Carcinogenesis vol.24 no.10 pp.1683–1690, 2003
DOI: 10.1093/carcin/bgg130

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

Fabrice Pierre[1,3], Sylviane Taché[1], Claude R.Petit[1], Roelof Van der Meer[2] and Denis E.Corpet[1]

[1]Ecole Nationale Vétérinaire Toulouse, UMR INRA-ENVT Xénobiotiques, 23 Capelles, 31076 Toulouse, France and [2]Nutrition and Health Programme, Wageningen Centre for Food Sciences, NIZO Food Research, PO Box 20, 6710 BA Ede, The Netherlands

[3]To whom correspondence should be addressed
Email: f.pierre@envt.fr

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 peroxyl 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 randomized to 11 groups fed with low-calcium (20 μmol/g) AIN76-based diets, containing 5% safflower oil. Haemin (0.25, 0.5 and 1.5 μmol/g) or haemoglobin (1.5 and 3 μmol haem/g) was added to five experimental diets, compared with a control diet without haem. Three other high-haemin diets (1.5 μmol/g) were supplemented with calcium (250 μmol/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 normalized 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 >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 ~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 10–20 times in rats consistently reduces the growth of pre-neoplastic 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

Abbreviations: ACF, aberrant crypt foci; MACF, major aberrant crypt foci; NOC, *N*-nitroso compounds; TBARs, thiobarbituric acid reactive substances.

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 5 mg/g of calcium, a 130 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 4 weeks of age. 124 animals were housed 2 rats/stainless steel, wire-bottomed cage. They were kept in a temperature of 22°C and light–dark cycle (12 h on and 12 h off), and were allowed free access to the standard AIN-76 diet and tap water. After 1 week of acclimatization, the rats received a single i.p. injection of azoxymethane (Sigma Chemical, St Quentin, France; 20 mg/kg i.p.) in NaCl (9 g/l). Seven days later, they were randomly allocated to 11 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 killed 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 I. The diet base was made by the UPAE (INRA, Jouy, France). Haem, bovine haemoglobin, ferric citrate, calcium phosphate and antioxidants (Sigma Chemical), 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 (230 μmol/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 four groups (control and high-haemin diet supplemented with calcium, antioxidant and olive oil). Its size was thus increased to $n = 2 \times 10$. In contrast, hypotheses that were not central to the present study, e.g. the effect of calcium in a control diet, were tested with fewer rats per group ($n = 8$).

Thiobarbituric acid reactive substances (TBARs) assay of diet

Initially, batches of diet were prepared for 3 weeks, stored at −20°C and given every other day. However, after 2 days at room temperature, the high-haemin diets smelt rancid and two rats on high-haemin high-calcium diet died within 2 weeks. We thus decided to prepare diets weekly. Diets were given daily, and were stored at −20°C for no longer than 7 days to reduce lipid peroxidation. Freshly prepared samples were left at room temperature for 24 h 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 μl of distilled water. This mixture was homogenized with 560 μl of butylhydroxytoluene (1% in ethanol) and 560 μl of thiobarbituric acid (1% in acetic acid 5%). After centrifugation (2700 *g* for 5 min), 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 μM). Lipid peroxidation of diets at 24 h (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 6 min, and the mucosal side was observed at ×32 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) (Figure 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 250 mM KOH, sonicated for 5 min (Cleanet, Hans Grieshaber, Switzerland) and mixed with 0.45 ml of distilled water, 3.75 ml of 2-propanol and 0.75 ml of HCl (1.15 M). This mix was homogenized, then centrifuged for 10 min at 1500 *g* and the supernatants were assayed for their haem content. Supernatants (50 μl) were mixed with 1 ml of glacial acetic acid. Subsequently, 50 μl of $FeSO_4 \cdot 7H_2O$ (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

Table I. Composition of experimental diets, given in g/kg of diet

Tested factor	Control	Haemin			Haemoglobin		Calcium		Anti-oxidants	Olive oil	
Group*	CD	LH	MH	HH	HG	TG	CDCA	HHCA	HHAO	CDOO	HHOO
Haemin	0	**0.16**[a]	**0.32**	**0.94**	0	0	0	**0.94**	**0.94**	0	**0.94**
Haemoglobin	0	0	0	0	**25**	**50**	0	0	0	0	0
Ferric citrate	0.36	0.29	0.24	0	0	0	0.36	0	0	0.36	0
Rutin + BHA[d]	0	0	0	0	0	0	0	0	**0.5 + 0.5**	0	0
$CaHPO_4 \cdot 2H_2O$	2.7	2.7	2.7	2.7	2.7	2.7	**33.75**	**33.75**	2.7	2.7	2.7
Safflower oil	50	50	50	50	50	50	50	50	50	**50**[c]	**50**[c]
Caseine	200	200	200	200	175	152	200	200	200	200	200
Corn starch	150	150	150	150	150	150	150	150	150	150	150
Sucrose	497	497	498	497.3	497	497	466	465.3	497.3	497.3	497.3
Cellulose	50	50	50	50	50	50	50	50	50	50	50
Methionine	3	3	3	3	3	3	3	3	3	3	3
Mineral mix[b]	35	35	35	35	35	35	35	35	35	35	35
Vitamin mix	10	10	10	10	10	10	10	10	10	10	10
Choline bitartrate	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 $CaHPO_4 \cdot 2H_2O$.
[c]Olive oil replaced safflower oil in CDOO and HHOO diets.
[d]BHA, butylated hydroxyanisole.

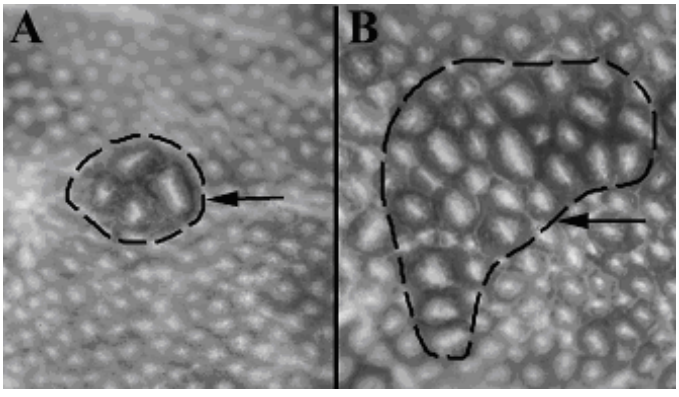

Fig. 1. Mucosal surface of colon stained with methylene blue visualized under a light microscope (×40). (**A**) Aberrant crypt focus with four crypts (control diet). (**B**) Major aberrant crypt focus with 20 crypts (high-haemin diet).

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 0.3 g of freeze-dried faeces. Samples were incubated at 37°C for 1 h and were thoroughly mixed during the incubation time. They were then centrifuged for 15 min at 20 000 *g*, 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 microlitres of faecal water were diluted in 90 µl of distilled water. This sample was mixed with 100 µl of sodium dodecyl sulfate (8.1%) and 1 ml of 2-thiobarbituric acid (TBA, 0.5% in acetic acid 10%). Samples were homogenized and heated at 95°C for 1 h. For blanks, TBA 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 4000 *g*. 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 was diluted in 250 µl of a 5:1 mix of 2-propanol and HCl (1 M). Samples were homogenized and centrifuged 2 min at 10 000 *g*. 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 (~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 80 µl. After a 5 min incubation at 37°C, 20 µl of washed human erythrocytes suspension (final haematocrit 5%) was 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 II, A). The daily food intake was similar in all groups (11 ± 0.8 g/day, full data not shown), and groups given haemin and haemoglobin diets had haem intakes matching the study design (Table II, A, column 6). However, the haem concentration was higher in the faeces of haemoglobin-fed rats than in haemin-fed rats (+45%, see Table II, last column). The two strategies to limit the peroxidation in a high-haemin context

Table II. 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; C: in a low-haemin context

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

*Dietary groups, see note to Table I.
[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).

(antioxidants and olive oil) increased significantly the concentration of haem in faeces (+44 and +86%, Table II, 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 noted previously by Sesink (23), and haem was thus diluted in the faeces. In contrast, haemoglobin was not laxative, and the antioxidants and olive oil normalized the laxative effect of haemin (Table II).

Effect of diets on carcinogenesis

The number and size of ACF in the colon of rats after 14 weeks on the experimental diets are reported in Table III 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 (Figure 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 III), 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 (Figure 2, $P < 0.0005$ by Student's *t*-test for both groups fed haemoglobin, but $P > 0.05$ by Dunnett'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 III). 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 III, 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 normalizing 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 III, C). In addition, macroscopic tumours were detected in the colon of five haem-fed diet 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 versus 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 IV). Dietary haemoglobin also increased haem concentration in faecal water, but less than haemin: a similar haem concentration was found in rats eating 32 μmol/day of dietary haemoglobin or 15 μmol/day of haemin (Tables I and IV). Haem can induce the formation of peroxyl 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 IV, 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 10-fold. Haemoglobin diets

Table III. Effect of experimental diets on the number and size of ACF and MACF 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; C: in a low-haemin context

Diets*	Haem in diet (μmol/g)	No. of rats	Total foci (ACF + MACF)			ACF[c]	MACF[d]
			Foci/colon	Size = aberrant crypts/focus	Aberrant crypts/colon	Foci/colon	Foci/colon
A							
CD	0	22	125 ± 25	2.6 ± 0.2	324 ± 70	125 ± 25	0.3 ± 0.8
LH	0.25	10	92 ± 16[a]	6.2 ± 2.0[a]	565 ± 193[a]	47 ± 23[a]	44 ± 20[a]
MH	0.5	10	120 ± 16	10.2 ± 0.9[a]	1233 ± 210[a]	12 ± 13[a]	108 ± 19[a]
HH	1.5	20	166 ± 26[a]	11.4 ± 1.1[a]	1901 ± 371[a]	7 ± 14[a]	158 ± 29[a]
HG	1.5	10	162 ± 27[a]	3.3 ± 0.4[b]	518 ± 119[b]	149 ± 23[b]	12 ± 8[b]
TG	3.0	10	167 ± 34[a]	2.7 ± 0.4	457 ± 119[b]	160 ± 32[a.b]	7 ± 6[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[b]	359 ± 38[b]	131 ± 23[b]	4 ± 2[b]
HHAO	1.5	10	142 ± 25	2.9 ± 0.3[b]	406 ± 85[b]	135 ± 25[b]	7 ± 7[b]
HHOO	1.5	8	138 ± 31	2.8 ± 0.3[b]	389 ± 115[b]	132 ± 25[b]	7 ± 8[b]
C							
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 I.
[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.

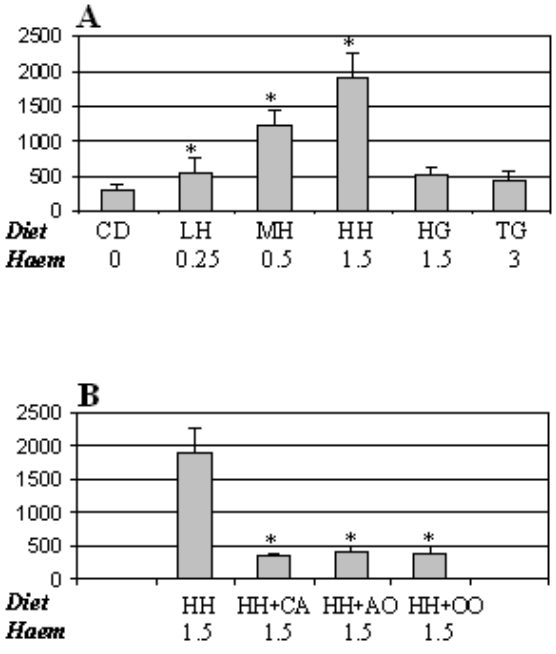

Fig. 2. Number of aberrant crypts in the colon of rats after 100 days on experimental diets. (**A**) Effect of haemin (LH, MH, HH) and haemoglobin (HG, TG). (**B**) Effect of calcium (CA), anti-oxidants (AO) and olive oil (OO) in a high-haemin context. *Significantly different from control diet CD (A) or from HH diet (B) ($P < 0.01$, by Dunnett's test). Haem concentration in diets (μmol/g).

also increased TBARs, but to a lesser extent. Furthermore, a cytotoxic factor was 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 IV). Calcium, antioxidants and olive oil completely suppressed the haemin-induced cytolytic activity of faecal water (Table IV, 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 IV, 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 μmol/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 (Figure 2), and the size of foci, by shifting ACF to MACF (Table III). 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 6-fold, when potent promoters like cooked casein or dextran sulfate increased this

Table IV. 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

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

*Dietary groups, see note to Table I.
[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).

number <2-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 ($LOO^{\bullet}$) from oxidized 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 that 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). 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 $LOO^{\bullet}$ radicals in the oil itself (41) and in the faecal matrix (42). Both antioxidant strategies halved TBARs in faecal water (Table IV, B). This inhibition of lipid peroxidation in faecal water was associated with inhibition of haemin-induced carcinogenesis (Table III, 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 (25,42), but contested by Sesink *et al*. (23). It is indeed surprising that both antioxidant strategies increased haem concentration in faeces (Table II), 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 IV, 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/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/day in humans, respectively. Because the diet contains 500–600 mg/day 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 II), but it was not soluble in the faecal water. This water thus contained no lipid peroxides and showed no cytolytic activity (Table IV). 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 III), an effect already observed by Bruce (W.R.Bruce, personal communication). 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 III). 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 II), but haem concentration in faecal water was higher in haemin-fed rats (Table IV). 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 IV) but very different numbers of MACF (Table III). Haemin and haemoglobin do not have the same fate in the gastrointestinal 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 at 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 (46). 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 IV). 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 (47). 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-dogs are cooked, free nitroso-haem is released from the denatured protein (48). 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.

Acknowledgements

We thank Xavier Blanc (UPAE, INRA) for the preparation of experimental diets, Denise S.M.L.Termont (NIZO) for the determination of cytolytic activity of faecal water, Alexandre Peuch for TBARs assays, Raymond Gazel and Florence Blas Y Estrada for the care of animals, Alain Paris for help in statistical analysis, Amanda Freeman for careful reading of the manuscript, and W.R.Bruce (U. of T.) for helpful advice with the study design. The studies were supported in part by the INRA, the DGER, and by a grant of the région Midi-Pyrénées.

References

1. Cummings,J.H. and Bingham,S.A. (1998) Diet and the prevention of cancer. *Br. Med. J.*, **317**, 1636–1640.
2. World Cancer Research Fund/American Institute for Cancer research (WCRF/AICR). Food, nutrition and the prevention of cancer: a global perspective. Washington (DC): WCRF/AICR, 1997.
3. Norat,T., Lukanova,A., Ferrari,P. and Riboli,E. (2002) Meat consumption and colorectal cancer risk: dose-response meta-analysis of epidemiological studies. *Int. J. Cancer*, **98**, 241–256.
4. Parnaud,G. and Corpet,D.E. (1997) Colorectal cancer: controversial role of meat consumption. *Bull. Cancer*, **84**, 899–911.
5. Mori,T., Imaida,K., Tamano,S., Sano,M., Takahashi,S., Asamoto,M., Takeshita,M., Ueda,H. and Shirai,T. (2001) Beef tallow, but not perilla or corn oil, promotion of rat prostate and intestinal carcinogenesis by 3,2′-dimethyl-4-aminobiphenyl. *Jpn. J. Cancer Res.*, **92**, 1026–1033.
6. Reddy,B.S., Narisawa,T. and Weisburger,J.H. (1976) Effect of a diet with high levels of protein and fat on colon carcinogenesis in F344 rats treated with 1,2-dimethylhydrazine. *J. Natl Cancer Inst.*, **57**, 567–569.
7. Nauss,K.M., Locniskar,M. and Newberne,P.M. (1983) Effect of alterations in the quality and quantity of dietary fat on 1,2-dimethylhydrazine-induced colon tumorigenesis in rats. *Cancer Res.*, **43**, 4083–4090.
8. Zhao,L.P., Kushi,L.H., Klein,R.D. and Prentice,R.L. (1991) Quantitative review of studies of dietary fat and rat colon carcinoma. *Nutr. Cancer*, **15**, 169–177.

9. Gallaher,D.D. and Chen,C.L. (1995) Beef tallow, but not corn bran or soybean polysaccharide, reduces large intestinal and fecal bile acid concentrations in rats. *Nutr. Cancer*, **23**, 63–75.
10. Suzuki,K., Suzuki,K. and Mitsuoka,T. (1992) Effect of low-fat, high-fat, and fiber-supplemented high-fat diets on colon cancer risk factors in feces of healthy subjects. *Nutr. Cancer*, **18**, 63–71.
11. McIntosh,G.H., Regester,G.O., Le Leu,R.K., Royle,P.J. and Smithers,G.W. (1995) Dairy proteins protect against dimethylhydrazine-induced intestinal cancers in rats. *J. Nutr.*, **125**, 809–816.
12. Soyars,K.E. and Fischer,J.G. (1998) Iron supplementation does not affect cell proliferation or aberrant crypt foci development in the colon of Sprague–Dawley rats. *J. Nutr.*, **128**, 764–770.
13. Davis,C.D. and Feng,Y. (1999) Dietary copper, manganese and iron affect the formation of aberrant crypts in colon of rats administered 3,2′-dimethyl-4-aminobiphenyl. *J. Nutr.*, **129**, 1060–1067.
14. Bingham,S.A., Pignatelli,B., Pollock,J.R., Ellul,A., Malaveille,C., Gross,G., Runswick,S., Cummings,J.H. and O'Neill,I.K. (1996) Does increased endogenous formation of *N*-nitroso compounds in the human colon explain the association between red meat and colon cancer? *Carcinogenesis*, **17**, 515–523.
15. Hughes,R., Cross,A.J., Pollock,J.R. and Bingham,S. (2001) Dose-dependent effect of dietary meat on endogenous colonic *N*-nitrosation. *Carcinogenesis*, **22**, 199–202.
16. Mirvish,S.S., Haorah,J., Zhou,L., Hartman,M., Morris,C.R. and Clapper,M.L. (2003) *N*-Nitroso compounds in the gastrointestinal tract of rats and in the feces of mice with induced colitis or fed hot dogs or beef. *Carcinogenesis*, **24**, 595–603.
17. Parnaud,G., Peiffer,G., Tache,S. and Corpet,D.E. (1998) Effect of meat (beef, chicken, and bacon) on rat colon carcinogenesis. *Nutr. Cancer*, **32**, 165–173.
18. Parnaud,G., Pignatelli,B., Peiffer,G., Tache,S. and Corpet,D.E. (2000) Endogenous *N*-nitroso compounds, and their precursors, present in bacon, do not initiate or promote aberrant crypt foci in the colon of rats. *Nutr. Cancer*, **38**, 74–80.
19. Sugimura,T. (1997) Overview of carcinogenic heterocyclic amines. *Mutat. Res.*, **376**, 211–219.
20. Sinha,R., Rothman,N., Brown,E.D., Salmon,C.P., Knize,M.G., Swanson,C.A., Rossi,S.C., Mark,S.D., Levander,O.A. and Felton,J.S. (1995) High concentrations of the carcinogen 2-amino-1-methyl-6-phenylimidazo-[4,5-b]pyridine (PhIP) occur in chicken but are dependent on the cooking method. *Cancer Res.*, **55**, 4516–4519.
21. Stavric,B. (1994) Biological significance of trace levels of mutagenic heterocyclic aromatic amines in human diet: a critical review. *Food Chem. Toxicol.*, **32**, 977–994.
22. Sinha,R. (2002) An epidemiologic approach to studying heterocyclic amines. *Mutat. Res.*, **506/507**, 197–204.
23. Sesink,A.L., Termont,D.S., Kleibeuker,J.H. and Van der Meer,R. (1999) Red meat and colon cancer: the cytotoxic and hyperproliferative effects of dietary heme. *Cancer Res.*, **59**, 5704–5709.
24. Sesink,A.L., Termont,D.S., Kleibeuker,J.H. and Van der Meer,R. (2001) Red meat and colon cancer: dietary haem-induced colonic cytotoxicity and epithelial hyperproliferation are inhibited by calcium. *Carcinogenesis*, **22**, 1653–1659.
25. Sawa,T., Akaike,T., Kida,K., Fukushima,Y., Takagi,K. and Maeda,H. (1998) Lipid peroxyl radicals from oxidized oils and heme-iron: implication of a high-fat diet in colon carcinogenesis. *Cancer Epidemiol. Biomarkers Prev.*, **7**, 1007–1012.
26. Fenaille,F., Mottier,P., Turesky,R.J., Ali,S. and Guy,P.A. (2001) Comparison of analytical techniques to quantify malondialdehyde in milk powders. *J. Chromatogr. A*, **921**, 237–245.
27. Bird,R.P. (1987) Observation and quantification of aberrant crypts in the murine colon treated with a colon carcinogen: preliminary findings. *Cancer Lett.*, **37**, 147–151.
28. Van den Berg,J.W., Koole-Lesuis,R., Edixhoven-Bosdijk,A. and Brouwers,N. (1988) Automating the quantification of heme in feces. *Clin. Chem.*, **34**, 2125–2126.
29. Ohkawa,H., Ohishi,N. and Yagi,K. (1979) Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal. Biochem.*, **95**, 351–358.
30. Govers,M.J., Termont,D.S., Lapre,J.A., Kleibeuker,J.H., Vonk,R.J. and Van der Meer,R. (1996) Calcium in milk products precipitates intestinal fatty acids and secondary bile acids and thus inhibits colonic cytotoxicity in humans. *Cancer Res.*, **56**, 3270–3275
31. Lapre,J.A. and Van der Meer,R. (1992) Diet-induced increase of colonic bile acids stimulates lytic activity of fecal water and proliferation of colonic cells. *Carcinogenesis*, **13**, 41–44.
32. Sesink,A. (2001) Red meat and colon cancer: a possible role for heme. PhD Thesis, Rijksuniversiteit Groningen, 31 Jan, 2001, pp. 1–130.
33. Vaghefi,N., Nedjaoum,F., Guillochon,D., Bureau,F., Arhan,P. and Bougle,D. (2002) Influence of the extent of hemoglobin hydrolysis on the digestive absorption of heme iron. An *in vitro* study. *J. Agric. Food Chem.*, **50**, 4969–4973.
34. Bartsch,H., Nair,J. and Owen,R.W. (2002) Exocyclic DNA adducts as oxidative stress markers in colon carcinogenesis: potential role of lipid peroxidation, dietary fat and antioxidants. *Biol. Chem.*, **383**, 915–921.
35. Wagener,F.A., Eggert,A., Boerman,O.C., Oyen,W.J., Verhofstad,A., Abraham,N.G., Adema,G., Van Kooyk,Y., De Witte,T. and Figdor,C.G. (2001) Heme is a potent inducer of inflammation in mice and is counteracted by heme oxygenase. *Blood*, 2001, **98**, 1802–1811.
36. Rhodes,J.M. and Campbell,B.J. (2002) Inflammation and colorectal cancer: IBD-associated and sporadic cancer compared. *Trends Mol. Med.*, **8**, 10–16.
37. Woo,J., Iyer,S., Cornejo,M.C., Mori,N., Gao,L., Sipos,I., Maines,M. and Buelow,R. (1998) Stress protein-induced immunosuppression: inhibition of cellular immune effector functions following overexpression of haem oxygenase (HSP 32). *Transplant Immunol.*, **6**, 84–93.
38. Magnuson,B.A. and Bird,R.P. (1993) Reduction of aberrant crypt foci induced in rat colon with azoxymethane or methylnitrosourea by feeding cholic acid. *Cancer Lett.*, **68**, 15–23.
39. Reddy,B.S., Maeura,Y. and Weisburger,J.H. (1983) Effect of various levels of dietary butylated hydroxyanisole on methylazoxymethanol acetate induced colon carcinogenesis in CF1 mice. *J. Natl Cancer Inst.*, **71**, 1299–1305.
40. Corpet,D.E. and Tache,S. (2002) Most effective colon cancer chemopreventive agents in rats: a systematic review of aberrant crypt foci and tumor data, ranked by potency. *Nutr. Cancer*, **43**, 1–21.
41. Kanazawa,A., Sawa,T., Akaike,T., Morimur,S., Kida,K. and Maeda,H. (2000) Generation of lipid peroxyl radicals from edible oils and their biological activities: a need for consideration for anti-radical components and purification processing. *Biofactors*, **13**, 187–193.
42. Owen,RW., Giacosa,A., Hull,WE., Haubner,R., Wurtele,G., Spiegelhalder,B. and Bartsch,H. (2000) Olive-oil consumption and health: the possible role of antioxidants. *Lancet Oncol.*, **1**, 107–112.
43. Baron,J.A., Beach,M., Mandel,J.S. *et al.* (1999) Calcium supplements for the prevention of colorectal adenomas. Calcium Polyp Prevention Study Group. *N. Engl. J. Med.*, **340**, 101–107.
44. Newmark,H.L., Lipkin,M. and Maheshwari,N. (1990) Colonic hyperplasia and hyperproliferation induced by a nutritional stress diet with four components of Western-style diet. *J. Natl Cancer Inst.*, **82**, 491–496.
45. Newmark,H.L. and Lipkin,M. (1992) Calcium, vitamin D, and colon cancer. *Cancer Res.*, **52**, 2067 s–2070 s.
46. Fly,A.D. and Czarnecki-Maulden,G.L. (2000) Iron bioavailability from hemoglobin and hemin in chick, rat, cat, and dog: a comparative study. *Nutr. Res.*, **20**, 237–248.
47. Baron,C.P. and Andersen,H.J. (2002) Myoglobin-induced lipid oxidation. A review. *J. Agric. Food Chem.*, **50**, 3887–3897.
48. Pearson,A.M. and Dutson,A.T. (1987) *Advances in Rat Research. Restructured Meat and Poultry Products*. Van Nostrand Reinhold Company, NY.

Received May 2, 2003; revised July 22, 2003; accepted July 24, 2003