Article

Circulating Bacterial DNA in Colorectal Cancer Patients: The Potential Role of *Fusobacterium nucleatum*

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Circulating Bacterial DNA in Colorectal Cancer Patients: The Potential Role of *Fusobacterium nucleatum*

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Abstract: Dysbiosis, as reflected by the altered composition of the microbiome favoring pathogenic species, is a major contributor to colorectal cancer (CRC) development, which could lead to bacterial translocation into the bloodstream. This study aimed to evaluate the presence of circulated bacterial DNA (cbDNA) in CRC patients, with or without surgical removal of the primary tumor. In total, 100 participants enrolled in this prospective clinicolaboratory study, equally divided into four groups; healthy controls (Group 1), patients with non-metastatic CRC with surgical removal of the primary tumor (Group 2), patients with metastatic CRC with surgically excised (Group 3) or non-excised (Group 4) primary tumor. DNA extracted from peripheral blood was analyzed using PCR with specific primers targeting *16S* rRNA, *Escherichia coli* (*E. coli*), and *Fusobacterium nucleatum* (*F. nucleatum*). Only the detection of *F. nucleatum* in the blood was significantly higher in Group 4 compared to Group 1 ($p < 0.001$), Group 2 ($p = 0.023$) and Group 3 ($p = 0.023$). The association of cbDNA with other clinical parameters or co-morbidities was also evaluated. These results highlighted the importance of bacterial translocation in CRC patients and the role of *F. nucleatum* as an intratumoral oncomicrobe associated with metastatic CRC.

Keywords: colorectal cancer; dysbiosis; bacterial translocation; surgery; intestinal microbiota; circulating bacterial DNA; metastasis; *Fusobacterium nucleatum*

1. Introduction

Colorectal cancer (CRC) is the third most common cancer worldwide, a leading cause of cancer mortality, accounting for 9% of all malignancies in adult patients [1]. Gradual accumulation of genetic mutations results in the formation of pre-cancerous lesions, which subsequently evolve into colorectal adenoma, and ultimately invasive CRC [2]. Immune and inflammatory responses are crucial in all stages of colorectal tumorigenesis [3]. Earlier CRC detection has been made feasible through current innovations in screening endoscopy, imaging modalities, and therapeutic strategies (surgery, radiotherapy, and chemotherapy), leading to enhanced survival rates [4].

Up to 22% of CRC patients are identified with metastatic disease at diagnosis, and various studies indicate that approximately 70% of patients would inevitably exhibit metastatic disease or

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recurrence, with up to 50% of cases involving synchronous or metachronous distant metastases [5]. Stage IV CRC patients typically have a poor prognosis, with a 14% 5-year survival rate. Hence, the development of efficient prognostic or diagnostic biomarkers is still a necessity.

The correlation between CRC and intestinal microbiota has been extensively evidenced. The diverse microbial ecosystem of the human intestinal microbiota constantly interacts with the host maintaining homeostasis through constant synergistic interactions with the host. However, disruptions in microbiota composition could favor the proliferation of pathogenic bacteria leading to detrimental effects, collectively termed "dysbiosis" [6]. Apart from alterations in microbial composition, dysbiosis also encompasses shifts in bacterial distribution, and dysregulated metabolism promoting colorectal tumorigenesis [7]. Such effects could lead to DNA damage, modulation of immunity, and inflammatory response [8]. Interestingly, surgical stress subsequently to curative resection for CRC affects the host-microbiota interaction, further promoting dysbiosis [9]. Alterations in various bacterial genera, including *Bacteroides*, *Bifidobacterium*, *Escherichia*, *Fusobacterium*, and *Lactobacillus*, among others, have been demonstrated after CRC surgery [10]. *Fusobacterium nucleatum* (*F. nucleatum*), an invasive, pro-inflammatory pathogen, indigenous to the oral microbiota [11], is one of the most researched bacterial species in CRC. *F. nucleatum* could trigger carcinogenesis mainly by stimulating the β-catenin cascade following the binding of Fusobacterium adhesion A (FadA) protein to E-cadherin [12]. Studies associate the higher abundance of *F. nucleatum* with reduced overall or cancer-specific survival in CRC patients [13].

Circulating bacterial DNA (cbDNA) in human blood has become evident [14]. Detection of cbDNA, through various molecular techniques [15,16], has also been reported as a reliable, non-invasive method for CRC screening and prediction of long-term outcomes in CRC patients, possibly participating in CRC pathogenesis [17,18]. However, there is limited data regarding the detection of cbDNA in patients with metastatic CRC, especially concerning any differences regarding the absence of the primary tumor due to previous surgical resection, and focusing on the presence of *F. nucleatum*.

In this context, the present study aimed to investigate the detection of cbDNA in the blood of patients with CRC using a polymerase chain reaction (PCR)-based method, compare patients with or without surgical resection of the primary tumor, as well as non-metastatic and metastatic disease, and to evaluate any associations with the patients' demographic and clinical parameters.

2. Results

2.1. Patients' Characteristics

The epidemiological and clinical characteristics of patients and healthy individuals are summarized in Table 1 and Data S1. Patient groups with CRC were older (mean age range 57.8 – 62.9 years old), and had higher body mass index (BMI) (mean BMI range 27.7 – 28.4 kg/m^2) compared to Group 1. Most of the total participants (63%) were males. By design, all patients in Groups 2 and 3 have undergone surgical resection of the primary tumor, whereas all patients in Groups 3 and 4 have metastatic disease (stage IV CRC). The majority of Group 2 patients (80%) had locally advanced (stage III) CRC. In Group 4 patients the tumor location was evenly distributed between the left colon and rectum (40% each), whereas the commonest CRC site regarding non-metastatic groups was the right colon (40% in Group 2 and 3 respectively). Pathologic staging of CRC was mostly intermediate (58.7% of all patients), while tumors presented microsatellite instability (MSI) in only 9.3% of CRC patients. The majority of patients were non-active smokers (64%) and reported no or infrequent alcohol consumption (89.3%) as determined by a negative Alcohol Use Disorders Identification Test-Concise (AUDIT-C) score (< 3 in females and < 4 in males). Regarding co-morbidities, most of the patients had cardiovascular disease (CVD) (45.3%), whereas diabetes and dyslipidemia were more common in Group 3 (44%) and Group 2 (36%), respectively. None of the Group 1 individuals had any co-morbidity.

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Table 1. Patients' and healthy individuals' demographic characteristics and clinical parameters.

Characteristics	Group 1 (n = 25)	Group 2 (n = 25)	Group 3 (n = 25)	Group 4 (n = 25)
		Mean (range)		
Age	20.5 (20-21)	61.5 (25-86)	62.9 (39-80)	57.8 (26-78)
BMI (kg/m^2)	23.1 (20.3-24.8)	28.4 (22.1-46.9)	27.7 (20.2-37.2)	27.9 (17.1-52.5)
		Frequency (percentage)		
Sex				
Male	18 (72%)	11 (44%)	18 (72%)	16 (64%)
Female	7 (28%)	14 (56%)	7 (28%)	9 (36%)
Surgery				
Yes	n/a *	25 (100%)	25 (100%)	0 (0%)
No	n/a	0 (0%)	0 (0%)	25 (100%)
Metastasis				
Yes	n/a	0 (0%)	25 (100%)	25 (100%)
No	n/a	25 (100%)	0 (0%)	0 (0%)
Tumor Location				
Right Colon	n/a	10 (40%)	10 (40%)	5 (20%)
Left Colon	n/a	9 (36%)	8 (32%)	10 (40%)
Rectum	n/a	6 (24%)	7 (28%)	10 (40%)
Stage				
II	n/a	5 (20%)	0 (0%)	0 (0%)
III	n/a	20 (80%)	0 (0 %)	0 (0%)
IV	n/a	0 (0%)	25 (100%)	25 (100%)
Grade				
Low	n/a	6 (24%)	2 (8%)	2 (8%)
Intermediate	n/a	13 (52%)	15 (60%)	16 (64%)
High	n/a	6 (24%)	8 (32%)	7 (28%)
Mismatch Repair (MMR) status				
Microsatellite Stability (MSS)	n/a	19 (76%)	23 (92%)	21 (84%)
Microsatellite Instability (MSI)	n/a	3 (12%)	2 (8%)	2 (8%)
Unreported	n/a	3 (12%)	0 (0%)	2 (8%)
Smoking				
Yes	0 (0%)	7 (28%)	12 (48%)	8 (32%)
No	25 (100%)	18 (72%)	13 (52%)	17 (68%)
Alcohol				
Yes	0 (0%)	1 (4%)	3 (12%)	4 (16%)
No	25 (100%)	24 (96%)	22 (88%)	21 (84%)
Diabetes				
Yes	0 (0%)	5 (20%)	11 (44%)	3 (12%)
No	25 (100%)	20 (80%)	14 (56%)	22 (88%)
Dyslipidemia				
Yes	0 (0%)	9 (36%)	5 (20%)	6 (24%)
No	25 (100%)	16 (64%)	20 (80%)	19 (76%)
Cardiovascular Disease (CVD)				
Yes	0 (0%)	12 (48%)	13 (52%)	9 (36%)
No	25 (100%)	13 (52%)	12 (48%)	16 (64%)

* n/a, not applicable.

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2.2. Detection of cbDNA

16S rRNA was detected in 84% of Group 1, 96% of Group 2, and 100% of Groups 3 and 4. The *β*-galactosidase gene of *Escherichia coli* (*E. coli*) was detected in 80% of Groups 1 and 2, 84% of Group 3 and 68% of Group 4. Finally, the *NusG* gene of *F. nucleatum* was detected in 12% of Group 1, 32% of Groups 2 and 3, and 68% in Group 4 (Table 2 and Figure 1).

Table 2. Frequency and percentages of circulating bacterial DNA (cbDNA) presence among control and patient groups.

Gene Target	Detection	Group 1 (n = 25)	Group 2 (n = 25)	Group3 (n = 25)	Group 4 (n = 25)
16S rRNA	Positive	21 (84%)	24 (96%)	25 (100%)	25 (100%)
	Negative	4 (16%)	1 (4%)	0 (0%)	0 (0%)
β-galactosidase gene of *Escherichia coli*	Positive	20 (80%)	20 (80%)	21 (84%)	17 (68%)
	Negative	5 (20%)	5 (20%)	4 (16%)	8 (32%)
NusG gene of *Fusobacterium nucleatum*	Positive	3 (12%)	8 (32%)	8 (32%)	17 (68%)
	Negative	22 (88%)	17 (68%)	17 (68%)	8 (32%)

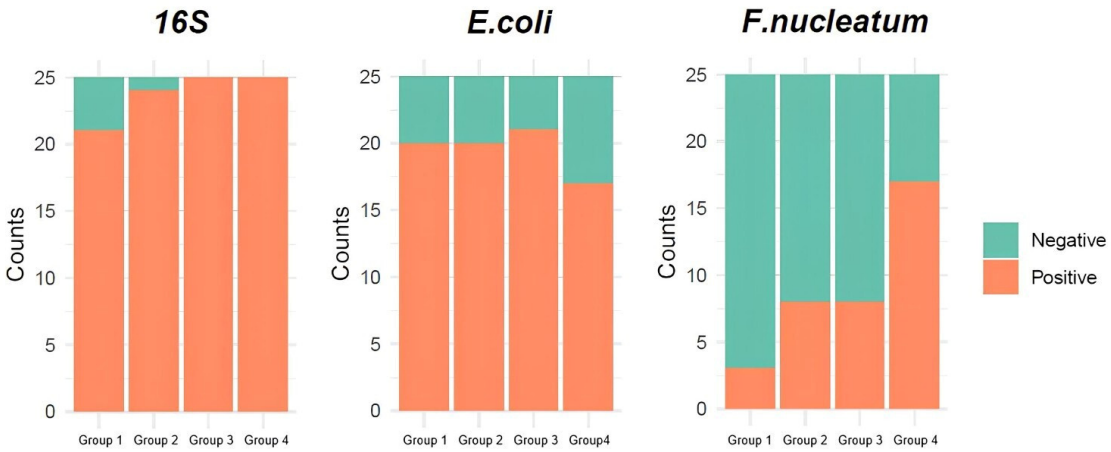

Figure 1. Frequencies of *16S* rRNA, *Escherichia coli* (*E. coli*), and *Fusobacterium nucleatum* (*F. nucleatum*) detection in blood among control and patient groups.

Pairwise comparisons between groups revealed significantly higher detection of *F. nucleatum* in Group 4 compared to Group 1 ($p < 0.001$, adj. $p < 0.001$), as well as compared to Group 2 or 3 ($p = 0.023$, adj. $p = 0.045$). However, there were no statistically significant differences regarding the detection of *16S* rRNA or *E. coli* among all groups (Table 3).

Table 3. Comparisons of cbDNA detection among control and patient groups.

Pairwise comparison	*16S* rRNA		*E. coli*		*F. nucleatum*	
	p-value	adj. *p*-value	*p*-value	adj. *p*-value	*p*-value	adj. *p*-value
Group 1 – Group 2	0.349	0.697	- *	-	0.171	0.205
Group 1 – Group 3	0.11	0.33	-	-	0.171	0.205
Group 1 – Group 4	0.11	0.33	0.52	-	<0.001	<0.001
Group 2 – Group 3	-	-	-	-	-	-
Group 2 – Group 4	-	-	0.52	-	0.023	0.045
Group 3 – Group 4	-	-	0.321	-	0.023	0.045

* $p = 1$.

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Further correlation of *E. coli* and *F. nucleatum* presence was performed after grouping the patients according to their clinical parameters. The detection of *E. coli* and *F. nucleatum* in blood was not significantly associated with differences in sex, metastasis, tumor location, grade, Mismatch Repair (MMR) status, smoking, alcohol consumption, dyslipidemia, or CVD (Table 4). Patients without surgical resection of the primary tumor presented significantly higher detection of *F. nucleatum* compared to those with surgical resection of the primary tumor (68% vs. 32%, $p = 0.006$). However, no such difference was observed concerning the detection of *E. coli* (68% vs. 82%, $p = 0.242$). Additionally, the detection of *E. coli* was significantly higher in patients with diabetes compared to those without (100% vs. 69.6%, $p = 0.004$), whereas such association was not evident with the presence of *F. nucleatum* (31.6% vs. 48.2%, $p = 0.286$).

Table 4. Association of cbDNA detection with patients' clinical parameters.

Parameter / Pairwise comparison	***E. coli***		***F. nucleatum***	
	p-value	adj. *p*-value	*p*-value	adj. *p*-value
Sex				
Female – Male	0.403	n/a [1]	0.348	n/a
Surgery				
No – Yes	0.242	n/a	0.006	n/a
Metastasis				
No (stage II/III) – Yes (stage IV)	0.777	n/a	0.217	n/a
Tumor Location				
Left Colon – Rectum	0.308	0.462	0.252	0.596
Right Colon – Rectum	0.292	0.462	- [2]	-
Left Colon – Right Colon	-	-	0.397	0.596
Grade				
High – Low	0.634	0.951	0.701	-
Intermediate – Low	0.427	0.951	-	-
High – Intermediate	-	-	0.605	-
MMR status				
MSI – MSS	-	n/a	-	n/a
Smoking				
No – Yes	0.15	n/a	0.634	n/a
Alcohol				
No – Yes	-	n/a	0.725	n/a
Diabetes				
No – Yes	0.004	n/a	0.286	n/a
Dyslipidemia				
No – Yes	-	n/a	-	n/a
CVD				
No – Yes	0.414	n/a	0.816	n/a

[1] n/a, not applicable; [2] $p = 1$.

3. Discussion

Lately, research groups have become increasingly interested in peripheral blood as a novel valuable source of cbDNA. PCR-based methods have been developed in earlier studies enabling the detection of cbDNA in CRC patients [19,20]. However, small cohorts were enrolled, and there was no discrimination between CRC patients' characteristics or proper integration of control subjects. More recent studies relying on advanced PCR-based methods [17,18,21,22] or next-generation sequencing (NGS) methods [23,24] shed light on the elusive subject of cbDNA detection in CRC patients.

In brief, the present study reveals that cbDNA of *16S* rRNA, *E. coli*, and *F. nucleatum* is present in the blood of healthy subjects and CRC patients. However, the origin of the cbDNA remains elusive. The presence of microbes in the blood may be attributed to occasional dissemination from various body reservoirs into the circulation, known as microbial translocation [25]. The main proposed

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mechanisms for this phenomenon are intestinal dysbiosis, dysfunction of the intestinal epithelial barrier, and increased permeability ("leaky-gut"). Notably, microbial components, including endotoxins, lipopeptides, and nucleic acids, among others, could also be present in the blood [26]. Additional mechanisms for bacterial translocation into the blood include the interaction of the microbiota with immune system cells, affecting multiple host functions [27], promoting chronic local and systemic inflammation [28], and utilizing dendritic cells or micro-fold cells [29]. Studies have shown that cbDNA was predominantly related to intestinal dysbiosis, although oral or skin microbiota could also serve as potential sources of cbDNA [23]. Studies have revealed great similarity of the *F. nucleatum* subtypes between saliva and tumor tissue samples in CRC [30], enhancing the hypothesis of orally-mediated intestinal dysbiosis. It is evident that in periodontitis several oral pathogens, including *F. nucleatum*, are incorporated into complex oral biofilms, facilitating the translocation of oral pathogens into the intestinal ecosystem by invading the bloodstream or swallowing saliva [31]. Residing into the colonic microbiota they further promote dysbiosis. In our cohort, it is reasonable to suggest that the majority of the cbDNA could probably have originated from the intestinal or oral microbial community. Nevertheless, the primary factor, between inflammatory response, alterations in microbiota composition, or increased intestinal permeability, leading to bacterial translocation remains unknown [32,33].

Our study demonstrated a high detection of *16S* rRNA (84%) and *E. coli* (80%) in the blood of healthy subjects, which was non-significantly different compared to CRC patients (96%-100% and 68%-84%, respectively). Similar to our study Giacconi et al. [21], by using real-time qPCR identified the presence of *16S* rRNA in all 40 control subjects and 50 CRC patients, although the bacterial load was higher in CRC patients compared to healthy subjects. Mutignani et al. [24], using NGS detected *16S* rRNA in all healthy controls and subjects with colorectal adenomas; CRC patients again presented higher cbDNA load. The abundance of *E. coli* did not differentiate between non-CRC and CRC subjects. Another study by Xiao et al. [23], also using NGS, analyzed the cbDNA between healthy controls, and patients with colorectal adenoma or CRC. A prominent and distinctive circulating cbDNA profile was identified between CRC patients and healthy subjects, highlighting 28 species deriving from intestinal or oral microbiota, which did not include *E. coli*. Messaritakis et al. [17], however, reported a higher PCR detection of *16S* rRNA in a larger cohort of 397 CRC patients (64.5%) compared to 32 healthy controls (15.6%). Notably, although there was no association concerning *E. coli* detection between these groups (p = 0.186) in accordance with our results, the percentages of positivity were significantly lower compared to our study (15.6% in the control group, and 26.2% in the CRC group).

The biological causes underlying these discrepancies are largely unknown. Concerning *16S* rRNA our study demonstrates a potentially substantial impairment of the intestinal permeability in healthy individuals leading to bacterial circulation, in accordance with previous reports [34]. Moreover, *E. coli* is an almost exclusively nonpathogenic commensal species of the intestinal microbiota, having been detected as a member of the intestinal microbiome of over 90% of healthy individuals [35]. The current literature regarding *E. coli* and CRC is largely ambiguous regarding its over- or under-representation in CRC-related microbiota compared to controls. This phenomenon is possibly due to the different abundances of the various phylotypes of *E. coli* (A, B1, B2, D), where *E. coli* strains belonging to phylotype A are mostly commensal, while strains of B2 phylotype are mainly considered as virulent species [36]. It is difficult to explain the lack of difference of *16S* rRNA or *E. coli* between CRC patients and healthy subjects since inflammatory responses in the CRC microenvironment could also affect bacterial dissemination [37]. This outcome could be partially attributed to a variety in size or shape inclinations in gut-blood bacterial translocation. It should further be emphasized that the present study compared healthy controls with stage IV CRC with or without surgical resection of the primary tumor and stage III CRC with surgical resection of the primary tumor, whereas in the aforementioned studies, the pool of CRC patients included patients with intestinal adenomas or stage I-III CRC without surgical resection of the primary tumor. Hence, our results are not directly comparable to these studies and should be carefully interpreted. The detection of bacterial by-products (metabolites or toxins) in the bloodstream may further aid the

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differentiation between tumor-free individuals and CRC patients. Future multi-omic studies integrating the analysis of cbDNA with microbiota profiling and metabolome could unveil the molecular mechanism of cbDNA alteration in CRC tumorigenesis.

In this study, we also report a significantly higher detection of *F. nucleatum* cbDNA in CRC patients (32%-68%) compared to healthy subjects (12%). This is in accordance with the higher identification of *F. nucleatum* in mucosal and fecal samples from CRC patients promoting intestinal dysbiosis [38–40]. To date, only the study by Xiao et al. [23] compared the *F. nucleatum* in the blood but found no significant difference between CRC patients and controls. However, this difference may be due to the relatively small sample size of their study in addition to the inclusion of only earlier stage CRC (II/III), and the different demographic or environmental variables.

Interestingly, in our setting, the detection of *F. nucleatum* cbDNA was significantly higher in the stage IV CRC patients without surgical resection of the primary tumor compared to stage II/III CRC or stage IV CRC patients with surgical resection of the primary tumor. This finding is in line with the current evidence that *F. nucleatum* constitutes a predominantly intratumoral oncomicrobe affecting the tumor microenvironment in promoting CRC pathogenesis [41], and its presence has also been extensively correlated with advanced CRC stages [42]. Nevertheless, it has been demonstrated that the surgical resection of the primary tumor in CRC patients could enhance or reduce the abundance of *F. nucleatum* in the gut [43–45]. Thus, our results indicate that the resection of the primary tumor could disrupt the active oral-gut axis in stage IV CRC reducing the circulation of *F. nucleatum*.

E. coli cbDNA was not significantly different between CRC patients with or without surgical resection of the primary tumor in our study. Only one study by Koulouridi et al. [18] investigated the detection of *E. coli* in the blood of stage III CRC patients with surgical resection of the primary tumor, reporting lower frequency (21.5%) in comparison with our results (82%). Studies in the gut microbiota of CRC patients reveal reduced populations of *E. coli* in cancerous tissue compared to adjacent healthy mucosa [46], while others report inconsistent alterations of *E. coli* abundance in surgically-treated CRC patients [45,47,48], which could merely explain these results. Research has revealed that in CRC, *E. coli* could invade the weakened intestinal vascular barrier, and be released into the portal circulation, colonizing the liver and promoting liver metastasis [49]. This fact, in combination with the observation that *E. coli* is not a strictly intratumoral microbe [47,50], could explain the similarity in the detection of *E. coli* cbDNA between stage IV CRC patients with or without surgical resection of the primary tumor. Notably, we further observed that neither *E. coli* nor *F. nucleatum* cbDNA was significantly altered between non-metastatic (stage II/III) and metastatic (stage IV) CRC patients. *F. nucleatum* has been thoroughly correlated with increased tumor invasion and lymph node or distant metastases [13,42], and *E. coli* has also been implicated in metastatic colorectal disease in combination with circulating tumor cells [51]. Similar to our results Giacconi et al. [21] revealed no correlation between increased cbDNA levels and the tumor stage or the presence of distant metastases. This finding might support the concept that cbDNA primarily plays a role in the early development of CRC. The study by Messaritakis et al. [17] reported a significantly higher presence of *E. coli* in stage IV CRC compared to stage II/III; however, cases with or without surgical resection of the primary tumor were pooled together not allowing an accurate comparison with our data. Our study did not include stage II/III CRC cases without surgical resection of the primary tumor, thus presenting a further limitation in elucidating the aforementioned discrepancies regarding cbDNA association with CRC stage or metastases.

Tumor location was not associated with cbDNA detection in our setting similar to previous PCR-based reports [17]. The utilization of NGS by Mutignani et al. [24] displayed enhanced bacterial transition from the intestinal environment to blood circulation in right colon cancer compared to rectal cancer. Regional discrepancies in genetic expression and immunological characteristics have also been emphasized [52]. Furthermore, intestinal microbiota composition-related tumorigenic mechanisms also vary between the left colon, right colon, or rectum [53]. These data indicate the potential usefulness of sensitive NGS-based methods in discerning bacterial translocation according to the CRC site.

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Regarding other demographic or clinical parameters of CRC patients, our study is unique in investigating any possible associations with cbDNA. Gut microbiota studies reveal a distinct microbiota profile in correlation with deficient mismatch repair [54], or high-graded CRC [55]. However, cbDNA detection in our cohorts revealed no significant differences in these characteristics. Several risk factors can contribute to intestinal dysbiosis, such as alcohol consumption [56], smoking, as well as obesity [57]. Studies have also shown the definite presence of cbDNA, in physiological conditions and various systematic diseases, including diabetes, metabolic, or cardiovascular diseases [29,58]. The CRC patients in this study display several clinical characteristics, together with the aforementioned diseases linked to dysbiosis. Between all these factors our study only revealed a strong correlation between higher *E. coli* cbDNA presence in CRC patients with diabetes. The abundance of *E. coli* in the gut microbiota of patients with diabetes is increased, serving as an opportunistic pathogen [59]. Nevertheless, the association of cbDNA detection with all the above clinical factors or co-morbidities may be influenced by the impact of the small sample size involved in our research.

Apart from the aforementioned limitations, it should be noted that our study, by design, is a non-randomized prospective study. We were not able to adjust for age between CRC and controls, with the latter belonging to a significantly lower age range, removing potential factors that could influence the results of cbDNA by controlling for these covariates. However, to mitigate the effect of the small sample size the gender and geographical distribution were equivalent between patient groups. The lack of thorough examination of detailed blood microbiota profiling among participants is another limitation, as we used primers targeting specific species. The primary focus of the present study was on the cbDNA, with no investigation conducted on the composition of other microorganisms, such as viruses or fungi, which have been recently discovered to be disrupted in fecal samples of CRC patients. Due to these factors, it is advisable to approach the outcomes cautiously, primarily to generate new hypotheses.

4. Materials and Methods

4.1. Patients Enrollment and Study Design

From September 2021 to April 2024, 75 patients aged > 18 years old with previously diagnosed and histologically confirmed CRC were included in the present, prospective, multi-centered study. For reference, 25 healthy individuals aged > 18 years old were also included, without any benign or malignant neoplastic colorectal disease (Group 1). The patients were equally divided into three groups. Group 2 (n = 25) included patients with non-metastatic CRC who have undergone surgical resection. Group 3 (n = 25) included patients with metastatic CRC who have undergone surgical resection of the primary tumor. Group 4 (n = 25) included patients with metastatic CRC without surgical resection of the primary tumor.

Further exclusion criteria, which applied to all groups included the administration of specific medications (antiemetic, antidiarrheal, antiparasitic, antiviral, antibiotic, probiotic, laxatives, non-steroidal anti-inflammatory, or corticosteroid drugs) within 4 weeks prior to sampling, radiographic studies with barium enema within 1 week prior sampling, active rectal bleeding, individuals with creatinine clearance (measured by Cockcroft-Gault equation) < 30 mL/min, pregnancy, presence of any active bacterial or viral infection, synchronous neoplastic disease elsewhere in the body, genetic disease, and CRC due to familial adenomatous polyposis or Lynch syndrome.

All patients were treated at the University Hospital of Heraklion (Department of Medical Oncology, Department of General Surgery, and Department of Surgical Oncology), at the Venizeleio General Hospital of Heraklion (Department of Oncology, Department of Surgery), and the Creta InterClinic Hospital (Department of Surgery).

4.2. Ethics Approval

The study has been approved by the Research Ethics Committee of Creta InterClinic Hospital (9/7/2021), of the Venizeleio General Hospital of Heraklion (23/14-07-2023), of the University Hospital

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of Heraklion (23743/02-10-2023), and of the University of Crete (119/24.10.2023). Informed consent was provided to and signed by all participants. The conducted procedures adhered to the ethical criteria set by the aforementioned committees.

4.3. Blood Sampling and DNA Extraction

Peripheral blood (3 mL in EDTA) was collected from all healthy subjects and CRC patients. Samples were collected just before the initiation of any adjuvant or first-line treatment. NucleoSpin© Blood DNA kit (Macherey-Nagel, Düren, Germany) was used for whole-blood genomic DNA extraction, according to the manufacturer's protocol. DNA quantification was performed by utilizing the NanoDrop ND-1000 v3.3 (Thermo Fisher Scientific, Waltham, MA, USA) spectrophotometer.

4.4. PCR Amplification of Microbial DNA

Two oligonucleotide primer pairs were used to detect cbDNA via PCR; the *β*-galactosidase gene of *E. coli* [17,18], and the *NusG* gene of *F. nucleatum* [60]. DNA integrity of the samples was performed by using glyceraldehyde phospho-dehydrogenase (GAPDH) as reference primers [17,18]. For reference marker in the detection of cbDNA, *16S* rRNA primers were also used in accordance with previous studies [17,18]. The PCR conditions, primer sequences, and fragment size of amplicons for each target gene are reported in Table S1.

4.5. Statistical Analysis

Descriptive and inferential statistics were performed regarding the patients' and healthy individuals' baseline variables (demographic characteristics, clinical parameters). Comparisons between categorical variables were calculated with Pearson's Chi-square test. Adjusted (adj.) p values through Bonferroni correction are also provided in cases of multiple comparisons. Statistical analysis was performed via SPSS v. 26 (IBM Corp. Armonk, New York, USA). Statistical significance was indicated at the conventional $p < 0.05$ threshold.

5. Conclusions

In conclusion, our study confirms the presence of cbDNA in healthy individuals and patients with CRC. High presence of *16S* rRNA and *E. coli* was revealed in all participants, providing strong evidence for bacterial translocation. Moreover, the significantly higher detection of *F. nucleatum* in the blood of patients with metastatic CRC without surgical resection of the primary tumor, confirms the role of this bacterium as an intratumoral pathogen associated with advanced stages of CRC. Future studies should focus on correlating these findings with patients' outcomes and survival, possibly elucidating the role of *F. nucleatum* as a prognostic biomarker in metastatic CRC. For the further investigation of the sources of cbDNA, fecal and oral samples should be analyzed in the future, along with blood microbiota, using more advanced NGS-based methods, assessing the relationship between CRC and microbiota dysbiosis.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org, Data S1: Raw data material; Table S1: PCR primers and conditions for DNA amplification.

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