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Carcinogenesis vol.34 no.12 pp.2757–2766, 2013
doi:10.1093/carcin/bgt290
Advance Access publication August 27, 2013

Dietary heme iron and the risk of colorectal cancer with specific mutations in *KRAS* and *APC*

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Red meat intake has been linked to increased colorectal cancer (CRC) risk. Although the underlying mechanisms remain unclear, experimental studies suggest a role for dietary heme iron. Because heme iron was shown to promote specific mutations, it would be insightful to link heme iron data to CRC with mutations in key genes in an observational, population-based study. We investigated the association between dietary heme iron intake and risk of CRC with mutations in *APC* (adenomatous polyposis coli) and *KRAS* (Kirsten ras) and P53 overexpression in the Netherlands Cohort Study. After 7.3 years of follow-up, excluding the first 2.3 years due to incomplete coverage of the pathology registry and to avoid preclinical disease, adjusted hazard ratios (including adjustment for total meat) and 95% confidence intervals were calculated, using 4026 subcohort members (aged 55–69 years at baseline), 435 colon and 140 rectal cancer patients. When comparing the highest with the lowest tertile of intake, heme iron intake was associated with an increased risk of CRC harboring activating mutations in *KRAS* (hazard ratio = 1.71, 95% confidence interval: 1.15–2.57; *P* for trend = 0.03) and CRC without truncating mutations in *APC* (hazard ratio = 1.79, 95% confidence interval: 1.23–2.60; *P* for trend = 0.003). We observed a positive association between heme iron intake and the risk of CRC with activating G>A mutations in *KRAS* (*P* for trend = 0.01) and overall G>A mutations in *APC* (*P* for trend = 0.005). No associations were found with CRC harboring G>T mutations in *KRAS/APC*. Heme iron intake was positively associated with the risk of P53 overexpressed tumors but not with tumors without P53 overexpression (Pheterogeneity = 0.12). Heme iron intake was associated with an increased risk of colorectal tumors harboring G>A transitions in *KRAS* and *APC* and overexpression of P53. These novel findings suggest that alkylating rather than oxidative DNA-damaging mechanisms are involved in heme-induced colorectal carcinogenesis.

Introduction

Although the accumulated evidence from prospective epidemiological studies supports a positive association between the consumption of red meat and colorectal cancer risk (1–3), very few human studies have comprehensively evaluated the potential underlying mechanisms.

Experimental studies suggest a role for dietary heme iron, which is present at 5-fold higher concentrations in red than white meat (4) and could explain why white meat, in contrast to red meat, is not associated with cancer risk (3). Nevertheless, epidemiological evidence for its carcinogenic potential is inconclusive (5–10). A recently conducted meta-analysis of five prospective studies showed a consistent, though modest, increased risk of 18% comparing the highest to the lowest category of heme iron intake (11).

Colorectal cancer is a heterogeneous endpoint, and the association between heme iron intake and colorectal cancer may become more evident when the underlying molecular events are taken into account. Most sporadic colorectal cancers are thought to arise through genetic pathways involving a stepwise pattern of mutations in the adenomatous polyposis coli (*APC*) and tumor protein 53 (*TP53*) tumor suppressor genes and the Kirsten ras (*KRAS*) oncogene (12). These key genes are frequently altered in colorectal cancer (12). Interestingly, experimental evidence suggests that heme iron and its metabolic products, may increase the overall mutation rate and promote specific point mutations in the DNA of colonic tissue. For example, heme was shown to catalyze the endogenous formation of *N*-nitroso compounds (NOC) (13), some of which are direct alkylating agents, whereas others need to be metabolized before yielding DNA alkylating intermediates (14). Such metabolites may lead to the induction of nitroso-compound-specific DNA adducts, such as *O*-6-methylguanine (15), and have shown to induce G>A transitions in a variety of genes including *KRAS* and *TP53* in both rodent and *in vitro* studies (16–18). Furthermore, both heme iron and NOC may also catalyze the formation of reactive oxygen species that can cause DNA damage (14,19), which, if not repaired, may induce G>T transversions (20). Lastly, heme is thought to promote colonic cytotoxicity and increased cell proliferation resulting in an increased overall mutation rate (21–23).

Linking heme iron exposure data to colorectal cancer with mutations in the colorectal cancer key genes *APC, TP53* and *KRAS* in an observational, population-based study may provide new insight in the involvement of these genes in meat-associated colorectal cancer risk. Although phenotypic expression studies in normal colonic tissue suggest that environmental factors can indeed influence protein expression patterns of these genes (24), previous studies linking meat consumption to (specific) mutations have yielded mixed results (25–29). However, we are the first to hypothesize that heme iron and/or its metabolites contribute to the observed colorectal cancer heterogeneity by increasing both the overall mutation rate and the occurrence of specific G>A and G>T genetic alterations in the *APC* and *KRAS* genes in colon and rectal tumors that are characteristic for past heme iron exposure. Because mutation status of the *TP53* gene was unavailable for the current analysis, we examined P53 overexpression status in relation to heme iron intake, a phenotype that has previously been inconsistently linked to meat intake (30,31). We further examined whether these associations differ by chlorophyll and dietary calcium intake because these may block the reactivity of heme iron in the gastrointestinal tract and thus prevent heme-induced colorectal carcinogenesis (32,33).

Methods

The Netherlands Cohort Study was initiated in September 1986 and includes 120 852 men and women aged 55–69 years at baseline, originating from 204 municipalities with computerized population registries. Full details of the study design have been described elsewhere (34). At the start of the study, participants completed a self-administered questionnaire on dietary habits, lifestyle characteristics, medical history and other potential risk factors for cancer. The case–cohort approach was used for reasons of efficiency in questionnaire processing and follow-up. Case subjects were enumerated from the entire cohort, whereas the accumulated person years of the entire cohort were estimated from a random subcohort of 5000 men and women, chosen immediately after baseline. The entire cohort is being monitored for cancer occurrence by annual record linkage to the Netherlands Cancer Registry and the Netherlands Pathology Registry (PALGA), together providing an almost complete coverage (35).

Abbreviations: CI, confidence intervals; CRC, colorectal cancer; FFQ, food-frequency questionnaire; HR, hazards ratio; NOC, N-nitroso compounds.

Dietary exposure assessment

All participants completed a 150-item semiquantitative food-frequency questionnaire (FFQ) at baseline, estimating the average frequency and amounts of foods and beverages consumed over the previous 12 months. Daily mean nutrient intakes were calculated from the FFQ dietary data by summing the multiplied frequencies and number of serving sizes of all food items with their tabulated nutrient contents from the Dutch food composition table (36).

The FFQ contained 14 items on consumption of meat with the hot meal, five items on consumption of processed meat used as sandwich fillings, and three items on fish. The heme iron content from these meat items and the meat used in mixed dishes was estimated as an animal-specific percentage of total iron, derived from data in the literature (e.g. 65, 39 and 26% for cooked beef, pork and chicken or fish, respectively), and has been reported in detail elsewhere (7).

The FFQ has been validated and tested for reproducibility (37,38). Crude (energy and gender adjusted between brackets) Pearson correlation coefficients (r) between the questionnaire and the 9-day diet record (kept over 3-day periods, 4–5 months apart) were 0.74 for energy, 0.72 (0.52) for total fat, 0.73 (0.58) for saturated fatty acids and 0.73 (0.75) for polyunsaturated fatty acids. The Spearman correlation coefficients for fresh meat, processed meat and fish were 0.46, 0.54 and 0.53, respectively (37).

Tissue samples and DNA isolation

Tumor material from incident colorectal cancer patients identified during 7.3 years of follow-up was collected after approval by the Ethical Review Board of Maastricht University, PALGA and the Netherlands Cancer Registry. The first 2.3 years of the follow-up period were excluded due to the incomplete coverage of PALGA and to avoid possible preclinical disease affecting exposure status. The tissue samples were distributed among 54 pathology laboratories throughout the Netherlands. Ninety percent (733 out of 815) of the available tissue samples contained sufficient tumor material for molecular analyses (39).

APC *and* KRAS *mutation analysis*

Mutation analysis of the mutation cluster region in the *APC* tumor suppressor gene (codons 1286–1520) and the exon 1 fragment of the *KRAS* oncogene (codons 12–13) was performed using a nested PCR approach, followed by direct sequencing of purified fragments. This procedure has been described in detail elsewhere (39,40). *KRAS* status was available for all, and *APC* status was available for 90% of the cases.

TP53 *expression analysis*

Immunohistochemical staining for P53 was performed according to the avidin–biotin–peroxidase complex method, using the DO-7 mouse monoclonal antibody (DAKO A/S, Denmark) as described previously (41). Immunostained slides and negative controls were evaluated semiquantitatively and independently by two observers without knowledge of clinical parameters. We defined cases positive for overexpression of *TP53* if 20% or more of the tumor cell nuclei showed positive staining with the antibody (41). For 99% of the cases, P53 expression data were available.

Statistical methods

Colorectal cancer was classified according to site: colon (ICD-O codes: 153.0–153.7); rectosigmoid (ICD-O code 154.0) and rectum (ICD-O code 154.1). Rectosigmoid cancer cases were not evaluated separately because of the small number of cases (n = 69) and the higher risk of misclassification (42). After 7.3 years of follow-up and exclusion of prevalent cancer cases at baseline other than skin cancer (n = 226), subjects with incomplete FFQs (n = 381), or subjects with missing information on confounders (n = 349), 4026 subcohort members and 644 colorectal (435 colon and 140 rectum cancer) cases remained eligible for analyses.

All analyses were conducted separately for colorectal, colon or rectum tumors with or without a truncating mutation in *APC*, an activating mutation in *KRAS* and overexpression of P53. Truncating *APC* mutations lead to the introduction of a stopcodon and result in a truncated and therefore inactive APC protein. Activating *KRAS* mutations are defined as mutations in codons 12 and 13 of the *KRAS* gene, leading to an altered amino acid resulting in an activated RAS protein.

We additionally classified the colorectal, colon and rectum tumors according to specific point mutations (G>A and G>T) in *APC* and *KRAS* according to two different methods, either focusing on overall mutations or restricting to functional (activating/truncating) mutations. Moreover, additional analyses were performed combining tumors with specific genetic aberrations in the *APC* and *KRAS* genes (e.g. tumors with no G>A mutation in both *KRAS* and *APC*).

Hazard ratios (HR) and corresponding 95% confidence intervals (CI) were estimated using the multivariate Cox proportional hazards model. The total person years at risk, estimated from the subcohort, were used in analyses (43). Standard errors were estimated using the robust Huber–White sandwich estimator to account for additional variance introduced by sampling from the cohort (44). The proportional hazards assumption was tested using the scaled Schoenfeld residuals.

Tests for heterogeneity were performed to evaluate differences in the association with heme iron between tumors with (specific) aberrations and tumors without any aberrations, using the competing risks procedure in STATA. However, the standard error for the difference of the log HRs from this procedure assumes independence of both estimated HRs, which would overestimate this standard error and thus overestimate the P-values for their difference; therefore, parameter estimates were based on a bootstrapping method that was developed for the case–cohort design (45). For each bootstrap sample, X subcohort members were randomly drawn from the subcohort of X subjects and Y cases from the total of Y cases outside the subcohort, both with replacement, out of the data set of $X + Y$ observations. The log HRs were obtained from this sample using the competing risks procedure and recalculated for each bootstrap replication. The CI and P-value of the differences in HR for the molecular subtypes were then calculated from the replicated statistics. Each bootstrap analysis was based on 1000 replications (46).

The covariates included in the multivariate analyses were either *a priori* selected risk factors of colorectal cancer or variables that changed one of the risk estimates by 10% or more. This resulted in a multivariable model including age at baseline (years), sex, intake of alcohol (0, 0.1–29.9, ≥30 g/day), vegetable consumption (g/day), total energy intake (kcal/day), body mass index (kg/m^2), family history of colorectal cancer (yes, no), smoking status (never, former, current) and non-occupational physical activity (<30, 30–60, 60–90, >90 min/day). In addition, we calculated a third model in which we additionally adjusted for the intake of total fresh meat (g/day) and processed meat (g/day) to assess the effect of heme iron independent of meat intake.

Subjects were classified into tertiles of heme iron consumption based on the subcohort (with the lowest tertile regarded as the reference group) and as a continuous variable (1 mg/day increment). Heme iron intake was adjusted for total energy intake using the residual regression method (47). For each analysis, trends were evaluated with the Wald test by assigning participants the median value for each level of the categorical exposure variables among the subcohort members and this variable was entered as a continuous term in the Cox regression model.

Possible effect modification by sex, chlorophyll, dietary calcium, body mass index, smoking status and physical activity was tested by entering cross-product terms in the model. However, none of these interaction terms was statistically significant (P for interaction >0.01).

All analyses were performed using STATA Statistical Software (Intercooled STATA, version 10; Stata-Corp LP, College Station, TX). All tests were two-tailed and differences were regarded as statistically significant at $P < 0.05$.

Results

The baseline characteristics of subcohort members, colorectal, colon and rectum cancer cases, of which the greater part did not differ significantly between the groups, are shown in Table I. Colorectal, colon and rectum cancer patients were older, more often men, had a higher percentage of former smokers and individuals with a family history of colorectal cancer compared with the subcohort.

No differences between colon and rectum cancer patients and subcohort members were observed regarding baseline heme iron intake in women (mean intake of 0.99, 0.99 and 0.97 mg/day, respectively). In men, daily heme iron intake was highest in colon cancer cases (1.23 mg), followed by the subcohort (1.15 mg) and lowest in rectum cancer cases (1.10 mg; results not shown). Also, no statistically significant differences in heme iron intake were observed between colon and rectum cancer cases characterized by specific molecular tumor characteristics in both men and women (results not shown). The correlation of heme iron intake and total fresh meat, processed meat and fresh and processed meat combined were 0.60, 0.42 and 0.68, respectively, for men and 0.59, 0.45 and 0.68, respectively, for women (results not shown). Among the colorectal cancer cases, 56% had overexpression of P53 in the cell nucleus, 33% had an activating *KRAS* mutation and 36% had a truncating *APC* mutation.

Age and sex adjusted (model 1), multivariable adjusted (model 2) and multivariable and fresh and processed meat intake adjusted (model 3) HRs and corresponding 95% CI for different molecular endpoints of colorectal, colon and rectum cancer according to tertiles of heme iron intake are presented in Table II. The HRs for heme iron

Table I. Baseline characteristics and dietary intakes of exposures of interest of colorectal cancer cases and subcohort members within the Netherlands Cohort Study

Characteristics	Subcohort	Colorectal cancer cases	Colon cancer cases	Rectum cancer cases
N	4026	644	435	140
Age (years)	61.3 ± 4.2[a]	62.8 ± 4.1	62.9 ± 4.1	62.3 ± 4.0
Sex (% male)	49.8	56.5	54.1	65.7
Dietary factors				
Total heme iron intake (mg/day)	1.1 ± 0.5	1.1 ± 0.5	1.1 ± 0.5	1.1 ± 0.4
Total fresh meat intake (g/day)[b]	99.5 ± 41.7	99.0 ± 37.5	99.5 ± 36.3	96.2 ± 38.4
Total processed meat intake (g/day)	14.3 ± 15.6	14.9 ± 16.3	14.8 ± 15.9	14.8 ± 13.8
Chlorophyll (mg/day)	52.2 ± 29.5	52.3 ± 27.4	51.3 ± 26.9	52.1 ± 27.4
Vegetables (g/day)	194.1 ± 82.1	193.6 ± 85.0	191.6 ± 81.5	193.1 ± 92.0
Energy intake (kJ/day)	8072 ± 2161	8094 ± 2018	8057 ± 2007	8343 ± 1891
Alcohol (%)				
0 (g/day)	23.3	23.1	25.5	19.3
0.1–29.9 (g/day)	67.6	64.6	62.8	65.7
≥30 (g/day)	9.1	12.3	11.7	15.0
Family history of colorectal cancer (% yes)	5.7	11.0	12.0	10.7
Body mass index (kg/m²)	25.0 ± 3.1	25.5 ± 3.1	25.5 ± 3.2	25.2 ± 2.9
Smoking status (%)				
Never	35.3	32.3	35.6	29.3
Former	36.4	45.3	44.1	45.0
Current	28.4	22.4	20.2	25.7
Non-occupational physical activity (%)				
<30 min/day	20.4	20.4	20.7	22.1
30–60 min/day	31.6	31.1	32.0	27.9
60–90 min/day	20.9	21.5	20.7	22.1
>90 min/day	27.0	27.2	26.7	27.9

[a]Mean ± SD (all such values).
[b]Intake of fresh meat items were based on raw meat weights.

and colorectal cancer were stronger when additionally adjusting the models for total fresh and processed meat. Heme iron intake was not associated with the risk of colorectal tumors harboring a stopcodon in *APC*. In contrast, a significantly increased HR between heme iron intake and the risk of colorectal tumors without mutations leading to the introduction of a stopcodon was observed [multivariable HR (model 3) highest versus lowest tertile: 1.79; 95% CI: 1.23, 2.60; *P* for trend = 0.003; HR per 1 mg/day increase: 1.40; 95% CI: 1.06–1.84]. Heme iron intake was significantly associated with colorectal tumors with activating *KRAS* gene mutations [multivariable HR (model 3) highest versus lowest tertile: 1.73; 95% CI: 1.08–2.77; *P* for trend = 0.03] and P53 overexpression in the cell nucleus [multivariable HR (model 3) for highest versus lowest tertile: 1.58; 95% CI: 1.10–2.27; *P* for trend = 0.008], but not with tumors without these aberrations. Comparable results were observed for colon cancer but not for rectum cancer (Table II).

Additional analyses were performed to evaluate the association between heme iron intake and the risk of colorectal, colon and rectum tumors harboring specific functional (truncating/activating) point mutations (G>A transitions and/or G>T transversions) in *APC* and *KRAS* (Table III). Again, associations were stronger after additional adjustment for total meat intake. No clear dose–response associations were observed between heme iron intake and the risk of colorectal, colon or rectum tumors harboring specific truncating mutations in *APC* nor for tumors without such G>A and G>T mutations. In contrast, heme iron intake was associated with an increased risk of colorectal tumors harboring activating G>A *KRAS* mutations [multivariable HR (model 3) highest versus lowest tertile of intake: 2.19; 95% CI: 1.20, 3.98; *P* for trend = 0.01; HR per 1 mg/day increase: 1.52; 95% CI: 1.03–2.24]. Such association was not found when focusing on activating G>T mutations or when looking at colorectal tumors without G>T and G>A mutations in *KRAS*. Similar associations were observed for colon but not for rectum cancer. In addition, heme iron intake was also associated with increased risk of colorectal tumors harboring activating/truncating G>A mutations in either *APC* and/or *KRAS* [multivariable HR (model 3): 1.79; 95% CI: 1.15, 2.78; *P* for trend = 0.008; HR per 1 mg/day increase: 1.43; 95% CI: 1.04–1.96], but not with tumors harboring G>T mutation, nor for tumors without such mutations in *APC* and *KRAS* combined.

When classifying the colorectal tumors according to specific point mutations, while focusing on overall mutations instead of restricting to functional mutations, we generally found similar results (Table IV). In addition, heme iron intake was significantly associated with an increased risk of colorectal tumors harboring overall G>A *APC* mutations [multivariable HR (model 3) highest versus lowest tertile of intake: 1.71; 95% CI: 1.16, 2.50; *P* for trend = 0.005; HR per 1 mg/day increase: 1.43; 95% CI: 1.08–1.88].

None of the tests for heterogeneity comparing the association with heme iron between molecular subgroups (e.g. tumors with specific mutations versus tumors without the specific mutations) in any of the analyses presented herein were statistically significant (all *P* for heterogeneity >0.05).

Discussion

To the best of our knowledge, this is the first prospective study to report a positive association between heme iron intake and the risk of colorectal tumors harboring G>A transitions in *KRAS* and *APC* and overexpression of P53.

Heme iron intake was dose-dependently associated with an increased risk of colorectal cancer with *KRAS* codon 12 and 13 mutations but not with wild-type *KRAS* tumors. More specifically, we observed a clear dose–response relation between heme iron consumption and colorectal cancers with specific G>A activating transitions in *KRAS*. Similar findings were observed with overall G>A mutations in *APC*, which is in line with a recent case-only study from EPIC-Norfolk (26). These G>A transitions are characteristic for alkylating agents such as NOC or their metabolites (16). The endogenous formation of NOC is catalyzed by heme (13). Some NOC have shown to react with DNA *in vitro* to give rise to NOC specific DNA adducts such as O6-carboxymethyldeoxyguanine (15), which was increased in exfoliated colonic cells from human volunteers who were fed a high red meat diet for 15 days (48). O6-carboxymethyldeoxyguanine adducts may cause DNA polymerase to misread the O6-guanine as

Table II. HR and 95% CI for colorectal, colon and rectum cancer with different molecular characteristics according to tertiles (*T*) of heme iron intake in the Netherlands cohort study on diet and cancer (7.3 years of follow-up)[a]

Molecular endpoint	Person Years	Colorectal cancer				Colon cancer				Rectum cancer			
		No. of cases	HR (95% CI)[b]	HR (95% CI)[c]	HR (95% CI)[d]	No. of cases	HR (95% CI)[b]	HR (95% CI)[c]	HR (95% CI)[d]	No. of cases	HR (95% CI)[b]	HR (95% CI)[c]	HR (95% CI)[d]
APC: without introduction of stopcodon													
*T*1	6496	98	1	1	1	72	1	1	1	19	1	1	1
*T*2	6484	133	1.35 (1.02–1.77)	1.31 (0.99–1.73)	1.49 (1.09–2.05)	99	1.36 (0.99–1.87)	1.32 (0.95–1.82)	1.44 (1.01–2.07)	24	1.26 (0.69–2.30)	1.26 (0.68–2.34)	1.62 (0.81–3.27)
*T*3	6480	137	1.41 (1.08–1.85)	1.39 (1.05–1.84)	1.79 (1.23–2.60)	102	1.43 (1.05–1.96)	1.40 (1.01–1.94)	1.68 (1.09–2.58)	25	1.32 (0.72–2.41)	1.34 (0.73–2.48)	2.16 (0.96–4.83)
P for trend			0.02	0.03	0.003		0.03	0.05	0.02		0.37	0.35	0.06
Continuous (1 mg/day intake increment)			1.17 (0.98–1.41)	1.17 (0.96–1.42)	1.40 (1.06–1.84)		1.17 (0.95–1.43)	1.16 (0.93–1.45)	1.27 (0.93–1.73)		1.00 (0.68–1.49)	1.02 (0.67–1.54)	1.38 (0.84–2.28)
APC: introduction of stopcodon													
*T*1	6496	68	1	1	1	37	1	1	1	20	1	1	1
*T*2	6484	64	0.93 (0.66–1.33)	0.89 (0.62–1.26)	0.89 (0.60–1.33)	36	0.97 (0.60–1.54)	0.95 (0.59–1.52)	0.98 (0.58–1.65)	19	0.94 (0.50–1.78)	0.87 (0.46–1.65)	0.91 (0.44–1.90)
*T*3	6480	77	1.14 (0.82–1.60)	1.06 (0.75–1.48)	1.03 (0.65–1.64)	51	1.39 (0.90–2.14)	1.33 (0.88–2.03)	1.36 (0.75–2.46)	12	0.61 (0.29–1.24)	0.54 (0.26–1.12)	0.58 (0.22–1.55)
P for trend			0.41	0.69	0.82		0.17	0.16	0.26		0.16	0.09	0.27
Continuous (1 mg/day intake increment)			1.24 (0.94–1.64)	1.16 (0.86–1.56)	1.22 (0.79–1.89)		1.44 (1.05–1.99)	1.41 (1.00–1.98)	1.60 (0.95–2.69)		0.67 (0.36–1.19)	0.28 (0.32–1.08)	0.58 (0.23–1.45)
P for heterogeneity: APC with stopcodon versus APC without stopcodon					0.16				0.28				0.27
KRAS: wild-type													
*T*1	6496	134	1	1	1	93	1	1	1	29	1	1	1
*T*2	6484	133	0.99 (0.77–1.27)	0.94 (0.73–1.22)	0.99 (0.74–1.32)	95	1.01 (0.76–1.46)	0.88 (0.72–1.33)	1.03 (0.73–1.43)	27	0.77 (0.45–1.32)	0.75 (0.44–1.30)	0.90 (0.49–1.65)
*T*3	6480	159	1.20 (0.94–1.53)	1.16 (0.90–1.49)	1.25 (0.89–1.76)	110	1.19 (0.90–1.59)	1.16 (0.86–1.56)	1.26 (0.85–1.86)	31	1.02 (0.61–1.67)	1.01 (0.59–1.70)	1.43 (0.71–2.88)
P for trend			0.13	0.20	0.16		0.21	0.29	0.23		0.92	0.92	0.29
Continuous (1 mg/day intake increment)			1.19 (0.99–1.44)	1.18 (0.96–1.45)	1.33 (0.99–1.77)		1.17 (0.94–1.47)	1.17 (0.92–1.49)	1.31 (0.92–1.87)		0.98 (0.66–1.47)	0.98 (0.64–1.51)	1.41 (0.85–2.35)
KRAS: activating mutation													
*T*1	6496	55	1	1	1	31	1	1	1	15	1	1	1
*T*2	6484	85	1.53 (1.08–2.17)	1.49 (1.04–2.12)	1.71 (1.15–2.57)	52	1.66 (1.06–2.62)	1.63 (1.03–2.59)	1.83 (1.09–3.09)	28	1.85 (0.96–3.56)	1.77 (0.91–3.46)	1.97 (0.91–4.25)
*T*3	6480	74	1.36 (0.95–1.95)	1.30 (0.90–1.87)	1.73 (1.08–2.77)	51	1.68 (1.07–2.65)	1.62 (1.02–2.56)	2.06 (1.12–3.79)	14	0.93 (0.44–1.99)	0.89 (0.42–1.87)	1.05 (0.42–2.68)
P for trend			0.12	0.21	0.03		0.03	0.05	0.03		0.73	0.59	0.93
Continuous (1 mg/day intake increment)			1.16 (0.90–1.48)	1.10 (0.85–1.42)	1.35 (0.93–1.94)		1.75 (1.06–2.91)	1.24 (0.94–1.64)	1.43 (0.98–2.09)		0.74 (0.44–1.24)	0.69 (0.41–1.16)	0.58 (0.27–1.25)
P for heterogeneity: mutated KRAS versus wild type KRAS					0.14				0.21				0.07
TP53: without overexpression													
*T*1	6496	79	1	1	1	56	1	1	1	14	1	1	1
*T*2	6484	106	1.33 (0.98–1.80)	1.28 (0.94–1.75)	1.29 (0.91–1.83)	80	1.43 (1.00–2.03)	1.35 (0.94–1.94)	1.36 (0.91–2.03)	16	1.14 (0.55–2.33)	1.20 (0.59–2.49)	1.36 (0.58–3.19)
*T*3	6480	93	1.19 (0.87–1.63)	1.15 (0.83–1.58)	1.15 (0.75–1.76)	72	1.29 (0.90–1.84)	1.24 (0.85–1.79)	1.24 (0.76–2.00)	16	1.16 (0.56–2.38)	1.29 (0.62. 2.70)	1.64 (0.59–4.57)
P for trend			0.31	0.47	0.63		0.17	0.32	0.48		0.70	0.50	0.34
Continuous (1 mg/day intake increment)			1.06 (0.85–1.32)	1.03 (0.81–1.30)	(0.95 (0.67–1.35)		1.10 (0.86–1.40)	1.05 (0.81–1.37)	0.94 (0.61–1.43)		0.94 (0.59–1.50)	1.04 (0.64–1.69)	1.15 (0.57–2.31)

Table II. *Continued*

Molecular endpoint	Person Years	Colorectal cancer				Colon cancer				Rectum cancer			
		No. of cases	HR (95% CI)[b]	HR (95% CI)[c]	HR (95% CI)[d]	No. of cases	HR (95% CI)[b]	HR (95% CI)[c]	HR (95% CI)[d]	No. of cases	HR (95% CI)[b]	HR (95% CI)[c]	HR (95% CI)[d]
TP53: overexpression													
T1	6496	108	1	1	1	68	1	1	1	30	1	1	1
T2	6484	109	1.00 (0.76–1.32)	0.97 (0.73. 1.28)	1.10 (0.80–1.50)	66	0.96 (0.68–1.37)	0.95 (0.67–1.36)	1.07 (0.72–1.59)	32	1.06 (0.64–1.76)	0.99 (0.59–1.66)	1.17 (0.67–2.07)
T3	6480	138	1.29 (0.98–1.68)	1.25 (0.95–1.63)	1.58 (1.10–2.27)	87	1.29 (0.93–1.79)	1.29 (0.92–1.80)	1.62 (1.03–2.55)	28	0.94 (0.56–1.58)	0.86 (0.51–1.46)	1.18 (0.60–2.30)
P for trend			0.05	0.09	0.008		0.11	0.12	0.03		0.79	0.57	0.65
Continuous (1 mg/day intake increment)			1.27 (1.04–1.56)	1.25 (1.01–1.55)	1.66 (1.25–2.21)		1.29 (1.01–1.66)	1.31 (1.01–1.70)	1.73 (1.24–2.42)		0.89 (0.59–1.36)	0.82 (0.52–1.28)	1.06 (0.61–1.86)
P for heterogeneity: *TP53* with overexpression versus *TP53* without overexpression					0.12				0.16				0.84

[a]T1 was the reference category. HRs were derived from Cox regression analyses.

[b]Adjusted for age at baseline (years) and sex.

[c]Adjusted for, age at baseline (years), sex, body mass index (kg/m^2), family history of colorectal cancer (yes, no), smoking (never, former, current), non-occupational physical activity (<30, 30–60, 60–90, >90 min/day), total energy intake (kcal/day), alcohol consumption (0, 0.1–29.9, ≥30 g/day) and total vegetable consumption (g/day).

[d]Adjusted for, age at baseline (years), sex, body mass index (kg/m^2), family history of colorectal cancer (yes, no), smoking (never, former, current), non-occupational physical activity (<30, 30–60, 60–90, >90 min/day), total energy intake (kcal/day), alcohol consumption (0, 0.1–29.9, ≥30 g/day), total vegetable consumption (g/day), total fresh meat intake (g/day) and total processed meat intake (g/day).

an adenine which results in miss-pairing with thymine, causing G>A transitions (49). Supporting these data, *N*-methyl *N*-nitrosourea, a potent and direct acting NOC, has shown to induce G>A transitions at codons 12 and 13 of the *KRAS* gene in rat colon tumors when infused intrarectally (17).

Heme iron also catalyzes the formation of hydroxyl radicals that can cause oxidative DNA lesions (14,19) that can promote G>T transversions (20). Moreover, NOC-metabolism has been shown to generate reactive oxygen species, which was associated with the deregulation of gene expression patterns that may be of relevance in human colorectal carcinogenesis (14). However, in both *KRAS* and *APC*, the magnitude of our findings for G>T mutations was smaller compared with our G>A specific associations, if present at all. Although the tests for heterogeneity between these molecular subgroups were not statistically significant, this may imply that the heme-stimulated alkylating effect of NOC might play a larger role in colorectal carcinogenesis than its oxidative potential. Indeed, it has been proposed previously that DNA alkylation is probably the most relevant genotoxic effect induced by NOC (50). However, G>A mutations can, next to alkylating damage, also result from other endogenous mechanisms. Nonetheless, the number of subjects with G>A and G>T mutations was relatively small and more research in larger samples is required.

Although heme iron intake was not significantly associated with the risk of tumors with (specific) mutations leading to the introduction of a stopcodon in *APC*, a significant positive trend between heme iron intake and tumors without an *APC* stopcodon was observed. These findings are supported by epidemiological studies examining red meat intake in relation to *APC* truncation status in the NLCS (28) and a Dutch colorectal adenoma case–control study (51) but contradict those from a cancer case–control study (52) and a case-only study (26).

The initial focus of our analyses was on functional mutations; inactivation of the *APC* gene and activation of *KRAS* are thought to drive the development of a carcinoma and are considered key events in early colorectal tumorigenesis (53). The potential impact of non-functional mutations in colorectal cancer development is not yet understood. Nevertheless, we only observed an association between heme iron intake and *APC* tumors without the introduction of a stopcodon. However, only half of all *APC* mutations led to the introduction of such a stopcodon that prompted us to examine the group of tumors harboring any *APC* mutation more closely for specific point mutation status, regardless of its functionality. These analyses showed an increased risk for tumors harboring overall G>A mutations in *APC*, which was not found when restricting to functional mutations only. This could suggest that the carcinogenic mechanism behind heme intake involves exposure-specific mutations that may not necessarily entail inactivation of *APC*. The wide spectrum of the *APC* mutation cluster region mutations could, in contrast to codon 12 and 13 mutations in the *KRAS* gene (harboring mainly G changes), serve as a potential useful fingerprint for long-term exposure to dietary heme iron. However, these conclusions are speculative and our findings need to be reproduced in future research.

In line with previous experimental evidence, we showed that heme iron intake was positively associated with the risk of P53 overexpressed colorectal tumors. However, because the absence of P53 overexpression does not necessarily imply the absence of *TP53* mutations and P53 overexpression may occur in the absence of mutations and *vice versa* (54,55), we are prudent when interpreting these findings.

The observed risk estimates were weaker and more unstable in rectum cases compared with colon cases. Although this may partly be attributed to the lower number of rectum cancer cases, previous studies suggest that dietary risk factors for colon and rectum cancer may differ as a result of, for instance, physiologic and biochemical subsite differences (9,56) that could affect the carcinogenic potential of meat mutagens such as heme iron. Nonetheless, tests for heterogeneity suggest that there is no statistical heterogeneity between the rectum and the colon endpoint, and results should be interpreted with caution.

Table III. HR and 95% CI for colorectal, colon and rectum cancer with specific functional point mutations in colorectal key genes according to tertiles (*T*) of heme iron intake in the Netherlands cohort study on diet and cancer (7.3 years of follow-up)[a]

Molecular endpoint	Person years	Colorectal cancer				Colon cancer				Rectum cancer			
		No. of cases	HR (95% CI)[b]	HR (95% CI)[c]	HR (95% CI)[d]	No. of cases	HR (95% CI)[b]	HR (95% CI)[c]	HR (95% CI)[d]	No. of cases	HR (95% CI)[b]	HR (95% CI)[c]	HR (95% CI)[d]
APC: without G>T and G>A mutations													
*T*1	6496	67	1	1	1	45	1	1	1	15	1	1	1
*T*2	6484	94	1.39 (1.01–1.92)	1.33 (0.96–1.85)	1.34 (0.93–1.93)	65	1.43 (0.97–2.11)	1.38 (0.93–2.06)	1.36 (0.88–2.11)	21	1.39 (0.71–2.71)	1.32 (0.67–2.60)	1.63 (0.75–3.58)
*T*3	6480	87	1.31 (0.94–1.82)	1.27 (0.91–1.79)	1.28 (0.81–2.03)	63	1.42 (0.96–2.10)	1.41 (0.94–2.12)	1.37 (0.79–2.36)	15	1.01 (0.49–2.07)	0.95 (0.46–1.97)	1.40 (0.53–3.69)
P for trend			0.13	0.20	0.36		0.09	0.11	0.31		0.96	0.83	0.54
Continuous (1 mg/ day intake increment)			1.22 (0.96–1.55)	1.21 (0.93–1.56)	1.23 (0.82–1.85)		1.25 (0.96–1.63)	1.28 (0.96–1.71)	1.24 (0.78–1.98)		0.79 (0.50–1.26)	0.73 (0.45–1.19)	0.82 (0.39–1.76)
APC: truncating G>A mutations													
*T*1	6496	44	1.00	1.00	1.00	25	1.00	1.00	1.00	12	1.00	1.00	1.00
*T*2	6484	40	0.90 (0.58–1.40)	0.84 (0.55–1.30)	1.04 (0.63–1.74)	23	0.91 (0.52–1.62)	0.87 (0.49–1.54)	1.02 (0.52–1.98)	12	0.99 (0.44–2.22)	0.89 (0.40–2.00)	1.13 (0.43–2.95)
*T*3	6480	45	1.03 (0.68–1.57)	0.92 (0.61–1.40)	1.34 (0.76–2.33)	27	1.09 (0.63–1.89)	0.98 (0.58–1.67)	1.29 (0.63–2.64)	8	0.67 (0.27–1.64)	0.59 (0.23–1.46)	0.87 (0.24–3.15)
P for trend			0.86	0.76	0.29		0.73	0.99	0.45		0.37	0.24	0.82
Continuous (1 mg/ day intake increment)			1.01 (0.71–1.43)	0.92 (0.64–1.30)	1.30 (0.84–2.01)		1.08 (0.69–1.69)	0.98 (0.63–1.55)	1.30 (0.73–2.33)		0.76 (0.36–1.64)	0.67 (0.30–1.51)	1.06 (0.40–2.83)
P for heterogeneity: truncating G>A in APC versus no G>A and G>T in APC					0.17				0.32				0.65
APC: truncating G>T mutations													
*T*1	6496	11	1	1	1	4	1	1	1	5	1	1	1
*T*2	6484	9	0.82 (0.34–1.98)	0.75 (0.31–1.85)	0.75 (0.28–2.03)	6	1.50 (0.42–5.35)	1.38 (0.38–5.00)	1.36 (0.32–5.74)	2	0.40 (0.08–2.06)	0.39 (0.07–2.12)	0.56 (0.09–3.60)
*T*3	6480	17	1.55 (0.72–3.32)	1.41 (0.66–3.01)	1.31 (0.46–3.73)	12	3.01 (0.97–9.34)	2.51 (0.79–7.95)	2.41 (0.54–10.78)	2	0.40 (0.08–2.07)	0.43 (0.09–1.97)	0.86 (0.09–7.79)
P for trend			0.23	0.31	0.52		0.04	0.09	0.20		0.29	0.29	0.87
Continuous (1 mg/ day intake increment)			1.62 (0.99–2.63)	1.53 (0.88–2.67)	1.55 (0.71–3.40)		2.00 (1.19–3.36)	1.89 (0.94–3.80)	1.74 (0.63–4.79)		0.60 (0.21–1.70)	0.64 (0.23–1.76)	2.05 (0.63–6.65)
P for heterogeneity: truncating G>T in APC versus no G>A and G>T in APC					0.27				0.92				0.99
KRAS: without G>T and G>A mutations													
*T*1	6496	137	1	1	1	94	1	1	1	33	1	1	1
*T*2	6484	141	1.02 (0.79–1.31)	0.98 (0.76–1.27)	1.02 (0.77–1.39)	100	1.06 (0.79–1.42)	1.02 (0.76–1.38)	1.06 (0.76–1.47)	27	0.81 (0.49–1.36)	0.79 (0.47–1.34)	0.96 (0.54–1.71)
*T*3	6480	161	1.18 (0.93–1.51)	1.15 (0.90–1.48)	1.23 (0.88–1.72)	111	1.19 (0.89–1.58)	1.16 (0.87–1.56)	1.23 (0.83–1.82)	32	0.98 (0.60–1.61)	0.98 (0.59–1.63)	1.41 (0.72–2.95)
P for trend			0.16	0.23	0.19		0.23	0.29	0.28		0.98	0.98	0.29
Continuous (1 mg/day intake increment)			1.16 (0.96–1.40)	1.15 (0.94–1.41)	1.27 (0.86–1.69)		1.15 (0.92–1.44)	1.15 (0.90–1.47)	1.24 (0.87–1.78)		0.94 (0.63–1.39)	0.94 (0.61–1.42)	1.34 (0.81–2.21)
KRAS: activating G>A mutations													
*T*1	6496	32	1	1	1	19	1	1	1	7	1	1	1
*T*2	6484	44	1.36 (0.86–2.17)	1.32 (0.82–2.12)	1.62 (0.95–2.77)	24	1.25 (0.68–2.30)	1.22 (0.66–2.27)	1.43 (0.71–2.89)	15	2.13 (0.87–5.25)	2.10 (0.84–5.28)	3.23 (1.12–9.34)
*T*3	6480	49	1.55 (0.99–2.44)	1.47 (0.93–2.34)	2.19 (1.20–3.98)	36	1.94 (1.10–3.41)	1.84 (1.04–3.27)	2.52 (1.17–5.39)	7	1.00 (0.35–2.87)	1.00 (0.34–2.93)	2.09 (0.59–7.47)
P for trend			0.06	0.10	0.01		0.02	0.03	0.01		0.86	0.89	0.28
Continuous (1 mg/day intake increment)			1.23 (0.93–1.62)	1.17 (0.87–1.57)	1.52 (1.03–2.24)		1.50 (1.11–2.04)	1.44 (1.03–1.99)	1.78 (1.17–2.69)		0.69 (0.36–1.30)	0.67 (0.35–1.31)	0.93 (0.36–2.43)
P for heterogeneity: activating G>A in KRAS versus no G>A and G>T in KRAS					0.55				0.30				0.25

Table III. *Continued*

Molecular endpoint	Person years	Colorectal cancer				Colon cancer				Rectum cancer			
		No. of cases	HR (95% CI)[b]	HR (95% CI)[c]	HR (95% CI)[d]	No. of cases	HR (95% CI)[b]	HR (95% CI)[c]	HR (95% CI)[d]	No. of cases	HR (95% CI)[b]	HR (95% CI)[c]	HR (95% CI)[d]
KRAS: activating G>T mutations													
*T*1	6496	19	1	1	1	10	1	1	1	5	1	1	1
*T*2	6484	32	1.67 (0.94–2.96)	1.62 (0.91–2.88)	1.77 (0.91–3.44)	22	2.18 (1.03–4.64)	2.16 (1.01–4.59)	2.48 (1.01–6.08)	8	1.60 (0.52–4.88)	1.48 (0.47–4.66)	1.10 (0.30–4.09)
*T*3	6480	21	1.12 (0.60–2.10)	1.07 (0.57–2.00)	1.27 (0.55–2.94)	12	1.23 (0.53–2.87)	1.24 (0.53–2.88)	1.62 (0.52–5.04)	5	1.00 (0.29–3.47)	0.86 (0.25–2.87)	0.43 (0.08–2.49)
P for trend			0.82	0.96	0.69		0.74	0.73	0.52		0.95	0.71	0.28
Continuous (1 mg/day intake increment)			1.14 (0.69–1.86)	1.09 (0.67–1.80)	1.36 (0.64–2.88)		1.01 (0.61–1.69)	1.02 (0.61–1.71)	1.18 (0.56–2.45)		0.99 (0.42–2.29)	0.83 (0.37–1.86)	0.29 (0.08–1.07)
P for heterogeneity: activating G>T in KRAS versus no G>A and G>T in KRAS					0.17				0.09				0.94
APC and *KRAS:* without G>T and G>A mutations													
*T*1	6496	59	1	1	1	41	1	1	1	14	1	1	1
*T*2	6484	66	1.11 (0.77–1.59)	1.06 (0.74–1.54)	1.04 (0.69–1.57)	49	1.19 (0.78–1.81)	1.14 (0.74–1.75)	1.11 (0.69–1.80)	12	0.85 (0.39–1.85)	0.84 (0.38–1.83)	0.99 (0.42–2.35)
*T*3	6480	66	1.13 (0.79–1.62)	1.11 (0.77–1.61)	1.06 (0.63–1.78)	46	1.13 (0.74–1.74)	1.12 (0.72–1.76)	1.07 (0.58–1.97)	12	0.88 (0.41–1.90)	0.88 (0.40–1.93)	1.22 (0.43–3.47)
P for trend			0.52	0.58	0.83		0.6	0.63	0.86		0.75	0.77	0.70
Continuous (1 mg/d intake increment)			1.09 (0.82–1.46)	1.10 (0.80–1.49)	1.05 (0.65–1.70)		1.06 (0.75–1.49)	1.08 (0.74–1.58)	1.00 (0.52–1.91)		0.82 (0.45–1.48)	0.82 (0.44–1.50)	1.10 (0.49–2.46)
APC and/or *KRAS:* activating and/or truncating G>A mutations													
*T*1	6496	65	1	1	1	40	1	1	1	15	1	1	1
*T*2	6484	75	1.15 (0.81–1.61)	1.09 (0.77–1.55)	1.33 (0.90–1.98)	41	1.02 (0.65–1.58)	0.99 (0.63–1.55)	1.15 (0.69–1.90)	25	1.66 (0.87–3.16)	1.56 (0.81–3.00)	2.13 (0.97–4.64)
*T*3	6480	86	1.34 (0.96–1.86)	1.25 (0.89–1.74)	1.79 (1.15–2.78)	58	1.47 (0.98–2.22)	1.38 (0.91–2.08)	1.83 (1.06–3.16)	15	1.00 (0.49–2.06)	0.94 (0.45–1.95)	1.61 (0.61–4.26)
P for trend			0.08	0.19	0.008		0.05	0.11	0.02		0.91	0.74	0.40
Continuous (1 mg/day intake increment)			1.16 (0.91–1.46)	1.09 (0.85–1.39)	1.43 (1.04–1.96)		1.29 (0.97–1.71)	1.22 (0.91–1.63)	1.54 (1.06–2.23)		0.85 (0.52–1.40)	0.79 (0.46–1.33)	1.12 (0.55–2.27)
P for heterogeneity: functional G>A in APC and/or KRAS versus no G>A and G>T in APC and KRAS					0.68				0.26				0.59
APC and/or *KRAS:* activating and/or truncating G>T mutations													
*T*1	6496	29	1	1	1	14	1	1	1	9	1	1	1
*T*2	6484	39	1.34 (0.82–2.17)	1.27 (0.78–2.08)	1.29 (0.74–2.24)	26	1.84 (0.96–3.55)	1.81 (0.94–3.49)	1.87 (0.89–3.94)	10	1.11 (0.45–2.73)	1.03 (0.41–2.59)	0.91 (0.32–2.59)
*T*3	6480	38	1.32 (0.81–2.16)	1.24 (0.76–2.03)	1.24 (0.64–2.41)	24	1.74 (0.89–3.37)	1.70 (0.87–3.29)	1.78 (0.73–4.35)	7	0.78 (0.29–2.10)	0.69 (0.27–1.80)	0.52 (0.13–2.06)
P for trend			0.28	0.42	0.58		0.12	0.14	0.25		0.60	0.42	0.32
Continuous (1 mg/day intake increment)			1.32 (0.92–1.89)	1.26 (0.86–1.83)	1.34 (0.75–2.39)		1.34 (0.91–1.98)	1.32 (0.87–1.98)	1.28 (0.69–2.36)		0.89 (0.44–1.77)	0.63 (0.21–1.91)	0.54 (0.19–1.59)
P for heterogeneity: functional G>T in *APC* and/or *KRAS* versus no G>A and G>T in *APC* and *KRAS*					0.90				0.52				0.97

[a]*T*1 was the reference category. HRs were derived from Cox regression analyses.

[b]Adjusted for age at baseline (years) and sex.

[c]Adjusted for age at baseline (years), sex, body mass index (kg/m^2), family history of colorectal cancer (yes, no), smoking (never, former, current), non-occupational physical activity (<30, 30–60, 60–90, >90 min/day), total energy intake (kcal/day), alcohol consumption (0, 0.1–29.9, ≥30 g/day) and total vegetable consumption (g/day).

[d]Adjusted for age at baseline (years), sex, body mass index (kg/m^2), family history of colorectal cancer (yes, no), smoking (never, former, current), non-occupational physical activity (<30, 30–60, 60–90, >90 min/day), total energy intake (kcal/day), alcohol consumption (0, 0.1–29.9, ≥30 g/day), total vegetable consumption (g/day), total fresh meat intake (g/day) and total processed meat intake (g/day).

The present study illustrates that when unraveling the mechanisms underlying the association between heme iron and colorectal cancer risk, it is valuable to take both (i) specific meat carcinogens such as heme iron and (ii) the molecular heterogeneity of the colorectal endpoint into account. Failure to do so may result in attenuated risk estimates as reflected in previous analyses from our cohort (7,27,28).

Remarkably, the associations presented herein were stronger after additional adjustment for fresh and processed meat intake. This may be explained by the previously reported suggestive inverse associations between total meat intake, particularly pork consumption and the risk of colon and rectal cancer harboring specific mutations in this population (27,28). However, heme iron captures the variation in red meat intake better than pork consumption alone, which is consumed at relatively high amounts in our population. By adjusting the associations for total meat intake, we attempted to partly adjust for all other mechanism by which meat intake may cause colorectal cancer, and

Table IV. HR and 95% CI for colorectal cancer with specific overall point mutations in colorectal key genes according to tertiles (*T*) of heme iron intake in the Netherlands cohort study on diet and cancer (7.3 years of follow-up)[a]

Molecular endpoint	Person years	Colorectal cancer			
		No. of cases	HR (95% CI)[b]	HR (95% CI)[c]	HR (95% CI)[d]
APC: without G>T and G>A mutations					
*T*1	6496	67	1	1	1
*T*2	6484	94	1.39 (1.01–1.92)	1.33 (0.96–1.85)	1.34 (0.93–1.93)
*T*3	6480	87	1.31 (0.94–1.82)	1.27 (0.91–1.79)	1.28 (0.81–2.03)
P for trend			0.13	0.20	0.36
Continuous (1 mg/day intake increment)			1.22 (0.96–1.55)	1.21 (0.93–1.56)	1.23 (0.82–1.85)
APC: G>A mutations					
*T*1	6496	93	1	1	1
*T*2	6484	99	1.06 (0.79–1.42)	1.03 (0.76–1.38)	1.23 (0.88–1.73)
*T*3	6480	117	1.27 (0.96–1.69)	1.21 (0.91–1.62)	1.71 (1.16–2.50)
P for trend			0.09	0.17	0.005
Continuous (1 mg/day intake increment)			1.12 (0.91–1.38)	1.08 (0.87–1.35)	1.43 (1.08–1.88)
P for heterogeneity: G>A in APC versus no G>A and G>T in APC					0.25
APC: G>T mutations					
*T*1	6496	12	1	1	1
*T*2	6484	9	0.75 (0.31–1.78)	0.67 (0.28–1.62)	0.70 (0.26–1.87)
*T*3	6480	19	1.59 (0.77–3.29)	1.41 (0.69–2.88)	1.43 (0.53–3.90)
P for trend			0.18	0.27	0.38
Continuous (1 mg/day intake increment)			1.62 (1.02–2.59)	1.52 (0.90–2.58)	1.63 (0.78–3.40)
P for heterogeneity: G>T in APC versus no G>A and G>T in APC					0.16
KRAS: without G>T and G>A mutations					
*T*1	6496	137	1	1	1
*T*2	6484	141	1.02 (0.79–1.31)	0.98 (0.76–1.27)	1.02 (0.77–1.39)
*T*3	6480	161	1.18 (0.93–1.51)	1.15 (0.90–1.48)	1.23 (0.88–1.72)
P for trend			0.16	0.23	0.19
Continuous (1 mg/day intake increment)			1.16 (0.96–1.40)	1.15 (0.94–1.41)	1.27 (0.86–1.69)
KRAS: G>A mutations					
*T*1	6496	33	1	1	1
*T*2	6484	45	1.35 (0.86–2.13)	1.29 (0.81–2.06)	1.60 (0.94–2.70)
*T*3	6480	51	1.57 (1.00–2.45)	1.46 (0.93–2.32)	2.19 (1.22–3.92)
P for trend			0.05	0.10	0.01
Continuous (1 mg/day intake increment)			1.25 (0.95–1.64)	1.18 (0.88–1.58)	1.55 (1.06–2.25)
P for heterogeneity: G>A in KRAS versus no G>A and G>T in KRAS					0.47
KRAS: G>T mutations					
*T*1	6496	19	1	1	1
*T*2	6484	32	1.67 (0.94–2.96)	1.62 (0.91–2.88)	1.77 (0.91–3.44)
*T*3	6480	21	1.12 (0.60–2.10)	1.07 (0.57–2.00)	1.27 (0.55–2.94)
P for trend			0.82	0.96	0.69
Continuous (1 mg/day intake increment)			1.13 (0.69–1.86)	1.09 (0.67–1.80)	1.36 (0.64–2.88)
P for heterogeneity: G>T in KRAS versus no G>A and G>T in KRAS					0.12
APC and *KRAS:* without G>T and G>A mutations					
*T*1	6496	59	1	1	1
*T*2	6484	66	1.11 (0.77–1.59)	1.06 (0.74–1.54)	1.04 (0.69–1.57)
*T*3	6480	66	1.13 (0.79–1.62)	1.11 (0.77–1.61)	1.06 (0.63–1.78)
P for trend			0.52	0.58	0.83
Continuous (1 mg/day intake increment)			1.09 (0.82–1.46)	1.10 (0.80–1.49)	1.05 (0.65–1.70)
APC and/or *KRAS:* G>A mutations					
*T*1	6496	111	1	1	1
*T*2	6484	128	1.14 (0.86–1.49)	1.11 (0.85–1.46)	1.30 (0.96–1.76)
*T*3	6480	142	1.30 (1.00–1.69)	1.25 (0.86–1.63)	1.68 (1.18–2.39)
P for trend			0.05	0.10	0.03
Continuous (1 mg/day intake increment)			1.15 (0.95–1.39)	1.11 (0.91–1.35)	1.41 (1.09–1.82)

Table IV. *Continued*

Molecular endpoint	Person years	Colorectal cancer			
		No. of cases	HR (95% CI)[b]	HR (95% CI)[c]	HR (95% CI)[d]
P for heterogeneity: G>A in APC and/or KRAS versus no G>A and G>T in APC and KRAS					0.84
APC and/or *KRAS:* G>T mutations					
*T*1	6496	30	1	1	1
*T*2	6484	39	1.29 (0.80–2.09)	1.22 (0.75–1.99)	1.26 (0.73–2.18)
*T*3	6480	40	1.35 (0.84–2.19)	1.26 (0.78–2.04)	1.32 (0.69–2.53)
P for trend			0.23	0.35	0.44
Continuous (1 mg/day intake increment)			1.33 (0.94–1.89)	1.26 (0.87–1.83)	1.37 (0.79–2.41)
P for heterogeneity: G>T in *APC* and/or *KRAS* versus no G>A and G>T in *APC* and *KRAS*					0.78

[a]T1 was the reference category. HRs were derived from Cox regression analyses.
[b]Adjusted for age at baseline (years) and sex.
[c]Adjusted for age at baseline (years), sex, body mass index (kg/m^2), family history of colorectal cancer (yes, no), smoking (never, former, current), non-occupational physical activity (<30, 30–60, 60–90, >90 min/day), total energy intake (kcal/day), alcohol consumption (0, 0.1–29.9, ≥30 g/day) and total vegetable consumption (g/day).
[d]Adjusted for age at baseline (years), sex, body mass index (kg/m^2), family history of colorectal cancer (yes, no), smoking (never, former, current), non-occupational physical activity (<30, 30–60, 60–90, >90 min/day), total energy intake (kcal/day), alcohol consumption (0, 0.1–29.9, ≥30 g/day), total vegetable consumption (g/day) total fresh meat intake (g/day) and total processed meat intake (g/day).

our findings may indeed suggest that heme iron itself is an important player in meat-induced carcinogenesis. In addition, when adjusting our analyses for total meat intake, an increase in heme iron intake results from a displacement in the composition of total fresh meat intake (e.g. pork to beef ratio). If other characteristics (e.g. cooking methods) differ for specific subtypes of meats and are relevant to colorectal cancer, this could result in residual confounding. In addition, other dietary characteristics specific to high heme iron consumers that are not captured by the covariates in the model could also result in residual confounding.

Our heme iron database was based on the total iron content of each meat item in the Dutch food composition database whereby an animal-specific proportion of heme iron relative to total iron was applied, as derived from analytical data reported in the literature for cooked meats. Heme iron values were available for all meat items in the FFQ, including specific Dutch items and cuts. Nevertheless, our method fails to account for effect of the cooking method and duration on the conversion of heme iron to non-heme iron; information that is available in the database developed at NCI (57,58). To evaluate the accuracy of our heme iron estimates, we compared the heme iron concentrations of meat items from our database to the NCI database, for those meat items for which an American equivalent was available (58). The concentrations heme iron in pork, chicken and processed meat were comparable across both databases, but the heme iron content of beef items (i.e. steak and hamburger) was considerable higher in the NLCS database.

We were not able to account for other, possibly correlated, meat mutagens such as heterocyclic amines and polycyclic aromatic hydrocarbons, which are formed in meats when cooked well at high temperatures (59,60). Although both groups of mutagens have shown to be implicated in DNA adduct formation (61,62) and colorectal carcinogenesis (59), they cannot explain the differential carcinogenic effect of white and red meat in the colon. Also a recent dietary intervention study with red meat showed that the observed increase in fecal genotoxicity was not likely to be explained by increased levels of heterocyclic amines or polycyclic aromatic hydrocarbons, but most probably due to heme iron mediated processes (63).

Our analyses have been performed using baseline FFQ data, resulting in an inability to assess and account for changes in intake and food compositions over time. However, the FFQ has shown to be representative for dietary habits over a period of at least 5 years (38). The prospective design reduced the potential for recall bias, and the nearly complete follow-up of cases and subcohort members makes selection bias unlikely. Detailed information on diet and potential risk factors of colorectal cancer enabled us to control for most known risk factors although misclassification of exposure may have occurred. Finally, although our results support prior biological hypotheses, we cannot rule out the possibility of chance findings given the relatively small subgroups of mutation specific colorectal tumors and the numerous associations investigated.

In conclusion, this is the first cohort study reporting the association between heme iron intake and the risk of colorectal cancer harboring specific mutations in key genes, suggesting that heme iron intake is especially associated with an increased risk of colorectal tumors harboring G>A transitions in *KRAS* and *APC* and overexpression of P53. These novel findings suggest that alkylating rather than oxidative DNA-damaging mechanisms are involved in heme-induced colorectal carcinogenesis.

Funding

The Dutch Cancer Society; Wereld Kanker Onderzoek Fonds (WCRF NL), grant 2008/11.

Acknowledgements

We are indebted to the participants of this study and further wish to thank the Netherlands Cancer Registry and registries and the Netherlands nationwide registry of pathology (PALGA). We also thank Dr A. Volovics and Dr A. Kester for statistical advice; S. van de Crommert, H. Brants, J. Nelissen, C. de Zwart, M. Moll, W. van Dijk, M. Jansen and A. Pisters for assistance; H. van Montfort, T. van Moergastel, L. van den Bosch, R. Schmeitz for programming assistance; Dr M. Brink for the collection of tissue samples; and Drs. Amanda Cross and Rashmi Sinha for providing the data from NCI heme iron database.

Conflict of Interest Statement: None declared.

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Received December 11, 2012; revised August 15, 2013; accepted August 20, 2013