# Heme iron from meat and risk of colorectal cancer: a meta-analysis and a review of the mechanisms involved

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## Abstract

Red meat and processed meat intake is associated with a risk of colorectal cancer, a major cause of death in affluent countries. Epidemiological and experimental evidence supports the hypothesis that heme iron present in meat promotes colorectal cancer. This meta-analysis of prospective cohort studies of colon cancer reporting heme intake included 566,607 individuals and 4,734 cases of colon cancer. The summary relative risk of colon cancer was 1.18 [95%C.I.: 1.06-1.32] for subjects in the highest category of heme iron intake compared with those in the lowest category. Epidemiological data thus show a suggestive association between dietary heme and risk of colon cancer. The analysis of experimental studies in rats with chemically-induced colon cancer showed that dietary hemoglobin and red meat consistently promote aberrant crypt foci, a putative pre-cancer lesion. The mechanism is not known, but heme iron has a catalytic effect on (i) the endogenous formation of carcinogenic N-nitroso compounds and (ii) the formation of cytotoxic and genotoxic aldehydes by lipoperoxidation. A review of evidence supporting these hypotheses suggests that both pathways are involved in heme iron toxicity.

## Introduction

Cancer of the colon and rectum, taken together, are the third most common type of cancer worldwide (1). In most publications, colon and rectal cancer are studied together and the term colorectal cancer (CRC) is used, which we also use here, except when the publications refer specifically to colon or rectal cancer. CRC is the second most common cause of cancer death in affluent countries. Dietary modifications might reduce this cancer burden by up to 70% (2). Three recent meta-analyses showed that total meat intake is not related to risk but that intake of red or processed meat is associated with a modest, but significant risk of CRC (3-5). Processed meat intake appears to be more closely linked with the risk of CRC than fresh red meat intake. In its 2007 report, the World Cancer Research Fund panel recommended that one should limit intake of red meat and avoid processed meat (1).

Several mechanisms may explain the relationship between the risk of CRC and the intake of red or processed meat. First, meat cooked at high temperature contains mutagenic heterocyclic amines. But heterocyclic amines might not be major players in CRC risk, since (i) consumption of chicken is a major contributor to intake of heterocyclic amines, but is not associated with the risk (6), and (ii) doses of heterocyclic amines that induce cancer in animals are 1000 to 100,000 times higher than the dose ingested by humans (7). A second

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hypothesis suggests that the high saturated fat content of red and processed meat increases the risk of CRC. But several studies, including a recent meta-analysis, showed no effect of saturated fat on colorectal carcinogenesis (8-11). A third hypothesis concerns the carcinogenic N-nitroso compounds (NOC), which can be formed in the gastrointestinal tract by N-nitrosation of peptide derived amines or amides. The role of NOC in human cancer is discussed below. Other more unlikely hypotheses involve the high protein, cholesterol and salt content of red or processed meat. For a review of all these mechanisms, see (12).

Sesink et al. suggested that heme iron, in the form of hemin (Chloroprotoporphyrin IX iron(III)) a ferric form of heme, may explain the link between the risk of colon cancer and red meat intake, and the lack of a link with white meat intake (13). Epidemiological and experimental evidence support heme toxicity. Heme consists of an iron atom contained in the center of a large heterocyclic organic ring called a porphyrin (Fig. 1). Heme is included in so-called hemoprotein i.e. hemoglobin, myoglobin (both involved in the oxygen supply), and in cytochromes (which catalyze electron transfer reactions). Red meat (such as beef, veal, lamb, mutton, pork and offal) owes it dark red color to the presence of a high concentration of myoglobin, and the heme content of red meat is 10-fold higher than that of white meat (such as chicken) (14). In processed red meat, heme iron is nitrosylated, because curing salt contains nitrate or nitrite (Fig. 1) (12).

The aims of the present mini-review were (i) to conduct a meta-analysis of epidemiological cohort studies on heme intake and the risk of colon cancer, (ii) to review experimental evidence supporting the above-cited heme hypothesis, (iii) to understand the mechanism of action of heme in carcinogenesis.

## Heme iron intake and risk of colon cancer: a meta-analysis of prospective cohort studies

The objective of this part of the review was to assess, through meta-analysis, the magnitude of the relation between heme iron intake and colon cancer. As most studies do not report data on rectal cancer, we decided to limit our analysis to colon cancer. The methodological procedure is described in the supplemental material to this article.

The characteristics of the five prospective cohort studies included in the meta-analysis are summarized in supplemental data (Table S1). This meta-analysis included data on 566,607 individuals and 4,734 cases of colon cancer. Although one cohort study found no association between heme and cancer (15), three found that a high intake of heme iron was linked with a higher risk of colon cancer (16-18), and one found a positive, but not significant, association between heme iron and colon cancer (19) (Fig. 2). In the Lee *et al.* study, the relative risk (RR) for both proximal and distal colon was 1.53 (95% Confidence Interval CI: 0.99-2.38). In the Balder *et al.* study, the association was positive in the two genders combined (RR=1.35, 95% CI: 1.03-1.77) (17). The summary RR of colon cancer in all five studies was 1.18 (95% (CI): 1.06-1.32) for subjects in the highest category of heme iron intake compared with those in the lowest category (Fig. 2). This meta-analysis showed a consistent association between high intake of heme iron and increased risk of colon cancer.

Two studies out of five considered calcium in the adjustments for the RR (16-18), and showed the strongest association between heme iron and colon cancer. This makes sense,

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since calcium inhibits heme-induced cytotoxicity, colonic epithelial hyperproliferation, and promotion of chemically induced carcinogenesis in animal models (20-22) (see below).

Two studies we excluded from the meta-analysis found similar results. An ecological study found a direct correlation between the dietary iron index and colon and rectal cancer (23). Ferrucci *et al.* observed a positive, but not significant, association between heme iron in diet and colorectal adenoma.

The present meta-analysis is the first to examine the relation between heme iron and colon cancer. But this study also has its limitations; first it includes only five cohort studies, and the way heme intake was measured differs in each study. Lee *et al.* and Larsson *et al.* calculated heme iron content in the diet by applying a factor of 0.4 to the total iron content of all meat items which essentially is reporting an overall red meat effect (16, 18). Balder *et al.* multiplied the heme iron content of each meat item by the mean daily intake of the relevant food item, estimated from the Dutch Food Composition Database (17), but the two methods yielded similar results (15). Cross *et al.* developed a new heme iron database based on measured values in conjunction with a detailed meat cooking questionnaire (19).

In conclusion, this meta-analysis showed a significant and consistent but modest increase in the risk of colon cancer associated with high heme iron intake. This study should be pursued by future prospective cohort studies, but this epidemiological result is in line with experimental *in vivo* results detailed below.

## Experimental evidence of colorectal cancer promotion by heme iron

Sawa *et al.* showed that dietary hemoglobin produces lipid peroxyl radicals and increases the incidence of nitrosomethylurea-induced colon cancer in rats fed polyunsaturated fat (24). Sesink *et al.* studied the effect of hemin-supplemented diet in non-initiated rats. Dietary hemin increases fat peroxidation and cytotoxic activity of fecal water, and epithelial proliferation by 70% (13). In hemin, the iron atom is stabilized by a freely exchangeable chloride. Pierre *et al.* also showed that hemin and hemoglobin increase the number of azoxymethane-induced aberrant crypt foci, which are putative preneoplastic lesions, in the colon of rats (21). In contrast with hemin, dietary hemoglobin does not increase the cytotoxicity of fecal water, and it is less potent than hemin in promoting colon carcinogenesis. Hemoglobin may be a suitable substitute for myoglobin in nutritional experiments with animal model, and a model agent for studies on the cytotoxicity of red meat (21).

Pierre *et al.* also fed three types of meat with different heme content (chicken, beef, and blood sausage) to rats treated with azoxymethane and fed a low-calcium diet (25). This study was the first to show that dietary meat can promote colon carcinogenesis, and that the effect depends on the heme concentration. The results of this study of meat contrast with those of several earlier studies, where red-meat based high-calcium diets failed to promote colon carcinogenesis, indicating probable protection by calcium (26). Subsequently, Pierre *et al.* tested the hypothesis, suggested by epidemiology, that nitrosyl heme in processed meat was more toxic than native heme in fresh meat (27). Cured meat can indeed promote colon carcinogenesis in rats (27). Dietary hemin, but not hemoglobin, could be used as a model agent to mimic the effects of processed meat in rats (27). In a recent study, Pierre *et al.* demonstrated that the nitrosylation of heme was a key event in the promoting effect of processed meat in rats (28).

Analysis of the results of experimental studies of rats with chemically-induced colon

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cancer (21, 22, 25, 29), showed that the global standardized effect size for number of aberrant crypt foci per colon was 1.73 [95%C.I.: 1.33-2.14] in rats given dietary heme iron in hemoglobin or beef meat, compared with control rats. The logistic regression approach showed a significant correlation between the number of aberrant crypts per colon and the concentration of heme in the diet (p-value=0.02) (see methods and figure in supplemental data). This experimental evidence that heme iron promotes carcinogenesis in rats is consistent with epidemiological evidence. Heme promotion may explain why the intake of red and processed meat is associated with a risk of colorectal cancer.

## Possible mechanisms of heme toxicity in the gastrointestinal tract

The mechanisms implicated in the promotion of colorectal cancer by heme are poorly understood. The mechanistic hypotheses are based on the catalytic effect of heme iron on (i) the formation of NOC and (ii) the formation of lipid oxidation endproducts.

### Heme iron catalyzes N-nitrosation

NOC are formed by N-nitrosation of amines and amides, produced primarily by bacterial decarboxylation of amino acids in the presence of a nitrosating agent (30). There was no *a priori* reason to think that nitrosation would require heme iron. The structure of nitrosamine is shown in Figure 1. NOC can be detected by thermal energy analysis following the release of nitric oxide from biological samples. This analytical procedure comprises nitrosyl iron and S-nitrosothiols in addition to nitrosamines and nitrosamides, which are collectively referred to as Apparent Total N-nitroso compounds (ATNC) (31).

*Animal and human studies*

Bacon-fed rats had a fecal concentration of ATNC 10 to 20 times higher than control rats (32). In addition, mice fed a diet of hot-dogs (18 %), had 4-5 times more ATNC, and mice fed a beef diet had 2-3 times more ATNC in their feces than controls fed no meat (33, 34).

Human volunteers given a high red meat diet excreted much more ATNC in their stools than controls given no or little red meat, or only white meat (31, 35, 36). The fecal concentration of ATNC was 60 times higher in volunteers given cured red meat than in volunteers given a vegetarian diet (37). Heme iron, and not inorganic iron or meat proteins, may be responsible for the nitrosation observed in the gut of volunteers fed red meat (38).

*Nature of ATNC*

A red meat diet increased nitrosyl iron and nitrosothiols in ileal outputs and in stools of volunteers, compared with a vegetarian diet, suggesting that these compounds contribute significantly to ATNC (39, 40). Nitrosothiols are rapidly formed from nitrite and thiol groups at low pH in the stomach and can be precursors for the formation of nitrosyl heme and NOC in the gut (39). The strong correlation between fecal nitrosyl iron and fecal heme suggests that nitrosyl heme is the main source of nitrosyl iron (39). Moreover, ATNC precursors from hot dogs were partially purified and separated by HPLC (41). One fraction was identified as 1-deoxy-N-1-glucosyl glycine by mass spectrometry, and the nitrosated fraction was shown to be mutagenic by the Ames test (41).

*Carcinogenicity of nitrosated compounds*

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The carcinogenicity of ATNC formed in the gut after eating heme from red or processed meat is unknown. Parnaud *et al.* found no initiation or promotion of preneoplastic lesions by ATNC in the colon of rats fed a bacon-based diet (32). Kunhle *et al* speculated that nitrosyl iron compounds and nitrosothiols may contribute to the tumorogenic potential of the diet (39). By contrast, in a commentary on Kunhle's article, Hogg speculated that the sequestration of the "nitrosating potential" of the diet as nitrosothiol or as nitrosyl iron may be a protective mechanism that would limit the formation of DNA alkylating agents (42).

However, several arguments suggest that ATNC may be important genotoxins. First, most NOC like nitrosamines, nitrosamides, and nitrosoguanidines, can yield alkylating agents during metabolism, and cause DNA damage. For instance N-methyl-N-nitrosurea intrarectally perfused induced G→A transitions in K-*ras* in 30% of rat colon carcinoma (43). In addition, nitrosated glycine derivatives reacted with DNA to give rise to promutagenic and toxic adducts including $O^6$-methylguanine and $O^6$-carboxymethylguanine (44). $O^6$-carboxymethylguanine adducts were found in stool exfoliated colonocytes from volunteers eating red meat, with a correlation between the level of adducts and of fecal ATNC, suggesting that ATNC are genotoxic (45). Moreover, potassium diazoacetate, a stable form of nitrosated glycine, was shown to induce mutations in the p53 gene in a functional yeast assay (46). The patterns of mutations were similar to the patterns observed in human colon tumors. This supports the hypotheses that nitrosation of compounds related to glycine contributes to p53 mutations in humans, and that $O^6$-carboxymethylguanine adducts in exfoliated colorectal cells are related to CRC (46).

**Heme iron catalyzes the oxidation of polyunsaturated fats**

The polyunsaturated fatty acid residues of phospholipids are extremely sensitive to oxidation. Lipid peroxidation is initiated by free-radical attack of membrane lipids and is catalyzed by heme with the following reaction: LOOH (lipid hydroperoxide) + Fe-ligand (heme) → LOOFe ligands → LO˙ (lipid alkoxy radical) + ˙OFe ligands (heme oxiradical) (47). The initial products of unsaturated fatty acid oxidation are lipid hydroperoxides, but they are relatively short lived. They are either reduced by glutathione peroxidase to unreactive fatty acid alcohols or they react with metals to produce a variety of reactive compounds like epoxides and aldehydes. The major aldehyde products of lipid peroxidation are malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) (48). These dietary lipid oxidation end products are risk factors for several human diseases (for review, see (49, 50)).

*Malondialdehyde (MDA)*

MDA is formed by oxidation of polyunsaturated fatty acids with two or more double bonds. MDA-induced DNA damage is mutagenic in bacterial, mammalian and human cells (51-53). MDA reacts with DNA to form adducts with deoxyguanosine, deoxyadenosine and deoxycytidine (for review, see (54)). The major DNA adduct formed by reaction of MDA with DNA is 1,$N^2$–malondialdehyde-deoxyguanosine ($M_1dG$). $M_1dG$ was detected in colorectal biopsies from normal mucosa of 162 participants in the United Kingdom FlexiScope Sigmoidoscopy Screening Trial and the EPIC study (55). The level of this adduct was modulated by dietary and life-style habits, and there is to higher M1dG levels in subjects with adenoma compared with adenoma-free subjects (P<0.005) (55).

*4-hydroxynonenal (4-HNE)*

In contrast with MDA, 4-HNE is weakly mutagenic but appears to be the main toxic product of lipid peroxidation (Fig. 1). 4-HNE has powerful effects on signal transduction pathways and some of its effects appear to be independent of DNA damage (48). Indeed, 4-HNE present in fecal water can induce apoptosis and necrosis of human colon carcinoma cells

through caspase 3 activation (56). Mutations in the *adenomatous polyposis coli* (*Apc*) gene on the chromosome 5q21 locus are considered to be one of the earliest events in the initiation of CRC (57). Moreover, *Apc* mutation was shown to reduce the level of caspase 3, 7 and 9 in mouse colonocytes, leading to resistance to apoptosis (58). An intestinal cell line derived from C57BL/6J mice (*Apc* +/+) and Min mice (*Apc* Min/+) retained the heterozygous Apc genotype and the disordered actin cytoskeleton network for the *Apc* Min/+ cell line (59, 60). By exposing this cell line to fecal water of heme-fed rats or to 4-HNE, Pierre *et al.* showed that apoptosis was suppressed in *Apc* Min/+ cells (61). The heterozygote *Apc* mutation is thus a strong selective advantage for colonic cells exposed to a lipoperoxidation-related genotoxic environment such as excess heme iron or 4-HNE (61).

In summary, heme catalyzes the formation of ATNC and of lipid oxidation end products, which may explain the promoting effects of red and processed meat on CRC. However, the pro-carcinogenic effect of heme can be inhibited by several molecules. First, calcium salts and chlorophyll can precipitate heme molecules and inhibit the cytotoxic and hyperproliferative effect of heme in the rat epithelium (17, 20-22, 62, 63). Moreover, the endogenous formation of ATNC is inhibited by vitamin C and E, and lipoperoxidation is inhibited by several polyphenols like quercetin, α-tocopherol or red wine polyphenols (64-68). The catalytic effects of heme and its inhibition are summarized in Figure 3.

## Conclusion

CRC is the leading cause of cancer death among non-smokers in affluent countries, and its prevention is thus a major goal for public health. Epidemiological studies demonstrate a modest but significant and consistent relation between red meat and processed meat intake and CRC risk. The dietary recommendations are to reduce red meat intake, and to avoid processed meat intake (1). However meat is an important source of proteins, providing all essential amino acids, and it is an excellent source of iron and zinc. Iron deficiency is the most widespread nutritional disorder in the world, especially among children and premenopausal women, and results in iron deficiency anemia (1). Knowledge of the mechanism of CRC promotion by meat may allow an alternative prevention strategy to be developed: inhibiting red and processed meat toxicity instead of stopping meat intake. Among the hypotheses explaining the association between meat intake and the risk of CRC, the effect of heme iron is supported by both epidemiological (Fig. 2) and experimental evidence (supplemental data Fig. S1). Several mechanisms may explain the effect of heme on CRC, and the two major hypotheses are that 1) heme catalyzes the endogenous formation of ATNC, and 2) heme catalyzes the peroxidation of dietary fats (Fig. 3). Calcium salts, chlorophyll, vitamin C and several polyphenols, may reduce these deleterious effects of heme. Specific recommendations might be made, for example "eat a yogurt after your steak". Moreover, vitamins or polyphenols could be added during the curing process. Ascorbic acid is already added during the processing of processed meats specifically to inhibit the formation of volatile NOC in the meat (69). We expect that this will reduce the risk of CRC without losing the benefit and the pleasure of eating meat.

## Acknowledgments

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**<u>Figure legends</u>**
**Figure 1: Structure of molecules cited in the review**
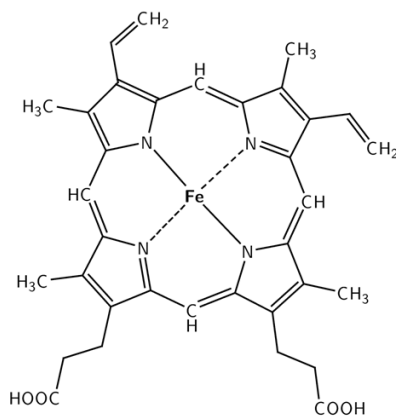
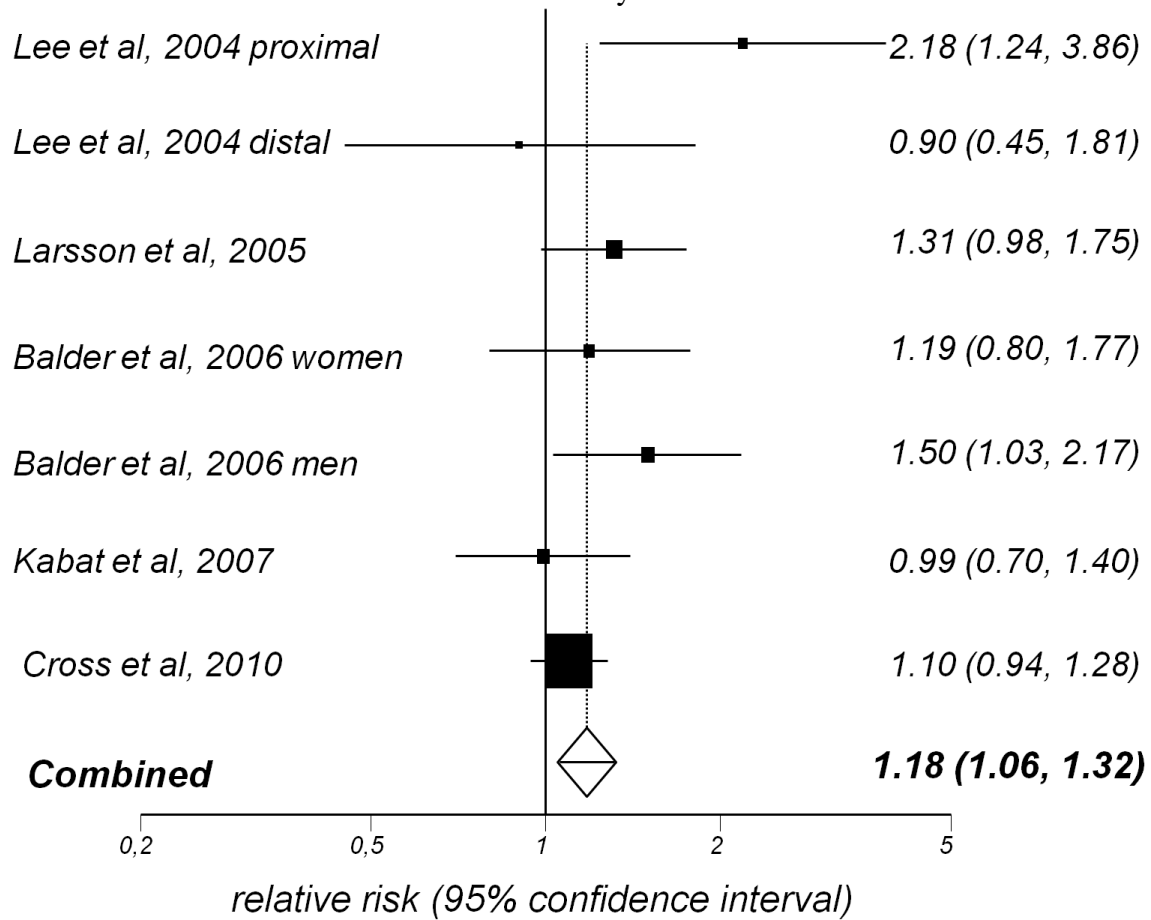

**Heme**
iron (II) protoporphyrin IX
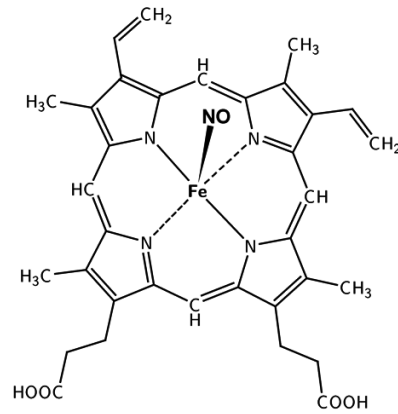
**Nitrosyl heme**
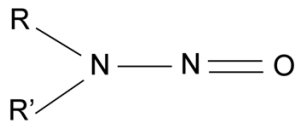
**Nitrosamine**
R, R' = alkyl or aryl
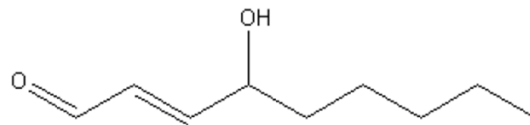
**4-hydroxynonenal (4-HNE)**

**Figure 1: Structure of molecules cited in the review**

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**Figure 2: Relative risks of colon cancer in prospective cohort studies, comparing the highest with the lowest category of heme iron consumption.**
Studies are ordered by year of publication. Squares represent study-specific RR and the size of squares is proportional to the statistical weight that each contributed to the summary estimate of relative risk (percentage weight of each study: Lee *et al*, 2004 proximal: 6.6%; Lee *et al*, 2004 distal: 4.6%; Larsson *et al*, 2005: 18%; Balder *et al*, 2006 women: 11.7%; Balder *et al*, 2006 men: 12.86%; Kabat *et al*, 2007: 14.2%; Cross *et al*, 2010: 32%). Horizontal lines represent 95% CIs. The diamond represents the summary estimate of the relative risk of all studies included in the meta-analysis.

| | |
|---|---|
| Lee et al, 2004 proximal | 2.18 (1.24, 3.86) |
| Lee et al, 2004 distal | 0.90 (0.45, 1.81) |
| Larsson et al, 2005 | 1.31 (0.98, 1.75) |
| Balder et al, 2006 women | 1.19 (0.80, 1.77) |
| Balder et al, 2006 men | 1.50 (1.03, 2.17) |
| Kabat et al, 2007 | 0.99 (0.70, 1.40) |
| Cross et al, 2010 | 1.10 (0.94, 1.28) |
| **Combined** | **1.18 (1.06, 1.32)** |

0,2    0,5    1    2    5

*relative risk (95% confidence interval)*

Test for heterogeneity: Cochran Q = 8.95; p-value = 0.18

**Figure 2: Relative risks of colon cancer in prospective cohort studies, comparing the highest with the lowest category of heme iron consumption**

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**Figure 3: Catalytic effects of heme on the formation of ATNC and lipid peroxidation, and their inhibition. Consequences for the development of colorectal cancer**
Heme catalyzes the formation of apparent N-nitroso compounds (ATNC) and lipid peroxidation endproducts, which partially explains the promoting effect of red and processed meat on colorectal cancer. The catalytic effects of heme can be inhibited by trapping the heme (calcium, chlorophyll). The endogenous formation of ATNC is inhibited by vitamin C and E, and it appears that polyphenols can inhibit lipid peroxidation.
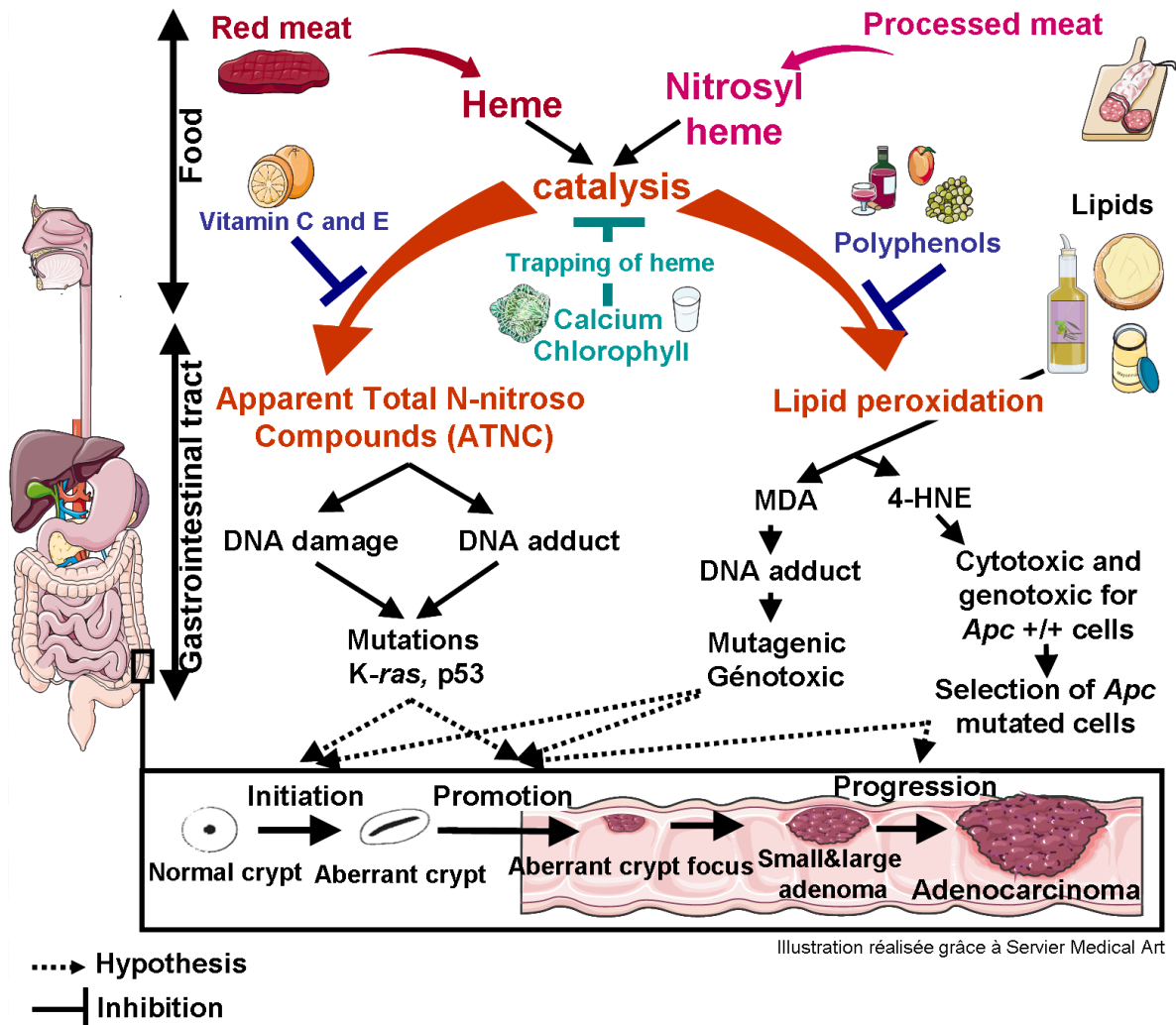


Figure 3: Catalytic effects of heme on the formation of ATNC and lipid peroxidation, and their inhibition. Consequences for the development of colorectal cancer

# Supplemental data

**Heme iron intake and risk of colon cancer: a meta-analysis of prospective cohort studies**

First, we selected prospective studies that assessed the relationship between heme iron intake and CRC. As minimum criteria, studies had to (i) use a prospective study design, and (ii) provide quantification of risks, including confidence intervals. Studies were identified by searching electronic databases (Medline, Google Scholar, The Cochrane Library and Web of Science) for articles published between 1970 and April 2010, using the search terms heme, haem, iron, heme iron, haem iron, colorectal, colon, rectal, cancer, prospective, cohort and exploded variants. References in the retrieved publications were checked for any other pertinent studies. Publications on the relation between red meat intake and colon cancer risk that did not take heme iron into account were excluded. We identified seven publications that reported results of epidemiological studies on heme iron intake related to the risk of colon, rectal or colorectal cancer (1-7). Two publications were excluded because they were not prospective cohort studies (5, 6). The remaining five publications were included in the meta-analysis.

We extracted the following data from each publication: the first author's last name, the year of publication, the country in which the study was performed, the sample size, the type of population, the age of the participant on entry in the cohort, the number of years of follow-up, the method of assessment of heme iron content in the diet, the categories of heme iron intake, the variable controlled for in the multivariate model, and the relative risk (RR) and 95% CI for colon cancer associated with heme iron intake. We had to limit the meta-analysis to colon cancer because data for colorectal cancer were not reported in two studies (1, 2). From each study, we extracted the adjusted RR of high versus low quintile of heme iron intake. The characteristics of the five prospective cohort studies included in the meta-analysis are listed in Table 1.

For the statistical analysis, we used the reported RR as a measure of association between heme iron intake and the risk of colon cancer. Reported RRs and corresponding confidence intervals (CIs) were transformed into their natural logarithms and weighted by the inverse of variance. For the study that provided separate RRs for proximal and distal colon cancer (1), and for men and women (3), we also presented the pooled RR, weighted by the inverse of variance to obtain a value for colon (distal and proximal) and for both genders respectively (data not shown). Heterogeneity among studies was assessed using Cochran's Q test (8). The possibility of a publication bias was assessed by funnel analysis. Statistical analyses were performed using StatDirect statistical software (version 2.7.7).

**Meta-analysis and quantitative review of experimental studies**

To assess the effect of heme iron on the incidence of preneoplastic lesions in carcinogen-induced rats, we evaluated a global standardized effect size from (9-12) with the endpoint "number of aberrant crypts per colon". We excluded groups fed a diet with additives (polyphenols, calcium) in order to study only the effect of heme. We excluded the hemin-fed groups because the results were not comparable with the other groups (hemoglobin and red meat). Although hemin is a good model for processed meat, it does not appear to mimic red meat properly (9, 11). We also excluded groups fed diets with chicken or black pudding, because there is almost no heme in chicken breast, and the heme concentration in blood pudding appeared to be too high to compare with other groups (10). We used standardized effect size, with continuous criteria, which enables small differences among the studies (e.g., choice of carcinogen) to be taken into account. Statistical analyses were performed using
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Revman 5, the Cochrane IMS statistical software. There was no heterogeneity among groups (p-value = 0.6), so a standard fixed effect was used. The findings of the present analysis revealed a consistent association between intake of hemoglobin and beef and an increase in the number of aberrant crypts per colon, with a global standardized effect size of 1.73 [1.33-2.14] (Fig. 1).

We then performed a logistic regression using R (http://www.r-project.org/), version 2.10.1. This analysis revealed a significant association between the number of aberrant crypts per colon and the concentration of heme in the diet (p-value=0.02). The R-squared was 0.62, which means that 62% of aberrant crypt foci variability was explained by the concentration of heme in the diet.
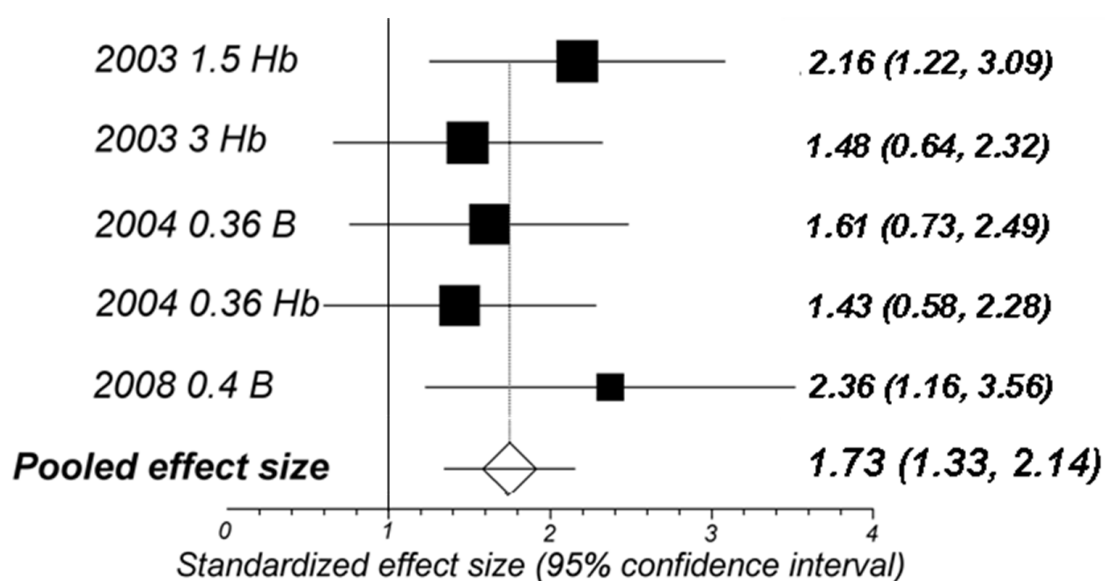


Test for heterogeneity: Cochran Q = 2.93; p-value = 0.57

**Figure S1: Standard mean difference of number of aberrant crypt in rats chemically induced colon cancer fed a diet containing heme**

**Figure 1:** Studies (left column of the graph) are ordered by year of publication (Pierre *et al*, 2003; Pierre *et al*, 2004, and Pierre *et al*, 2008) and increasing dietary heme concentration (1.5, 3, 0.36 and 0.4 µmol/g). Hb: diet contained haemoglobin, and B: diet contained beef. Squares represent group-specific standardized effect size, which is a measure of the strength of the relationship between two variables, commonly used in analysis of experimental data, in order to combine them in a summary effect size (pooled effect size). The size of the squares is proportional to the statistical weight that each study contributed to the pooled effect size. Horizontal lines represent 95% confidence intervals (CIs). The diamond represents the pooled effect size of all studies included in the analysis, and its 95% CI.

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| Study and country | Study participants; age at cohort entry | Follow-up years (mean) | Number of colon cancer cases | Exposure assessment | Assessment of heme iron content | Adjusted RR[*] [95% Confidence interval][†] for colon cancer |
|---|---|---|---|---|---|---|
| *Lee et al, 2004* Iowa Women's Health Study, USA | 34 708 post-menopausal women aged 55-69 years | 1986-2000 (15 years ) | 741 438 proximal 303 distal | Food-frequency questionnaire | A factor of 0.4 was applied to the total iron content of all meat items | Proximal: 2.18 (1.24-3.86)[‡] P=0.01 Distal: 0.90 (0.45-1.81)[‡] P=0.77 |
| *Larsson et al, 2005* Swedish Mammography Cohort | 61 433 women aged 40-75 years | 1987-2004 (14.8 years) | 547 | Food-frequency questionnaire | A factor of 0.4 was applied to the total iron content of all meat items | 1.31 (0.98-1.75)[§] P=0.03 |
| *Balder et al, 2006* Netherlands Cohort Study on Diet and Cancer (NLCS) | 120 852 women and men; Subcohort: 4371 women and men (2156 men 2215 women) aged 55-69 years | 1986-1995 (9.3 years) | 1023 539 men 484 women | Food-frequency questionnaire | Estimated heme iron content of each meat × mean daily intake of the relevant food items | Men: 1.50 (1.03-2.17)[**] [††] P=0.02 Women: 1.19 (0.80-1.77) [**][††] P=0.43 |
| *Kabat et al, 2007* Canadian National Breast screening study (NBSS) | 48 666 women aged 40-59 years | 1982-1999 (16.4 years) | 428 | Food-frequency questionnaire | The methods of Lee et al. and of Balder et al. were compared and gave similar results. | 0.99 (0.70-1.40)[‡‡] P=0.99 |
| *Cross et al, 2010* NIH-AARP Diet and Health Study, USA | 300 948 women and men aged 50-71 years | 1995-2002 (7.2 years) | 1995 | Food-frequency questionnaire<br><br>Risk-factor questionnaire | Heme iron database based on measured values from meats cooked using different methods and cooked to varying degrees of doneness, used in conjunction with the detailed meat cooking questionnaire to quantitatively assess heme iron intake | 1.10 (0.94-1.28)[§§] P= 0.138 |

[*] Relative risk (RR) was calculated using Cox proportional hazards models

[†] High versus low quintile of heme iron consumption

[‡] Adjustments : Age; total caloric intake; body mass index; physical activity; smoking status, alcohol consumption; history of diabetes; hormone replacement therapy; intake of multivitamins, saturated fat, soluble fibers, insoluble fibers, calcium, vitamin E, folates from food and multivitamin supplements

[§] Adjustments: Age; body mass index; educational level; total energy; intake of saturated fat, dietary fibers, calcium, folates

[**] Cases diagnosed during the first 2 years of follow-up were excluded

[††] Adjustments: Age at baseline; body mass index; family history of colorectal cancer; smoking status; physical activity; total energy intake; alcohol consumption; total consumption of vegetables

[‡‡] Age; body mass index; menopausal status; use of oral contraceptives; hormone replacement therapy; dietary intake of fat, fiber, folic acid, total calories; pack-years of smoking; alcohol intake; education; physical activity

[§§] Gender; educational level; body mass index; smoking; total energy intake (kcals/day); fiber (g/1000kcal); dietary calcium (mg/1000kcal)

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