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Dietary nitrosyl-heme from processed meats and its association with colorectal cancer risk: findings from the EPIC cohort study

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Abstract

Background Processed meat (PM) consumption is an established risk factor for colorectal cancer (CRC). It has been hypothesized that nitrosyl-heme, formed by the addition of nitrites during meat processing, may enhance the carcinogenic effects of PMs. This study aims to investigate the association between nitrosyl-heme intake and CRC risk within the European Prospective Investigation into Cancer and Nutrition(EPIC) study.

Methods This prospective study included 367,463 participants(70.3% women) from seven countries from the EPIC-study. Dietary data were collected via baseline questionnaires, and nitrosyl-heme exposure was estimated using biochemical data from 52 Spanish PMs, extrapolated to country-specific items. Sex-specific multivariable-adjusted hazard ratios(HRs) and 95% confidence intervals(CIs) were calculated using Cox proportional hazards models.

Results Over a 15-year median follow-up, 5,115 incident CRC cases were identified. Comparing the highest vs. the lowest sex-specific tertile of nitrosyl-heme intake we found no significant association with CRC risk (HR_{T3vsT1}:1 .01;95%CI:0.93–1.09). Subgroup analyses by tumor subtype and interactions with lifestyle factors also showed no associations.

Conclusions This study offers insights into nitrosyl-heme exposure in European populations but found no link to CRC risk. Further research is needed to understand nitrosyl-heme's role in CRC.

Keywords Nitrosyl-heme, Colorectal cancer, Processed meat

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Introduction

Colorectal cancer (CRC) is the third most common cancer globally, with over 1.93 million cases and 935,000 deaths each year. It is also the second leading cause of cancer-related deaths worldwide for both men and women [1]. Its incidence is expected to increase, mainly in people under 50 years of age, in the coming years, particularly in high-income countries [2]. In 2015, the International Agency for Research on Cancer (IARC) classified processed meat (PM) as a carcinogen for humans and concluded that each 50-g of PM consumed on average daily increases the risk of CRC by 18% [3].

Most epidemiological and experimental evidence on the potential compounds from PMs supports a significant role of heme iron, an important type of iron found in red meat, in promoting CRC associated with both red and PM [4–6]. However, in PM, by adding nitrite and/or nitrate salts, the heme molecule is nitrosylated, promoting the formation of nitrosyl-heme, which is released from myoglobin during cooking and/or curing [7]. The nitrosylation of heme iron could be potentially more toxic than the heme molecule itself [7, 8].

One of the leading hypothesis for explaining how nitrosyl-heme contributes to the promotion of CRC carcinogenesis is that this compound, combined with the high levels of N-nitroso compound precursors in PM, increases exposure to N-nitroso compounds, which are formed both externally and within the body [8]. This combination could be the cause behind the more pronounced carcinogenic effects in PM compared to red meat.

Despite heme iron and the risk of CRC research has been extensively studied, research on nitrosyl-heme remains scarce, especially concerning evidence from cohort studies. Only two prospective studies have attempted to examine the relationship between nitrosyl-heme intake and CRC risk. One such study, conducted in a French cohort, found positive associations with the proximal colon cancer subtype. However, this study used a fixed coefficient (0.67) to estimate nitrosyl-heme content across all PM products [9]. In contrast, the second study [10], conducted by our group within the European Prospective Investigation into Cancer and Nutrition (EPIC)-Spain cohort, used direct laboratory measurements of nitrosyl-heme in PM products to estimate intake levels within the cohort. This study found no overall association between nitrosyl-heme and 577 CRC cases in Spanish subjects, nor when analysed by cancer subtypes.

Given the biological plausibility of a link between nitrosyl-heme intake and CRC risk observed in vivo [11], in animal studies [12], and in epidemiological studies [13], we found it important to investigate further in a larger and heterogeneous population. So, the aim of this study was to assess the relationship between nitrosyl-heme intake and the risk of CRC in men and women from the EPIC Europe cohort.

Materials and methods

Study population

The EPIC cohort is a multicentre prospective study designed to examine the relationship between lifestyle factors and cancer in Europe, including diet. The study involved over 500,000 volunteers (mostly aged 35–70 years and approximately two-thirds were women) who were recruited between 1992 and 1998 from 23 centres in 10 European countries (Spain, France, Italy, Denmark, Germany, Norway, Sweden, the Netherlands, Greece and UK) [14, 15]. The rationale, data collection and study population were described by Riboli et al. 1997 [15].

The following exclusions were made: 1) Participants who had cancer other than non-melanoma skin cancer when the study began, or those whose diagnosis date is unknown or who have incomplete follow-up data ($n = 29{,}331$). 2) Subjects with no dietary information (i.e. did not complete the questionnaire) or extreme ranking on the ratio energy intake/energy requirement (top and bottom 1%) ($n = 13{,}281$). 3) Participants from Greece, Sweden and Norway due to data restriction issues ($n = 111{,}248$). In total, 153,860 participants were excluded. The final number of EPIC cohort participants available for this analysis was 367,463 (109,120 men and 258,343 women) (Fig. 1).

Ethical approval was secured from both the IARC Ethical Committee and the local ethics committees associated with each EPIC centre (reference number 20–02). Every participant in the cohort provided written informed consent before enrolment.

Dietary and lifestyle factors assessment

Questionnaire information on sociodemographic characteristics, lifestyle factors, and medical history was gathered during personal interviews through the use of a lifestyle questionnaire. In Oxford and France, participants provided self-reported values for baseline weight and standing height. For participants from other locations, these measurements were measured directly. Regular physical activity (PA) was assessed using the validated EPIC-PAQ questionnaire [16]. Dietary intake was assessed through country-specific validated questionnaires, with most countries employing extensive quantitative or semiquantitative food-frequency questionnaires (FFQs) [15]. Additionally, all participants completed questionnaires on education, alcohol intake, smoking status, reproductive health, and previous disease history.

Nitrosyl-heme was expressed as hemin form (651.94 g/mol molecular weight). To assess its exposure, we

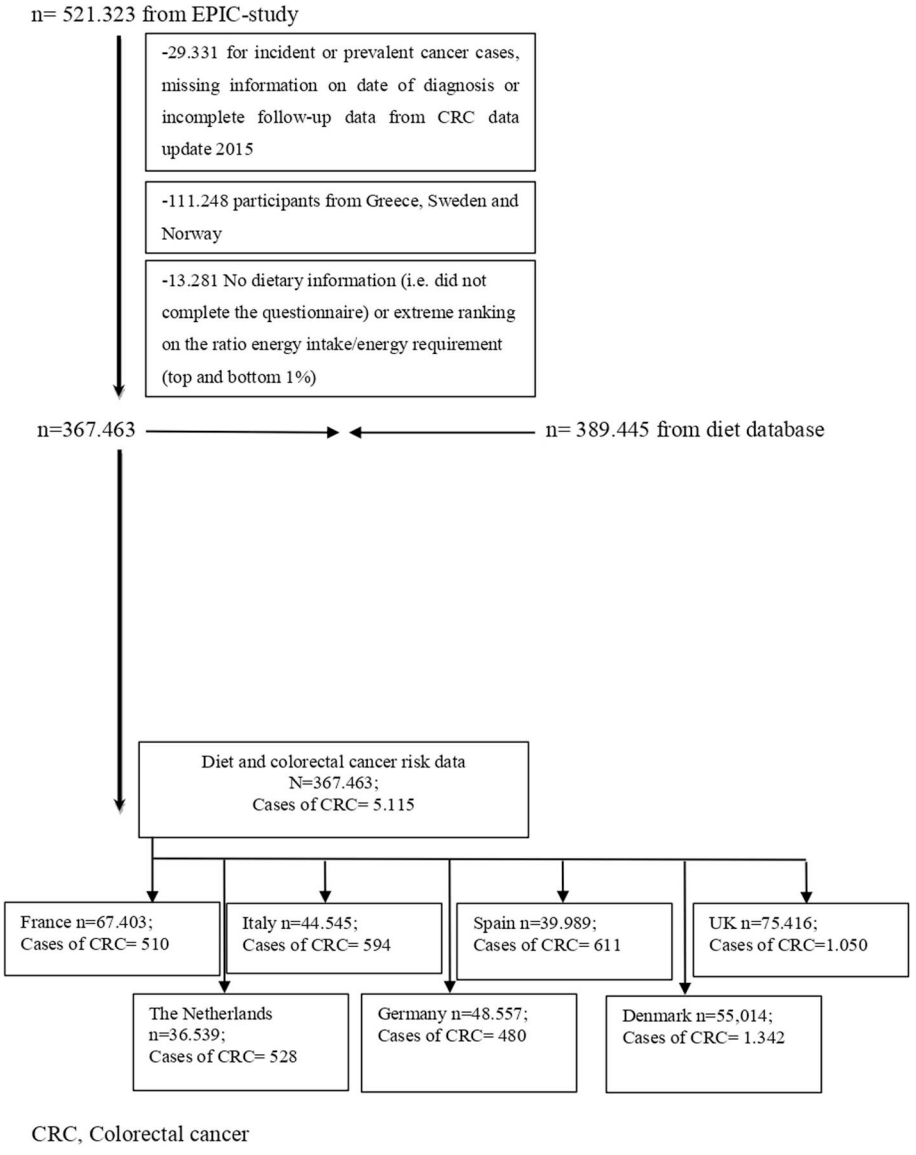

Fig. 1 Flowchart of participants from the European prospective invetigation into cancer and nutrition study in the analyses

employed food composition data obtained in our prior study [17] concerning nitrosyl-heme levels, which were determined using the *HPLC* methodology. Our study included a total of 52 food items derived from the EPIC-DH questionnaires. We estimated food and nutrient intake, including consumption of PMs using the EPIC Nutrient Database (ENDB), and we calculated the daily intake of nitrosyl-heme by multiplying the intake of each PM item (in grams/day) by its assigned content in nitrosyl-heme and summing the results across all PMs. Finally, we extrapolated data on nitrosyl-heme, obtained from the 52 Spanish PMs, to PMs obtained in each country from the EPIC questionnaires. In order to carry out this extrapolation more rigorously, the ingredients of each PM were taken into account according to the country, as well as the addition of nitrate salts in the items or lack thereof. We used a direct approach for items that showed similarity in ingredients, recipes, and available literature, while an indirect approach was utilised in cases where a direct approximation was not feasible. This indirect approach was based on: 1. Averages of two or more items with similar ingredients or recipes; 2. The median of all Spanish items in cases where the generic item lacked specific information; and 3. The most consumed item by the country when estimating a generic item was challenging. Ultimately, extrapolation was conducted for a total of 157 European PMs, with 138 items being extrapolated using a direct approach (87.90%).

Follow-up for cancer incidence

The identification of new cancer cases was done by linking records with cancer registries or using a mix of health

data sources such as health insurance records, pathology registries, or actively following up with participants and their relatives. We defined CRC based on the definitions by the International Classification of Diseases for Oncology (ICD-O; codes: C18 (for colon cancer) and C19-C20 (for rectal cancer)). Proximal colon cancer included those tumours of the area of the splenic flexure (cecum, transverse colon and ascending colon) and were coded as C18.0-C18.5. Distal colon cancers were classified when tumours originated in the descending (C18.6) or sigmoid colon (C18.7). For overlapping tumours were categorised as C18.8 and for those with unspecified location as C18.9. For colon cancer the latter three were included in its definition (proximal, distal and overlapping). Rectal cancer comprised new tumours occurring from the rectosigmoid junction to the rectum (C19: rectosigmoid junction, C20: rectum).

Statistical analyses

For descriptive analyses, frequencies for categorical variables of baseline characteristics were calculated. Because nitrosyl-heme content was right-skewed, median and its respective range interquartile was reported.

We used restricted cubic splines (RCS) to evaluate linearity in the trend in the CRC with dietary nitrosyl-heme intake by sex. Despite testing various transformations (logarithm, square root), the relationship remained nonlinear. Finally, we used the Akaike Information Criterion to select the best representation of the relationship between dietary nitrosyl-heme intake and CRC risk, favouring the three knots. Analyses were conducted using Cox regression to estimate Hazard ratios(HRs) and 95% confidence intervals(CIs) separately for the associations between sex-specific tertiles of nitrosyl-heme and CRC risk. Additional analyses separating men and women were also conducted. HRs were determined by comparison with the lowest tertile of intake. Age served as the time scale, and different models were run: Model 1: stratified by age at recruitment in 10-year categories and centre with adjustments for sex and energy intake; Model 2: was model 1 additionally adjusted by height, weight, waist circumference, education, smoking status, PA, lifetime alcohol consumption, dietary fibre and calcium. Family CRC history was not included in the model because it was not available in all centres. For Model 2, a sensitivity analysis was conducted excluding the first five years of follow-up in order to assess the potential influence of reverse causality,which describes how the outcome can influence the proposed exposure variables, thus altering the direction of their relationship. We also performed an additional sensitivity analysis by removing participants from the UK (mostly vegetarians in the EPIC-Oxford centre). We assessed interactions with smoking, BMI, PA, fibre and calcium intake, energy and alcohol consumption. Finally, we ran homogeneity of location subtype risk using Wald tests.

Rstudio (4.1.3) was used for analyses, with significance set at $p < 0.05$.

Results

Among the 367,463 participants included in this analysis (258,343 women), 5,115 subjects were diagnosed with incident CRC (2,887 women) after a median of follow-up of almost 15 years. Table 1 summarises the baseline characteristics of both men and women. In both sexes, higher BMI and waist circumference showed, on average, higher intakes of nitrosyl-heme., as well as in current smokers and in German subjects. In men, nitrosyl-heme daily intake consumption seemed to be higher with elevated levels of PA and in heavy alcohol intake (Supplementary Table 1). Among women, the group classified as moderately active in PA exhibited the highest nitrosyl-heme intake, as well as those who reported never consuming large amounts of alcohol (Supplementary Table 2).

Associations between sex-specific nitrosyl-heme intake and CRC risk by site are reported in Table 2. There was no statistically significant association between nitrosyl-heme and CRC risk (Model2:, HR_{T3vsT1} 1.01, 95% CI = 0.93–1.09). Additionally, no evidence of an association was found when analysing distal and proximal colon adenomas and ($HR_{T3vsT1} = 1.02$, 95CI%: 0.87–1.20); *P*-trend = 0.83, and ($HR_{T3vsT1} = 0.97$, 95CI%: 0.83–1.14); *P*-trend = 0.74, respectively. Regarding colon and rectal cancer, no association was found either ($HR_{T3vsT1} = 1.01$, 95CI%: 0.90–1.13); *P*-trend = 0.86 and ($HR_{T3vsT1} = 0.95$, 95CI%: 0.82–1.10); *P*-trend = 0.48, respectively. Model 2: Model 1 and further adjusted by: height (in cm), weight (in kg), education level (none, primary school, technical/professional school, secondary school, longer education, and unknown), smoking status (never, former, and current), waist circumference (low: <88 cm in women & <102 cm in men; high: ≥88cm in women & ≥102cm in men), physical activity (inactive, moderately inactive, moderately active, active, unknown/missing), lifetime alcohol consumption (as continuous variable), fibre (as continuous variable), calcium (very low: ≤743.68 mg/day; low: >743.68mg/day–<1,000 mg/day and normal: ≥1,000 mg/day)

For Model2, Wald tests showed no significant differences in the association between daily sex-specific nitrosyl-heme intake and the risk of colon versus rectal cancer, or between distal versus proximal colon cancer (Table 2). In women, there were significant differences between distal and proximal colon cancer but not between colon and rectal cancer (Table 3). Within this group, the crude/energy-adjusted model suggested a borderline association with colorectal cancer risk, which disappeared after

Table 1 Population characteristics according nitrosyl-heme intake in the European prospective investigation into cancer and nutrition study

		N (%)	Nitrosyl-heme intake (µg/d)		
			p25	Median	p75
Total		367,463	239.20	659.26	1,445.11
Sex					
	Men	109,120 (29.7)	379.73	1,006.80	2,047.18
	Women	258,343 (70.3)	208.56	559.25	1,205.15
Age (y)					
	<33	17,227 (4.7)	2.996	58.20	497.53
	≥33-<44	59,890 (16.3)	173.00	610.98	1,466.00
	≥44 - <54	139,629 (38.0)	305.73	746.0	1,554.68
	≥54- <64	121,426 (33.0)	300.00	729.27	1,518.21
	≥64	29,291 (8.0)	181.86	458.49	974.99
BMI (kg/m2)					
	<25	191,966 (52.2)	187.71	571.82	1,297.76
	≥25–30	127,652 (34.7)	301.60	742.35	1,568.57
	≥30	47,845 (13.0)	328.57	800.18	1,726.04
Country					
	France	67,403 (18.3)	406.71	883.86	1,689.43
	Italy	44,545 (12.1)	196.99	412.48	766.32
	Spain	39,989 (10.9)	242.00	603.77	1,218.80
	UK	75,416 (20.5)	21.40	181.86	431.69
	The Netherlands	36,539 (9.9)	348.74	797.21	1,519.65
	Germany	48,557 (13.2)	1,002.37	1,888.89	3,030.28
	Denmark	55,014 (15.0)	494.03	1,018.92	1,783.28
Waist circumference (cm)[a]					
	Normal	249,985 (68.0)	197.05	575.23	1,320.12
	High	69,886 (19.0)	341.49	803.62	1,672.99
	Missing/Unknown	47,592 (13.0)	409.89	896.97	1,716.81
Cambridge physical activity index (Met-h/week)					
	Inactive	76,536 (20.8)	201.03	532.98	1,208.60
	Moderately Inactive	125,365 (34.1)	239.71	654.83	1,419.98
	Moderately Active	88,121 (24.0)	261.95	715.68	1,531.78
	Active	70,697 (19.2)	263.37	745.71	1,603.73
	Missing/Unknown	6,744 (1.8)	307.80	780.75	1,559.92
Educational level					
	None	14,874 (4.0)	205.09	549.65	1,157.88
	Primary School	86,998 (23.7)	325.53	746.76	1,561.17
Technical, professional		80,035 (21.8)	286.08	753.23	1,617.91
	Secondary School	75,015 (20.4)	254.19	656.43	1,364.08
	Longer Education	93,882 (25.5)	165.00	603.65	1,464.21
	Not specified	16,659 (4.5)	61.19	309.78	707.92
Smoking status					
	Never	183,880 (50.0)	205.82	583.17	1,303.24
	Former	99,571 (27.1)	245.71	678.08	1,496.41
	Current	77,838 (21.2)	343.07	834.39	1,727.81
	Missing/Unknown	6,174 (1.7)	279.52	678.35	1,380.55
Alcohol lifetime (g/day)[b]					
	Non alcohol	24,396 (6.6)	171.43	442.83	942.86
	Never heavy	32,945 (89.7)	245.79	674.85	1,465.78
	Heavy	13,610 (3.7)	300.35	879.82	1,953.99
Family history of colorectal cancer					
	Yes	12,120 (30.5)	281.57	655.24	1,316.68
	No	9,300 (2.5)	286.14	664.64	1,319.19

Table 1 (continued)

		N (%)	Nitrosyl-heme intake (µg/d)		
			p25	Median	p75
	Not specified	246,043 (67.0)	220.79	662.18	1,521.6
Calcium (mg/day)[c]					
	Very Low	91,863 (25.0)	227.08	642.33	1,454.05
	Low	106,118 (28.9)	248.95	654.90	1,422.57
	Normal	169,482 (46.1)	240.00	673.44	1,454.52
Fibre (g/day)					
	<30	301,493 (82.0)	257.14	662.75	1,417.94
	≥30	65,970 (18.0)	141.91	642.01	1,583.51

[a]Waist circumference (cm) (normal: <88 cm in women & <102 in men; high ≥88cm in women & ≥102 in men; missing/unknown)

[b]Alcohol lifetime (g/day) (0=no alcohol in the past, i.e. at all ages in the past the alcohol use is 0; 1=never heavy (all past points in time g/d alcohol <60 (men) or <30 (women)) as the reference; 2=heavy alcohol use in the past (≥60 (men) or ≥30 (women) at least at one age in the past)

[c]Categorization of calcium intake was performed based on the population distribution in the calcium variable (continuous) and according to the daily intake recommendations found in the following document: https://ods.od.nih.gov/factsheets/Calcium-DatosEnEspanol/: (very low: ≤743.68 mg/day; low: >743.68mg/day–<1,000 mg/day and normal: ≥1,000 mg/day). *BMI* body mass index

Table 2 Multivariable HR (95% CI) of colorectal adenomas by site according to sex-specific tertile of nitrosyl-heme intake

	Tertiles of nitrosyl-heme intake[a,b]					
	HR (95% CI) T1	HR (95% CI) T2	HR (95% CI) T3	*p*-trend	Continuous (per 400 µg increment)[c]	AIC[f]
Colorectal Cancer						
N Cases	1,630	1,894	1,591		5,115	
Model1	Referent	1.01 (0.94–1.08)	1.03 (0.95–1.11)	0.52	1.01 (1.00–1.02)	63,976.9
Model2	Referent	0.99 (0.93–1.06)	1.01 (0.93–1.09)	0.87	1.00 (0.99–1.01)	63,806.8
Distal colon Cancer[d]						
N Cases	455	514	427		1,396	
Model1	Referent	1.02 (0.89–1.16)	1.02 (0.87–1.20)	0.77	1.00 (0.98–1.02)	17,405.9
Model2	Referent	1.01 (0.88–1.15)	1.02 (0.87–1.20)	0.83	0.99 (0.97–1.01)	17,338.5
Proximal colon Cancer[d]						
N Cases	487	531	410		1,428	
Model1	Referent	1.04 (0.91–1.19)	1.00 (0.85–1.17)	0.98	0.99 (0.97–1.01)	17,713.9
Model2	Referent	1.03 (0.90–1.17)	0.97 (0.83–1.14)	0.74	0.98 (0.96–1.01)	17,651.4
Colon Cancer[e]						
N Cases	1,018	1,131	908		3,057	
Model1	Referent	1.04 (0.95–1.14)	1.03 (0.93–1.15)	0.55	1.00 (0.99–1.02)	38,042.1
Model2	Referent	1.02 (0.93–1.12)	1.01 (0.90–1.13)	0.86	0.99 (0.98–1.01)	37,895.9
Rectal Cancer[e]						
N Cases	489	610	531		1,630	
Model1	Referent	1.06 (0.93–1.20)	0.97 (0.84–1.12)	0.64	1.01 (1.00–1.03)	20,665.2
Model2	Referent	1.04 (0.91–1.18)	0.95 (0.82–1.10)	0.48	1.01 (0.99–1.02)	20,639.3

Model 1 stratified by age and centre, and adjusted for sex and energy intake.

There were 233 and 184 overlapping/non-specified colon or in situ cancer cases in men and women, respectively.

[a]The analyses were run using sex-specific tertiles. Nitrosyl-heme (µg/day) for women: T1, 0–≤307.49; T2, >307.49–≤927.92; T3: >927.92; Nitrosyl-heme (µg/day) for men: T1, 0–≤560.80; T2, >560.80–≤1,620.57; T3:>1,620.57

[b]Person-years by nitrosyl-heme tertiles: T1, 1,784881.17 person-years; T2, 1,725064.45 person-years and T3, 1,595336.01 person-years

[c]Continuos variables: the HR (95% CI) corresponds to an increase of: 400 µg/day for nitrosyl-heme

[d]P homogeneity proximal vs. distal colon cancer = 0.16 for nitrosyl-heme iron intake

[e]P homogeneity colon vs. rectum cancer = 0.32 for nitrosyl-heme iron intake

[f]*AIC* Akaike information criterion

full multivariable adjustment. In men, no significant differences were found for any tumour subtypes (Table 3).

We tested for potential interactions between sex-specific tertiles of nitrosyl-heme intake and CRC risk; however, the tests for interactions were not statistically significant (Table 4). Analyses excluding participants with < 5 years of follow-up did not materially change the associations. In addition, excluding participants from the UK (health-conscious) from the regression models also did not materially change the findings (Supplementary

Table 3 Multivariable HR (95% CI) of colorectal adenomas by site and sex according to tertile of nitrosyl-heme intake

	Women				Tertiles of nitrosyl-heme intake[a,b,c,d]		Men					
	HR (95% CI) T1	HR (95% CI) T2	HR (95% CI) T3	*p*-trend	Continuous (per 400 µg increment)[c]	AIC[h]	HR (95% CI) T1	HR (95% CI) T2	HR (95% CI) T3	*p*-trend	Continuous (per 400 µg increment)[e]	AIC[h]
Colorectal Cancer												
N Cases	913	1,080	894		2,887		717	814	697		2,228	
Model1	Referent	1.01 (1.00–1.21)	1.11 (1.00–1.24)	0.045*	1.02 (1.00–1.03)	33,804.5	Referent	1.04 (0.93–1.16)	1.03 (0.90–1.17)	0.69	1.00 (0.99–1.02)	24,628.9
Model2	Referent	1.07 (0.98–1.18)	1.06 (0.94–1.18)	0.33	1.01 (0.99–1.02)	33,261.2	Referent	0.98 (0.88–1.09)	0.94 (0.82–1.08)	0.40	0.99 (0.98–1.01)	23,920.0
Distal colon Cancer[f]												
N Cases	244	289	242		775		211	225	185		621	
Model1	Referent	1.10 (0.92–1.32)	1.16 (0.95–1.43)	0.15	1.02 (0.99–1.03)	9,090.3	Referent	0.99 (0.81–1.22)	1.06 (0.82–1.35)	0.68	1.00 (0.97–1.02)	6,678.5
Model2	Referent	1.09 (0.91–1.31)	1.13 (0.91–1.40)	0.26	0.01 (0.98–1.05)	9,094.8	Referent	0.93 (0.76–1.14)	0.95 (0.74–1.23)	0.72	0.98 (0.96–1.01)	6,724.8
Proximal colon cancer[f]												
N Cases	297	353	261		911		190	178	149		517	
Model1	Referent	1.12 (0.95–1.31)	1.04 (0.86–1.27)	0.60	1.00 (0.96–1.03)	10,532.8	Referent	0.94 (0.75–1.18)	0.96 (0.72–1.26)	0.73	0.99 (0.96–1.02)	5,667,9
Model2	Referent	1.10 (0.93–1.29)	1.00 (0.82–1.21)	0.98	0.99 (0.95–1.02)	10,518.4	Referent	0.89 (0.71–1.12)	0.89 (0.67–1.17)	0.38	0.98 (0.95–1.01)	5,636.2
Colon Cancer[g]												
N Cases	581	692	542		1,815		717	814	697		1,242	
Model1	Referent	1.13 (1.00–1.26)	1.13 (0.99–1.30)	0.07	1.01 (0.99–1.03)	21,106.0	Referent	0.97 (0.84–1.13.84.13)	1.02 (0.85–1.21.85.21)	0.88	1.00 (0.98–1.02.98.02)	13,598.3
Model2	Referent	1.09 (0.96–1.23.96.23)	1.08 (0.94–1.24.94.24)	0.28	1.00 (0.98–1.05.98.05)	21,070.5	Referent	0.92 (0.79–1.06.79.06)	0.93 (0.78–1.11.78.11)	0.40	0.99 (0.97–1.01.97.01)	13,492.5
Rectal Cancer[g]												
N Cases	264	286	257		807		225	324			823	

Table 3 (continued)

	Women				Tertiles of nitrosyl-heme intake[a,b,c,d]		Men					
	HR (95% CI) T1	HR (95% CI) T2	HR (95% CI) T3	*p*-trend	Continuous (per 400 µg increment)[c]	AIC[h]	HR (95% CI) T1	HR (95% CI) T2	HR (95% CI) T3	*p*-trend	Continuous (per 400 µg increment)[e]	AIC[h]
Model1	Referent	0.99 (0.83–1.18.83.18)	1.03 (0.84–1.26.84.26)	0.75	1.03 (1.00–1.06.00.06)	9,621.1	Referent	1.23 (1.02–1.48.02.48)	1.10 (0.88–1.37.88.37)	0.48	1.00 (0.98–1.03.98.03)	9,285.0
Model2	Referent	0.97 (0.81–1.16.81.16)	0.99 (0.8–1.22.8.22)	0.90	1.03 (1.00–1.06.00.06)	9,639.9	Referent	1.17 (0.97–1.41.97.41)	1.02 (0.81–1.27.81.27)	0.97	1.00 (0.98–1.02.98.02)	9,257.7

[a]Nitrosyl-heme intake (µg/day) for women: T1, 0–≤307.49; T2, >307.49–≤927.92; T3: >927.92

[b]Person-years by nitrosyl-heme tertiles: T1, 1784881.17 person-years; T2, 1725064.45 person-years and T3, 1595336.01 person-years

[c]Nitrosyl-heme intake (µg/day) for men: T1, 0–≤560.80; T2, >560.80–≤1,620.57; T3:>1,620.57

[d]Person-years by nitrosyl-heme tertiles: T1, 1784881.17 person-years; T2, 1725064.45 person-years and T3, 1595336.01 person-years

For women:

Model 1 stratified by age and centre, and adjusted for energy intake

Model 2: Model 1 and further adjusted by: height (in cm), weight (in kg), education level (none, primary school, technical/professional school, secondary school, longer education, and unknown), smoking status (never, former, and current), waist circumference (low: <88 cm in women; high: >=88cm), physical activity (inactive, moderately inactive, moderately active, active, unknown/missing), lifetime alcohol consumption (as continuous variable), fibre (as continuous variable), calcium (very low: ≤ 747.61 mg/day; low: >747.61mg/day–<1,000 mg/day and normal: ≥1,000 mg/day)

[e]Continuos variables: the HR (95% CI) corresponds to an increase of: 400 mcg/day for nitrosyl-heme

[f]P homogeneity proximal vs. distal colon cancer = 0.02 for nitrosyl-heme iron intake

[g]P homogeneity colon vs. rectum cancer = 0.10 for nitrosyl-heme iron intake

There were 129 and 130 overlapping/non-specified colon or in situ cancer cases in women, respectively

[h]*AIC* Akaike information criterion

For men:

Model 1 stratified by age and centre, and adjusted for energy intake

Model 2: Model 1 and further adjusted by: height (in cm), weight (in kg), education level (none, primary school, technical/professional school, secondary school, longer education, and unknown), smoking status (never, former, and current), waist circumference (low: <102 cm in men; high: >=102cm in men), physical activity (inactive, moderately inactive, moderately active, active, unknown/missing), lifetime alcohol consumption (as continuous variable), fibre (as continuous variable), calcium (very low: ≤ 734.50 mg/day; low: >734.50 mg/day–<1,000 mg/day and normal: ≥1,000 mg/day)

[e]Continuos variables: the HR (95% CI) corresponds to an increase of: 400 µg/day for nitrosyl-heme

[f]P homogeneity proximal vs. distal colon cancer = 0.29 for nitrosyl-heme iron intake

[g]P homogeneity colon vs. rectum cancer = 0.10 for nitrosyl-heme iron intake

There were 66 and 51 overlapping/non-specified colon or in situ cancer cases in women, respectively

[h]*AIC* Akaike information criterion

Table 4 Interactions between sex-specific tertiles of nitrosyl-heme intake and potential effect modifiers in relation to colorectal cancer risk

Sex-specific tertiles of nitrosyl-heme intake[a,b]					
Colorectal Cancer	**Cases (%)**	**HR (95% CI) T1**	**HR (95% CI) T2**	**HR (95% CI) T3**	***p*-interaction**
N Cases	5,115	1,630	1,894	1,591	
BMI (kg/m^2)[c]					
Model2	5,115 (100.0)	Referent	1.43 (0.93–2.19.93.19)	1.00 (0.65–1.54.65.54)	0.1457
Fibre (g/day)[c]					
Model2	5,115 (100.0)	Referent	1.18 (0.96–1.45.96.45)	1.12 (0.89–1.40.89.40)	0.3452
Calcium (mg/day)[c]					
Model2	5,115 (100.0)	Referent	1.08 (0.95–1.24.95.24)	1.01 (0.87–1.17.87.17)	0.8499
Cambridge physical activity index (Met-h/week)					
Model2					0.8683
Inactive	1,288 (25.2)	Referent	0.94 (0.82–1.08.82.08)	0.98 (0.83–1.16.83.16)	
Moderately Inactive	1,686 (33.0)	Referent	0.98 (0.87–1.11.87.11)	0.94 (0.81–1.09.81.09)	
Moderately Active	1,061 (20.7)	Referent	1.01 (0.86–1.18.86.18)	0.95 (0.79–1.15.79.15)	
Active	989 (19.3)	Referent	1.02 (0.86–1.21.86.21)	0.98 (0.81–1.19.81.19)	
Missing/Unknown	91 (1.8)	Referent	1.18 (0.70–1.99.70.99)	0.79 (0.42–1.50.42.50)	
Smoking Status					
Model2					0.7176
Never	2,119 (41.4)	Referent	1.04 (0.96–1.17.96.17)	1.02 (0.89–1.16.89.16)	
Former	1,688 (33.0)	Referent	0.95 (0.84–1.08.84.08)	0.96 (0.83–1.11.83.11)	
Current	1,240 (24.2)	Referent	1.02 (0.88–1.18.88.18)	0.95 (0.80–1.12.80.12)	
Missing/Unknown	68 (1.3)	Referent	0.73 (0.37–1.41.37.41)	0.91 (0.44–1.87.44.87)	
Energy intake (kcal/day)[c]					
Model2	5,115 (100.0)	Referent	1.22 (0.96–1.56.96.56)	1.18 (0.91–1.53.91.53)	0.259
Alcohol lifetime (g/day)[cd]					
Model2	5,115 (100.0)	Referent	0.96 (0.73–1.26.73.26)	0.84 (0.60–1.17.60.17)	0.7544

[a]The analyses were run using sex-specific tertiles. Nitrosyl-heme intake (µg/day) for women: T1, 0–≤307.49; T2, >307.49–≤927.92; T3: >927.92; Nitrosyl-heme intake (µg/day) for men: T1, 0–≤560.80; T2, >560.80–≤1,620.57; T3:>1,620.57

[b]Person-years by nitrosyl-heme tertiles: T1, 1,784881.17 person-years; T2, 1,725064.45 person-years and T3, 1,595336.01 person-years

Model 1 stratified by age and centre, and adjusted for sex and energy intake

Model 2: Model 1 and further adjusted by: height (in cm), weight (in kg), education level (none, primary school, technical/professional school, secondary school, longer education, and unknown), smoking status (never, former, and current), waist circumference (low: <88 cm in women & <102 cm in men; high: >=88cm in women &>=102cm in men), physical activity (inactive, moderately inactive, moderately active, active, unknown/missing), lifetime alcohol consumption (as continuous variable), fibre (as continuous variable), calcium (very low: ≤743.68 mg/day; low:>743.68mg/day–<1,000 mg/day and normal: ≥1,000 mg/day)

[c]Expressed as a continuous variable. This approach preserves the full variability of the covariates and maximises statistical power while avoiding arbitrary cut-offs. **d** Alcohol lifetime (g/day) (0=no alcohol in the past, i.e. at all ages in the past the alcohol use is 0; 1=never heavy (all past points in time g/d alcohol <60 (men) as the reference;2=heavy alcohol use in the past (≥60 (men) at least at one age in the past)

BMI body mass index

All *p*-interactions were >0.05

Table 3). In addition, when examining PM intake across different follow-up periods (2 to 20 years), the associations with CRC risk remained stable over time, with no indication of time-dependent effects (Supplementary Table 4).

Discussion

This study is the first to extrapolate nitrosyl-heme values for a wide range of European PMs using data from nitrosyl-heme levels in Spanish PMs. In this prospective European cohort, no significant association was observed between nitrosyl-heme intake and CRC risk, either overall or by tumour subtypes. However, sex-stratified subanalysis showed significant differences between distal and proximal colon cancer in women.

Our findings contrast with those of *Bastide* et al. [9], who have examined the relationship between nitrosyl-heme intake and CRC risk. *Bastide's* study found a significant positive association between nitrosyl-heme intake and proximal colon cancer (comparing the highest to the lowest quartile) among women in a French cohort. However, in our study, although the observations did not reach statistical significance, a trend towards a higher risk of distal colon cancer in women was observed, as well as a trend towards a greater risk of both colon and CRC, though these associations diminished at higher intake levels. Several methodological differences may account for our differing results. *Bastide* et al. used a fixed coefficient for nitrosyl-heme content across all PM products, potentially resulting in less precise estimations. In contrast, our approach involved direct laboratory measurements of nitrosyl-heme in Spanish PMs, to provide more accurate assessments. One possible explanation for the significant differences observed in these tumour subtypes in women may be linked to variations in hormonal and metabolic pathways compared to men [18].

Despite potential mechanisms linking nitrosyl-heme intake to CRC risk [19–24], our findings suggest nitrosyl-heme intake in this cohort might not significantly increase CRC risk. Although the EPIC study has previously reported a positive association between CRC risk and PM intake across all participating countries [25], the overall lack of a significant association observed in our analysis suggests that other factors may play a more influential role in colorectal carcinogenesis. This null association between nitrosyl-heme intake and CRC risk could be explained by the interplay of multiple dietary components. Protective nutrients such as fibre and calcium may mitigate potential harmful effects of nitrosyl-heme, while other compounds, including nitrates/nitrites and carcinogens formed during certain cooking methods, may further modulate the risk. In addition, the absence of information on meal timing and eating occasions could obscure potential interactions among these dietary

factors. Altogether, these findings highlight the complexity of dietary influences on CRC risk and the need for further research to disentangle these interactions.

Our study has some limitations. First, dietary intakes were collected only once at baseline which may not fully capture changes over time. Second, the EPIC study sample is not representative of the entire European population. Additionally, the dietary data from the 1990s may not reflect consumption trends, which could affect the accuracy of PM intake reported after years of follow-up. However, issues that could affect the representativeness of the current samples were considered, such as changes in ingredients or procedure. Finally, although we aimed to provide an accurate estimation of nitrosyl-heme content in European PMs, no validation procedure from biomarkers or by comparison to 24-h dietary recall were already available. Nevertheless, the large number of PMs analysed through direct extrapolation strengthens our findings.

This study's strengths include its prospective design within the EPIC study, minimising selection and recall biases. Its large sample size and extended follow-up bolster the reliability and applicability of findings across diverse European populations. Additionally, robust adjustments for numerous confounders related to diet, lifestyle, and health status were incorporated into the statistical models. Laboratory measurements using the *HPLC* method to quantify nitrosyl-heme levels in Spanish PMs further strengthen the study's methodology.

Conclusion

Given the biological plausibility between nitrosyl-heme and CRC risk, and despite not finding an association, these findings emphasise the need for further research, including new data on nitrosyl-heme in typical/regional PMs from different countries, repeated measurements of PM intake in longitudinal studies, the identification of biomarkers of PM intake and a better understanding of the role of hormonal and metabolic pathways according to sex, to elucidate their involvement in carcinogenesis.

Abbreviations

Cis	Confidence intervals
CRC	Colorectal cancer
EPIC	European prospective investigation into cancer and nutrition
ENDB	EPIC nutrient database
FFQ	Food-frequency questionnaires
HRs	Hazard ratios
IARC	International agency for research on cancer
PA	Physical activity
PM	Processed meat
RCS	Restricted cubic splines

Supplementary Information

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Supplementary Material 1.

Disclaimer

Where authors are identified as personnel of the International Agency for Research on Cancer/World Health Organization, the authors alone are responsible for the views expressed in this article and they do not necessarily represent the decisions, policy or views of the International Agency for Research on Cancer/World Health Organization.

Authors' contributions

PJ, LL-B and LRB designed research; PJ, LL-B and LR-B conducted research; LL-B and LR-B analysed data; and LR-B wrote the paper; all authors did manuscript revision. PJ had primary responsibility for final content. All authors read and approved the final manuscript.

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Data availability

EPIC data and biospecimens are available for investigators who seek to answer important questions on health and disease in the context of research projects that are consistent with the legal and ethical standard practices of the International Agency for Research on Cancer (IARC), WHO, and the EPIC centres. The primary responsibility for accessing the data, obtained in the frame of the present publication, belongs to the EPIC centres that provided them. Access to EPIC data can be requested to the EPIC Steering Committee, as detailed in the EPIC-Europe Access Policy.

Declarations

Ethics approval and consent to participate

The study was conducted in accordance with the Declaration of Helsinki, and was approved by the Ethics Committee of the International Agency for Research on Cancer (IARC) and local ethical committees pertaining to EPIC Centers.

Consent for publication
Written informed consent was obtained from all participants involved in the study.

Competing interests
The authors declare no competing interests.

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