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Fusobacterium nucleatum associates with stages of colorectal neoplasia development, colorectal cancer and disease outcome.

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Abstract:	Commensal bacteria in the colon may play a role in colorectal cancer (CRC) development. Recent studies from North America show Fusobacterium nucleatum (Fn) infection is over represented in disease tissue versus matched normal tissue in CRC patients. Using qPCR of DNA extracted from colorectal tissue biopsies and surgical resections of 3 European cohorts totalling 122 CRC patients we found overabundance of Fn in cancerous compared to matched normal tissue ($p<0.0001$). To determine whether Fn infection is an early event in CRC development, we assayed Fn in colorectal adenoma tissue (CRA) from 52 Irish patients. While for all CRAs Fn level was marginally significantly higher in disease versus normal tissue ($p=0.06$) it was significantly higher for high grade dysplasia ($p=0.0148$). As a secondary objective, we determined CRC patients with low Fn levels had a significantly longer overall survival time than patients with moderate and high levels of the bacterium ($p=0.0083$). Investigation of Fn as a potential non-invasive biomarker for CRC screening showed

	that while Fn was more abundant in stool samples from CRC patients compared to adenomas or controls, levels in stool did not correlate with cancer or adenoma tissue levels from the same individuals. This is the first study examining Fn in the colonic tissue and stool of European CRC and CRA patients, and suggests Fn as a novel risk factor for disease progression from adenoma to cancer, possibly affecting patient survival outcomes. Our results highlight the potential of Fn detection as a diagnostic and prognostic determinant in CRC patients.
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Title Page

Fusobacterium nucleatum associates with stages of colorectal neoplasia development, colorectal cancer and disease outcome

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Abstract

Commensal bacteria in the colon may play a role in colorectal cancer (CRC) development. Recent studies from North America show *Fusobacterium nucleatum (Fn)* infection is over represented in disease tissue versus matched normal tissue in CRC patients. Using qPCR of DNA extracted from colorectal tissue biopsies and surgical resections of 3 European cohorts totalling 122 CRC patients we found overabundance of *Fn* in cancerous compared to matched normal tissue ($p<0.0001$). To determine whether *Fn* infection is an early event in CRC development, we assayed *Fn* in colorectal adenoma tissue (CRA, #11) from 52 Irish patients. While for all CRAs *Fn* level was marginally significantly higher in disease versus normal tissue ($p=0.06$) it was significantly higher for high grade dysplasia ($p=0.0148$). As a secondary objective, we determined CRC patients with low *Fn* levels had a significantly longer overall survival time than patients with moderate and high levels of the bacterium ($p=0.0083$). Investigation of *Fn* as a potential non-invasive biomarker for CRC screening showed that while *Fn* was more abundant in stool samples from CRC patients compared to adenomas or controls, levels in stool did not correlate with cancer or adenoma tissue levels from the same individuals. This is the first study examining *Fn* in the colonic tissue and stool of European CRC and CRA patients, and suggests *Fn* as a novel risk factor for disease progression from adenoma to cancer, possibly affecting patient survival outcomes. Our results highlight the potential of *Fn* detection as a diagnostic and prognostic determinant in CRC patients.

Keywords: Colorectal cancer; neoplastic progression; bacterial infection.

Introduction

Colorectal cancer (CRC) is the third most commonly diagnosed cancer worldwide [1,2]. As most CRCs may develop slowly over years from precursor lesions, screening and early diagnosis are key to disease prevention [3].

The human intestine is estimated to be colonized by more than 1000 bacterial phylotypes, which are thought to play important roles in nutrition and immune status, as well as many disease processes [4-6]. An altered presence of specific micro-organisms has been associated with a number of diseases of the gastro intestinal tract (GIT), such as inflammatory bowel disease (IBD) [7], irritable bowel syndrome (IBS, #14) [8,9] and CRC [10-12]. While its role remains unclear, *Helicobacter pylori* has long been associated with CRC [13] and intraepithelial *Escherichia coli* was also found to be present in the colonic mucosa of tumour and normal tissue from CRC patients [14]. In recent years several groups have appreciably expanded the evidence linking infectious agents to colonic disease development [15]. These micro-organisms are thought to create a microenvironment more favourable to CRC development [16]. It now seems that not only can the bacterial composition of the gut assist initiation and progression of colorectal tumours, but also that tumours can affect the representation of bacteria in their vicinity [10,12,17].

Microbiome sequencing studies have proven very successful in uncovering novel candidate bacterial species in tumour and stool samples from CRC patients [10,18,19]. In particular, two North American studies in 2012 showed over-representation of *Fusobacterium nucleatum* (*Fn*) in CRC tumours versus surrounding normal tissue [20,21]. *Fn* is a highly invasive, gram-negative anaerobic bacterium and part of the oral and gut commensal flora [22] that has been linked to several diseases, such as periodontitis [23], appendicitis [24], Lemierre's disease [25], and inflammatory bowel disease [26]. *Fn* may contribute to CRC development by invading colonic mucosa and inducing local inflammation and increased expression of cytokines, leading to colorectal disease [20,26-29]. More convincing evidence that *Fn* infection directly contributes to colorectal carcinogenesis rather than being a consequence of disease progression derives from two recent reports showing that *Fn* invasion, through its unique FadA adhesion, recruits tumour-infiltrating immune cells and generates an oncogenic/pro-inflammatory microenvironment conducive for colorectal neoplasia [30,31].

The aims of our study were to evaluate the presence of *Fn* in tumour tissue from European CRC patients and to further assess bacterial presence in pre-cancerous tissues and stool samples in order to determine whether *Fn* load associates with the adenoma to cancer progression. We also assessed *Fn* as a prognostic biomarker by correlating survival outcomes in CRC patients with bacterial load.

Materials and Methods

Sample Cohorts

The subjects in this study were derived from three different patient cohorts in Czech Republic (CZ, #13), Germany (DE) and in Ireland (IE). The Czech cohort comprises tumour (n=49) and matched normal tissue samples (n=32) from CRC patients diagnosed and treated at the Departments of Surgery and Oncology, at the Teaching Hospital and Medical School in Pilsen, and Charles University in Prague, during the period 2008-2010. In the German CRC cohort (n=45), tumour tissue and matched normal tissue were collected from patients undergoing surgery at the University Hospital in Munich. In the Irish cohort, CRC (n=28) and adenoma (n=52) patient tissue along with the corresponding matched normal tissue were collected from the Departments of Gastroenterology and Surgery, The Adelaide and Meath Hospital in Dublin. Stool samples from CRC (n=7) patients, adenoma (n=24; HGD n=10, TVA n=12, TA n=2) patients, and normal controls (n=25) were also collected from the Departments of Gastroenterology and Surgery, The Adelaide and Meath Hospital in Dublin. The clinical characteristics of our study cohorts are summarised in Table 1.

Fresh tumour samples were obtained from patients during resection of the primary tumour or by biopsy prior to any treatment regime (all centres) and adenoma biopsies were obtained at colonoscopy during routine CRC screening, without previous CRC diagnosis (Dublin only). The tissue samples were macrodissected and either snap frozen in liquid nitrogen (Munich, Prague, Pilsen) or fixed in an RNA stabilization solution ('RNA later', Ambion, Dublin) for long term storage at –80°C. The control mucosa samples were taken from the macroscopically unaffected resection margins of colon tissues or at a distant site in the colorectal tract during colonoscopy. The resection margins were microscopically evaluated and only samples free of malignant cells were further analysed. Histology was verified by an experienced pathologist at each centre. Clinical follow up was obtained for the Czech CRC and Irish CRA cohort, from the hospital clinicians. We excluded patients with a history of a bowel disorder such as inflammatory bowel disease, those with polyposis syndromes, and Lynch syndrome.

Informed consent was obtained from all participating subjects for these studies in accord with the Helsinki declaration. All samples are coded to protect patient anonymity. The study was approved by the Ethical Committee of the St. James's Hospital and Federated Dublin Voluntary Hospitals Joint Research Ethics Committee (Ireland), the Human Subjects Committee of the Technische Universität Munich (Germany) and the Ethical Commission of the Medical Faculty and Teaching Hospital in Pilsen (Czech Republic).

DNA extraction

In the Irish cohort, 20-30 mg of tissue were lysed on ice in 400 µL of lysis buffer (50 mmol/L HEPES pH 7.5, 150 mmol/L NaCl, 5 mmol/L EDTA) and protease inhibitor (Calbiochem, Hampshire, UK) followed by sonication on ice for 3x30 seconds. Lysates were centrifuged at 10,000 *g* for 10 minutes at 4°C. gDNA was then extracted using the Norgen All-in-one kit (cat. No. 24200). DNA was quantified using a Nanodrop 2000c spectrophotometer (Thermo Scientific, Asheville, NC, USA).

In the German cohort, 20-30 mg of frozen tissue samples were lysed by digestion with proteinase K and lysis buffer and DNA was extracted from the lysate using the QIAmp DNA Mini Kit (Qiagen, Hilden, Germany)

following the manufacturer's instructions. After elution the DNA was quantified using a Nanodrop 1000 photospectrometer (Peqlab Biotechnologie GmbH, Erlangen, Germany).

Finally, for the Czech cohort, DNA was similarly extracted using the AllPrep DNA/RNA/Protein Mini Kit and by following the manufacturer's instructions. (Qiagen, Hilden, Germany). The DNA concentration was determined using Quant-iTT dsDNA BR Assay Kit (Life Technologies Czech Republic s.r.o., Prague, Czech Republic) and Infinite M200 fluorescence reader (Tecan Group Ltd., Mannedorf, Austria).

For stool samples, DNA was extracted from 220 mg sections taken from three different locations per whole stool specimen using the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Purified DNA was eluted in 200 mL of the supplied elution buffer and the DNA yield was quantified by ultraviolet spectrometry (260/280 nm). Triplicate aliquots of extracted stool DNA per patient sample were therefore available for downstream analysis.

Quantitative Real time PCR (qPCR)

qPCR to quantify levels of *Fn* in both disease and matched normal tissue from adenoma and CRC patients, as well as in stool samples, was performed on the Applied Biosystems (ABI, #31) 7500 Real Time PCR system. Levels of *Fn*, and that of the internal control prostaglandin transporter (*PGT*) gene, were measured simultaneously from the same gDNA preparation. Each 12 µL reaction consisted of 40 ng template DNA, 400 nM of each primer set, 400 nM of each probe, and 1 x final concentration of *TaqMan*™ universal mastermix (cat. No. 4304437). Reaction conditions were 2 minutes at 50°C, 10 minutes at 95°C, 60 cycles of 15 seconds at 95°C and 1 minute at 57°C. Cycle threshold (CT) was calculated using the SDS software on the ABI 7500 system. A qPCR assay for levels of the common *Bacteroides* genus was used as an internal control in the stool samples [32]. Samples that showed no amplification within 60 cycles were censored and assumed no template present or below detection limit. All samples and controls were run in duplicate. 0.01 ng of *Fn*, strain CC53, provided by Dr Robert Holt (Genome Sciences Centre, Vancouver, Canada) was used as a positive control.

Fn levels are given as relative quantification (RQ) and were determined by $2^{-\Delta CT}$, where ΔCT is the difference in threshold cycle number for the test and reference (*PGT* for tissue and *Bacteroides* for stool) gene assay. Fold increase of *Fn* quantification in disease tissue over matched normal colorectal tissue was calculated as $2^{-\Delta\Delta CT}$. Previously published primer and probes were used [20,32] with the following sequences:

Fusobacteria forward primer, 5' CAACCATTACTTTAACTCTACCATGTTCA 3'; Fusobacteria reverse primer, 5' GTTGACTTTACAGAAGGAGATTATGTAAAAATC 3'; Fusobacteria FAM probe, 5' TCAGCAACTTGTCCTTCTTGATCTTTAAATGAACC 3';

PGT forward primer, 5' ATCCCCAAAGCACCTGGTTT 3'; PGT reverse primer, 5' AGAGGCCAAGATAGTCCTGGTAA 3'; PGT FAM probe, 5' CCATCCATGTCCTCATCTC 3'.

Bacteroides forward primer 5' TTCAGGCTAGCGCCCATT 3'; Bacteroides reverse primer 5' GGAACTGAGACACGGTCCAAAC 3'; Bacteroides FAM probe 5'CCAATATTCCTCACTGCTGCCTCCCGTA 3'.

Statistical analysis

Fn levels determined by qPCR are given as $2^{-\Delta CT}$ where ΔCT is median of the difference in CT between test and reference gene. This relative quantification (RQ) was log-transformed to be analysed as $\log_2(1/2^{-\Delta CT})$. Wilcoxon signed rank test for paired analysis was used to compare levels of *Fn* in disease versus matched normal tissue. Mann-Whitney test was applied to compare two non-paired groups. Kruskal-Wallis test was used to compare median levels of *Fn* between more than two groups such as cohort origin or CRA subgroups. Odds ratios (OR) were assessed by binary logistic regression to assess influence of *Fn* levels on disease group. Further, associations between *Fn* levels and disease progression were analysed by Spearman correlation analysis.

The ratio of *Fn* levels between tumour and matched normal colorectal tissue is given as fold increase $2^{-\Delta\Delta CT}$ where ΔΔCT is median of the difference of $RQ_{Disease}$ and RQ_{Normal}. Log-rank test was used to investigate differences between survival distributions of subject groups. To stratify subjects by level of fold increase in *Fn*, we first grouped those subjects with undetected *Fn* in their tumour tissue. The remaining patients with detectable *Fn* in their tumour tissue were separated into tertiles of low, moderate and high fold increase. Cox proportional regression was applied to the three subject groups to determine hazard ratio (HR) of *Fn* on subject survival. Investigating if the trichotomised subject groups showed any further differences besides survival distributions, chi-square test was used to compare proportions in categorical variable such as chemotherapy or TNM staging.

All statistical analyses were performed in Matlab (MathWorks, Natick, MA, USA) apart from Cox regression which was calculated using SPSS (IBM, Armonk, NY, USA) and log-rank test which was assessed using R (R project, www.r-project.org). P values ≤ 0.05 were considered statistically significant.

Results

Fusobacterium is more abundant in colorectal cancer tumour versus normal tissue

We confirmed by qPCR that relative quantification of *Fn* is significantly raised in the diseased tissue compared to the matched normal tissue in all three European cancer cohorts (see Table 1; CZ cohort p=0.0016; DE cohort p=0.0001; IE cohort p=0.0063) (Fig. 1). Since levels of *Fn* are similar in all three cohorts (RQ_{Tumour} p=0.10; RQ_{Normal} p=0.35) and there is no significant difference in fold increase (CZ 266-fold, DE 43-fold, IE 9-fold, p=0.94), we also pooled the cohorts. Again, we found significantly higher levels in tumour tissue (pooled cohorts RQ_{Normal} 2^{-19} vs. RQ_{Tumour} 2^{-10}, p<0.0001). Average *Fn* levels are increased by over 45 fold in the pooled cohorts.

The details on cancer stage, location and mutation status are summarized in Table 1. We found no significant modification of *Fn* levels dependent on cancer location (colon vs. rectum), cancer pathology and clinical categories, or mutation status (*KRAS*, *BRAF* and *TP53*).

Fusobacterium levels increase with adenoma to cancer progression

We then investigated *Fn* infection in an Irish patient cohort with precancerous adenomas. Marginally significant *Fn* over-representation was observed in the adenoma group when diseases tissue was compared to matched normal tissue ($RQ_{Disease}$ 2^{-29} vs. RQ_{Normal} 2^{-30}, p=0.06). However, average *Fn* quantification in disease tissue were 2^{-10} and 2^{-29} (p=0.0015) in cancer and adenoma respectively, while the matched normal levels were 2^{-28} and 2^{-30} (p=0.0010) respectively. This increase in *Fn* load in the disease tissue from CRA to CRC gives a modest but significant association with cancer risk compared to adenomas ($OR_{Disease}$=1.05, p=0.0096).

This indication that *Fn* levels may increase during the transition from adenoma to cancer encouraged us to investigate levels of *Fn* within increasingly dysplastic adenoma stages. Adenoma samples were grouped into tubular adenoma (TA, n=9), tubulovillous adenoma (TVA, n=26) and high grade dysplasia (HGD, n=17). In tubular adenoma and in tubulovillous adenoma, we found levels of *Fn* to be similar in normal and disease tissue (TA $RQ_{Disease}$ 2^{-29} vs. RQ_{Normal} 2^{-30}, p=0.95; TVA $RQ_{Disease}$ 2^{-30} vs. RQ_{Normal} 2^{-29}, p=0.62). However, *Fn* levels in high grade dysplasia are increased in disease tissue (HGD $RQ_{Disease}$ 2^{-25} vs. RQ_{Normal} 2^{-31}, p=0.0148) (Fig. 2).

Fn levels are significantly different between the stages of neoplastic progression (CRC vs. HGD vs. TVA vs. TA; $RQ_{Disease}$ p=0.0094; RQ_{Normal} p=0.0039). Post-hoc tests showed levels are similarly high in cancer and high grade dysplasia tissue ($RQ_{Disease}$ p=0.12) while they are lower in adenomas when compared to cancers ($RQ_{Disease}$ TVA vs. CRC, p=0.0013; TA vs. CRC, p=0.0287). Strikingly, levels of *Fn* increase through adenomatous stage progression and from adenoma to cancer in both the disease and normal tissue ($RQ_{Disease}$ cor=0.37, p=0.0008; RQ_{Normal} cor= 0.24, p=0.0328).

Fusobacterium levels associate with patient survival

As our data indicated that *Fn* level in disease tissue was associated with disease progression, we next examined *Fn* load in relation to cancer patient outcome. Using 3-5 year patient follow up, which was available for 32 patients of the Czech cohort, we compared fold increase of *Fn* of tumour and matched normal colorectal tissue

with overall patient survival. We found a significant difference in survival distributions between patients without detected *Fn* in tumour tissue or low fold increase and those with high fold increase (fold increase < 2^5 vs. fold increase > 2^{16}, p=0.0083) (Fig. 3). Median survival of subjects with high *Fn* fold increase is 2 years, whereas all subjects with low tumour to normal ratio survive more than 3 years. The point estimate of the Hazard Ratio for a patient with high *Fn* fold increase is almost 20 times higher than for a patient with no or low fold increase (HR=19.96, 95% CI=1.42-281.42, p=0.0266). Besides difference in survival, there are no further significant differences between patients with no/low or high *Fn*, neither in chemo-/radiotherapy nor in TNM/Dukes staging.

Fusobacterium levels in stool samples associate with colorectal disease but do not reflect tissue levels

To test *Fn* as a non-invasive biomarker for CRC screening, we measured bacterial levels in DNA extracted from 56 patient stool samples from the Irish cohort CRC (n = 7), CRA (n = 24), and controls (n = 25). *Fn* levels in CRC patient stool samples were significantly higher than those from subjects with CRA (CRC RQ_{stool} 2^{-15} vs. CRA RQ_{stool} 2^{-21}, p=0.0316) and controls (CRC RQ_{stool} 2^{-15} vs. RQ_{stool} 2^{-21}, p=0.0201) (Fig. 4a). Levels in control subjects were equivalent to those from the CRA patients (p=0.94). *Bacteroides* level was used a reference bacterial assay for the stool sample qPCRs. Comparing *Fn* levels in stool samples with those from the matched tissue samples from the same CRC and CRA patients, where available, revealed no correlation between the sample types (CRC rho=0.36 p=0.11, CRA rho=0.24 p=0.42) (see Fig. 4b).

Discussion

The results of this study confirm for the first time in European cohorts previous reports from North America [20,21] that *Fn* is over-represented in tumour tissue compared to normal tissue in colorectal cancers. More strikingly this work indicates that *Fn* load increases with disease progression from adenoma to cancer and may also be related to survival from cancer.

The cohorts examined in this study included samples from three distinct European populations; one from Ireland, one from Germany, and one from the Czech Republic. In all populations *Fn* was over-represented in disease tissue compared to matched normal tissue.

Fn levels show a distinct increase through the major stages of colorectal neoplasia progression. There was a gradual increase in *Fn* level from TA to TVA through to HGD, which is associated with a high risk, unless treated, of progressing to cancer. These data suggest that *Fn* load is possibly associated with both adenoma pathology progression and the transition from adenoma to cancer. While preparing this manuscript, a similar pattern of *Fn* over abundance in adenomas was also reported by two studies from North America [29,30]**.** Kostic et al found *Fn* overabundance in both tissue and stool of CRA patients, when compared to matched normal tissue and healthy controls respectively [30]. McCoy and colleagues reported adenoma subjects had a significantly higher abundance of *Fn* compared to controls. However here they examined mucosa biopsies taken from the anal verge of the normal rectal mucosa of adenoma patients compared to normal controls rather than matched disease and control tissue [29].

Potentially, any impact of *Fn* infection on adenoma development and progression to more neoplastic lesions will be considerable as 95% of all cancers arise from adenomas but only a small number of adenomas become cancerous [33,34]. Unfortunately there are no reliable predictive markers of whether an adenoma will advance to cancer. Of potential relevance in this context, we also found *Fn* load in the stool of CRC patients to be significantly higher than that of adenoma patients ($p=0.0316$) and also healthy control patients ($p=0.0201$), although we had a small number of stool samples from CRC patients available for analysis. Interestingly, a recent Chinese study examining faecal microbial communities in healthy and CRC groups observed a marked increase in the relative abundance of *Fn* in the CRC cancer cohort compared with the healthy control group [35]. Similar trends were reported in a North American study examining *Fn* abundance in stool samples from healthy subjects, CRA subjects, and CRC subjects [30]. However, in our study, *Fn* levels from stool samples and the corresponding disease or matched normal tissue from the same CRC or CRA patient showed no correlation, which would limit the value of stool screening for *Fn* as an individualized CRC screening biomarker. Previously, bacterial communities in the colon have also been found to differ significantly to those in the faeces [36]. The potential of *Fn* as a non-invasive CRC screening biomarker needs to be tested in a large study.

The overabundance of *Fn* in disease vs. normal tissue in both cancers and adenomas prompted us to examine *Fn* levels in relation to patient outcome. We report for the first time a significant association between *Fn* level and patient outcome. Kaplan-Meier analysis indicated that patients with high levels of *Fn* had a significantly shorter survival time, when compared to patients with low levels of *Fn*. This suggests that *Fn* may have value as a

prognostic indicator and that efforts to eradicate *Fn* infection could be considered for CRC patients. However, our finding is based upon a modest number of patients with an average follow-up time of 3-5 years and thus we had limited power to test *Fn* level as an independent prognostic factor. The use of *Fn* as an independent prognostic biomarker is an intriguing and novel prospect that requires validation in a much larger patient group over a longer time-frame.

For those patients with follow-up information available there was no significant association of bacterial levels with adenoma recurrence. However, due to the limited follow up time for our adenoma cohort we could not more comprehensively access the correlation of *Fn* levels with either adenoma recurrence or with progression to cancer (there were no cancers diagnosed in these patients which was to be expected given the time frame and the fact that these adenomas were removed during colonoscopy in the period 2008-2010). A large follow-up study examining *Fn* levels with adenoma recurrence risk would be very desirable, especially for those patients with advanced adenomas, as this may provide a screening biomarker for selecting adenomas that are more likely to recur, with implications for increasing follow-up stringency and/or anti-microbial treatments for these individuals.

Chronic inflammation is an important factor contributing to carcinogenesis, so it is conceivable that the presence of *Fn* may represent an opportunistic passenger infection at an immune-compromised site, and as disease progresses more bacteria colonise the site. However, colonic mucosal inflammation linked to *Fn* invasion and increased expression of cytokines has been suggested as a possible mechanism leading to colorectal disease [29]. *Fn* effectively adheres to and invades epithelial cells eliciting an immune response [27,37] and strains isolated from inflamed human biopsy material from patients with IBD exhibit a more invasive phenotype [26], while a further, recent IBD related study found invasiveness of *Fn* correlated with increased mRNA expression of pro-inflammatory cytokine TNF-α and IL-1β [28]. The same strain of *Fn* assayed in our study has been isolated from CRC tumour tissue and shown to be invasive in a CaCo-2 cell assay [20]. McCoy and colleagues [29] showed a significant positive correlation between *Fn* abundance and TNF-α and IL-10 expression in rectal mucosa biopsies from adenoma patients compared to normal controls. During preparation of this manuscript, two new studies contributed the most convincing evidence so far that *Fn* infection directly contributes to colorectal carcinogenesis. Employing both in vitro and in vivo methods these studies highlighted *Fn* involvement in promotion of tumorigenesis [30,31]. Using HCT116 Xenograft mice, Rubinstein and colleagues demonstrated that *Fn* invasion of epithelial cells via FadA binding to E-Cadherin promotes inflammation and drives oncogenic signalling [31]. Kostic and co-workers studied *Fn* infection in the $Apc^{Min/+}$ mouse model and arrived at similar conclusions, implicating *Fn* in the generation of a proinflammatory microenvironment. *Fn* infection not only increased tumour burden in these mice but also increased infiltrating immune cells in the area [30]. Together, these data strongly support a role of *Fn*-induced mucosal inflammation in colorectal disease.

In summary the findings presented here highlight the emergence of *Fn* as a risk factor for disease progression from adenoma to cancer, and as a possible indicator of survival outcomes in CRC patients. Further investigation is needed to establish whether *Fn* is actively involved in driving the adenoma to cancer transition in humans or is merely an opportunistic passenger, and how *Fn* infection may affect survival from disease.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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Table 1: Overview of clinical data of European cohorts (tissue samples).

NA not applicable or missing; SD standard deviation; CZ Czech; DE Germany; IE Ireland; CRC colorectal cancer; CRA colorectal adenoma; RQ relative quantification

Cohort	CZ	DE	IE	IE
Diagnosis	CRC	CRC	CRC	CRA
Total n of tissue samples	49	45	28	52
Gender n [male/female]	35/14	24/21	16/12	33/19
Age mean±SD [years]	70±10	67±11	61±11	63±8
Location n [colon/rectum]	NA	27/18	21/7	30/22
T staging n [T0/T1/T2/T3/T4]	0/0/1/42/6	1/1/10/26	2/7/2/13/4	NA
N staging n [Tx/N0/N1/N2/N3]	0/47/2/0/0	1/19/15/10/0	2/19/4/3/0	NA
M staging n [Mx/M0/M1]	0/49/0	43/0/2	25/0/3	NA
Dukes staging n [A/B/C/D]	0/47/2/0	NA	9/8/7/4	NA
Grade n [1/2/3]	6/36/6	1/28/13	NA	NA
KRAS n [wildtype/mutation]	3/2	37/7	15/12	29/22
BRAF n [wildtype/mutation]	4/0	39/5	24/2	50/1
TP53 n [wildtype/mutation]	NA	27/17	11/2	15/0
Fusobacterium in disease tissue median (inter quartile range) RQ	2^{-13} (2^{-4} to 2^{-21})	2^{-6} (2^{-2} to 2^{-15})	2^{-10} (2^{-4} to 2^{-27})	2^{-29} (2^{-12} to 2^{-31})
Fusobacterium in normal tissue median (inter quartile range) RQ	2^{-21} (2^{-16} to 2^{-25})	2^{-14} (2^{-7} to 2^{-29})	2^{-28} (2^{-9} to 2^{-29})	2^{-30} (2^{-27} to 2^{-31})

Figure legends

Fig. 1 *Fusobacterium* in diseased and matched normal colorectal tissue in European CRC cohorts

Boxplots show median and inter quartile range of relative *Fn* quantification. Relative quantification is significantly higher in colorectal tumour tissue than in matched normal colorectal epithelium tissue, in all CRC cohorts (CZ p=0.0016; DE p=0.0001; IE p=0.0063). All cohorts show similar quantification levels, there is neither significant difference in tumour tissue (p=0.10) nor in normal tissue (p=0.35). (See supplemental figure 1 for visualisation of raw data points for each matched tissue sample).

Fn Fusobacterium nucleatum; CRC colorectal cancer; N normal colorectal epithelium tissue; T colorectal tumour tissue; D diseased adenoma tissue; CZ Czech; DE Germany; IE Ireland; n.s. not significant; * significant

Fig. 2 *Fusobacterium* in diseased and matched normal colorectal tissue of CRC and CRA subjects

Boxplots show median and inter quartile range of relative *Fn* quantification. Relative quantification differs significantly between diseased tissue of CRC and CRA (p=0.0094). Post-hoc tests show significant difference between quantification in tumour tissue and tissue of less advanced forms of adenoma (CRC vs. TA p=0.0287; CRC vs. TVA p=0.0013) while more severe adenomas show similar *Fn* levels as tumour tissue (CRC vs. HGD p=0.12). Also, as in CRC, HGD shows elevated *Fn* quantification in disease tissue compared to matched normal colorectal epithelium tissue (p=0.0148), whereas there is no significant difference between tissues in lower adenomas (TVA p=0.62; TA p=0.95). (See supplemental figure 2 for visualisation of raw data points for each matched tissue sample).

Fn Fusobacterium nucleatum; CRC colorectal cancer; CRA colorectal adenoma; HGD high grade dysplasia; TVA tubulovillous adenoma; TA tubular adenoma; N normal colorectal epithelium tissue; T colorectal tumour tissue; D diseased adenoma tissue; IE Ireland; n.s. not significant; * significant

Fig. 3 Overall survival of colorectal cancer subjects associates with increase in *Fusobacterium* quantification

Kaplan-Meier curves showing overall survival of colorectal cancer (CRC) subjects trichotomised into groups of 'no/low' (blue dotted), 'moderate' (black dashed) and 'high' (red solid) fold increase of *Fn* quantification in tumour tissue over matched normal epithelial tissue. Subjects with fold increase in *Fn* $> 2^{16}$ show poor prognosis with median survival of 2 years. When no *Fn* was detected in tumour tissue, or fold increase was $< 2^{5}$, then survival at 2 years was still 100%. Survival distributions differ significantly between groups of 'no/low' and 'high' *Fn* fold increase (p=0.0083).

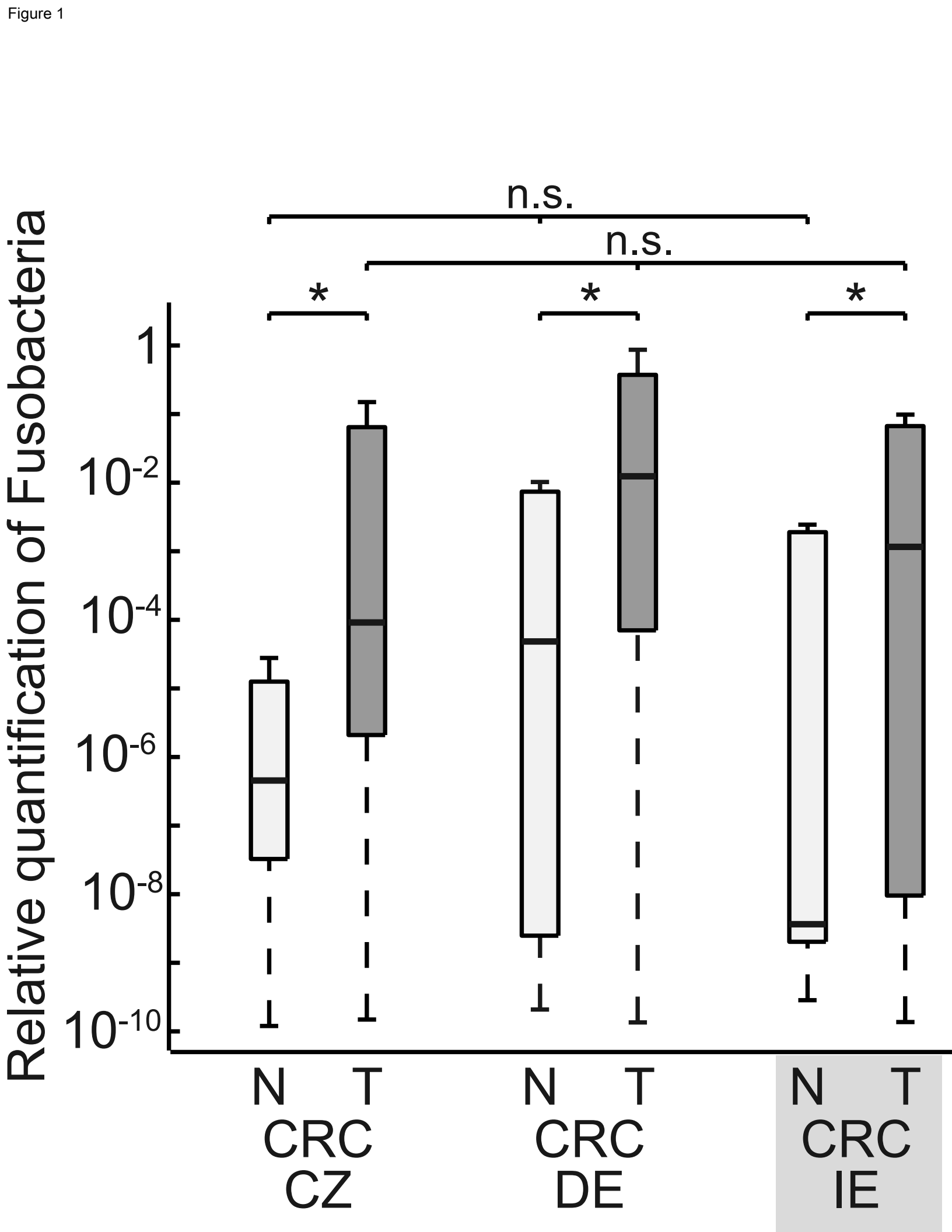
Fig. 4 *Fusobacterium* in stool and matched tissue samples of CRC, CRA and healthy subjects

a) Boxplots show median and inter quartile range of relative *Fn* quantification in stool samples of CRC, CRA and healthy control subjects. Levels are higher in CRC than in CRA (p=0.0316) and controls (p=0.0201), while CRA has similar *Fn* levels as healthy controls (p=0.94).

b) Relative quantifications of *Fn* in stool and matched disease tissue samples show no pattern of significant or high correlation (CRC rho=0.36 p=0.11, CRA rho=0.24 p=0.42, HGD rho=0.32 p=0.48, TVA rho=0.24 p=0.70).

Fn Fusobacterium nucleatum; CRC colorectal cancer; CRA colorectal adenoma; HGD high grade dysplasia; TVA tubulovillous adenoma; TA tubular adenoma; RQ relative quantification; n.s. not significant; * significant

Figure 1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Relative quantification of Fusobacteria
1
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10-4
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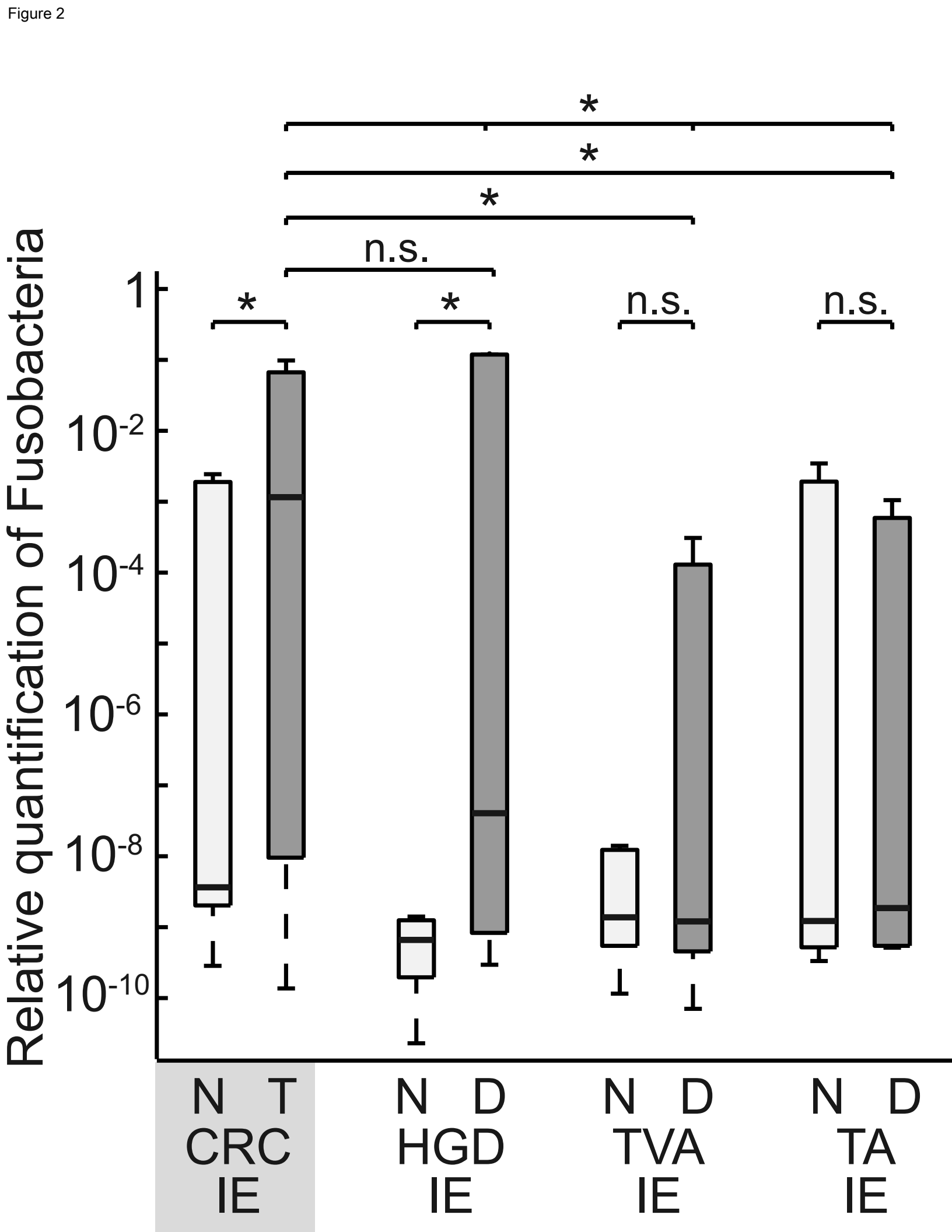
Figure 2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1
10-2
10-4
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Relative quantification of Fusobacteria
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CRC
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N D
TVA
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TA
IE

Figure 3

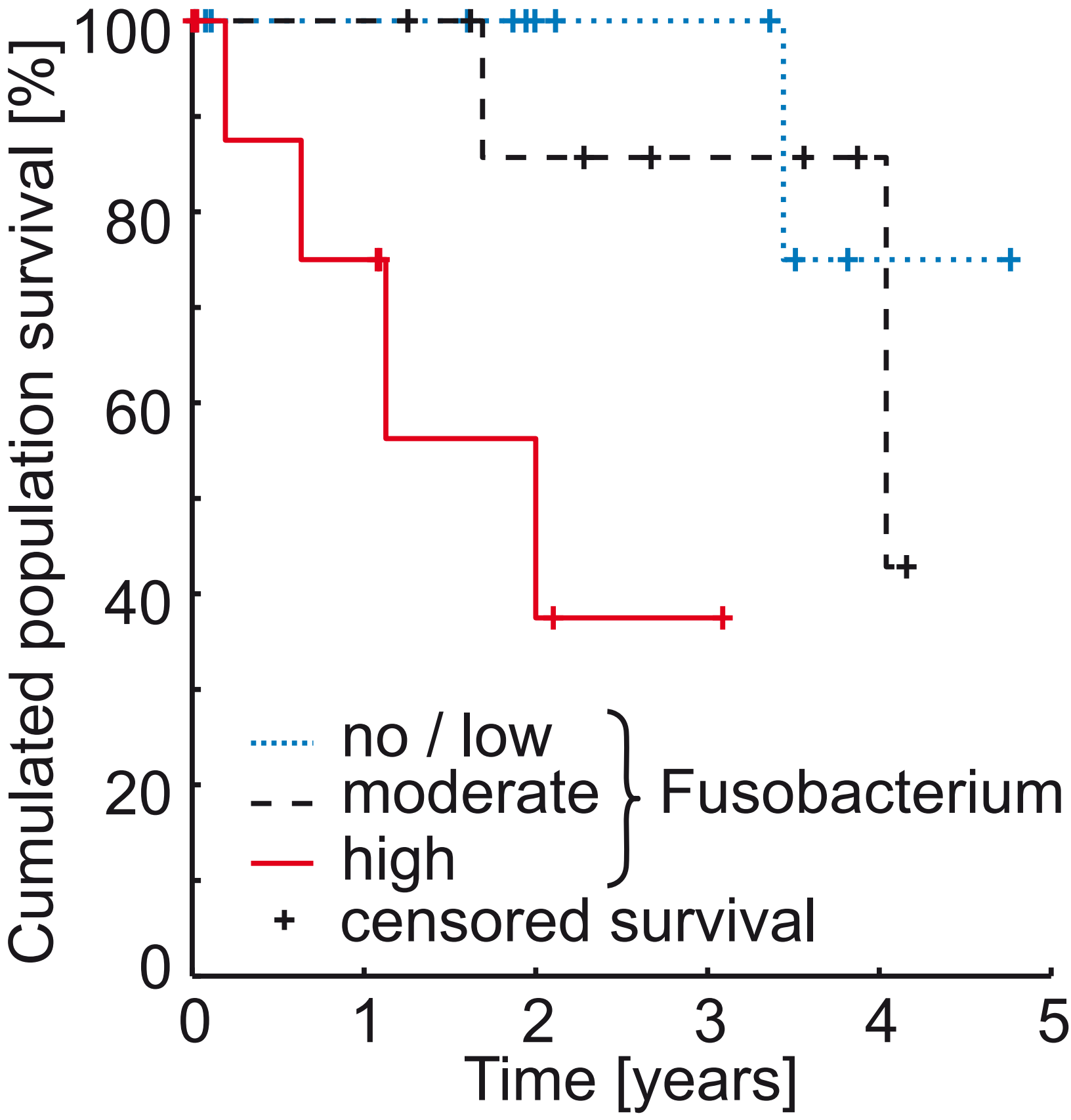
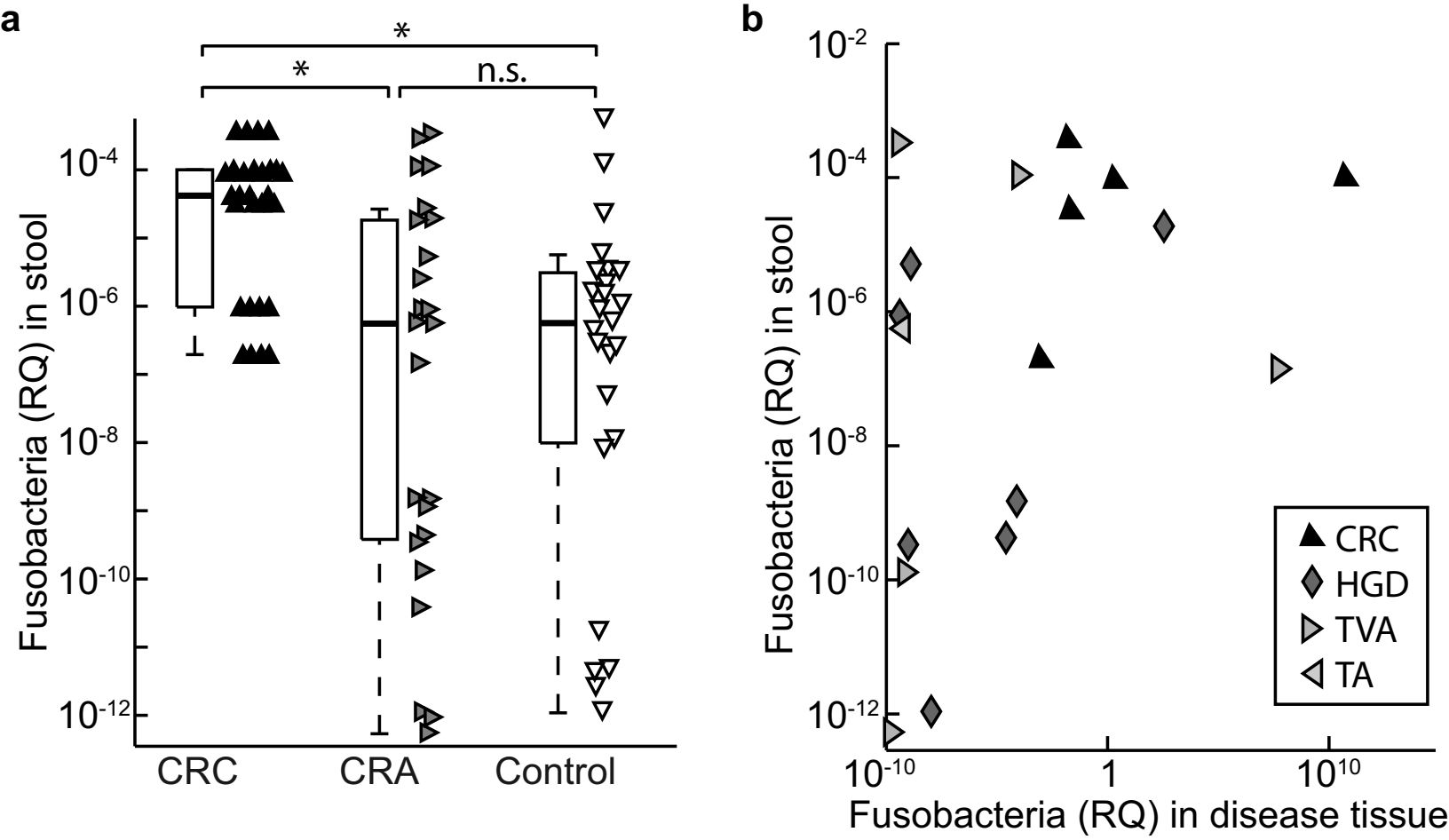

Cumulated population survival [%]
100
80
60
40
20
0
0
1
2
3
4
5
Time [years]
no / low
moderate
high
Fusobacterium
censored survival

Figure 4


a
*
*
n.s.
Fusobacteria (RQ) in stool
10-4
10-6
10-8
10-10
10-12
CRC
CRA
Control
b
Fusobacteria (RQ) in stool
10-2
10-4
10-6
10-8
10-10
10-12
10-10
1
1010
Fusobacteria (RQ) in disease tissue
CRC
HGD
TVA
TA

Electronic Supplementary Material

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