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## **Meat and cancer: haemoglobin and haemin in a low calcium diet promote colorectal carcinogenesis at the aberrant crypt stage in rats**

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### Abbreviations:

ACF, Aberrant crypt foci; HO-1, Haem-hoxygenase-1; LOO·, Alkylperoxyl radicals; MACF, Major ACF; MDA, Malondialdehyde; NOC, N-nitroso compounds; TBARs, Thiobarbituric acid reactive substances.

**Abstract:**

High intake of red meat, but not of white meat, is associated with an increased risk of colon cancer. However, red meat does not promote cancer in rodents. Haemin, added to low-calcium diets, increases colonic proliferation, and haemoglobin, added to high-fat diets, increases the colon tumour incidence in rats, an effect possibly due to peroxy radicals. We thus speculated that haem might be the promoting agent in meat, and that prevention strategies could use calcium and antioxidants. These hypotheses were tested in rats at the aberrant crypt foci (ACF) stage at 100 days. F344 rats (n=124) were given an injection of azoxymethane and were then randomised to 11 groups fed with low-calcium (20 $\mu$ mol/g) AIN76-based diets, containing 5% safflower oil. Haemin (0.25, 0.5 and 1.5 $\mu$ mol/g) or haemoglobin (1.5 and 3  $\mu$ mol haem/g) was added to five experimental diets, compared to a control diet without haem. Three other high-haemin diets (1.5 $\mu$ mol/g) were supplemented with calcium (250 $\mu$ mol/g), antioxidant butylated hydroxyanisole and rutin (0.05% each), and olive oil, which replaced safflower oil. Faecal water was assayed for lipid peroxidation by thiobarbituric acid reactive substances (TBARs) test, and for cytolytic activity. Haemin strikingly increased the ACF size, dose-dependently, from 2.6 to 11.4 crypts/ACF (all  $p < 0.001$ ). The high-haemin diet also increased the number of ACF per colon ( $p < 0.001$ ). Promotion was associated with increased faecal water TBARs and cytotoxicity. Calcium, olive oil, and antioxidants each inhibited the haemin-induced ACF promotion, and normalised the faecal TBARs and cytotoxicity. The haemoglobin diets increased the number of ACF and faecal TBARs, but not the ACF size or the faecal cytotoxicity. In conclusion, dietary haemin is the most potent known ACF promoter. Haemoglobin is also a potent promoter of colorectal carcinogenesis. The results suggest that myoglobin in red meat could promote colon cancer. Diets high in calcium, or in oxidation-resistant fats, may prevent the possible cancer-promoting effect of red meat.

## Introduction

Colorectal cancer is the second most common cause of cancer death in affluent countries. Dietary modifications might reduce the incidence by more than 70%, but the necessary changes are not precisely known (1). People in western countries are advised to reduce their intake of red meat (2), although epidemiological results are not entirely consistent. The recent dose-response meta-analysis of epidemiological studies by Norat *et al.* suggests that red meat and processed meat intakes are associated with increased risks of colorectal cancer. The mean relative risk associated with the consumption of 120 g/day of red meat is 1.24 (95% confidence interval, 1.08-1.41). The risk fraction attributable to current levels of red meat intake is about 10-25% in regions where red meat intake is high. If average red meat intake is reduced to 70 g/week in these regions, colorectal cancer risk would hypothetically decrease by 7-24% (3). In contrast, white meat (e.g., chicken) and fish intakes are not associated with risk.

Surprisingly, several studies show that rats or mice fed a beef-based diet have fewer tumours than control animals fed a casein or a soy-protein based diet. The evidence from animal studies is far from conclusive (4). Studies testing more specifically the effect of meat components (fat, protein and iron), on colorectal carcinogenesis yielded conflicting results. Many studies tested the effect of saturated fat on the incidence of colon cancer: whereas several studies show that beef tallow promotes cancer in F344 rats (5,6), no promotion was observed in SD rats (7,8). Furthermore, the hypothesis that saturated fat enhances faecal bile acid excretion, a speculated promoting stimulus, is not supported by experimental studies in rats and in volunteers (9,10). Similarly, no clear picture emerges from the studies on protein-rich (11) and high-iron diets. For instance, a diet fortified with iron may, or may not, enhance colon carcinogenesis (12, 13). In addition, meat can deliver specific carcinogens to the colon: *N*-nitroso compounds (NOC) and heterocyclic amines. Red meat consumption enhances the excretion of NOC in the faeces of volunteers (14, 15), and possibly in mice (16). However, a bacon-based diet that increases NOC excretion 10 to 20-times in rats consistently reduces the growth of preneoplastic lesions (17,18). Heterocyclic amines, produced during meat cooking, are potent mutagens and carcinogens that are eaten daily by meat eaters (19). However, some facts suggest that heterocyclic amines may not be major determinants of colon cancer in humans: (i) chicken is an important contributor of heterocyclic amines intake but its consumption is not related to cancer risk (20), (ii) rodent carcinogenic doses are 100-1000 times higher than human exposure (21), and (iii) the colon cancer risk in humans is associated with cooking methods, but not directly with the heterocyclic amines intake (22).

Recently, a new hypothesis implying haem was proposed to explain the association of colon cancer risk with red meat intake, and the lack of association with white meat intake (23). The haem content of red meat is 10-fold higher than that of white meat. Haem is the iron-bearing prosthetic group of haemoproteins. They include myoglobin in red meat which is made of a single globin chain holding one haem, and haemoglobin in red blood cells which is made of the association of four chains, each holding a haem. These proteins are digested in the upper gastrointestinal tract, delivering haem with ferrous iron oxide to the colon. Haemin has often been used in place of haem delivered from haemoprotein digestion. Haemin is the ferric porphyrin component in haemoglobin, with a freely exchangeable axial chloride group. Sesink *et al.* studied the effect of haemin-supplemented diet in non-initiated rats. They showed that dietary haemin increases epithelial proliferation (23). This short-term increase in epithelial proliferation is inhibited by calcium, and is associated with cytolytic activity of faecal water (24). Furthermore, Sawa *et al.* showed *in vitro* that haem induces a non-enzymatic

peroxidation of polyunsaturated fatty acids. In contrast, virgin olive oil resists oxidation because it contains monounsaturated oleic acid and antioxidant polyphenols. *In vivo*, a diet containing both haemoglobin and polyunsaturated fatty acids increases the number of carcinogen-induced adenomas in the colon of rats (25). We thus think that haem could explain the epidemiological correlation between red meat and colon cancer risk. These studies may also explain why red meat did not promote carcinogenesis in previous animal studies: the high level of calcium in experimental diets and/or the resistance of dietary fat to peroxidation, may have hindered the promoting effect of haem. Indeed, the AIN-76 standard rat diet contains 5µg/g of calcium, a 200 µmol/g concentration that matches the 180 µmol/g that inhibits the haemin-induced proliferation (24).

The present study was designed to investigate the effects of diets containing haem, as haemin or haemoglobin, on colon carcinogenesis in rats. Four hypotheses were tested: (i) haemin promotes carcinogenesis, dose-dependently, when added to a low-calcium diet with 5% safflower oil; (ii) free haem (haemin) has not the same effect as protein-bound haem (haemoglobin); (iii) the inhibition of lipid peroxidation inhibits the promotion by haemin; (iv) a high calcium diet reduces the promotion by haemin. The results show that dietary haem dose-dependently increased colorectal carcinogenesis at the aberrant crypt stage in rats, and that haemin was more toxic than haemoglobin. Furthermore, calcium, olive oil or antioxidants suppressed the promotion by haemin. These results suggest preventive strategies against the supposed promotion of colon cancer by haem present in red meat.

## Materials and methods

### *Animals and experimental procedure*

Animal care followed the guidelines of the European Council on animals used in experimental studies. Female F344 rats were obtained from Iffa-Credo (Lyon, France) at four weeks of age. One hundred and twenty-four animals were housed two rats per stainless steel, wire-bottomed cage. They were kept in a temperature of 22°C and light-dark cycle (12h on and 12h off), and were allowed free access to the standard AIN-76 diet and tap water. After one week of acclimatisation, the rats received a single i.p. injection of azoxymethane (Sigma Chemical, St Quentin, France; 20mg/kg ip) in NaCl (9 g/l). Seven days later, they were randomly allocated to eleven groups and fed the experimental diets. Body weights were monitored weekly throughout the experimentation, and food and water intakes were measured once a month. Faeces were collected between days 50-51 and 63-64 of the study, and were frozen at -20°C. The animals were sacrificed 97-99 days after the start of experimental diets. The colons were removed and fixed in 10% buffered formalin (Sigma Chemical) before aberrant crypt foci (ACF) scoring.

### *Diets*

Eleven groups of rats were fed for 14 weeks dry powdered AIN76-based purified diets. The control group (N=22 rats) was given a low-calcium diet (20 µmol/g) containing 5% safflower oil. The experimental diets were based on this control diet. Their composition, balanced for fat, protein and iron, is given in Table 1. The diet base was made by the UPAE (INRA, Jouy, France). Haem, bovine haemoglobin, ferric citrate, calcium phosphate, and antioxidants (Sigma Chemical, St Quentin, France), safflower oil (Farberdistelol, Cereol, Mannheim, Germany), and olive oil (Vierge extra, Lesieur, Neuilly, France), were added into the powdered diet in our laboratory. The dose-effect of haemin was studied in three groups of rats (N= 10, 10 and 20 rats) given haemin-supplemented diets (0.25, 0.5, and 1.5 µmol/g respectively). The effect of haemoglobin was studied in two groups of 10 rats, given a diet supplemented with haemoglobin (0.36 and 0.72 µmol/g). Haemoglobin, 0.36 µmol, and haemin, 1.5 µmol, have the same haem content, which enabled the effects of free haemin and of haemoprotein to be compared. To investigate the effect of calcium and of antioxidants, two groups of eight rats were fed a high-haemin diet (1.5 µmol/g) supplemented either with calcium (230 µmol/g) or with butylated hydroxyanisole and rutin (0.05% each). Lastly, the effect of oil was investigated by replacing safflower oil with olive oil (5%) in a high-haemin diet given to eight rats. Because calcium and olive oil might be protective, even in a normal diet without haemin, two other groups of eight rats received haemin-free control diets, supplemented with calcium (230µmol/g), or olive oil (5%). The number of rats per group was calculated to enhance the statistical power. Accordingly, the basic group size (N= 10) was multiplied by the square root of the number of planned pairwise comparisons. For instance, the group of rats fed the high-haemin diet was compared with 4 groups (control, and high-haemin diet supplemented with calcium, antioxidant and olive oil). Its size was thus increased to N=2x10. In contrast, hypotheses which were not central to the present study, e.g., the effect of calcium in a control diet, were tested with fewer rats per group (N=8).

### *TBARs assay of diet*

Initially, batches of diet were prepared for three weeks, stored at -20°C and given every other day. However, after two days at room temperature, the high-haemin diets smelt rancid and two rats on high-haemin high-calcium diet died within two weeks. We thus decided to prepare diets weekly. Diets were given daily, and were stored at -20°C for no longer than seven days to reduce lipid peroxidation. Freshly prepared samples were left at

room temperature for 24 hours and tested for lipid peroxidation at T0 and T24 h as described by Fenaille *et al.* (26). The diet sample (70 mg) was mixed with 700  $\mu$ l of distilled water. This mixture was homogenised with 560  $\mu$ l of butylhydroxytoluene (1% in ethanol) and 560  $\mu$ l of thiobarbituric acid (1% in acetic acid 5%). After centrifugation (2700Xg for 5min), the supernatant (1 ml) was heated for 60 min at 70°C. After cooling, the amount of TBARs was determined as malondialdehyde (MDA) equivalents by measure of the absorbance of the mix at 532 nm against standards (1,1,3,3-tetramethoxypropane at 0, 100, 200, 400, 600, 800 and 1000  $\mu$ M). Lipid peroxidation of diets at 24 hours (mg MDA/kg) was as follows: 2.18 for LH diet, 4.17 for MH diet, 9.91 for HH diet, 9.51 for HHCA diet, 9.20 for HHOO diet, and not detectable for the three haemin-free control diets, haemoglobin diets and high-haemin diet added with antioxidants (HHAO).

#### ***Assay of ACF***

At the end of the study, colons were excised, flushed with Krebs solution (Sigma Chemical), opened longitudinally and fixed flat between coded filter papers in 10% buffered formalin (Sigma Chemical). ACF were scored by Bird's procedure (27). The colons were stained with methylene blue (0.1%) for six minutes, and the mucosal side was observed at x32 magnification. A single observer scored all colons blindly. The number of ACF per colon, and the number of aberrant crypts per focus, were recorded. On some colons, no typical ACF were seen, but instead zones where all crypts looked aberrant with slit-like opening, increased blue staining, enlarged size and pericryptal zone. The major differences between these zones and the typical ACF were their larger size and the lack of a defined outline. We decided to name these lesions Major Aberrant Crypt Foci (MACF) (Fig 1).

#### ***Analysis of haem in total faeces***

The amount of haem in faeces was determined according to Van den Berg *et al.* (28). An acidified methanol-chloroform extract (final concentration of HCl 1 M) was carried out on 20 mg of faeces. After centrifugation, the chloroform phase was recovered and dried under nitrogen. Samples were dissolved in 0.45 ml 250mM KOH, sonicated for 5 min (Cleanet, Hans Grieshaber, Switzerland) and mixed with 0.45ml of distilled water, 3.75 ml of 2-propanol and 0.75 ml of HCL (1.15 M). This mix was homogenised, then centrifuged for 10 min at 1500Xg and the supernatants were assayed for their haem content. Supernatants (50  $\mu$ l) were mixed with 1 ml of glacial acetic acid. Subsequently, 50  $\mu$ l of FeSO<sub>4</sub>·7H<sub>2</sub>O (0.12 M freshly prepared) and HCl (4.5 M) were added. Samples were immediately mixed and incubated at 60°C for 30 min. Two millilitres of 2-propanol/water (1/1) were added before fluorescence measurement using excitation and emission wavelengths of 400 and 594 nm (JY3D, Jobin-Yvon, France). Blanks were obtained using the same protocol, but without the incubation at 60°C.

#### ***Preparation of faecal water for biochemical assays***

Faecal water was prepared by reconstituting freeze-dried faeces by adding 1 ml of distilled water to 0.3g of freeze-dried faeces. Samples were incubated at 37°C for one hour and were thoroughly mixed during the incubation time. They were then centrifuged for 15 min at 20000Xg, and supernatants were collected and stored at -20°C until use. Each sample was collected from one cage. One sample thus corresponded to the pooled faeces from two rats.

#### ***TBARs assay of faecal water***

TBARs were measured in faecal water according to Ohkawa *et al.* (29). Ten  $\mu$ l of faecal water were diluted in 90  $\mu$ l of distilled water. This sample was mixed with 100  $\mu$ l of sodium dodecyl sulfate (8.1%) and 1 ml of 2-

thiobarbituric acid (TBA, 0.5% in acetic acid 10%). Samples were homogenised and heated at 95°C for one hour. For blanks, thiobarbituric acid was omitted and replaced by 1 ml of acetic acid (10%). After heating, TBARs were extracted with 1 ml of butanol, then centrifuged for 10 min at 4000Xg. The amount of TBARs was determined as malondialdehyde equivalents by measure of the absorbance of the butanol extract at 532 nm against a standard (1,1,3,3-tetramethoxypropane at 0, 100, 200, 400, 600, 800 and 1000 µM).

#### ***Haem assay***

Haem was assayed in the faecal water according to Sesink *et al.* (23). Briefly, 50 µl of faecal water were diluted in 250 µl of a 5:1 mix of 2-propanol and HCl (1 M). Samples were homogenised and centrifuged 2 min at 10000Xg. Supernatants were assayed for haem as described above.

#### ***Cytolytic activity of faecal water***

The cytolytic activity of faecal water was quantified by potassium-release from erythrocytes as described by Govers *et al.* (30). Briefly, faecal water was obtained by reconstituting freeze-dried faeces with double distilled water (approximately 30% dry weight). Final osmolarity of each sample was 300 mosmol/L. Then, 10 or 20 µl of faecal water were mixed with saline buffer to 80µl. After a 5 min incubation at 37°C, 20 µl of washed human erythrocytes suspension (final haematocrit 5%) were added. After incubating for 15 min at 37°C, the cytolytic activity was measured.

#### ***Statistics***

Results, given as mean ± SD, were analysed using Systat 5 software for Windows. Differences between groups were analysed by one-way analysis of variance (ANOVA). When ANOVA showed a statistically significant effect ( $p < 0.05$ ), comparison of each experimental group with the control group was made using Dunnett's test which corrects for multiple comparisons. Most groups were compared with the control group fed a diet without haem. However, according to the tested hypotheses, groups given a high-haemin diet supplemented with a potential inhibitor of haem-promotion were compared with the group fed the high-haemin diet (1.5 µmol/g). Differences were considered significant when two-sided P was  $< 0.05$ .

## Results

### *General observations*

Rats given a high-haemin diet gained less weight than control rats (-10%). In contrast, for the same or a higher haem intake, haemoglobin diets did not depress the growth of rats (Table 2, panel A). The daily food intake was similar in all groups ( $11 \pm 0.8$  g/d, full data not shown), and groups given haemin and haemoglobin diets had haem intakes matching the study design (Table 2, panel A, column 6). However, the haem concentration was higher in the faeces of haemoglobin-fed rats than in haemin-fed rats (+45%, see Table 2 last column). The two strategies to limit the peroxidation in a high-haemin context (antioxidants and olive oil) increased significantly the concentration of haem in faeces (+44% and +86%, Table 2, panel B, last column). These differences in haem faecal concentrations were likely due to differences in faecal daily weight: high-haemin diets increased the faecal excretion (+33%), a laxative effect previously noted by Sesink (23), and haem was thus diluted in the faeces. In contrast, haemoglobin was not laxative, and the antioxidants and olive oil normalised the laxative effect of haemin (Table 2).

### *Effect of diets on carcinogenesis*

The number and size of aberrant crypt foci in the colon of rats after 14 weeks on the experimental diets are reported in Table 3 and figure 2. Haemin strikingly increased the size of foci, dose-dependently, from 2.6 in control rats to 6.2, 10.2 and 11.4 crypts/focus in rats fed the haemin diets (all  $p < 0.001$ ). Haemin also increased strongly and dose-dependently the number of aberrant crypts (Fig. 2). The high-haemin diet, but not the low- and medium-haemin diets, increased the number of total foci per colon from 125 to 166 ( $p < 0.001$ ). The haemoglobin diets similarly increased the number of foci from 125 to 162 and 167 ( $p = 0.001$ , Table 3), but not their size, although the high-haemoglobin diet contained twice the amount of haem as the high-haemin diet. Haemoglobin also increased the total number of aberrant crypts in the colon of rats, but the increase was marginally significant (Fig. 2,  $p < 0.0005$  by Student t test for both groups fed haemoglobin, but  $p > 0.05$  by Dunnet's test).

Surprisingly, the rats given the low-haemin diet had less foci than control rats given no haem at all (125 foci in controls, 92 in low-haemin group,  $p < 0.01$ ). However, since these foci contained more aberrant crypts than those of control rats, even the low-haemin diet increased the total number of aberrant crypts per colon (324 aberrant crypts in controls, 565 in low-haemin group,  $p < 0.01$ ). Indeed, the specific analysis of ACF and MACF data shows that haemin promoted or induced the large MACF instead of classical ACF, while haemoglobin promoted the classical ACF and not the MACF (Table 3). Both tested levels of haemoglobin produced the same effect on carcinogenesis. It is possible that the lowest level was high enough to reach a plateau of promotion.

Calcium, antioxidants, and olive oil completely inhibited the promoting effect of haemin on the size of foci (Table 3, panel B), and reduced the number of aberrant crypts ( $p < 0.01$ ) in the colon of rats. These three dietary interventions specifically suppressed the haemin-induced MACF, thus normalising the type of ACF. No difference was seen between rats given high-calcium and olive oil control diets and rats given the no-haemin control diet (Table 3, panel C). In addition, macroscopic tumours were detected in the colon of five haem-diet fed rats: two rats given the high-haemin diet, one rat given the medium-haemin diet, and one rat given the high-haemoglobin diet (5/60 haem-fed rats vs. 0/38 control rats,  $p = 0.15$  by Fisher's exact test with the approximation of Woolf). Histology showed all tumours were typical adenomas.

### ***Faecal water characteristics***

We measured the characteristics of faecal water because, according to studies on bile acids, the soluble fraction of colonic contents would interact more strongly with the mucosa than the insoluble fraction (31). As expected, the haem concentration in faecal water depended directly on the level of haemin in the diet (Table 4). Dietary haemoglobin also increased haem concentration in faecal water, but less than haemin: A similar haem concentration was found in rats eating 32  $\mu\text{mol/d}$  of dietary haemoglobin or 15  $\mu\text{mol/d}$  of haemin (Tables 1 and 4). Haem can induce the formation of peroxy radicals in fats, which may be cytotoxic and cleave DNA *in vivo* (25). Lipid peroxidation was thus measured in faecal water by the TBARs assay. As shown in Table 4, lipid peroxidation correlated with haem concentration in faecal water ( $r=0.96$  for the 11 groups,  $r=0.87$  for the 116 rats, both  $p<0.0001$ ). The high-haemin diet thus increased TBARs in the faecal water by ten-fold. Haemoglobin-diets also increased TBARs, but to a lesser extent. Furthermore, a cytotoxic factor is found in the faecal water of haemin-fed rats (23). Accordingly, the cytolytic activity of faecal water increased 50-fold in rats fed a high-haemin diet, but, surprisingly absolutely no increase was seen in haemoglobin-fed rats (Table 4). Calcium, antioxidants and olive oil completely suppressed the haemin-induced cytolytic activity of faecal water (Table 4, panel B). The dietary calcium completely inhibited the effects of haemin. Rats fed the high-haemin high-calcium diet had no haem in faecal water, and no increased peroxidation. Sesink *et al.* suggest that this inhibition comes from the binding and precipitation of haem by calcium phosphate (24). Antioxidants and olive oil halved the haemin-induced lipid peroxidation, and reduced the haem concentration, in faecal water of rats given a high-haemin diet. Lastly, calcium, antioxidants or olive oil did not change faecal water values in rats given a diet without haemin (Table 4, panel C).

## Discussion

Dietary haem significantly increased the colon carcinogenesis in rats at the aberrant crypt stage. This result supports the hypothesis that haem can explain the association between red meat consumption and colon cancer risk. The low-haemin diet in this study (0.25 $\mu$ mol/g of diet) mimics the haem content of a meat-based Western diet (32). Indeed, this study showed that (i) haem as haemin or haemoglobin increased the number of azoxymethane-induced ACF in the colon of rats; (ii) haemin effect, the strongest promotion ever reported on ACF, was associated with cytotoxicity and lipid peroxidation in faecal water; (iii) haemin effect was inhibited by dietary calcium, by dietary antioxidants, and by olive oil; and (iv) haemoglobin, which raised lipid peroxidation in faecal water but not cytotoxicity, was less potent than haemin to promote colon carcinogenesis. The effect of haemin, antioxidants, calcium and haemoglobin is discussed below.

**Haemin** was used to explore the effect of haem from red meat because meat haemoproteins are digested in the stomach and the small bowel, and provide free haem to the colon (33). Haemin strikingly increased the number of azoxymethane-induced aberrant crypts (Fig. 2), and the size of foci, by shifting ACF to MACF (Table 3). Macroscopic tumours were seen in rats given haemin for 100 days. To our knowledge, the magnitude of this promotion was the highest ever seen: haemin increased the number of aberrant crypts six-fold, when potent promoters like cooked casein or dextran sulfate increase this number less than two-fold. In faecal water, dietary haemin raised dose-dependently the cytolytic activity, the lipid peroxidation, and the haem concentration: could these changes explain the observed promotion? A faecal cytolytic factor, not yet identified but different from haemin, would increase the epithelial proliferation (23). This factor might be the ACF promoter. Also, the peroxidation of polyunsaturated fatty acids produces aldehydes such as malondialdehyde and 4-hydroxynonenal, which form mutagenic DNA-adducts (34). Sawa *et al.* showed that haem generates alkylperoxyl radicals (LOO $\cdot$ ) from oxidised oil, particularly from safflower oil. Such radicals can cleave DNA, which would contribute to the colon cancer risk (25), and may also explain the observed ACF promotion. Last, faecal water contained soluble haem, which might directly promote carcinogenesis. Indeed, haem can induce gut inflammation through the up-regulation of adhesion molecules, the increase in vascular permeability and the granulocytes infiltration (35). Thus, the chronic inflammation caused by the continuous haem intake might have promoted the ACF growth (36). In addition, haemin may have weakened the tumour immunosurveillance. Indeed, haem induces haem-oxygenase-1 (HO-1), and the over-expression of HO-1 results in the inhibition of T-cell and NK-cell mediated lysis (37). A strange finding was that fewer ACF were scored in the gut of rats fed a low-haemin diet, than in rats fed the control diet. However, other agents which promote the growth of ACF, and increase the incidence of chemically-induced cancer, decrease the number of ACF (e.g., cholic acid) (38). To prevent the deleterious effect of haemin, several strategies of dietary changes have been tested in this study: the addition of antioxidant agents, the substitution of safflower oil by oxidation-resistant olive oil, and the addition of haemin-blocking calcium phosphate to the diet.

**The antioxidants** we added to safflower oil, or naturally present in olive oil, completely inhibited the haemin-induced promotion of colon carcinogenesis. Safflower oil is highly susceptible to oxidation, and the high-haemin diet with 5% safflower oil quickly turned rancid (see TBARs assay of diets, in the Materials and Methods section). The addition of two antioxidants into the diet, one soluble in fat, the other soluble in water, completely inhibited the peroxidation of diet. These antioxidants also show direct chemopreventive properties when given during the carcinogen treatment (39), or at concentrations higher than 0.05% (40). However, we

think that, in the present conditions (very low levels given post-initiation), their effect was due to the inhibition of haemin-induced peroxidation. Olive oil was added to another diet because it contains the relatively oxidation-resistant oleic acid, and phenolic compounds, which scavenge  $\text{LOO}\cdot$  radicals in the oil itself (41) and in the faecal matrix (42). Both antioxidant strategies halved TBARs in faecal water (Table 4, panel B). This inhibition of lipid peroxidation in faecal water was associated with inhibition of haemin-induced carcinogenesis (Table 3, panel B). It is thus possible that lipid peroxidation itself is a link between dietary haem and cancer enhancement, as suggested by Sawa and by Owen (25, 41), but contested by Sesink *et al.* (23). It is indeed surprising that both antioxidant strategies increased haem concentration in faeces (Table 2), but decreased factors apparently not related to oxidation: haem concentration was reduced by 30-40%, and cytolytic activity was fully suppressed, in the faecal water of rats given the diets with olive oil or with antioxidants (Table 4, panel B). The mechanism by which olive oil and antioxidants suppressed the haemin-induced promotion thus appears complex and warrants further studies.

**Calcium** phosphate was added to a high-haemin diet, as an alternative strategy to reduce the effect of haem. Many studies suggest that calcium can protect against colon carcinogenesis. For instance dietary calcium reduces the recurrence of adenomatous polyps in patients (43). In volunteers, calcium precipitates the intestinal fatty acids and the secondary bile acids, and thus inhibits colonic cytotoxicity, and, possibly, the promotion by high-fat diets (30). Newmark *et al.* (44) suggest that 5 mg/g calcium in the AIN-76A rodent diet is equivalent to 2700 mg calcium per day in humans, that is three times the Recommended Dietary Allowance. Using the same proportionality here, the low-calcium (0.79 mg/g) and the high-calcium (9.9 mg/g) diets were equivalent to 427 and 5346 mg calcium per day in humans, respectively. Because the diet contains 500-600 mg/d calcium on average in the USA (45), the low-calcium diet in this study mimics the intake of a considerable fraction of the American population. In rats given the high-haemin diet, dietary calcium suppressed colon ACF in rats. Calcium reduces the haemin-induced colonic hyper-proliferation, because calcium phosphate precipitates haemin in the gut (24). Faecal haem was high in rats given a high-calcium diet (Table 2), but it was not soluble in the faecal water. This water thus contained no lipid peroxides and showed no cytolytic activity (Table 4). Clearly the precipitation of haem by calcium is enough to explain the protection against haem-induced carcinogenesis. In conclusion, both strategies were effective against haemin promotion. Red meat contains no haemin, but myoglobin. We thus also studied the effect of dietary haemoglobin, a haemoprotein similar to myoglobin, but easier to obtain.

**Haemoglobin** increased the number of aberrant crypts and of ACF in rats (Table 3), an effect already observed by Bruce (46). The magnitude of the effect (1.6-fold increase) is second only to haemin, dextran sulphate and cooked casein. This result provides a possible explanation for the association observed between red meat consumption, and the colon cancer risk in humans. In contrast with haemin, haemoglobin did not increase significantly the number of aberrant crypts per focus, and was thus less potent than haemin to promote the ACF growth (Table 3). The difference between haemin and haemoglobin was mainly due to the MACFs: frequent in the colon of haemin-fed rats, occasional in the colon of haemoglobin-fed rats. How could this difference be explained? Haemin and haemoglobin led to similar faecal haem excretion: 33% of the haem intake was excreted in both cases (Table 2), but haem concentration in faecal water was higher in haemin-fed rats (Table 4). However, this difference cannot explain the differential effect on MACF: rats in groups HH and TG had similar levels of haem in faecal water (Table 4) but very different numbers of MACF (Table 3). Haemin and

haemoglobin do not have the same fate in the gastro-intestinal tract. Haemin (free haem) is poorly soluble in water, and forms insoluble polymers at low pH in the stomach (33). Haemin thus escapes small intestinal absorption and reaches the colon. In the colon, haemin induces lipid peroxidation and yields the cytotoxic compound described by Sesink *et al.*: this fat soluble compound is found in the faeces of haemin-fed rats, it shows a specific absorption a 400 nm, but it differs from haemin, porphyrin or bilirubin (23). On the other hand, haemoglobin is water soluble. The hydrolysis of its globin moiety in the upper digestive tract yields haem in a form which may be more available than haemin (33). Indeed, dietary haemoglobin is three times more efficient than haemin in providing iron for red blood cell synthesis in rats (47). In contrast with haemin-fed rats, animals given a haemoglobin-diet had no cytolytic activity at all in faecal water, which shows that the fates of haemin and of haemoglobin are not the same (Table 4). It also suggests that the MACFs were specifically produced by the haemin-induced cytotoxic factor (23). Haemin has been used in experimental studies to investigate the effect of red meat consumption. The underlying hypothesis was that, because globin is digested in the upper tract, haemoprotein and haemin would deliver similar haem-compounds to the colon. The present results clearly show that this is not true. Myoglobin is the main pigment in beef and pork muscles, whereas some haemoglobin is found in white meat. Both haemoproteins hold the haem similarly, and both can initiate the peroxidation of fats (48). We thus suggest that haemoglobin may be a suitable substitute for myoglobin, and thus for red meat studies in rats. In cured meat, however, nitrite reacts with myoglobin to yield nitrosomyoglobin. When ham and hot-dog are cooked, free nitroso-haem is released from the denatured protein (49). Haemin might thus be a suitable model to study the effect of processed meat on colon cancer. Calcium, olive oil and antioxidants protective effects were associated with the inhibition of haemin-induced lipid peroxidation. Lipid peroxidation was associated with the promotion of aberrant crypts by both haemin and by haemoglobin. We thus suppose that calcium, olive oil and antioxidants could counteract the haemoglobin-promotion. The advice to increase consumption of olive oil and of calcium-rich dairy products might be easier to follow than the advice to abstain from consumption of red meat. Indeed, the association between red meat intake and colon cancer was more consistently shown in North-American studies than in European studies. Now, in Europe, the calcium and the olive oil intakes are higher than in the USA (24).

In conclusion, dietary haemin and haemoglobin increased colorectal carcinogenesis in rats. No known ACF promoter is more potent than haemin. Haemoglobin is close to myoglobin, and it was much less toxic than haemin. This study supports the hypothesis that red meat could promote colon cancer because of its myoglobin content. A diet high in calcium, or with oxidation-resistant fats, may prevent the possible cancer-promoting effect of red meat.

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Table 1: Composition of experimental diets, given in g per kg of diet.

Tested factor	Control	Haemin		Haemoglobin			Calcium		Anti-oxidants		Olive oil	
		CD	LH	MH	HH	HG	TG	CDCA	HHCA	HHAO	CDOO	HHOO
<b>Haemin</b>	0	<b>0.16<sup>a</sup></b>	<b>0.32</b>	<b>0.94</b>	0	0	0	<b>0.94</b>	<b>0.94</b>	0	<b>0.94</b>	
<b>Haemoglobin</b>	0	0	0	0	<b>25</b>	<b>50</b>	0	0	0	0	0	
<b>Ferric Citrate</b>	0.36	0.29	0.24	0	0	0	0.36	0	0	0.36	0	
<b>Rutin+BHA<sup>d</sup></b>	0	0	0	0	0	0	0	0	<b>0.5 + 0.5</b>	0	0	
<b>CaHPO<sub>4</sub>.2H<sub>2</sub>O</b>	2.7	2.7	2.7	2.7	2.7	2.7	<b>33.75</b>	<b>33.75</b>	2.7	2.7	2.7	
<b>Safflower oil</b>	50	50	50	50	50	50	50	50	50	<b>50<sup>c</sup></b>	<b>50<sup>c</sup></b>	
<b>Caseine</b>	200	200	200	200	175	152	200	200	200	200	200	
<b>Corn starch</b>	150	150	150	150	150	150	150	150	150	150	150	
<b>Sucrose</b>	497	497	498	497.3	497	497	466	465.3	497.3	497.3	497.3	
<b>Cellulose</b>	50	50	50	50	50	50	50	50	50	50	50	
<b>Methionine</b>	3	3	3	3	3	3	3	3	3	3	3	
<b>Mineral mix<sup>b</sup></b>	35	35	35	35	35	35	35	35	35	35	35	
<b>Vitamin mix</b>	10	10	10	10	10	10	10	10	10	10	10	
<b>Choline bitartrate</b>	2	2	2	2	2	2	2	2	2	2	2	

\* Groups: CD, Control Diet; LH, Low Haemin diet; MH, Medium Haemin diet; HH, High Haemin diet; HG, High haemoGlobin diet; TG, Top haemoGlobin diet; HHAO, High Haemin and AntiOxidants diet; HHCA, High Haemin and Calcium diet; HHOO, High Haemin and Olive Oil diet; CDCA, Control and Calcium diet; CDOO, Control and Olive Oil diet.

a: Values are g/kg diet, main differences between diets are printed in bold.

b: Mineral mix without CaHPO<sub>4</sub>.2H<sub>2</sub>O

c: olive oil replaced safflower oil in CDOO and HHOO diets

d: BHA, butylated hydroxyanisole



Table 2: Effect of diet on body weight, haem intake, concentration in faeces and daily output **A**: Effect of haemin and haemoglobin; **B**: Effect of inhibitors in a high-haemin context, and, **C**: in a low-haemin context.

<b>A</b>	Haem in diet	No of Rats	Final body weight g/rat	Faecal output (dry weight) g/day	Haem intake $\mu\text{mol/day}$	Haem in faeces $\mu\text{mol/g}$
Diets*	$\mu\text{mol/g}$					
CD	0	22	197 $\pm$ 9	0.6 $\pm$ 0.1	0	0.04 $\pm$ 0.09
LH	0.25	10	192 $\pm$ 8	0.5 $\pm$ 0.1	2.9 $\pm$ 0.1 <sup>a</sup>	0.95 $\pm$ 0.24
MH	0.5	10	187 $\pm$ 8 <sup>a</sup>	0.5 $\pm$ 0.1	5.5 $\pm$ 0.3 <sup>a</sup>	2.1 $\pm$ 0.7 <sup>a</sup>
HH	1.5	20	178 $\pm$ 9 <sup>a</sup>	0.8 $\pm$ 0.1 <sup>a</sup>	15.2 $\pm$ 2.2 <sup>a</sup>	6.6 $\pm$ 1.3 <sup>a</sup>
HG	1.5	10	193 $\pm$ 9	0.6 $\pm$ 0.1	16.8 $\pm$ 2.0 <sup>a</sup>	9.6 $\pm$ 0.9 <sup>a</sup>
TG	3.0	10	190 $\pm$ 10	0.6 $\pm$ 0.2	31.8 $\pm$ 3.2 <sup>a</sup>	14.7 $\pm$ 2.3 <sup>a</sup>
<b>B</b>						
HH	1.5	20	178 $\pm$ 9	0.8 $\pm$ 0.1	15.2 $\pm$ 2.2	6.6 $\pm$ 1.3
HHCA	1.5	6	183 $\pm$ 5	0.8 $\pm$ 0.2	17.8 $\pm$ 1.4 <sup>b</sup>	7.9 $\pm$ 0.6
HHAO	1.5	10	186 $\pm$ 9	0.6 $\pm$ 0.1 <sup>b</sup>	14.8 $\pm$ 1.3	9.5 $\pm$ 1.5 <sup>b</sup>
HHOO	1.5	8	180 $\pm$ 7	0.6 $\pm$ 0.1 <sup>b</sup>	16.5 $\pm$ 0.8	12.3 $\pm$ 1.6 <sup>b</sup>
<b>C</b>						
CD	0	22	197 $\pm$ 9	0.6 $\pm$ 0.1	0	0.04 $\pm$ 0.09
CDCA	0	8	193 $\pm$ 5	1.0 $\pm$ 0.1 <sup>a</sup>	0	0.01 $\pm$ 0.02
CDOO	0	8	188 $\pm$ 9	0.6 $\pm$ 0.1	0	0.04 $\pm$ 0.07

\* Dietary groups, see note to table 1.

a, a': significantly different from control diet CD (a:  $p < 0.01$ , a':  $p < 0.05$  by Dunnett's test)

b: significantly different from high-haemin diet HH ( $p < 0.01$ , by Dunnett's test)

c: haem metabolized was extrapolated by subtracting haem output from haem intake

Table 3: Effect of experimental diets on the number and size of aberrant crypt foci and major aberrant crypt foci in the colon of rats after 100 days of nutritional experimentation. **A**: Effect of haemin and haemoglobin; **B**: Effect of inhibitors in a high-haemin context, and, **C**: in a low-haemin context.

Diets*	Haem in diet μmol/g	No of Rats	Total Foci (ACF + MACF)			ACF <sup>c</sup> Foci / colon	MACF <sup>d</sup> Foci / colon
			Foci / colon	Size = aberrant crypts / focus	Aberrant crypts / colon		
CD	0	22	125 ± 25	2.6 ± 0.2	324 ± 70	125 ± 25	0.3 ± 0.8
LH	0.25	10	92 ± 16 <sup>a</sup>	6.2 ± 2.0 <sup>a</sup>	565 ± 193 <sup>a</sup>	47 ± 23 <sup>a</sup>	44 ± 20 <sup>a</sup>
MH	0.5	10	120 ± 16	10.2 ± 0.9 <sup>a</sup>	1233 ± 210 <sup>a</sup>	12 ± 13 <sup>a</sup>	108 ± 19 <sup>a</sup>
HH	1.5	20	166 ± 26 <sup>a</sup>	11.4 ± 1.1 <sup>a</sup>	1901 ± 371 <sup>a</sup>	7 ± 14 <sup>a</sup>	158 ± 29 <sup>a</sup>
HG	1.5	10	162 ± 27 <sup>a</sup>	3.3 ± 0.4 <sup>b</sup>	518 ± 119 <sup>b</sup>	149 ± 23 <sup>b</sup>	12 ± 8 <sup>b</sup>
TG	3.0	10	167 ± 34 <sup>a</sup>	2.7 ± 0.4	457 ± 119 <sup>b</sup>	160 ± 32 <sup>a,b</sup>	7 ± 6 <sup>b</sup>
<b>B</b>							
HH	1.5	20	165 ± 26	11.4 ± 1.1	1901 ± 371	7 ± 14	158 ± 29
HHCA	1.5	6	135 ± 22	2.7 ± 0.4 <sup>b</sup>	359 ± 38 <sup>b</sup>	131 ± 23 <sup>b</sup>	4 ± 2 <sup>b</sup>
HHAO	1.5	10	142 ± 25	2.9 ± 0.3 <sup>b</sup>	406 ± 85 <sup>b</sup>	135 ± 25 <sup>b</sup>	7 ± 7 <sup>b</sup>
HHOO	1.5	8	138 ± 31	2.8 ± 0.3 <sup>b</sup>	389 ± 115 <sup>b</sup>	132 ± 25 <sup>b</sup>	7 ± 8 <sup>b</sup>
<b>C</b>							
CD	0	22	125 ± 25	2.6 ± 0.2	324 ± 70	125 ± 25	0.3 ± 0.8
CDCA	0	8	125 ± 11	2.8 ± 0.4	354 ± 28	124 ± 12	1.2 ± 2.3
CDOO	0	8	123 ± 18	2.4 ± 0.3	302 ± 63	123 ± 18	0.2 ± 0.7

\* Dietary groups, see note to table 1.

a: significantly different from control diet :CD (p<0.01, by Dunnett's test)

b: significantly different from HH diet (p<0.01, by Dunnett's test)

c: the median ACF contained 2.6 ± 0.2 aberrant crypts

d: the median MACF contained 11.5 ± 0.7 aberrant crypts

Table 4: Effect of experimental diets on concentration of haem, lipid peroxidation and cytotoxicity in faecal water. **A**: Effect of haemin and haemoglobin diets; **B**: Effect of antioxidant; high calcium and olive oil diet on a haemin context, **C**: Effect of calcium and olive oil on control diet.

<b>A</b>	Haem in diet	Haem in faecal water	Lipid peroxidation: TBARs in faecal water	Cytotoxicity of faecal water
Diets*	$\mu\text{mol/g}$	$\mu\text{M}$	MDA equivalents ( $\mu\text{M}$ )	% K release
CD	0	$2.6 \pm 4.5$	$38 \pm 14$	$2 \pm 0$
LH	0.25	$36 \pm 17^{a,b}$	$67 \pm 32^b$	$34 \pm 7^a$
MH	0.5	$135 \pm 44^{a,b}$	$168 \pm 13^{a,b}$	$97 \pm 1^a$
HH	1.5	$258 \pm 91^a$	$346 \pm 26^a$	$100 \pm 1^a$
HG	1.5	$158 \pm 48^{a,b}$	$187 \pm 21^{a,b}$	$2 \pm 0$
TG	3.0	$262 \pm 68^a$	$260 \pm 28^{a,b}$	$2 \pm 1$
<b>B</b>				
HH	1.5	$258 \pm 91$	$346 \pm 26$	$100 \pm 1$
HHCA	1.5	$4.7 \pm 4.6^b$	$49 \pm 6^b$	$2 \pm 0^b$
HHAO	1.5	$160 \pm 46^b$	$138 \pm 17^b$	$2 \pm 0^b$
HHOO	1.5	$186 \pm 61^b$	$166 \pm 27^b$	$5 \pm 4^b$
<b>C</b>				
CD	0	$2.6 \pm 4.5$	$38 \pm 14$	$2 \pm 0$
CDCA	0	$3.8 \pm 2.7$	$28 \pm 9$	$5 \pm 3$
CDOO	0	$1.6 \pm 1.8$	$45 \pm 4$	$3 \pm 3$

\* Dietary groups, see note to table 1.

Figure 1 : Mucosal surface of colon stained with methylene blue visualised under a light microscope (x40). (A) Aberrant Crypt Focus with four crypts (control diet), (B) Major Aberrant Crypt Focus with twenty crypts (high haemin diet).

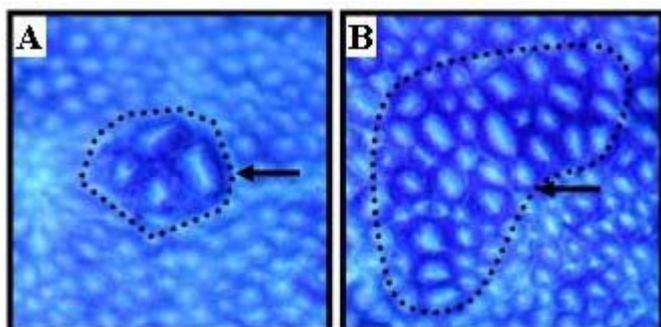


Figure 2: Number of aberrant crypts in the colon of rats after 100 days on experimental diets. A: Effect of haemin (LH, MH, HH) and haemoglobin (HG, TG); B: Effect of calcium (CA), anti-oxidants (AO) and olive oil (OO) in a high-haemin context. \*: significantly different from control diet CD (panel A) or from HH diet (panel B) ( $p < 0.01$ , by Dunnett's test). *Haem* concentration in diets ( $\mu\text{mol/g}$ )

