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Partial Associations of Dietary Iron, Smoking and Intestinal Bacteria with Colorectal Cancer Risk

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Abstract

Smoking and high red meat intake have been associated with colorectal cancer (CRC) risk. Increased iron exposure may be a common factor, favoring the colonization of certain bacterial pathogens that preferentially grow in an iron-rich luminal environment. We analyzed the data from a population-based case-control study of CRC and measured antibody levels against flagelin of *Salmonella* (FliC), one of the irontrophic bacteria, in two independent blood collections. The risk of CRC synergistically increased by combined exposures to heme iron intake and pack-years (PY) of cigarette smoking (P-value for the interaction = 0.039 on the continuous scale). There was a marginally significant interaction between heme iron intake and PY in increasing FliC antibody in the US control subjects (P=0.055), although no iron or smoking data were available for Dutch samples. Furthermore, FliC antibody levels were significantly higher in patients with colorectal polyps and cancer than in controls in both Dutch (3.93 vs. 2.23) (P=0.014) and US samples (6.65 vs. 4.37) (P<0.001). Potential roles of iron from cigarette smoking and dietary heme in CRC through altering irontrophic luminal bacterial population may warrant further investigation.

Keywords

smoking; iron; intestinal bacteria; colorectal cancer; Salmonella

Introduction

The International Agency for Research on Cancer recently concluded that there is now sufficient evidence that tobacco smoking causes cancer of the colorectum (1). The mechanistic basis through which cigarette smoking specifically increases the risk of colorectal cancer (CRC) is, however, poorly understood. Tobacco contains not only a variety of chemical carcinogens (1,2), but also a prominent amount of iron as a component of mainstream cigarette smoke (3). Exposure to tobacco smoke, in fact, has been shown to increase systemic iron load (4). Thus, iron from cigarette smoke may contribute to intracolonic iron by swallowing tobacco smoke as well as through increased mucosal iron levels through systemic circulation. The other major source of luminal iron in humans is

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acquired via diet, i.e., red meat, fortified cereals, and supplements. Importantly, most dietary iron is not absorbed and is concentrated in feces at a level calculated to be 10-fold higher than in most tissues, making luminal iron exposures potentially important (5).

While intraluminal iron promotes the generation of hydroxy radicals with interaction with intestinal bacteria (5), increased luminal iron content may also change intestinal microbial composition. Iron fortification indeed led to a more pathogenic bacterial gut profile in African children (6) and an increased number of Enterobacteriaceae was associated with high ferritin and transferrin saturation in pregnant women in Spain (7). Depletion of luminal iron in rodents induced significant compositional alterations in colonic microbiota and prevented the development of chronic inflammation (8). Furthermore, smoking has been associated with an increase in irontrophic bacteria (9,10). Microbiologically, high iron acquisition capacity is a well known virulence determinant for many bacteria, including *Enterobacteriaceae* (11). Some of these irontrophic bacteria, such as *Salmonella*, produce bacterial toxins that exert genotoxicity and have been linked to biliary tract cancer (12). In addition, *Salmonella* are known to use multiple high-affinity iron acquisition systems (13,14).

Here we evaluated the effect of potential interaction between dietary iron and cigarette smoking on CRC risk in a population-based case-control study in the US. We also examined the association between CRC risk and a serological marker of irontrophic bacteria (anti-*Salmonella* Flagellin antibody) in a subset of the population-based study and in an independent study from the Netherlands.

Materials and methods

Study design

This study was designed as secondary analyses of blood samples and epidemiologic data collected for the published studies described elsewhere (15–18). The associations of CRC with smoking and dietary iron were assessed using the data from a population-based case-control study in Metropolitan Detroit USA (15,16). The bacterial serology study was a joint project between Radboud University Nijmegen Medical Centre (RUNMC), the Netherlands, and Wayne State University (WSU), USA, using collection of deidentified samples at RUNMC and a subset of the Detroit study participants (17,18). The study was approved by the Medical Ethical Committee of Nijmegen/Arnhem (#2006/078) and the WSU Human Investigation Committee (#0409000504).

Detroit case-control study

In brief, eligible study subjects were residents in the Metropolitan Detroit Tri-County (Wayne, Oakland and Macomb) area, between 45 and 80 years of age at time of ascertainment, with a working telephone and no prior history of any invasive cancer, in-situ CRC or colectomy. Eligible CRC cases were histologically diagnosed between January 1, 2003 and September 30, 2005, and were identified through the Metropolitan Detroit Cancer Surveillance System. Frequency-matched population controls were selected through random digit dialing. A total of 1,335 cases (41.7%) and 1,682 controls (59.4%) consented to the study, and 1,205 cases and 1,547 controls of these remained eligible after completion of the study. The cases and controls were well balanced concerning the matching variables, age, race and county of residence, but gender-matching was incomplete (50% and 57% females in the cases and controls, respectively). The subjects were interviewed over the telephone using structured questionnaires regarding their usual diet and other risk factors for CRC for the time-period preceding cancer diagnosis (approximately 2 years prior to the interview). A validated semi-quantitative food frequency questionnaire (FFQ), Block 98.2 (Block Dietary

Data Systems, Berkeley, CA), was used to estimate daily nutrient (including individual fatty acid groups) intake. Energy-adjusted nutrient intake was calculated by means of the residual method described by Willett and Stampfer (19). Total iron and other vitamin/mineral intake was computed as the sum of energy-adjusted dietary intake and intake from supplements.

Blood samples

Blood samples were derived from the same pool of the samples used previously (18) that comprised a subset of the Detroit case-control study samples and a subset of archived serum samples at Department of Laboratory Medicine Radboud University Nijmegen Medical Centre (Nijmegen, The Netherlands) and included controls, colorectal polyps (any type), and local stage (I and II) of CRC but excluded stage III and IV cases. Selection procedures of these patients were also described previously (17). The Detroit samples consisted of 33 CRC cases, 11 controls with colorectal polyps and 47 controls without history of colorectal polyps and cases and controls were matched for age and gender. The Nijmegen samples consisted of 37 CRC and 12 polyp patients who had been admitted to the Radboud University Nijmegen Medical Centre and 27 healthy blood donors (>50 years of age). Serum and plasma sampleswere stored at −80 °C until use.

Salmonella anti-Flagellin (FliC) IgG Enzyme-linked immunosorbent assay (ELISA) measurements

The FliC ELISA assay was developed and performed at Department of Laboratory Medicine as described (17,18). In short, ELISA plates were coated with FliC (InvivoGen) for at least 18 hours at 4°C, after which the wells were extensively blocked by 1% bovine serum albumin (BSA) *(blank)* in PBS-Tween20 (0.1%) for 2 hours at 37°C. For each antigen-coated well, a second well on the same plate was incubated in coating buffer *without* FliC and subsequently blocked with 1% BSA (*blank*). Next, serum or plasma samples (0.1% dilution in PBS-0.1%Tween20/1%BSA) were added to the wells and incubated overnight at 4°C. After washing, incubation was prolonged for 90 minutes with horseradish peroxidase (HRP)-labeled goat antihuman IgG (1:25.000; Jackson Immunoresearch) at room temperature. The optical density of HRP-converted 3,3′,5,5′-tetramethylbenzidine (TMB)-substrate was quantified at a wavelength of 450 nm in a spectrophotometer. Samples were measured in duplicate (both for FliC and blank) and titers of a specific sample were calculated as the mean $OD450_{FliC} - OD450_{blank}$ and expressed as arbitrary *Salmonella typhimurium* units (STU) based on a reference sample from a *S. typhimurium*-infected patient that was measured in every plate. Titers were set to zero in case of a negative outcome of the calculation.

Statistical analysis

Odds ratios (ORs) and 95% confidence intervals (CIs) for CRC associated with cigarettes smoking and iron intake were estimated using an unconditional logistic regression model (20), adjusting for selected covariates as described below. Because of the unbalanced gender matching, we first calculated bi-variable (gender-adjusted) ORs, instead of univariable ORs. Then, additional covariates were selected from established risk factors for CRC in the literature (21,22). They were tested one at a time in a model that included basic demographic variables, age, gender and educational levels, and those showing an association at the 10% level were included to estimate multivariable ORs. As a result, the final multivariable model included age, gender, educational level, total energy, calcium, fiber intake, family history of CRC, regular (>= 3 times per week for 6 months or longer) non-steroidal anti-inflammatory drug (NSAID) use, body mass index (body weight (kg)/body height $(m)^2$), and physical activity index in their 30s, which was the weighted sum of time the subject spent per 24 hours as described previously (23). Major dietary sources of iron, such as red meat, were not included in the model simultaneously because their inclusions (i.e., controlling their intake

levels) would alter the interpretation of regression coefficients for iron intake. After exclusion of subjects with missing covariates and exposure variables, the final analytical samples in this study consisted of 1136 cases and 1470 controls.

The ever smokers were defined as those who smoked at least one cigarette per day for 6 months or longer and further divided into current and former smokers at time of the survey. We also calculated pack-years of cigarette smoking based on total number of years of smoking and average number of cigarettes smoked per day, which was grouped into 0, 0.01 to <20, 20 to <40, and 40+. Iron intake was grouped into quintile levels based on distributions of the cases and controls combined and the lowest quintile was used as the reference category to calculate ORs. Tests for linear trend in the logit of risk associated with ordinal dietary intake were performed using median intake of each level as well as using continuous values. The interactions between pack-years of cigarette smoking and dietary iron intake were tested by the inclusion of multiplicative interaction terms between these variables.

For the serology study, the ORs and 95% CI for CRC, polyps and both combined were also estimated by unconditional logistic models, with above median FliC ELISA titers in each US and Dutch population as the cut point for exposure. In the 45 control subjects with no polyps in the Detroit population for whom smoking and dietary information was available, FliC ELISA titers were summarized according to pack-years of smoking, quartiles (instead of quintile due to the small sample size) of dietary iron intake and their combinations, and the association of FliC titers with pack-years of smoking, dietary iron and their multiplicative interaction term were analyzed by a linear regression model. The Dutch study was not included in this analysis as it did not collect epidemiology data. All statistical analyses were performed using SAS version 9.

Results

In bivariate analyses of the case-control data, smoking status was not associated with risk of CRC, although there was a slight insignificant increase in ever smokers (OR=1.10, 95% CI 0.93–1.38). When the ORs were estimated according to levels of pack-years (PY), there was a significant linear trend in CRC risk with increasing PY. The OR associated with 40 PY or greater was 1.29 (95% CI 1.02–1.62). Inclusion of other covariates known to be associated with CRC in the model significantly weakened these associations, making them no longer statistically significant (Table 1).

Among dietary sources of iron, only heme iron intake was significantly positively associated with the risk of CRC in bivariate model using non-energy-adjusted intake (P for trend 0.01, OR for the top vs. bottom quintile was 1.47, 95%Ci 1.14–1.89). Again, when adjusting for other covariates, these associations became virtually null for all types of iron intake examined (Table 2).

Table 3 presents the OR and 95% CI for CRC according to combinations of heme iron intake and PY of smoking in multivariable models. An increasing trend in risk of CRC with heme iron intake was only seen in smokers with 40 or more PY, and it was only significant with the continuous interaction term. Likewise, an increasing trend in CRC risk with PY was only seen in individuals within the highest quintile of dietary heme iron, and the linear trend was only significant for the interaction terms when using median values of each level (0, 4, 15, 24,37 and 70 for PY, and 2.31, 3.30, 3.90, 4.61 and 5.97 for heme iron). Overall, the interaction between PY and dietary heme on CRC risk was significant on a continuous scale (P=0.039). There were no significant interactions between smoking and total dietary iron or total iron from both foods and supplements (data not shown).

In the Detroit control group, *Salmonella* flagellin (FliC) antibody levels tended to be higher in the subjects with 20 or more PY and with heme intake higher than 3.9 g per day. Although neither association itself was statistically significant, the interaction between these two was marginally statistically significant in a linear regression model (P=0.055) (Figure 1). Smoking and dietary data were not available for the Nijmegen samples. However, the mean FliC titer in the Dutch control group was significantly lower than that in the US controls (2.23 vs. 4.37), and in the corresponding cases (3.93 vs. 6.65). The fact that per capita consumptions of both cigarettes and meat were lower in the Netherland than in the US may partly account for these differences (24, 25).

In both the Dutch and American samples, *Salmonella* FliC antibody levels were significantly higher in CRC cases and in all cases combined (CRC + polyps) compared with controls without polyps. The ORs for all cases were approximately 3.5 and 2.0 for Detroit and Nijmegen populations, respectively (Figure 2). Age and gender were broadly balanced between these cases and controls, and they were not significantly associated with the antibody titers in either population, except a positive association with age in the Detroit cases only (not in the controls).

Discussion

Multiple factors drive the progression from healthy mucosa to colorectal carcinoma, and accumulating evidence, including ours (17,18), points to a potential role of intestinal bacteria in disease initiation and progression. A number of environmental and host factors have been suggested to influence gut bacterial populations: diet (26–29) and smoking (9) are among those with growing research interest. Our study is the first to show the interaction of these elements on the risk of CRC. Despite the small sample size, the present study was able to detect significant differences in *Salmonella* FliC antibody titers between the cases and controls in two independent populations, and suggested those subjects with higher titers may have a 2–3 fold increased risk of having a colorectal tumor. The etiological role of *Salmonella* or other irontrophic bacteria, however, remains to be determined.

Previous studies of the intestinal microbiome showed that i) *Salmonella*, *Citrobacter* and *Cronobacter* were among the low abundant intestinal species or were even completely absent in healthy individuals (30,31), while these were consistently detected in non-malignant colonic mucosa samples from CRC patients (32); ii) Shigella spp displayed an increased abundance in the intrinsic (non-malignant) microbiome of adenoma patients (33), iii) *Citrobacter* species have the potential to initiate tumors in an animal model for CRC (34). On the contrary, Marchesi et al found the decreased presence of members of the *Enterobacteriaceae*, such as *Salmonella*, *Citrobacter*, *Shigella*, and *Cronobacter spp*. in tumor tissue of the CRC patients (35). This leads to the hypothesis that these bacteria are part of the intrinsic microbiome that increases the susceptibility to CRC, but they can be outgrown by other commensal-like bacteria upon disease progression.

The results of this study suggest the importance of heme iron from animal meat, and not total iron intake, in CRC risk. Independent of iron, heme is known to exert cytotoxic effects via catalysis of lipid peroxidation, impairing organization of lipid bilayers and organelles, destabilizing the cellular cytoskeleton and promoting damage to cellular macromolecules (36–38). In experimental animals, increased colonic cell proliferation (39) and increased cytotoxicity of fecal water have been demonstrated with a heme-containing diet compared with control diets (40). These properties of heme may increase CRC risk directly in conjunction with cigarette smoking, as observed in the joint analysis of the present study.

Although our results were not necessarily unequivocal, they allowed us to formulate a hypothesis that irontrophic bacteria cultivated by higher iron load in the host through smoking and diet may increase the risk of CRC. This interaction was significant only on the continuous scale, suggesting that the interaction at a high range of exposures is important in defining the relationship. Increased heme iron intake could increase the risk of CRC via changes in the intestinal microbiota. The ability to use heme as a source of iron is a characteristic of growing number of bacterial pathogens (41,42). A recent study suggests that intestinal bacterial activity is necessary for dietary heme to induce hyperproliferation of colorectal epithelial cells (43). An increase in dietary heme intake can also lead to an increase in non-heme iron in the intestinal lumen. A heme-containing diet increased fecal ferritin excretion significantly in comparison with a non-heme diet with the same iron content (43). *Salmonella* can sequester ferritin-iron through siderophore-based iron uptake systems (45) and thus gain iron from such increased luminal ferritin concentrations originating from exfoliated mucosal cells with high ferritin content.

An interesting feature of the present study is that we used an immunological biomarker of *Salmonella* colonization, and not an assay of bacterial levels. This raises concerns over specificity of our findings to risk of CRC, but *Salmonella* colonization at other anatomic sites is rare (46). The presence of antibodies also does not ensure that the bacteria were present at the time of blood sampling and may merely indicate a past infection/colonization. On the other hand, use of a biomarker is a considerable strength in that it indicates not only intestinal colonization but also intestinal translocation to result in a systemic response.

Another concern is whether the presence of tumors increases the permeability of the intestinal tract. In our previous study, we measured the humoral immune response to endotoxin, an intrinsic component of the cell wall from the majority of Gram-negative intestinal bacteria (17). The relative endotoxin antibody expression in patients with stage I/II tumors was markedly lower than in polyp patients. This suggests that increased anti-FliC levels in early CRC patients likely cannot be fully attributed to a general loss of intestinal barrier function.

We also acknowledge limitations inherent to the case-control study design and in use of FFQs for dietary assessment (15,16) (47). The observed differences in this study may have been further weakened by the fact that 20% of the population over the age of 50 is estimated to carry asymptomatic adenomatous polyps (48). Larger studies are therefore warranted as well as analysis of prospectively collected blood samples.

Nevertheless, the results here shed light on a new group of intestinal bacteria that deserve further investigation for their potential role in CRC initiation and progression. Furthermore, the results of this study suggest a possibility that the composition of the bacterial population may be modifiable through changes in individuals' behaviors, i.e., smoking and diet, which presents an ample opportunity for primary prevention of CRC. In addition, detection of such bacteria in patients with colorectal polyps may help identify patients who are likely to at higher risk for CRC and thus need close surveillance.

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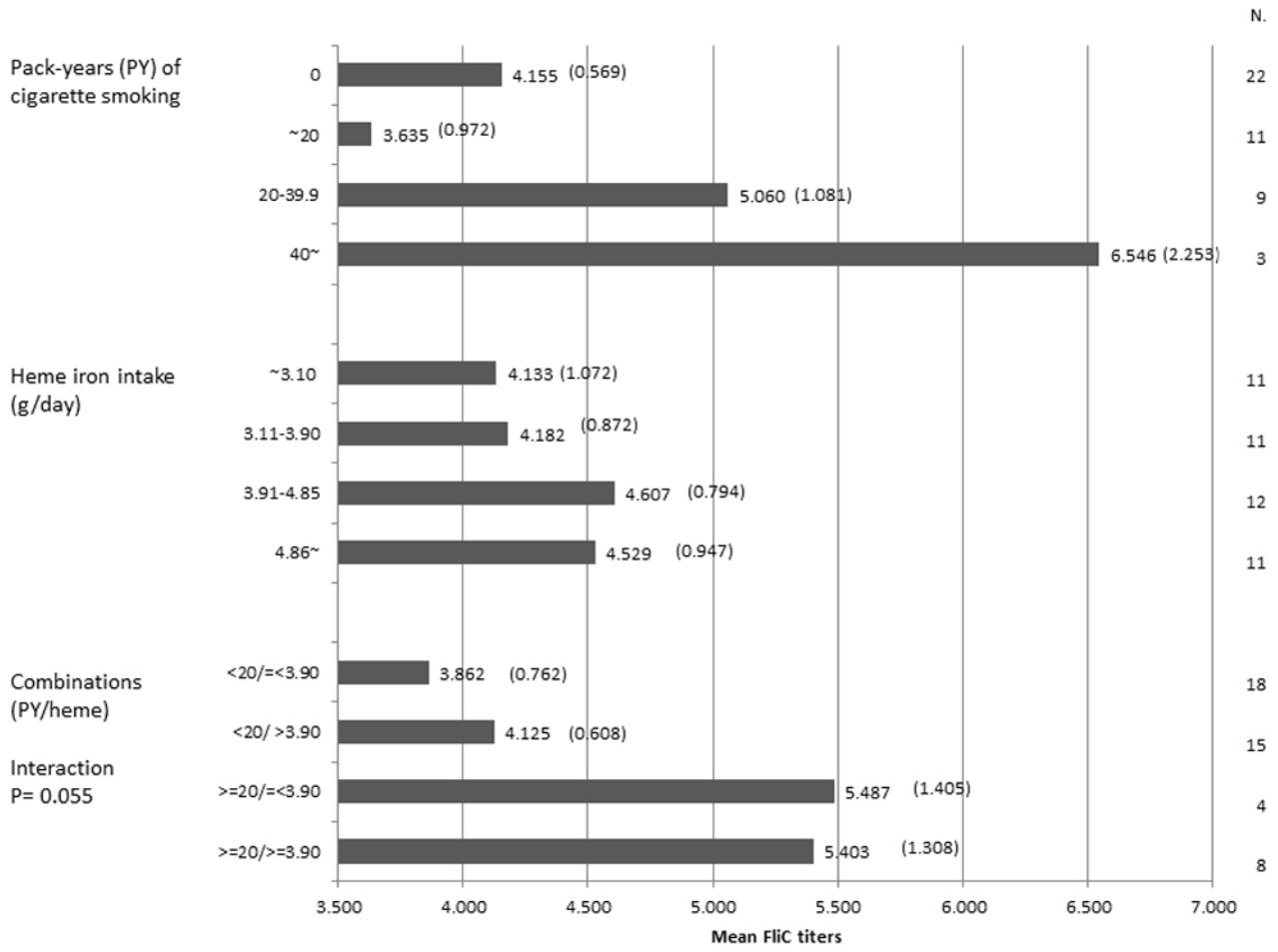
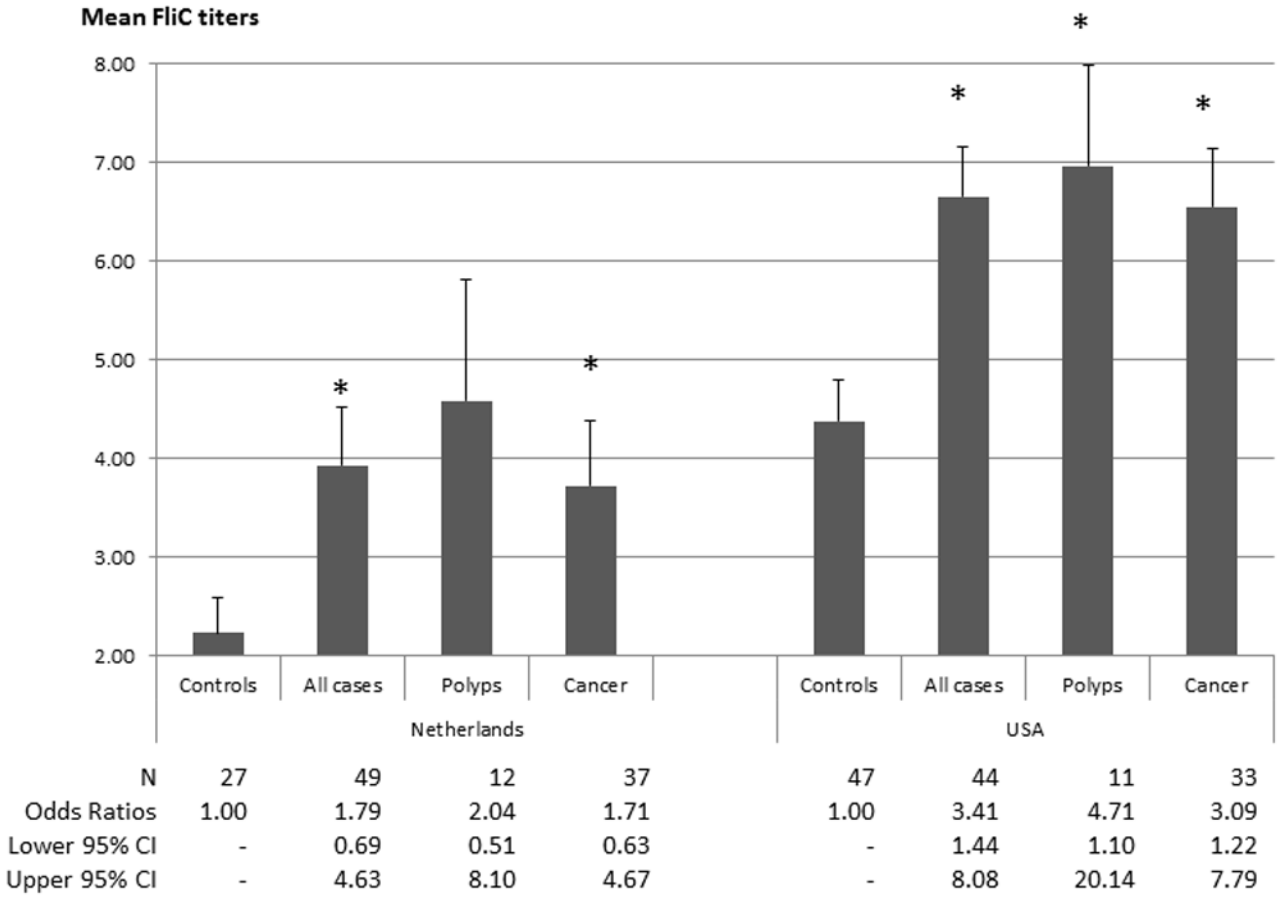


Figure 1.

Mean antibody titers to Salmonella flagellin and standard errors (SE) according to pack-years of cigarette smoking, dietary hem levels and their combinations in US control group (N=45). The numbers next to each bar indicate the mean titer and SE.



N	27	49	12	37	47	44	11	33
Odds Ratios	1.00	1.79	2.04	1.71	1.00	3.41	4.71	3.09
Lower 95% CI	-	0.69	0.51	0.63	-	1.44	1.10	1.22
Upper 95% CI	-	4.63	8.10	4.67	-	8.08	20.14	7.79

Figure 2.

Mean antibody titers to Salmonella flagellin, their standard errors (SE), odds ratios (ORs) and 95% confidence intervals (CI) for colorectal tumors associated with above-median titer in each population (cutoff point= 2.00 for the Netherlands and 4.85 for US). The extended lines from the top of the bars indicate SEs and asterisks indicate statistical difference ($P<0.05$) from the controls.

Table 1

Bi-variable and multi-variable odds ratios (OR) and 95% confidence intervals (CI) according to history of cigarette smoking

Variables	Categories	Controls	Cases	OR1	95% CI	OR2	95% CI
Smoking status	Never	664	477	1.00	-	1.00	-
	Ever	806	659	1.10	(0.93–1.38)	1.05	(0.89–1.23)
	Former	510	422	1.10	(0.92–1.32)	1.09	(0.91–1.31)
	Current	296	237	1.08	(0.88–1.33)	0.97	(0.78–1.21)
Pack years	0	664	477	1.00	-	1.00	-
	0.01–19.9	359	257	0.97	(0.90–1.19)	0.98	(0.80–1.20)
	20–39.9	239	199	1.12	(0.90–1.40)	1.11	(0.88–1.39)
	40+	208	203	1.29	(1.02–1.62)	1.10	(0.86–1.40)
Trend P				P=0.018	(0.021)	P=0.328	(0.401)

*Trend tests were based on median values of each category as well as continuous values (in the parentheses)

OR1: adjusted for gender

OR2: adjusted for age, gender, total energy, total calcium, dietary fiber intake, physical activities in their 30s, body mass index, family history of colorectal cancer, highest education achieved, and NSAID use

Table 2

Bi-variable and multi-variable odds ratios (OR) and 95% confidence intervals (CI) according to quintiles of iron intake

Type of iron		Controls	Cases	OR1	95% CI	Controls	Cases	OR2	95% CI
Total Iron *	Q1	273	242	1.00	-	269	252	1.00	-
	Q2	284	242	0.95	(0.74–1.21)	276	245	1.12	(0.87–1.45)
	Q3	298	225	0.82	(0.64–1.05)	293	229	1.02	(0.79–1.33)
	Q4	321	196	0.69	(0.54–0.88)	323	198	0.87	(0.67–1.13)
	Q5	294	231	0.85	(0.66–1.08)	309	212	1.06	(0.81–1.40)
	Trend P			P=0.031	(0.111)			P=0.476	(0.849)
Dietary iron	Q1	286	225	1.00		265	256	1.00	-
	Q2	300	234	0.98	(0.77–1.25)	304	217	0.90	(0.70–1.17)
	Q3	315	204	0.81	(0.63–1.03)	304	218	0.93	(0.72–1.21)
	Q4	296	221	0.91	(0.71–1.16)	298	223	0.97	(0.74–1.26)
	Q5	273	252	1.09	(0.85–1.40)	299	222	1.07	(0.81–1.40)
	Trend P			p=0.426	(0.394)			P=0.409	(0.638)
Heme iron	Q1	321	200	1.00	-	294	227	1.00	-
	Q2	295	226	1.20	0.93–1.54	301	220	0.96	(0.74–1.24)
	Q3	290	230	1.23	0.96–1.57	298	225	0.98	(0.75–1.28)
	Q4	303	221	1.11	0.86–1.42	292	229	1.00	(0.77–1.30)
	Q5	261	259	1.47	1.14–1.89	286	235	0.98	(0.76–1.27)
	Trend P			P=0.011	(0.008)			P=0.992	(0.624)

* Including supplements

Trend tests were based on median values of each category as well as continuous values (in the parentheses)

OR1: adjusted for gender

OR2: adjusted for age, gender, total energy, total calcium, dietary fiber intake, physical activities in their 30s, body mass index, family history of colorectal cancer, highest education achieved, and NSAID use

Cutoffs for total, dietary and heme iron intake are 11.8/17.4/26.1/33.3, 9.9/12.8/16.2/20.9, and 2.11/2.93/3.94/5.60 g/day and the corresponding cutoffs for intake adjusted for the mean calorie intake in the population (~2000 kcal) and the corresponding cutoffs for energy-adjusted intake are 13.706/16.79/26.23/33.405, 12.66/14.346/16.09/18.89 and 2.915/3.607/4.2402/5.158 g/day.

Table 3

Multivariable odds ratio (OR) and 95% confidence intervals (CI) according to combination of pack-years of cigarette smoking and heme iron intake

	Pack-years of cigarette smoking													
	0			<20			<40			40+			Trend P	
Heme iron quintiles	Controls/Cases	OR	95% CI	Controls/Cases	OR	95% CI	Controls/Cases	OR	95% CI	Controls/Cases	OR	95% CI		
Q1	137/98	1.00	-	79/55	0.89	(0.57–1.39)	37/34	1.16	(0.67–2.02)	41/40	0.95	(0.56–1.61)	0.925	(0.532)
Q2	146/88	0.80	(0.55–1.18)	72/52	0.94	(0.59–1.49)	48/45	1.21	(0.74–2.00)	35/35	1.09	(0.62–1.90)	0.353	(0.232)
Q3	142/112	1.02	(0.70–1.49)	73/47	0.88	(0.55–1.40)	45/33	0.97	(0.57–1.67)	37/33	0.91	(0.52–1.60)	0.622	(0.632)
Q4	120/96	1.03	(0.70–1.52)	69/54	1.03	(0.65–1.63)	51/40	0.97	(0.59–1.62)	52/39	0.83	(0.49–1.38)	0.426	(0.502)
Q5	119/83	0.86	(0.58–1.28)	66/49	0.88	(0.55–1.41)	58/47	0.93	(0.57–1.51)	43/56	1.40	(0.85–2.30)	0.068	(0.119)
Trend P		0.696	(0.576)		0.697	(0.812)		0.478	(0.644)		0.362	(0.047)		
Interaction													P=0.435	(0.039)

*Trend tests were based on median values of each category as well as continuous values (in the parentheses)