



# Molecular and Pathology Features of Colorectal Tumors and Patient Outcomes Are Associated with *Fusobacterium nucleatum* and Its Subspecies *animalis*

Ivan Borozan<sup>1</sup>, Syed H. Zaidi<sup>1</sup>, Tabitha A. Harrison<sup>2</sup>, Amanda I. Phipps<sup>2</sup>, Jiayin Zheng<sup>2</sup>, Stephen Lee<sup>1</sup>, Quang M. Trinh<sup>1</sup>, Robert S. Steinfeldt<sup>2</sup>, Jeremy Adams<sup>1</sup>, Barbara L. Banbury<sup>2</sup>, Sonja I. Berndt<sup>3</sup>, Stefanie Brezina<sup>4</sup>, Daniel D. Buchanan<sup>5,6,7,8</sup>, Susan Bullman<sup>9</sup>, Yin Cao<sup>10,11</sup>, Alton B. Farris III<sup>12</sup>, Jane C. Figueiredo<sup>13</sup>, Marios Giannakis<sup>14,15</sup>, Lawrence E. Heisler<sup>1</sup>, John L. Hopper<sup>6</sup>, Yi Lin<sup>2</sup>, Xuemei Luo<sup>1</sup>, Reiko Nishihara<sup>16,17,18</sup>, Elaine R. Mardis<sup>19</sup>, Nickolas Papadopoulos<sup>20</sup>, Conghui Qu<sup>2</sup>, Emma E.G. Reid<sup>1</sup>, Stephen N. Thibodeau<sup>21</sup>, Sophia Harlid<sup>22</sup>, Caroline Y. Um<sup>23</sup>, Li Hsu<sup>2,24</sup>, Andrea Gsur<sup>4</sup>, Peter T. Campbell<sup>23</sup>, Steven Gallinger<sup>1,25,26</sup>, Polly A. Newcomb<sup>2,27</sup>, Shuji Ogino<sup>14,17,28,29,30</sup>, Wei Sun<sup>2</sup>, Thomas J. Hudson<sup>1</sup>, Vincent Ferretti<sup>1,31</sup>, and Ulrike Peters<sup>2,27</sup>

## ABSTRACT

**Background:** *Fusobacterium nucleatum* (*F. nucleatum*) activates oncogenic signaling pathways and induces inflammation to promote colorectal carcinogenesis.

**Methods:** We characterized *F. nucleatum* and its subspecies in colorectal tumors and examined associations with tumor characteristics and colorectal cancer-specific survival. We conducted deep sequencing of *nusA*, *nusG*, and bacterial *16s rRNA* genes in tumors from 1,994 patients with colorectal cancer and assessed associations between *F. nucleatum* presence and clinical characteristics, colorectal cancer-specific mortality, and somatic mutations.

**Results:** *F. nucleatum*, which was present in 10.3% of tumors, was detected in a higher proportion of right-sided and advanced-stage tumors, particularly subspecies *animalis*. Presence of *F. nucleatum* was associated with higher colorectal cancer-specific mortality (HR, 1.97;  $P = 0.0004$ ). This association was restricted to non-hypermethylated, microsatellite-stable tumors (HR, 2.13;  $P = 0.0002$ )

and those who received chemotherapy [HR, 1.92; confidence interval (CI), 1.07–3.45;  $P = 0.029$ ]. Only *F. nucleatum* subspecies *animalis*, the main subspecies detected (65.8%), was associated with colorectal cancer-specific mortality (HR, 2.16;  $P = 0.0016$ ), subspecies *vincentii* and *nucleatum* were not (HR, 1.07;  $P = 0.86$ ). Additional adjustment for tumor stage suggests that the effect of *F. nucleatum* on mortality is partly driven by a stage shift. Presence of *F. nucleatum* was associated with microsatellite instable tumors, tumors with *POLE* exonuclease domain mutations, and *ERBB3* mutations, and suggestively associated with *TP53* mutations.

**Conclusions:** *F. nucleatum*, and particularly subspecies *animalis*, was associated with a higher colorectal cancer-specific mortality and specific somatic mutated genes.

**Impact:** Our findings identify the *F. nucleatum* subspecies *animalis* as negatively impacting colorectal cancer mortality, which may occur through a stage shift and its effect on chemoresistance.

<sup>1</sup>Ontario Institute for Cancer Research, Toronto, Ontario, Canada. <sup>2</sup>Public Health Sciences Division, Fred Hutchinson Cancer Research Centre, Seattle, Washington. <sup>3</sup>Division of Cancer Epidemiology and Genetics, National Cancer Institute, National Institutes of Health, Bethesda, Maryland. <sup>4</sup>Institute of Cancer Research, Department of Medicine I, Medical University of Vienna, Vienna, Austria. <sup>5</sup>Colorectal Oncogenomics Group, Department of Clinical Pathology, The University of Melbourne, Parkville, Victoria, Australia. <sup>6</sup>The University of Melbourne Centre for Cancer Research, Victorian Comprehensive Cancer Centre, Parkville, Victoria, Australia. <sup>7</sup>Familial Cancer Clinic, Genetic Medicine, The Royal Melbourne Hospital, Parkville, Victoria, Australia. <sup>8</sup>Centre for Epidemiology and Biostatistics, Melbourne School of Population and Global Health, The University of Melbourne, Parkville, Victoria, Australia. <sup>9</sup>Human Biology Division, Fred Hutchinson Cancer Research Center, Seattle, Washington. <sup>10</sup>Division of Public Health Sciences, Department of Surgery, Washington University School of Medicine in St. Louis, St. Louis, Missouri. <sup>11</sup>Siteman Cancer Center, Washington University School of Medicine in St. Louis, St. Louis, Missouri. <sup>12</sup>Pathology and Laboratory Medicine, Emory University School of Medicine, Atlanta, Georgia. <sup>13</sup>Department of Medicine, Samuel Oschin Comprehensive Cancer Institute, Cedars-Sinai Medical Center, Los Angeles, California. <sup>14</sup>Department of Medical Oncology, Dana-Farber Cancer Institute and Harvard Medical School, Boston, Massachusetts. <sup>15</sup>Broad Institute of MIT and Harvard, Cambridge, Massachusetts. <sup>16</sup>Department of Oncologic Pathology, Dana-Farber Cancer Institute and Harvard Medical School, Boston, Massachusetts. <sup>17</sup>Department of Nutrition, Harvard T.H. Chan School of Public Health, Boston, Massachusetts. <sup>18</sup>Program in MPE Molecular Path-

ological Epidemiology, Department of Pathology, Brigham and Women's Hospital and Harvard Medical School, Boston, Massachusetts. <sup>19</sup>The Steve and Cindy Rasmussen Institute for Genomic Medicine, Abigail Wexner Research Institute, Nationwide Children's Hospital, Columbus, Ohio. <sup>20</sup>Ludwig Center for Cancer Genetics and Therapeutics, Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins Medicine, Baltimore, Maryland. <sup>21</sup>Division of Laboratory Genetics, Department of Laboratory Medicine and Pathology, Mayo Clinic, Rochester, Minnesota. <sup>22</sup>Oncology, Department of Radiation Sciences, Faculty of Medicine, Umeå University, Umeå, Sweden. <sup>23</sup>Behavioral and Epidemiology Research Group, American Cancer Society, Atlanta, Georgia. <sup>24</sup>Department of Biostatistics, School of Public Health, University of Washington, Seattle, Washington. <sup>25</sup>Lunenfeld-Tanenbaum Research Institute, Sinai Health, University of Toronto, Toronto, Ontario, Canada. <sup>26</sup>General Surgery, Surgery and Critical Care Program, University Health Network Toronto General Hospital, University of Toronto, Toronto, Ontario, Canada. <sup>27</sup>Department of Epidemiology, School of Public Health, University of Washington, Seattle, Washington. <sup>28</sup>Department of Epidemiology, Harvard T.H. Chan School of Public Health, Harvard University, Boston, Massachusetts. <sup>29</sup>Cancer Immunology Program, Dana-Farber/Harvard Cancer Center, Boston, Massachusetts. <sup>30</sup>Cancer Epidemiology Program, Dana-Farber/Harvard Cancer Center, Boston, Massachusetts. <sup>31</sup>CHU Sainte-Justine Research Center, University of Montreal, Montreal, Quebec, Canada.

**Note:** Supplementary data for this article are available at Cancer Epidemiology, Biomarkers & Prevention Online (<http://cebp.aacrjournals.org/>).

## Introduction

The rapid advances in DNA sequencing technologies have allowed unbiased and comprehensive identification of pathogens in human tissues (1, 2). It is thought that approximately 15% of all cancers are attributable to microorganisms, including bacteria (3, 4). Microbes are not only expected to play a role in susceptibility to some cancers but also in therapeutic response (5). A well characterized example is gastric cancers, of which 770,000 of all new cancer cases, worldwide, were attributable to *Helicobacter pylori* infections in 2012 (4, 6).

In a subset of colorectal cancer cases, *Fusobacterium nucleatum*, an inhabitant of the oral cavity and gastrointestinal tract, has been found to be enriched in tumor tissues (7–15). Multiple mechanisms have been described by which the *F. nucleatum* may promote colorectal cancer. These include activation of WNT/ $\beta$ -catenin signaling (16), creation of a proinflammatory microenvironment (17), and modulation of the tumor microenvironment to evade the antitumoral immune response (18). The presence of *F. nucleatum* in colorectal tumors is known to be associated with poor prognosis and resistance to chemotherapy (19). However, less is known about tumor and patient attributes in relation to *F. nucleatum* in colorectal cancer. A few studies have shown associations of *F. nucleatum* with somatic mutation burden, tumor site, and tumor stage (7, 8, 20). However, these studies have relatively small sample sizes and are limited with respect to survival data, somatic mutation information, and epidemiologic data. Moreover, these studies did not investigate *F. nucleatum* composition in colorectal tumors at the subspecies level. Additionally, as somatic mutations in cancer have been observed to affect the tumor microbiome in other cancer types, it is important to examine associations of *F. nucleatum* with existing mutation data for the same colorectal tumors (21).

Here, we conducted targeted deep sequencing to assess the presence of *F. nucleatum* across 1,994 colorectal tumors, which were characterized for somatic mutations in 205 genes (22). In this large dataset from the Genetics and Epidemiology of Colorectal Cancer Consortium (GECCO) and the Colon Cancer Family Registry (CCFR), we characterized *F. nucleatum* and its subspecies, and examined associations with colorectal cancer–specific survival, somatically mutated genes, mutational burden, tumor site, and tumor stage. This comprehensive study provides insights into the role of *F. nucleatum* in colorectal cancer.

## Materials and Methods

### Study populations

The studies include the Colorectal Cancer Study of Austria (CORSA), Ontario Familial Colorectal Cancer Registry (OFCCR), Seattle-Colon Cancer Family Registry (SCCFR), and Cancer Prevention Study-II (CPS-II). Study descriptions and sample selection are described in the Supplementary Materials and Methods. Institutional review boards approved the study, and all patients (or their proxies) provided written informed consent to allow collection of specimens

I. Borozan and S.H. Zaidi contributed equally to this article.

**Corresponding Authors:** Ulrike Peters, Public Health Sciences Division, Fred Hutchinson Cancer Research Center, 1100 Fairview Avenue North, P.O. Box 19024, Seattle, WA 98109-1024. Phone: 206-667-2450; Fax: 206.667.7850; E-mail: upeters@fredhutch.org; and Vincent Ferretti, CHU Sainte-Justine Research Center, University of Montreal, 3175 Côte-Sainte-Catherine Road,

and data used in this study. Patients or the public were not involved in the design, conduct, reporting, or dissemination plans of our research.

### DNA extraction, sequencing, and identification of somatic mutations

Details of materials and *F. nucleatum* sequencing are described in the Supplementary Materials and Methods. Briefly, tumor tissue was macrodissected from formalin-fixed, paraffin-embedded (FFPE) tissue sections guided by an hematoxylin and eosin (H&E)–stained slide marked for the tumor region, and DNA was extracted using the QIAamp DNA Mini or QIAamp DNA FFPE tissue kits. Paired-end high-depth sequencing was conducted on HighSeq 2500 (Illumina) using a custom-designed AmpliSeq panel. Details of sequencing, identification of somatic mutations, and determination of microsatellite instability (MSI) status have been previously described (22).

### Detection and quantification of *F. nucleatum* DNA in colorectal cancer tumors

To detect *Fusobacterium* DNA in colorectal cancer tumors, *Fusobacterium*-specific *nusA* and *nusG* genes (NC 003454), and the hypervariable regions, V2, V3, and V6 of the bacterial 16S rRNA genes were sequenced. To identify the *Fusobacterium*-specific sequence reads and quantify their abundance in each sample, the following steps were taken: (i) Unmapped reads from each BAM file containing sequence reads aligned to the human reference sequence (GRCh37/hg19 assembly) were retrieved; (ii) sequence reads with low complexity were removed; (iii) unmapped sequence reads were aligned in single-end mode using the Bowtie2 aligner (23) to a reference sequence database (parameters used: `-very-sensitive-local -k 100 -score-min L,0,1.2`) composed of five *F. nucleatum* complete genomes (NC003454.1, NC009506.1, NC021277.1, NC021281.1, and NC022196.1) and 17,657 16S rRNA genes; (iv) aligned reads were collapsed using the Picard's MarkDuplicates tool; (v) reads with ambiguous alignments were reassigned to the most probable *F. nucleatum* genome of origin using a statistical model based on the read alignment scores (24).

To quantify the amount of *F. nucleatum* in tumor samples, we only considered reads that aligned to *F. nucleatum* reference sequences with greater than 95% identity match. The number of *F. nucleatum* mapped reads found in each tumor was normalized to the total number of reads obtained for a given sample so that the abundance of *F. nucleatum* for each sample was measured in parts per million (ppm) of the total number of reads sequenced. To limit the number of potential false positives, we applied a strict threshold of  $\geq 0.5$  ppm to define a positive presence of *F. nucleatum*. This stringent cutoff was used to remove nonspecific alignments or sequencing artifacts (Supplementary Materials and Methods; Supplementary Figs. S1 and S2). *F. nucleatum* next-generation sequencing (NGS) data was validated using TaqMan qPCR assay (Supplementary Materials and Methods; Supplementary Fig. S3).

### Analysis of mutated genes

We defined a gene as being somatically mutated if it carried any nonsilent mutation, which included all nonsynonymous, frameshift and in-frame indels, splicing, and nonsense mutations. We further

Montréal (Qc) H3T 1C5, Canada. Phone: 514-345-4931, ext. 6507; E-mail: vincent.ferretti.hsj@ssss.gouv.qc.ca

Cancer Epidemiol Biomarkers Prev 2021;XX:XX-XX

doi: 10.1158/1055-9965.EPI-21-0463

©2021 American Association for Cancer Research

refined the mutation definition for a subset of genes with more detailed information available for mutational functional relevance. Mutations in *APC* were restricted to truncating mutations within the first 1,600 codons with known functional consequences (25). To specifically examine the effect of mutations in *KRAS*, *BRAF*, *ERBB2*, and *ERBB3*, only tumors with mutually exclusive mutations in the same signaling pathway were analyzed. For *KRAS*, codons G12, G13, Q61, K117, and A146 were included, in which mutations are known to confer oncogenic signaling. For *BRAF*, only the V600E oncogenic mutation was included. For *PIK3CA*, only nonsynonymous mutations were included. For *POLE*, only nonsynonymous and nontruncating mutations in the exonuclease domain were included. In consideration of statistical power for somatic gene mutation analyses, we restricted analyses to genes on our panel that have been well established as being mutated in colorectal cancer (*KRAS*, *TP53*, *POLE*, *BRAF*, *APC*, *PIK3CA*, and *SMAD4*) and previously implicated with *F. nucleatum* (*KRAS*). *ERBB2* and *ERBB3* genes were also included since they belong to the same tyrosine kinase pathway as *KRAS* and *BRAF*.

### Statistical analysis

To investigate how different factors such as patients' clinical and tumor molecular features relate to the presence or absence of *F. nucleatum* in tumor tissue logistic regression models were used. To assess associations with receipt of chemotherapy, cancer stage, CpG island methylator phenotype status (CIMP), sex, MSI status, and hypermutation status, two different logistic regression models were fitted. The first (simpler) model contained age at diagnosis and sex as covariates, while a second (larger) model further included tumor site, hypermutation status, MSI status, and the presence (or absence) of mutations in *POLE*, *TP53*, and *ERBB3* genes. An ANOVA was performed for the age at diagnosis and tumor burden using the `anova()` function from the R package `stats` (where age at diagnosis and tumor burden were treated as dependent variables and *F. nucleatum* status (presence/absence) as an independent variable).

We adjusted *P* values using the Benjamini–Hochberg method (Supplementary Materials and Methods) to control the FDR. To assess the association between the presence of *F. nucleatum* subspecies with colorectal cancer–specific mortality in 1,320 cases with available survival outcomes data, we used Cox proportional hazards regression analysis, adjusting for sex, age at diagnosis, tumor site, tumor stage, hypermutation status, tumor burden, MSI status, and *POLE*, *TP53*, and *ERBB3* mutation status. We checked the proportional hazards assumption and used stratification for the Cox model to allow for nonproportionality as necessary. To deal with missing data in the case of univariable and multivariable logistic regressions (results in **Table 1**), cases with missing data for tumor location were removed before the analysis. For the Cox proportional hazards regression (results in **Tables 2** and **3**), when fitting the model only the relevant complete cases were used in the analysis. For each *F. nucleatum* subspecies status, we generated adjusted survival curves that were obtained by averaging conditional survival curves based on the Cox model over covariate distribution, as well as the corresponding pointwise confidence intervals (CI) based on 1,000 bootstrapping samples. We performed a series of sensitivity analyses in which different propensity score based methods were used for modeling the association of *F. nucleatum* subspecies with survival (Supplementary Materials and Methods). For mutated genes significantly associated with *F. nucleatum*, we estimated the average treatment effect (ATE) by marginalizing over the covariate distribution to quantify the difference in proportions of *F. nucleatum* presence between mutated and nonmutated.

### Data availability

All data relevant to the study are included in the article or uploaded as supplementary information.

## Results

### Characterization of *Fusobacterium* in colorectal cancer cases

We detected *F. nucleatum*–specific DNA in 10.3% of 1,994 colorectal cancer tumors ( $n = 206$ ; **Fig. 1**). Among the sequence reads mapping to *Fusobacterium*, 92% mapped to *F. nucleatum*. Other species, including *F. periodonticum*, each mapped to  $\leq 2\%$  of reads (**Fig. 2**; Supplementary Table S1). Further characterization of the *F. nucleatum* mapping reads identified subspecies *F. nucleatum animalis* (68.5%), *F. nucleatum nucleatum* (13.9%), *F. nucleatum vincentii* (13.9%), and *F. nucleatum polymorphum* (3.7%; Supplementary Table S1).

As survival, tumor feature, and chemotherapy data were not available for all tumors (see **Table 1** and Supplementary Materials and Methods), the total number of samples used in analyses varied.

### Association of *F. nucleatum* with clinical features

*F. nucleatum* was found more often in right-sided tumors compared with left-sided tumors [odds ratio (OR), 1.77;  $P = 0.00072$ ; **Table 1**]. Within the proximal region, tumors in the cecum were most likely to be positive for *F. nucleatum* (OR, 1.5, 95% CI, 1.0–2.3;  $P = 0.035$ ). Tumors diagnosed at stage II (OR, 1.77;  $P = 0.027$ ) or stage III (OR, 1.84;  $P = 0.022$ ) were more likely to be positive for *F. nucleatum* compared with stage I tumors. Furthermore, *F. nucleatum* was more prevalent among women than men (OR, 1.42;  $P = 0.031$ ). However, none of these associations remained significant in the multivariate adjusted model and after accounting for multiple testing. Age at diagnosis was not associated with presence of *F. nucleatum* in tumors ( $P = 0.59$ ).

We performed subsequent analyses to examine associations at the subspecies level. Consistent with our overall findings, presence of the predominant subspecies, *F. nucleatum* subspecies *animalis*, was associated with right-sided versus left-sided tumors (OR, 2.47;  $P = 2.5e-5$ ), female versus male sex (OR, 1.52;  $P = 0.04$ ), and stage II (OR, 2.3;  $P = 0.02$ ), stage III (OR, 2.55;  $P = 0.009$ ) versus stage I tumors, and microsatellite-unstable versus tumors not microsatellite unstable (OR, 2.84;  $P = 4.86e-06$ ; Supplementary Table S2). Except for sex, these associations remained significant in the multivariate adjusted model and accounting for multiple testing. *F. nucleatum* subspecies *vincentii*, and *F. nucleatum* subspecies *nucleatum* were not associated with the above features (Supplementary Table S2).

### Impact of *F. nucleatum* on survival

We observed that patients with *F. nucleatum* present in tumors were more likely to die of colorectal cancer than those without *F. nucleatum* present (HR, 1.97, CI, 1.35–2.86,  $P = 0.0004$ ). This association remains statistically significant, even after adjustment for tumor stage ( $P = 0.018$ ; **Fig. 3A** and **3B**; **Table 2**). A subanalysis restricted to non-hypermutated, microsatellite stable (MSS) tumors was consistent with this overall finding (HR, 2.13; 95% CI, 1.44–3.15;  $P = 0.0002$ ). In contrast, among cases with hypermutated tumors with MSI, the presence of *F. nucleatum* was not significantly associated with survival (HR, 0.84; 95% CI, 0.21–3.34;  $P = 0.81$ ). As *F. nucleatum* has been linked to chemoresistance (19), we stratified the association between *F. nucleatum* and colorectal cancer–specific mortality by chemotherapy. We observed that *F. nucleatum* was associated with increased

**Table 1.** Patient characteristics, tumor features, and the presence of *F. nucleatum* in colorectal cancer tissue.

Characteristic	Fuso-negative, N (%)	Fuso-positive, N (%)	OR (95% CI) <sup>a</sup>	FDR, P <sup>b</sup>	OR (95% CI) <sup>c</sup>	FDR, P <sup>b</sup>
Age at diagnosis <sup>d</sup>						
Mean	63.3 ± 12.2	63.8 ± 12.5	-		-	
N	1,659	192	-		-	
Sex						
Men	836 (50)	80 (42)	Ref		Ref	
Women	824 (50)	112 (58)	1.42 (1.05-1.93; P = 0.023)	0.031	1.31 (0.96-1.79; P = 0.09)	0.18
Total	1,660	192				
Chemotherapy						
No	616 (48.5)	66 (44.3)	Ref		Ref	
Yes	653 (51.5)	83 (55.7)	1.25 (0.88-1.79; P = 0.22)	0.27	1.29 (0.90-1.87; P = 0.17)	0.21
Total	1,269	149				
Cancer stage						
Stage I	462 (35)	35 (24)	Ref		Ref	
Stage II	356 (27)	47 (33)	1.77 (1.11-2.85; P = 0.017)	0.027	1.72 (1.06-2.82; P = 0.029)	0.099
Stage III	345 (26)	48 (33)	1.84 (1.15-2.98; P = 0.012)	0.022	1.88 (1.16-3.10; P = 0.011)	0.061
Stage IV	142 (11)	14 (10)	1.36 (0.68-2.58; P = 0.36)	0.36	1.52 (0.75-2.95; P = 0.23)	0.25
Total	1,305	144				
Tumor site						
Rectal	419 (25.2)	31 (16.1)	Ref		Ref	
Distal colon	533 (32.1)	51 (26.6)	1.27 (0.8-2.04; P = 0.31)	0.34	1.22 (0.77-1.97; P = 0.40)	0.4
Proximal colon	708 (42.7)	110 (57.3)	2.04 (1.36-3.16; P = 8e-4)	0.0017	1.46 (0.94-2.33; P = 0.098)	0.18
Left (rectal + distal)	952	82	Ref		Ref	
Right (proximal colon)	708	110	1.77 (1.31-2.4; P = 0.00026)	0.00072	1.29 (0.92-1.82; P = 0.14)	0.19
Total	1,660	192				
Mutation burden <sup>d</sup>						
Mean	23.2 ± 47.8	34.4 ± 49.02				
Total	1,677	194				
Hypermutation status						
Nonhypermutated	1,394 (84.0)	138 (71.9)	Ref		Ref	
Hypermutated	266 (16.0)	54 (28.1)	1.98 (1.39-2.78; P = 0.0001)	0.0004	0.51 (0.27-0.95; P = 0.036)	0.099
Total	1,660	192				
Microsatellite instability status						
MSS	1,434 (86)	134 (70)	Ref		Ref	
MSI	226 (14)	58 (30)	2.27 (1.56-3.3; P = 1.8e-5)	9.9e-5	3.22 (1.72-5.97; P = 0.00023)	0.0025
Total	1,660	192				
CpG island methylator phenotype status						
CIMP <sup>-</sup>	1,049	107	Ref		Ref	
CIMP <sup>+</sup>	191	48	1.11 (1.06-1.16; P = 3.2e-6)	3.5e-5	1.04 (0.99-1.10; P = 0.12)	0.19
Total	1,240	155				

Abbreviation: Ref, reference.

<sup>a</sup>Adjusted for the age at diagnosis and sex.<sup>b</sup>P values were adjusted for multiple testing using the Benjamini-Hochberg method.<sup>c</sup>Adjusted for age at diagnosis; sex; tumor site; hypermutation status; MSI status; and mutations in *POLE*, *TP53*, and *ERBB3* (for models investigating hypermutation and MSI status, those variables were only included once in the model).<sup>d</sup>An ANOVA was performed for age at diagnosis and tumor burden (age at diagnosis P = 0.59 and mutation burden P = 0.002).

colorectal cancer-specific mortality in colorectal cancer cases receiving chemotherapy (HR, 1.92; CI, 1.07-3.45; P = 0.029), but not in those without chemotherapy (HR, 0.42; CI, 0.06-3.16; P = 0.40).

We further examined colorectal cancer-specific survival within *F. nucleatum* subspecies. The presence of *F. nucleatum* subspecies *animalis* was associated with higher mortality (HR, 2.16; 95% CI, 1.34-3.47; P = 0.0016; **Table 3**); however, the presence of *F. nucleatum* subspecies *vincentii* and *F. nucleatum* subspecies *nucleatum* were not (HR, 1.07; 95% CI, 0.48-2.43; P = 0.86; **Fig. 3C**). When further adjusting for tumor stage in the subspecies analysis, *F. nucleatum* subspecies *animalis* exhibited the same survival trend, but did not remain statistically significant. This may be due to a limited sample size (**Table 3**) or may suggest that the effect of *F. nucleatum* and the

subspecies *animalis* on colorectal cancer-specific mortality is partly driven by a shift in stage.

Importantly, all results from the Cox proportional hazards regression analyses described above were consistent with results from our sensitivity analyses using different propensity score-based methods (Supplementary Tables S3, S4, and S5).

### ***F. nucleatum*, MSI, and tumor hypermutation features**

*F. nucleatum* was more often present in hypermutated tumors compared with nonhypermutated tumors (OR, 1.98; P = 0.0004; **Table 1**). Mutations in DNA mismatch repair genes (*MLH1*, *MLH3*, *MSH2*, *MSH6*, *PMS2*) and in the exonuclease domain of *POLE* contribute to hypermutation in colorectal cancer (26). DNA mismatch



**Table 2.** Presence of *F. nucleatum* in colorectal cancer tissue and colorectal cancer-specific survival.

<i>F. nucleatum</i>	Cases (n)	Events (n)	HR (95% CI) <sup>a</sup>	HR (95% CI) <sup>b</sup>
Negative	1,174	216	1.00 (Ref)	1.00 (Ref)
Positive	142	34	1.97 (1.35–2.86)	1.68 (1.09–2.59)
<i>P</i>			0.0004	0.018

<sup>a</sup>Multivariable analysis adjusted for sex; age at diagnosis; tumor site; hypermutation status; tumor burden; mutations in *POLE*, *TP53*, and *ERBB3*; and MSI status.

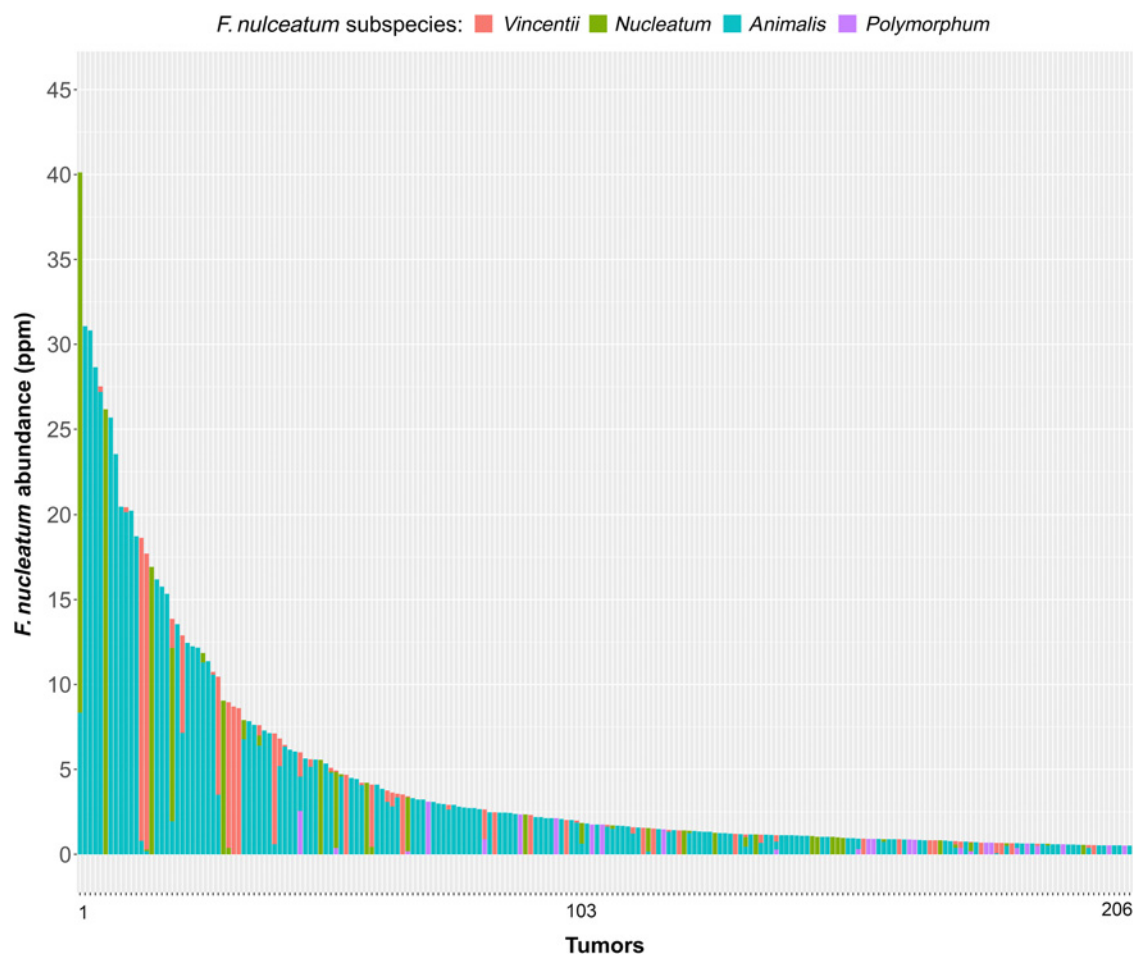
<sup>b</sup>Multivariable analysis adjusted for sex; age at diagnosis; tumor site; hypermutation status; tumor burden; mutations in *POLE*, *TP53*, and *ERBB3*; MSI status; and tumor stage.

**Table 3.** Presence of *F. nucleatum* subspecies in colorectal cancer tissue and colorectal cancer-specific survival.

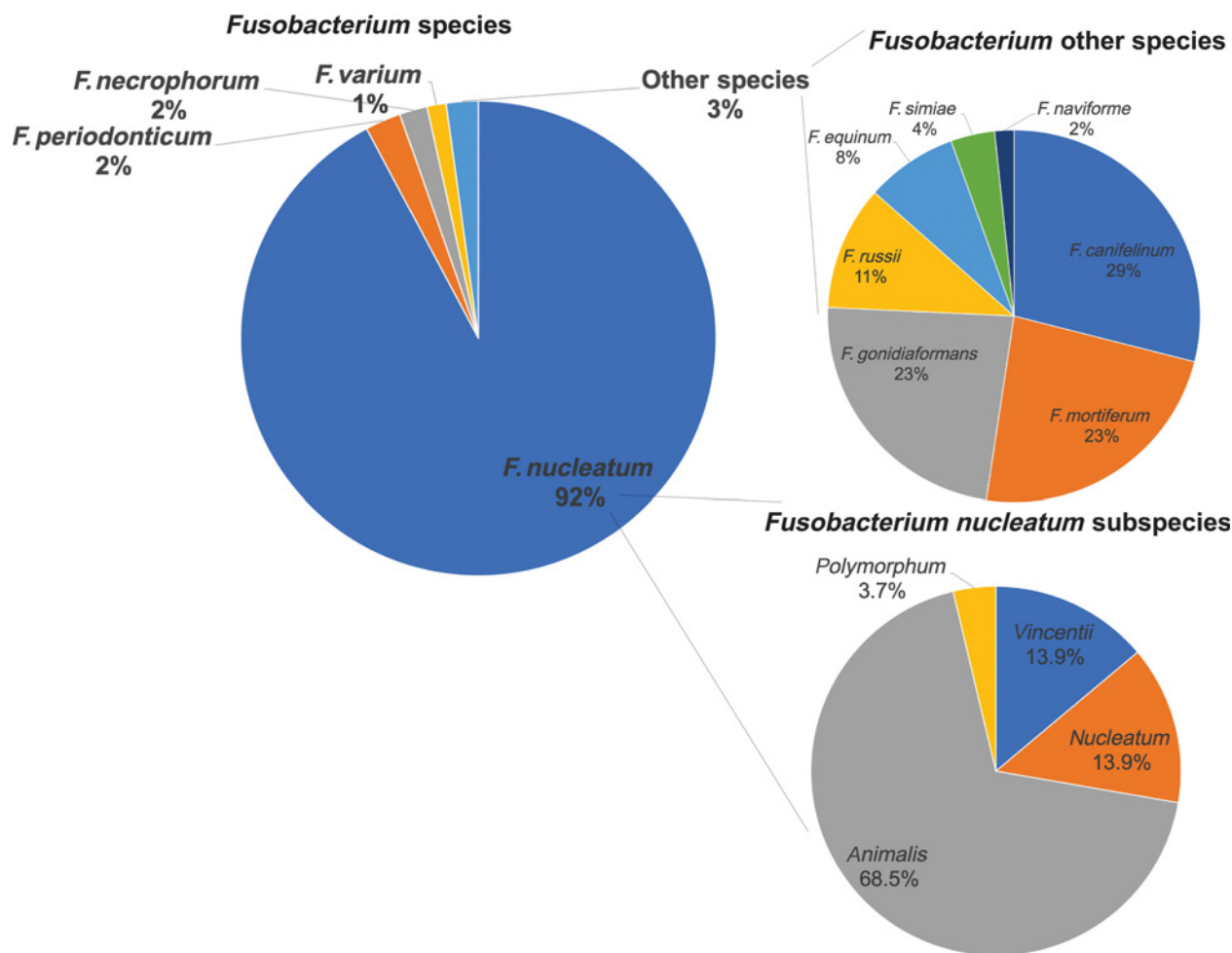
<i>F. nucleatum</i> subsp <i>animalis</i>	Cases (n)	Events (n)	HR (95% CI) <sup>a</sup>	HR (95% CI) <sup>b</sup>
Negative	1,196	224	1.00 (Ref)	1.00 (Ref)
<i>animalis</i>	80	20	2.16 (1.34–3.47)	1.75 (0.99–3.10)
<i>P</i>			0.0016	0.056
<i>vincentii</i> + <i>nucleatum</i>	40	6	1.07 (0.48–2.43)	1.02 (0.42–2.51)
<i>P</i>			0.86	0.96

<sup>a</sup>Multivariable analysis adjusted for sex; age at diagnosis; tumor site; hypermutation status; tumor burden; mutations in *POLE*, *TP53*, and *ERBB3*; and MSI status.

<sup>b</sup>Multivariable analysis adjusted for sex; age at diagnosis; tumor site; hypermutation status; tumor burden; mutations in *POLE*, *TP53*, and *ERBB3*; MSI status; and tumor stage.

**Figure 1.**

*F. nucleatum* DNA abundance detected in 1,994 colorectal cancer tumors. *F. nucleatum* DNA abundance in units of ppm detected in 206 colorectal tumor DNA samples with minimum abundance  $\geq 0.5$  ppm. The *F. nucleatum* subspecies are shown in different colors.

**Figure 2.**

Percent of sequencing reads mapping to *Fusobacterium* species and subspecies. A total of 206 colorectal cancer tumors positive for *F. nucleatum* were used in the plots.

repair-deficient, MSI tumors were more likely to have *F. nucleatum* present compared with the MSS tumors (OR, 2.27;  $P = 9.9 \times 10^{-5}$ ; **Table 1**). Additionally, *F. nucleatum* was associated with CIMP status (OR, 1.11;  $P = 3.5 \times 10^{-5}$ ; **Table 1**). We found that tumors with *POLE* exonuclease domain mutations were also more likely to have *F. nucleatum* present (OR, 4.14;  $P = 0.029$ ; **Table 4**). The association for MSI status and *POLE* mutation status remained significant in the multivariate adjusted model and accounting for multiple testing.

At the subspecies level, *F. nucleatum* subspecies *animalis* was more often present in hypermutated tumors compared with nonhypermutated tumors (OR, 2.59;  $P = 2.5 \times 10^{-5}$ ; Supplementary Table S2). Tumors with MSI were also more likely to have *F. nucleatum* subspecies *animalis* present than the MSS tumors (OR, 2.84;  $P = 2.5 \times 10^{-5}$ ).

### *F. nucleatum* and tumor gene mutations

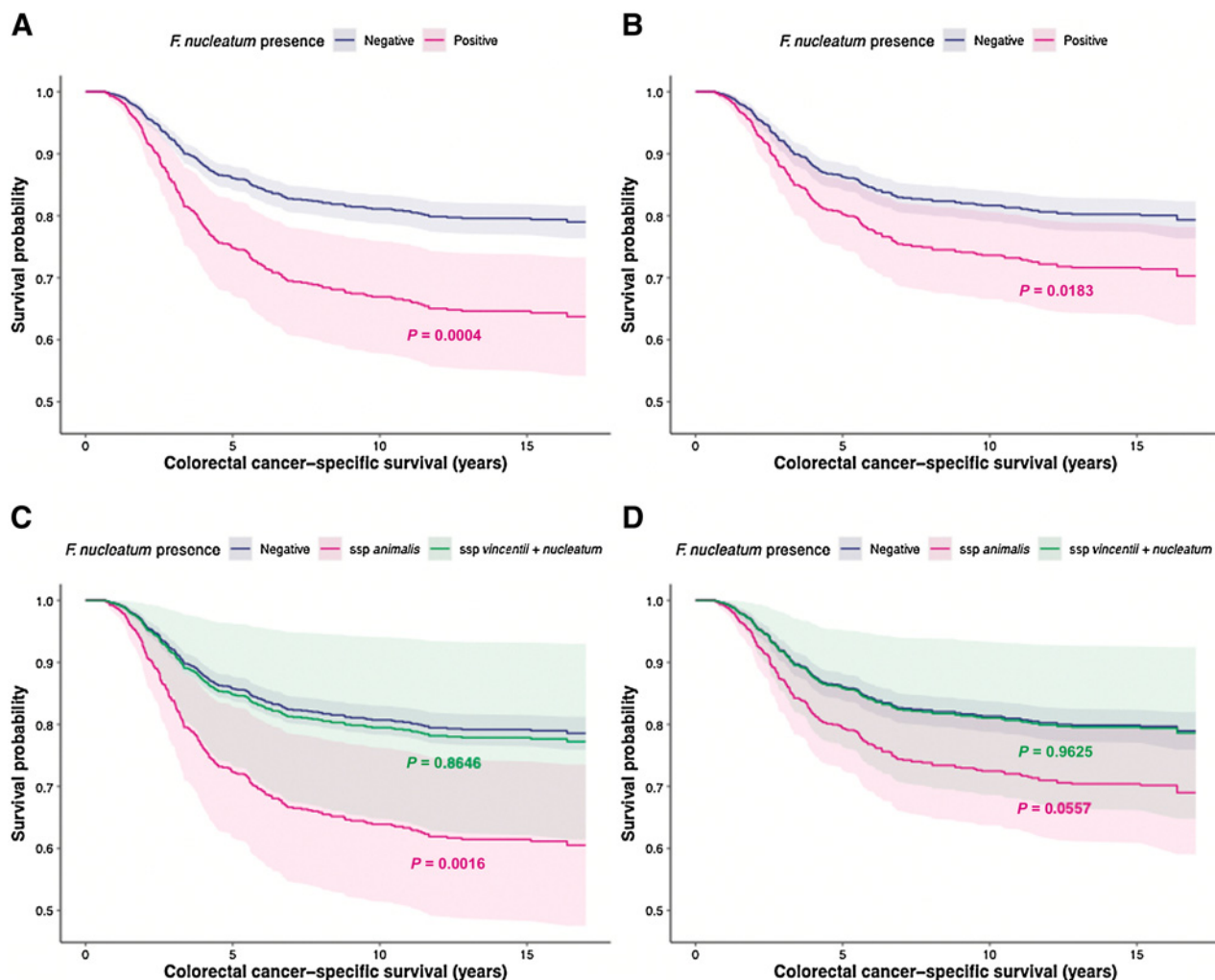
When testing the association of *F. nucleatum* with mutation status of somatic genes frequently mutated in colorectal cancer, while accounting for multiple testing, we observed that the presence of *F. nucleatum* was associated with mutations in *ERBB3* (OR, 4.33;  $P = 0.005$ ; **Table 4**). The average treatment effect of *F. nucleatum* on *ERBB3* was 18.8% ( $P = 0.007$ ; Supplementary Table S6) with 8.2% for

*ERBB3* nonmutated and 27.0% for *ERBB3* mutant. Mutations in *TP53* appeared to be negatively associated with the presence of *F. nucleatum*, but results were not statistically significant after accounting for multiple comparisons (OR, 0.64;  $P = 0.064$ ; **Table 4**). Mutation status of *APC*, *PIK3CA*, *KRAS*, *BRAF*, *ERBB2*, and *SMAD4* was not associated with *F. nucleatum* prevalence.

At the subspecies level, the presence of *F. nucleatum* subspecies *animalis* was associated with mutations in *ERBB3* (OR, 3.76;  $P = 0.043$ ; Supplementary Table S7). No significant associations were found between presence of *F. nucleatum* subspecies *animalis* and mutation status for *APC*, *TP53*, *KRAS*, *PIK3CA*, *KRAS*, *BRAF*, *ERBB2*, or *SMAD4*.

## Discussion

We used NGS to comprehensively characterize the presence of *F. nucleatum* in 1,994 colorectal cancer cases. Presence of *F. nucleatum* was associated with tumor site, tumor stage, sex, microsatellite instability, and mutation status of a subset of genes frequently mutated in colorectal cancer. Colorectal cancer-specific mortality increased with the presence of *F. nucleatum*, an association that was restricted to



**Figure 3.**

Adjusted survival curves for colorectal cancer-specific survival based on Cox models. **A**, According to the presence of *F. nucleatum* DNA in colorectal cancer tissues adjusted for sex; age at diagnosis; tumor site; hypermutation status; tumor burden; mutations in *POLE*, *TP53*, and *ERBB3*; and MSI. **B**, According to the presence of *F. nucleatum* DNA in colorectal cancer tissues adjusted for sex; age at diagnosis; tumor site; hypermutation status; tumor burden; mutations in *POLE*, *TP53*, and *ERBB3*; MSI; and tumor stage. **C**, According to the presence of *F. nucleatum* subspecies DNA in colorectal cancer tissues adjusted for sex; age at diagnosis; tumor site; hypermutation status; tumor burden; mutations in *POLE*, *TP53*, and *ERBB3*; and MSI. **D**, According to the presence of *F. nucleatum* subspecies DNA in colorectal cancer tissues adjusted for sex; age at diagnosis; tumor site; hypermutation status; tumor burden; mutations in *POLE*, *TP53*, and *ERBB3*; MSI; and tumor stage. *P* values resulting from the multivariate analyses are shown on the plots. Pointwise confidence intervals are based on 1,000 bootstrapping samples. ssp, subspecies.

nonhypermutated, microsatellite-stable tumors and to patients receiving chemotherapy. *F. nucleatum* subspecies *animalis* is the most common subspecies in colorectal cancer, and is most significantly associated with patient tumor characteristics, colorectal cancer-specific mortality, and somatic mutations.

We used 18 primer pairs to amplify *F. nucleatum*-specific *nusA*, *nusG* genes, and *16S rRNA*, and generated consistent results across our studies at the species and subspecies levels. The percent of cases we observed as having *F. nucleatum* present (10.3%) is in line with most previous studies, which identified *F. nucleatum* in 9% to 13% of colorectal cancer cases (7, 9, 19, 27, 28).

Tumors containing *F. nucleatum* were more likely to present in the right side of the colon, at advanced stages, and in women. The higher prevalence of *F. nucleatum* bearing tumors in the cecum, the far right of the colon, is consistent with at least one previous publication (8).

More tumors in the cecum with *F. nucleatum* may have occurred due to its direct connection to the appendix, which is known to harbor *Fusobacteria* (29). We discovered that right-sided tumor location, and advanced tumor stages (particularly stage II and III), were significantly associated with the presence of *F. nucleatum* subspecies *animalis*, but not with other abundant *F. nucleatum* subspecies. These findings are relevant for colorectal cancer prognosis and treatment.

We found the presence of *F. nucleatum* associated with increased colorectal cancer-specific mortality. This observation is consistent with previous publications (7, 20, 30–32). Our finding of an association of *F. nucleatum* with higher colorectal cancer-specific mortality in patients receiving chemotherapy further supports its role in chemoresistance (19). Moreover, we identified the *F. nucleatum* subspecies *F. nucleatum* subspecies *animalis*, but not *F. nucleatum* subspecies *vincentii* or *F. nucleatum* subspecies *nucleatum*, to be associated with

**Table 4.** Associations between somatically mutated genes and the presence of *F. nucleatum* in colorectal tumors.

Mutation status (mutant vs. nonmutated)	Univariate OR (95% CI)	FDR, $P^a$	Multivariable OR (95% CI) <sup>b</sup>	FDR $P^a$
<i>KRAS</i> <sup>c</sup>	1.35 (0.94–1.94, $P = 0.099$ )	0.16	1.42 (0.97–2.07, $P = 0.067$ )	0.15
<i>TP53</i>	0.58 (0.43–0.79, $P = 0.00044$ )	0.002	0.64 (0.44–0.94, $P = 0.021$ )	0.064
<i>POLE</i> <sup>d</sup>	2.82 (1.1–6.4, $P = 0.019$ )	0.057	4.14 (1.42–11.19, $P = 0.0064$ )	0.029
<i>ERBB2</i>	0.95 (0.28–2.43, $P = 0.93$ )	0.93	0.65 (0.18–1.77, $P = 0.44$ )	0.665
<i>ERBB3</i>	6.22 (2.87–12.89, $P = 1.49e-6$ )	1.34e-5	4.33 (1.84–9.85, $P = 0.00056$ )	5.0e-3
<i>APC</i>	0.77 (0.57–1.05), $P = 0.098$ )	0.16	1.02 (0.73–1.42, $P = 0.93$ )	0.93
<i>BRAF</i>	1.58 (0.89–2.70, $P = 0.12$ )	0.16	0.88 (0.43–1.73, $P = 0.72$ )	0.81
<i>PIK3CA</i>	1.37 (0.9–2.02, $P = 0.12$ )	0.16	1.22 (0.80–1.83, $P = 0.33$ )	0.60
<i>SMAD4</i>	1.08 (0.64–1.72, $P = 0.75$ )	0.85	1.11 (0.65–1.7, $P = 0.68$ )	0.81

Note: In the regression analysis, *F. nucleatum* was used as a binary outcome variable (negative = 0 vs. positive = 1) and gene mutation status as the independent variable. Details of genes with mutations are presented in the Materials and Methods section.

<sup>a</sup> $P$  values were adjusted for multiple testing using the Benjamini-Hochberg method.

<sup>b</sup>Logistic regression model adjusted for sex, age at diagnosis, tumor site, MSI, and hypermutation status.

<sup>c</sup>Codons G12/13, Q61, K117, and A146.

<sup>d</sup>Exonuclease domain mutations.

colorectal cancer–specific mortality. This difference is likely not driven by statistical power as the association with colorectal cancer–specific survival is qualitatively different (HR, 2.16 vs. 1.07). Our approach was capable of detecting all 4 *F. nucleatum* subspecies, of these *F. nucleatum* subspecies *polymorphum* was the least abundant and present in fewer patients. These findings strongly suggest a pathogenic role of *F. nucleatum animalis* in colorectal cancer.

Comparing stage-unadjusted and stage-adjusted results suggests that the effect of *F. nucleatum* and its subspecies *animalis* on survival is mediated, in part, through a shift towards a higher tumor stage given that the stage-adjusted association is weaker. We consider the stage-adjusted analyses as secondary analyses that provide mechanistic insights into the association. While studies that aim to identify predictors for survival tend to default to stage-adjusted analyses to explore if the marker adds information above the commonly used stage information, our study is focused on establishing and characterizing the association between *F. nucleatum* status and survival.

The availability of tumor sequencing data allowed us to examine associations between the presence of *F. nucleatum* and select tumor molecular features. *F. nucleatum*, and its subspecies *F. nucleatum animalis*, were more commonly found in tumors with MSI. Furthermore, we found that *F. nucleatum* was associated with the presence of mutations in the proofreading exonuclease domain of *POLE*, independent of MSI status. Hypermutated tumors are known to exhibit abundant neoantigens and thereby elicit an immune response; however, mechanisms of immunosuppression by *F. nucleatum* in colorectal cancer also have been described (18). As *F. nucleatum* was not associated with colorectal cancer–specific mortality in hypermutated tumors, it is unlikely that *F. nucleatum* impacts improved survival in this tumor subtype.

We further examined the association of *F. nucleatum* with frequently mutated genes belonging to the following pathways implicated in colorectal cancer: WNT, p53, receptor-tyrosine kinase, TGF- $\beta$ , and PI-3-kinase pathways (33). Among these genes, only an association between *KRAS* and *F. nucleatum* was described in a previous study (32). Consistent with this prior study, we found suggestive evidence (although not surpassing the multiple comparison test) that the presence of *F. nucleatum* is associated with mutations in *KRAS* (32). As *KRAS* belongs to the receptor tyrosine kinase pathway, we further examined the association of *F. nucleatum* with mutually exclusive

mutations in *BRAF*, *NRAS*, *ERBB2*, and *ERBB3*, all belonging to the same pathway. Interestingly, the presence of *F. nucleatum*, and its subspecies *F. nucleatum animalis*, was significantly associated with mutations in *ERBB3*, after adjusting for covariates. Nonsynonymous oncogenic mutations in *ERBB3* have been described in colorectal cancer and other cancers (34–36). Confirmed oncogenic mutations, V104M/L, A232V, P262S, G284R, and Q809R (34, 35) as well as all known and predicted oncogenic mutations described in OncoKB curated dataset (37) were present in our colorectal cancer tumors. Among these mutations, the V104M/L substitutions in the extracellular domain of the ERBB3 receptor were the most common alterations. Expression of ERBB family of receptors on the surface of epithelial cells exposes these receptors directly to gut bacteria. Several pathogenic bacteria are known to exploit host cell signaling pathways to promote their adherence and internalization. For example, for entry of *N. meningitidis* and *N. gonorrhoeae* into epithelial cells, translocation of ERBB receptors to the apical surface and their recruitment have been demonstrated (38). *F. nucleatum* has also been shown to internalize in the epithelial and endothelial cells of colon and other tissues (11, 39–41). Internalization of *F. nucleatum* into epithelial cells and recruitment of the ERBB receptors by other bacteria for host cell entry may suggest a similar mechanism for *F. nucleatum*. Additional studies are required to test this hypothesis and potential blockade of ERBB receptors.

The second most commonly mutated gene in colorectal cancer is *TP53*, having mutations in approximately 53% of tumors (22, 33). Although our result is slightly above the significance threshold after adjusting for multiple testing, our analysis suggests that *F. nucleatum* is more likely present in tumors with wild-type *TP53*, in line with the results from a previous study (15). Consistent with our findings, *TP53* deletion and inhibition in the host were shown to enhance the clearance of extracellular bacteria during pneumonia by increasing the function of microbicidal neutrophils (42). Some pathogenic bacteria are also known to inhibit p53 by inducing its degradation resulting in the alteration of the p53 stress response (43). This phenomenon was first described in gastric cells cocultured with *Helicobacter pylori* (44). By interfering with the wild-type p53 function, bacterial pathogens extend the life of host cells to access sufficient nutrients during intracellular replication (45). Although the exact role of *F. nucleatum* in the p53 pathway remains unknown, similar



mechanisms of rapid clearance of *F. nucleatum* from tumors with mutated *TP53* and the requirement of wild-type p53 for successful infection could be operating in colorectal cancer tumors.

In colorectal cancer, *F. nucleatum* has been shown to activate WNT/ $\beta$ -catenin signaling, generate a proinflammatory microenvironment, and modulate the tumor microenvironment to evade the antitumoral immune response. Interaction of the FadA protein of *F. nucleatum* to E-cadherin mediates *F. nucleatum* attachment and invasion into the epithelial cells leading to the activation of  $\beta$ -catenin signaling, increased expression of transcription factors, oncogenes, WNT genes, and inflammatory genes, as well as growth stimulation of colorectal cancer cells (16). *F. nucleatum* has also been shown to generate a proinflammatory microenvironment by the expansion of myeloid-derived immune cells that is conducive for colorectal cancer neoplasia progression (17). Enrichment of the *F. nucleatum* in colonic adenomas indicates its involvement in the early development of colorectal cancer (16, 17). Interaction of the Fap2 protein of *F. nucleatum* with the TIGIT receptor on natural killer cells, T cells, and tumor-infiltrating lymphocytes inhibits antitumor immune activities of these cells (18). Collectively, modulation of the tumor microenvironment, oncogenic signaling in tumor cells, and immune evasion by *F. nucleatum* exacerbate tumor progression affecting survival.

Our findings advance our understanding of the potential contributions of the gut microbiome to colorectal cancer outcomes. Potential interventions that could stem from this work include the use of antibiotics, pre- and probiotics, vaccines, fecal transplants, and dietary/exercise interventions. Previous work in colorectal cancer patient-derived xenograft mice, has shown that treatment with a broad-spectrum antibiotic reduces *F. nucleatum* load, cancer cell proliferation, and overall tumor growth (13). These findings demonstrate the potential therapeutic value of antimicrobial interventions in patients with *F. nucleatum*-associated colorectal cancer. However, as the use of broad-spectrum antibiotics in patients with colorectal cancer may have negative consequences via impact on the commensal microbial community, further studies to identify a narrow-spectrum antibiotic targeting *F. nucleatum* are warranted. Other alternative approaches include the use of “predatory” bacteria such as *Bdellovibrio bacteriovorus* to combat *F. nucleatum* (46) or other oncogenic bacteria and dietary interventions (9, 47, 48) that alter the gut microbiome.

The enrichment of *F. nucleatum* in colorectal cancer, as well as its role in colorectal cancer tumorigenesis, have been described in several studies (7–13, 30). One primary strength of the current study is the use of NGS of archival FFPE tumor tissues, which enabled identification of *F. nucleatum* at the subspecies level. We adjusted for multiple comparisons, allowing for careful evaluation of findings from prior studies that lacked this more stringent approach. Another advantage of this study, which had robust findings across different propensity score-based methods, is the analysis of well characterized cohorts with long follow-up periods and data on colorectal cancer-specific survival, tumor characteristics, somatic mutations. Because *F. nucleatum* was found in only 10% of tumors, some analyses of subspecies and less frequently mutated genes were limited due to sample size, even in the context of this large study. Thus, additional tumor sequencing is required to further improve statistical power.

Our study produced important and significant results, including that right-sided tumors are more likely to contain *F. nucleatum*, and that presence of *F. nucleatum* is significantly associated with advanced-stage tumors, colorectal cancer-specific mortality, and specific tumor molecular features. *F. nucleatum* was also associated with increased colorectal cancer-specific mortality in chemotherapy-receiving colorectal cancer cases. Additionally, we found that *F. nucleatum* was more

prevalent in tumors with *KRAS* and *ERBB3* mutated genes and wild-type *TP53*. Although multiple mechanisms have been described for *F. nucleatum*'s role in colorectal cancer, its connection to *TP53* and *ERBB3* pathways remains unknown. Particularly intriguing is the novel finding pointing toward *F. nucleatum* subspecies *animalis* as the most likely pathogenic subspecies of *F. nucleatum*. In this regard, our results may shed new light on the role of *F. nucleatum* in colorectal cancer and aid in designing better approaches for the diagnosis and treatment of patients with colorectal cancer harboring *F. nucleatum* in their tumors.

## Authors' Disclosures

S. Bullman reports personal fees from GlaxoSmithKline and Biomx outside the submitted work; in addition, S. Bullman has a patent for 62/534,672 pending. M. Giannakis reports grants from Bristol-Myers Squibb, Merck, Servier and Janssen outside the submitted work. R. Nishihara is an employee and shareholder of Pfizer. N. Papadopoulos reports other support not relevant to this manuscript outside the submitted work; in addition, N. Papadopoulos is a cofounder of Thrive, ManaTbio, and Personal Genome Diagnostics; owns equity in Exact Sciences, ManaTbio, and Personal Genome Diagnostics; is a consultant to Thrive and NeoPhore; is an advisor to CAGE Pharma and Vidium; and holds equity in Cage. The companies named above, as well as other companies, have licensed previously described technologies related to the work described in this paper from Johns Hopkins University. N. Papadopoulos is an inventor on some of these technologies. Licenses to these technologies are or will be associated with equity or royalty payments to the inventors as well as to Johns Hopkins University. Additional patent applications on the work described in this paper are being filed by Johns Hopkins University. The terms of all these arrangements are being managed by Johns Hopkins University in accordance with its conflict of interest policies. S. Ogino reports grants from NIH during the conduct of the study. No disclosures were reported by the other authors.

## Authors' Contributions

**I. Borozan:** Conceptualization, data curation, formal analysis, investigation, methodology, writing—original draft, writing—review and editing. **S.H. Zaidi:** Conceptualization, data curation, formal analysis, validation, investigation, methodology, writing—original draft, project administration, writing—review and editing. **T.A. Harrison:** Resources, data curation, formal analysis, validation, investigation, methodology, writing—original draft, project administration, writing—review and editing. **A.I. Phipps:** Formal analysis, investigation, methodology, writing—original draft, writing—review and editing. **J. Zheng:** Formal analysis, investigation, methodology, writing—original draft. **S. Lee:** Validation, investigation, methodology. **Q.M. Trinh:** Data curation, investigation, methodology. **R.S. Steinfeldt:** Data curation, investigation. **J. Adams:** Data curation. **B.L. Banbury:** Formal analysis, investigation. **S.I. Berndt:** Investigation. **S. Brezina:** Investigation. **D.D. Buchanan:** Investigation. **S. Bullman:** Investigation. **Y. Cao:** Investigation, writing—review and editing. **A.B. Farris III:** Resources. **J.C. Figueiredo:** Investigation. **M. Giannakis:** Investigation, writing—review and editing. **L.E. Heisler:** Data curation. **J.L. Hopper:** Investigation, writing—review and editing. **Y. Lin:** Formal analysis, investigation, writing—original draft. **X. Luo:** Data curation. **R. Nishihara:** Investigation. **E.R. Mardis:** Supervision. **N. Papadopoulos:** Supervision. **C. Qu:** Data curation, formal analysis, investigation. **E.E.G. Reid:** Validation, methodology. **S.N. Thibodeau:** Resources, methodology. **S. Harlid:** Investigation. **C.Y. Um:** Resources, investigation. **L. Hsu:** Formal analysis, investigation. **A. Gsur:** Resources, investigation. **P.T. Campbell:** Resources, investigation. **S. Gallinger:** Resources, supervision, funding acquisition, investigation. **P.A. Newcomb:** Resources, investigation. **S. Ogino:** Conceptualization, investigation, writing—original draft, writing—review and editing. **W. Sun:** Data curation, investigation, methodology. **T.J. Hudson:** Conceptualization, resources, supervision, funding acquisition, investigation, methodology, writing—original draft. **V. Ferretti:** Resources, supervision, investigation, methodology. **U. Peters:** Conceptualization, resources, data curation, formal analysis, supervision, funding acquisition, validation, investigation, methodology, writing—original draft, project administration, writing—review and editing.

## Acknowledgments

GECCO is supported in part by NCI/NIH awards U01 CA137088 (NCI, U. Peters), U01 CA164930 (NCI, U. Peters), and R01 CA176272 (NCI, P.A. Newcomb and A.T. Chan). The CCFR is supported in part by NIH/NCI awards U01 CA167551 (NCI, M.A. Jenkins), U01 CA074794 (NCI, P.A. Newcomb), U24 CA074794 (NCI,

P.A. Newcomb), and R01 CA076366 (NCI, P.A. Newcomb). OFCCR is supported by U01 CA074783 (S. Gallinger), U24 CA074783 (S. Gallinger), and the Ontario Research Fund GL201-043 (B.W. Zanke). American Cancer Society funds the creation, maintenance, and update of the CPS-II cohort. CORSA: "Österreichische Nationalbank Jubiläumssfondsprojekt" 12511 (A. Gsur) and Austrian Research Funding Agency (FFG) grant 829675 (A. Gsur). S. Ogino was supported by NCI (R35 CA197735), a Nodal Award from Dana-Farber Harvard Cancer Center, and Cancer Research UK Grand Challenge Award (through the OPTIMISTIC Team). M. Giannakis was supported by the Cancer Research UK Grand Challenge Award and a Stand Up to Cancer Colorectal Cancer Dream Team Translational Research Grant (SU2C-AACR-DT22-17). D.D. Buchanan is supported by an NHMRC R.D. Wright Career Development Fellowship. Additional funding was provided by the Ontario Institute for Cancer Research. We thank all those who agreed to participate in the CORSA study, including the patients and the control persons, as well as all the physicians and students. The authors thank the CPS-II participants and Study

Management Group for their invaluable contributions to this research. The authors would also like to acknowledge the contribution to this study from central cancer registries supported through the Centers for Disease Control and Prevention National Program of Cancer Registries, and cancer registries supported by the National Cancer Institute Surveillance, Epidemiology and End Results program. The authors would like to thank all those at the GECCO Coordinating Center for helping bring together the data and people that made this project possible.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received April 11, 2021; revised June 27, 2021; accepted October 21, 2021; published first November 1, 2021.

## References

- Gwinn M, MacCannell D, Armstrong GL. Next-generation sequencing of infectious pathogens. *JAMA* 2019;321:893-4.
- Forbes JD, Knox NC, Ronholm J, Pagotto F, Reimer A. Metagenomics: The next culture-independent game changer. *Front Microbiol* 2017;8:1069.
- Parkin DM. The global health burden of infection-associated cancers in the year 2002. *Int J Cancer* 2006;118:3030-44.
- Plummer M, de Martel C, Vignat J, Ferlay J, Bray F, Franceschi S. Global burden of cancers attributable to infections in 2012: a synthetic analysis. *Lancet Glob Health* 2016;4:e609-16.
- Helmkink BA, Khan MAW, Hermann A, Gopalakrishnan V, Wargo JA. The microbiome, cancer, and cancer therapy. *Nat Med* 2019;25:377-88.
- Bouvard V, Baan R, Straif K, Grosse Y, Secretan B, El Ghissassi F., et al. A review of human carcinogens—Part B: biological agents. *Lancet Oncol* 2009;10:321-2.
- Mima K, Nishihara R, Qian ZR, Cao Y, Sukawa Y, Nowak JA, et al. Fusobacterium nucleatum in colorectal carcinoma tissue and patient prognosis. *Gut* 2016; 65:1973-80.
- Mima K, Cao Y, Chan AT, Qian ZR, Nowak JA, Masugi Y, et al. Fusobacterium nucleatum in colorectal carcinoma tissue according to tumor location. *Clin Transl Gastroenterol* 2016;7:e200.
- Mehta RS, Nishihara R, Cao Y, Song M, Mima K, Qian ZR, et al. Association of dietary patterns with risk of colorectal cancer subtypes classified by fusobacterium nucleatum in tumor tissue. *JAMA Oncol* 2017;3:921-7.
- Brennan CA, Garrett WS. Fusobacterium nucleatum - symbiont, opportunist and oncobacterium. *Nat Rev Microbiol* 2019;17:156-66.
- Castellarin M, Warren RL, Freeman JD, Dreolini L, Krzywinski M, Strauss J, et al. Fusobacterium nucleatum infection is prevalent in human colorectal carcinoma. *Genome Res* 2012;22:299-306.
- Kostic AD, Gevers D, Pedamallu CS, Michaud M, Duke F, Earl AM, et al. Genomic analysis identifies association of Fusobacterium with colorectal carcinoma. *Genome Res* 2012;22:292-8.
- Bullman S, Pedamallu CS, Sicinska E, Clancy TE, Zhang X, Cai D, et al. Analysis of Fusobacterium persistence and antibiotic response in colorectal cancer. *Science* 2017;358:1443-8.
- Repass J. Reproducibility Project: Cancer Biology. Replication Study: Fusobacterium nucleatum infection is prevalent in human colorectal carcinoma. *Elife*;7: e258012018.
- 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 Res* 2014;74:1311-8.
- Rubinistein 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: 195-206.
- Kostic AD, Chun E, Robertson L, Glickman JN, Gallini CA, Michaud M, et al. Fusobacterium nucleatum potentiates intestinal tumorigenesis and modulates the tumor-immune microenvironment. *Cell Host Microbe* 2013; 14:207-15.
- Gur C, Ibrahim Y, Isaacson B, Yamin R, Abed J, Gamliel M, et al. Binding of the Fap2 protein of Fusobacterium nucleatum to human inhibitory receptor TIGIT protects tumors from immune cell attack. *Immunity* 2015;42:344-55.
- Yu T, Guo F, Yu Y, Sun T, Ma D, Han J, et al. Fusobacterium nucleatum promotes chemoresistance to colorectal cancer by modulating autophagy. *Cell* 2017;170: 548-63.
- Chen Y, Lu Y, Ke Y, Li Y. Prognostic impact of the Fusobacterium nucleatum status in colorectal cancers. *Medicine (Baltimore)* 2019;98:e17221.
- Greathouse KL, White JR, Vargas AJ, Bliskovsky VV, Beck JA, von Muhlinen N, et al. Interaction between the microbiome and TP53 in human lung cancer. *Genome Biol* 2018;19:123.
- Zaidi SH, Harrison TA, Phipps AI, Steinfeld R, Trinh QM, Qu C, et al. Landscape of somatic single nucleotide variants and indels in colorectal cancer and impact on survival. *Nat Commun* 2020;11:3644.
- Langmead B, Salzberg SL. Fast gapped-read alignment with Bowtie 2. *Nat Methods* 2012;9:357-9.
- Hong C, Manimaran S, Shen Y, Perez-Rogers JF, Byrd AL, Castro-Nallar E, et al. PathoScope 2.0: a complete computational framework for strain identification in environmental or clinical sequencing samples. *Microbiome* 2014;2:33.
- Vogelstein B, Papadopoulos N, Velculescu VE, Zhou S, Diaz LA, Kinzler KW. Cancer genome landscapes. *Science* 2013;339:1546-58.
- Hino H, Shiomi A, Kusuhara M, Kagawa H, Yamakawa Y, Hatakeyama K, et al. Clinicopathological and mutational analyses of colorectal cancer with mutations in the POLE gene. *Cancer Med* 2019;8:4587-97.
- Mima K, Sukawa Y, Nishihara R, Qian ZR, Yamauchi M, Inamura K, et al. Fusobacterium nucleatum and T cells in colorectal carcinoma. *JAMA Oncol* 2015;1:653-61.
- Nosho K, Sukawa Y, Adachi Y, Ito M, Mitsuhashi K, Kurihara H, et al. Association of Fusobacterium nucleatum with immunity and molecular alterations in colorectal cancer. *World J Gastroenterol* 2016;22:557-66.
- Rogers MB, Brower-Sinning R, Firek B, Zhong D, Morowitz MJ. Acute appendicitis in children is associated with a local expansion of fusobacteria. *Clin Infect Dis* 2016;63:71-8.
- Lee D-W, Han S-W, Kang J-K, Bae JM, Kim H-P, Won J-K, et al. Association between fusobacterium nucleatum, pathway mutation, and patient prognosis in colorectal cancer. *Ann Surg Oncol* 2018;25:3389-95.
- Flanagan L, Schmid J, Ebert M, Soucek P, Kunicka T, Liska V, et al. Fusobacterium nucleatum associates with stages of colorectal neoplasia development, colorectal cancer and disease outcome. *Eur J Clin Microbiol Infect Dis* 2014; 33:1381-90.
- Yamaoka Y, Suehiro Y, Hashimoto S, Hoshida T, Fujimoto M, Watanabe M, et al. Fusobacterium nucleatum as a prognostic marker of colorectal cancer in a Japanese population. *J Gastroenterol* 2018;53:517-24.
- CGA Network. Comprehensive molecular characterization of human colon and rectal cancer. *Nature* 2012;487:330-7.
- Jaiswal BS, Kljavin NM, Stawiski EW, Chan E, Parikh C, Durinck S, et al. Oncogenic ERBB3 mutations in human cancers. *Cancer Cell* 2013;23:603-17.
- Loree JM, Bailey AM, Johnson AM, Yu Y, Wu W, Bristow CA, et al. Molecular landscape of ERBB2/ERBB3 mutated colorectal cancer. *J Natl Cancer Inst* 2018; 110:1409-17.
- Ross JS, Fakih M, Ali SM, Elvin JA, Schrock AB, Suh J, et al. Targeting HER2 in colorectal cancer: The landscape of amplification and short variant mutations in ERBB2 and ERBB3. *Cancer* 2018;124:1358-73.

37. Chakravarty D, Gao J, Phillips SM, Kundra R, Zhang H, Wang J, et al. Oncokb: a precision oncology knowledge base. *JCO Precis Oncol* 2017;2017:PO.17.00011.
38. Ho J, Moyes DL, Tavassoli M, Naglik JR. The role of erbb receptors in infection. *Trends Microbiol* 2017;25:942–52.
39. Biedermann A, Kriebel K, Kreikemeyer B, Lang H. Interactions of anaerobic bacteria with dental stem cells: an in vitro study. *PLoS One* 2014;9:e110616.
40. Kriebel K, Biedermann A, Kreikemeyer B, Lang H. Anaerobic co-culture of mesenchymal stem cells and anaerobic pathogens - a new in vitro model system. *PLoS One* 2013;8:e78226.
41. Xu M, Yamada M, Li M, Liu H, Chen SG, Han YW. FadA from *Fusobacterium nucleatum* utilizes both secreted and nonsecreted forms for functional oligomerization for attachment and invasion of host cells. *J Biol Chem* 2007;282:25000–9.
42. Madenspacher JH, Azzam KM, Gowdy KM, Malcolm KC, Nick JA, Dixon D, et al. p53 Integrates host defense and cell fate during bacterial pneumonia. *J Exp Med* 2013;210:891–904.
43. Zaika AI, Wei J, Noto JM, Peek RM. Microbial regulation of p53 tumor suppressor. *PLoS Pathog* 2015;11:e1005099.
44. Wei J, Nagy TA, Vilgelm A, Zaika E, Ogden SR, Romero-Gallo J, et al. Regulation of p53 tumor suppressor by *Helicobacter pylori* in gastric epithelial cells. *Gastroenterology* 2010;139:1333–43.
45. Siegl C, Rudel T. Modulation of p53 during bacterial infections. *Nat Rev Microbiol* 2015;13:741–8.
46. Negus D, Moore C, Baker M, Raghunathan D, Tyson J, Sockett RE. Predator versus pathogen: how does predatory *Bdellovibrio bacteriovorus* interface with the challenges of killing gram-negative pathogens in a host setting? *Annu Rev Microbiol* 2017;71:441–57.
47. Liu Y, Ajami NJ, El-Serag HB, Hair C, Graham DY, White DL, et al. Dietary quality and the colonic mucosa-associated gut microbiome in humans. *Am J Clin Nutr* 2019;110:701–12.
48. Huang P, Liu Y. A reasonable diet promotes balance of intestinal microbiota: prevention of precancer. *Biomed Res Int* 2019;2019:3405278.

# Cancer Epidemiology, Biomarkers & Prevention

**AACR** American Association  
for Cancer Research

## Molecular and Pathology Features of Colorectal Tumors and Patient Outcomes Are Associated with *Fusobacterium nucleatum* and Its Subspecies *animalis*

Ivan Borozan, Syed H. Zaidi, Tabitha A. Harrison, et al.

*Cancer Epidemiol Biomarkers Prev* Published OnlineFirst November 4, 2021.

<b>Updated version</b>	Access the most recent version of this article at: doi: <a href="https://doi.org/10.1158/1055-9965.EPI-21-0463">10.1158/1055-9965.EPI-21-0463</a>
<b>Supplementary Material</b>	Access the most recent supplemental material at: <a href="http://cebp.aacrjournals.org/content/suppl/2021/10/27/1055-9965.EPI-21-0463.DC1">http://cebp.aacrjournals.org/content/suppl/2021/10/27/1055-9965.EPI-21-0463.DC1</a>

<b>E-mail alerts</b>	<a href="#">Sign up to receive free email-alerts</a> related to this article or journal.
<b>Reprints and Subscriptions</b>	To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at <a href="mailto:pubs@aacr.org">pubs@aacr.org</a> .
<b>Permissions</b>	To request permission to re-use all or part of this article, use this link <a href="http://cebp.aacrjournals.org/content/early/2021/12/02/1055-9965.EPI-21-0463">http://cebp.aacrjournals.org/content/early/2021/12/02/1055-9965.EPI-21-0463</a> . Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.