterial DNA copies in the very same reaction with clear cost-effective advantages in the clinical environment. This is a very well described technique[26] and, by using MGB probes (compared to the conventional non-MGB ones), it is possible to achieve enhanced binder profiles, as they form stable complexes with the DNA being quantified, therefore reducing the time expended in optimizing the PCR assays and increasing their reliability. Another advantage of real-time PCR with MGB probes relies in the increased sensitivity of the reaction with the detection of up to 10^1 to 10^3 target bacterial cells.

The use of the PCR technique for the detection of *Fusobacterium* spp in routine clinical practice is of the utmost importance as there is increasing evidence[27,29] that demonstrates the amount of Fn DNA in CRC tissue to correlate with lower patient survival, and therefore hypothesizing its value as a putative prognostic factor. This could have an impact in cancer treatment through the use of antibiotics or probiotics as a complementary strategy to standard oncology therapies, or even nonsteroidal anti-inflammatory drugs as a preventive armamentarium for CRC development in patients with adenomatous lesions rich in *Fusobacterium* spp, as this *bacterium* has a NF-kb proinflammatory gene signature with increased expression of *PTSG2/COX-2* gene.[10] In addition to its value as an independent prognostic factor, this technique could also be useful as a diagnostic marker of patients with overabundance of *Fusobacterium* spp in their feces and hence with increased risk of developing adenomas or colorectal carcinomas.

The main shortcoming of the present study is the small sample size. In the PCR analysis of 5 samples, the numbers of total bacterial DNA remained below the inferior detection limit of the assays (<15 copies/μL), thus impairing the ascertainment of their *Fusobacterium* relative abundance. As such, these are not truly zero values and may be caused by technical limitations of the PCR technique used. In fact, despite being high, the recovery rate of DNA between cells in the biopsy samples and those in the PCR DNA mixture, with the method we have chosen, is 78.8%.[26] Of note, having obtained human *β-globin* gene amplification in all the 28 samples, we concluded there was no PCR inhibition and confirmed the efficiency of the DNA extraction step.

Conclusion

We aimed for a research done entirely on clinical grounds in view of implementing the use of real-time PCR with MGB probes for the detection of *Fusobacterium* spp in our routine clinical practice. This was the first Portuguese study using this method to determine the *Fusobacterium* spp load in both colorectal adenomas or carcinomas and paired normal tissue, making it

possible to measure total and specific bacterial DNA copies in the very same reaction with clear cost-effective advantages for the clinical setting. By using MGB probes, it is possible to achieve enhanced binder profiles, thereby reducing the time expended in optimizing the PCR assays. Another advantage of this technique is the increased sensitivity of the reaction. The validation of the technique for the detection of *Fusobacterium* is relevant as a diagnostic and prognostic tool with possibilities of having an impact in cancer treatment.

The present research laid the groundwork and helped us gaining insights for a future study in which we aim to determine the amount of *Fusobacterium* spp using the same technique but in a larger sample size composed of adenomas with high-grade dysplasia and a higher number of serrated adenoma specimens, with the goal of investigating if this *bacterium* is in fact richer in patients of our institution harboring such lesions than in those with low-grade dysplasia.

Acknowledgments

Ethics approval: The study was approved by Centro Hospitalar de São João ethics committee. It is in compliance with Helsinki declaration: http://www.wma.net/en/30publications/10policies/b3/index.html.

Author contributions

CJNS collected conceived and designed the study, collected patients' material, analyzed the results, and drafted the manuscript. YOE helped in drafting the manuscript, in the study design and was responsible for bacterial DNA extraction from biopsy samples, generation of PCR standard curves, and RT-PCR with MGB probes analysis. SR, RC, RR, and OL helped collecting adenoma tissue and paired normal mucosa from patients undergoing colonoscopy screening. FC was responsible for the histological classification of both adenomas and CRC clinical specimens. JSS helped in the DNA extraction from biopsy samples, generation of PCR standard curves, RT-PCR with MGB probes analysis, and in the choice of primers and probes used. All authors read and approved the final manuscript.

Conflicts of interest

The authors declare that they have no competing interests.

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