



**HAL**  
open science

## **Beef meat and blood sausage promote the formation of azoxymethane-induced mucin-depleted foci and aberrant crypt foci in rat colons.**

Fabrice H.F. Pierre, Amanda Freeman, Sylviane Taché, Roelof van Der Meer,  
Denis E. Corpet

### ► **To cite this version:**

Fabrice H.F. Pierre, Amanda Freeman, Sylviane Taché, Roelof van Der Meer, Denis E. Corpet. Beef meat and blood sausage promote the formation of azoxymethane-induced mucin-depleted foci and aberrant crypt foci in rat colons.. *The Journal of Nutrition*, 2004, 134 (10), pp.2711-6. hal-00411761

**HAL Id: hal-00411761**

**<https://hal.science/hal-00411761>**

Submitted on 28 Aug 2009

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

1 **Beef meat and blood sausage promote azoxymethane-induced**  
2 **mucin-depleted foci and aberrant crypt foci in rat colons<sup>1</sup>**

3  
4 Fabrice Pierre<sup>2</sup>, \*Amanda Freeman, Sylviane Taché, #Roelof Van der Meer and Denis E.  
5 Corpet

6 Ecole Nationale Vétérinaire Toulouse, UMR INRA-ENVT Xénobiotiques, 23 Capelles,  
7 31076 Toulouse, France, \*Department of Veterinary Science, University of Melbourne,  
8 Australia, and #Wageningen Centre for Food Sciences, NIZO Food Research, PO Box 20,  
9 6710 BA Ede, The Netherlands

10 **Running title:** Red meat promotes colon carcinogenesis in rats

11 **Foot notes:**

12 1- The study was supported by the INRA, the DGER, and the French region Midi-Pyrénées

13 2- To whom requests for reprints should be addressed, at Ecole Nationale Vétérinaire  
14 Toulouse, UMR INRA-ENVT Xénobiotiques, 23 Capelles, 31076 Toulouse, France. Phone:  
15 +33 05 61 19 32 89; Fax: +33 05 61 49 12 63; E-mail: [f.pierre@envt.fr](mailto:f.pierre@envt.fr)

16 3- Abbreviations: ACF: aberrant crypt foci, MDF: mucin-depleted foci, MTT: 3-(4,5-  
17 dimethyldiazol-2-yl)-2,5 diphenyl tetrazolium bromid, TBARS: thiobarbituric acid reactive  
18 substances

19 4- Data were presented at the 4<sup>th</sup> NACRe symposium: Freeman, A., Taché, S., Corpet, D.E.,  
20 Pierre, F. (2003) Viande et cancer : Promotion des lésions précancéreuses du colon du rat par  
21 le poulet, le bœuf et le boudin noir. 13-14 November, Paris, France.

22 Number of word : 5952; Number of tables: 3; Number of figure: 2

23 **Abstract**

24

25 Red meat is associated with colon cancer risk. Puzzlingly, meat does not promote  
26 carcinogenesis in rodent studies. However, we demonstrated previously that dietary heme  
27 promotes aberrant crypt foci (ACF), in rats given a low-calcium diet. Here, we test the  
28 hypothesis that heme-rich meats promote colon carcinogenesis in rats treated with  
29 azoxymethane, in low-calcium diets (20 mmol/kg). Three meat diets were formulated to  
30 contain varying concentrations of heme by the addition of raw chicken, beef, or black  
31 pudding (blood sausage). The no-heme control diet was supplemented with ferric citrate, and  
32 a heme control diet with hemoglobin, to match iron or heme concentration in the beef diet.  
33 After 100 d colons were scored for ACF and mucin-depleted foci (MDF). Fecal water was  
34 assayed for lipoperoxides and cytotoxicity. Only diets with heme promoted MDF, but all  
35 meat diets promoted ACF. The number of MDF/colon was  $0.55 \pm 0.68$  in controls, but  $1.2 \pm 0.6$   
36 ( $p=0.13$ ),  $1.9 \pm 1.4$  ( $p<0.01$ ) and  $3.0 \pm 1.2$  ( $p<0.001$ ) in chicken, beef and black pudding-fed  
37 rats. MDF promotion was significantly greater for the high-heme black pudding diet, than for  
38 the median-heme beef diet. The number of ACF/colon was  $71 \pm 16$  in controls, but  $90 \pm 18$ ,  
39  $99 \pm 13$  and  $103 \pm 14$  in chicken, beef, and black pudding-fed rats (all  $p<0.001$ ). No ACF or  
40 MDF difference was seen between beef and the matching heme control diet. MDF promotion  
41 correlated with high fecal water lipoperoxides and cytotoxicity ( $r=0.65$ ,  $p<0.01$ ). This is the  
42 first study to show the promotion of experimental carcinogenesis by dietary meat, and the  
43 association with heme intake.

44

45 **Keywords:** Colorectal carcinogenesis, Heme, Lipoperoxidation, Red meat, Chicken

46

47 **Introduction**

48 Colorectal cancer is a major killer in affluent countries, and recommendations are to reduce  
49 red meat intake to reduce the risk (1). The meta-analysis of epidemiological studies by Norat  
50 *et al.*, found a moderate but significant association between red meat intake and colorectal  
51 cancer risk (2). In puzzling contrast with epidemiological studies, experimental studies do not  
52 support the hypothesis that red meat would increase colorectal cancer risk. Among the twelve  
53 rodent studies reported in the literature, none demonstrated a specific promotional effect of  
54 red meat (3-14). McIntosh *et al.* (3) showed that rats given a diet containing kangaroo meat,  
55 soybean protein or casein have similar incidence of dimethylhydrazine-induced tumors.  
56 Clinton *et al.* (4) also found the colon tumor incidence to be the same for beef meat (raw or  
57 grilled) and soybean diet fed rats. Nutter *et al.* (5) found beef proteins to afford significant  
58 protection in mice compared with milk protein. Reddy *et al.* (6) and Pence *et al.* (7) found  
59 high-protein and high-fat diets, whatever the protein source, to increase colon tumor  
60 incidence in rats, but beef meat affords a significant protection compared with casein (7).  
61 Pence *et al.* (8) found that well-cooked beef meat decreases the risk of colon cancer in rats  
62 compared to casein in a high-fat context, but increases the risk in a low-fat context. Lai *et al.*  
63 (9) found that a lean beef diet does not increase tumor incidence in rats compared with a  
64 casein-iron citrate diet. Alink *et al.* (10) showed that human diets (with meat) produced more  
65 colon carcinomas in rats than rodent diets (with no meat). Alink's results do not support  
66 specific meat promotion, however, as the human diets contained more fat and less fiber than  
67 the rodent diets. Mutanen *et al.* (11) did not find beef meat diet to increase significantly the  
68 number of intestinal tumor in Min mice, although it contained five times more fat than the  
69 control diet. Ketunen *et al.* (12) found less tumors in female Min mice given beef meat than  
70 in controls. Parnaud *et al.* (13) did not find red meat to promote azoxymethane-induced  
71 aberrant crypt foci (ACF) compared to casein-fed controls. Belobrajdic *et al.* (14) found

72 kangaroo meat to promote ACF in comparison with whey protein, but whey is a known  
73 protector of colon carcinogenesis (15).

74

75 Sesink *et al.* speculated that heme, found in red meat myoglobin, would enhance colon  
76 carcinogenesis. They demonstrated that pure hemin added to rats diet increases colonic  
77 epithelial proliferation (16), and that calcium phosphate inhibits the hemin-induced  
78 proliferation (17). In line with Sesink's hypothesis, we have shown that hemin diets increase  
79 the number and size of azoxymethane-induced ACF in rats fed a low-calcium diet, while  
80 hemoglobin diets increase ACF number only (18). Dietary hemin also produces cytotoxic  
81 fecal water and high amounts of thio-barbituric acid reactive substances (TBARS), indicative  
82 of lumen lipoperoxidation (16), while dietary hemoglobin increases fecal TBARS only (18).  
83 ACF are putative preneoplastic lesions, and the effect of agents on ACF correlates with the  
84 effect on tumor incidence in most studies (19), but not all. Recently, alternative short term  
85 biomarkers of colon carcinogenesis have been proposed: mucin-depleted foci (MDF)(20).  
86 MDF are easy to score and may predict tumor outcome better than ACF (20,21).

87

88 The present study was designed to test the hypothesis that heme in the food matrix can  
89 promote colon carcinogenesis in a low-calcium context. The diets used in previous animal  
90 studies (3-13) contained high levels of calcium, which may explain they did not show a  
91 promoting effect of red meat. Three types of meat were chosen with different heme contents:  
92 Chicken, beef and black pudding. A fourth diet, containing pure hemoglobin, was added.  
93 This diet acted as a control as it contained the same concentrations of heme as the beef diet,  
94 and the myoglobin in beef is very close in structure to hemoglobin. Besides the ACF  
95 endpoint, we also scored MDF.

96 **Materials and methods**

97 *Animals*

98 Sixty Fischer 344 female rats were purchased at 4 w of age from Iffa Credo (St.Germain  
99 l'Arbresle, France). Animal care was in accordance with the guidelines of the European  
100 Council on animals used in experimental studies. They were distributed randomly in pairs  
101 into stainless steel wire bottomed cages. The room was kept at a temperature of 22°C on a  
102 12-h light-dark cycle. Animals were allowed 7 d of acclimatization to the room and to the  
103 control diet (cf. Table I) before being injected i.p. with the carcinogen azoxymethane (Sigma  
104 chemical, St.Quentin, France; 20 mg/Kg body weight) in NaCl (9 g/L). Seven days after the  
105 injection the rats were allowed free access to their respective diet for 100 d. Feed was  
106 changed every second or third day and water once a week. Animal body weights were  
107 monitored weekly. Feed intake per cage of two rats was also monitored at periodic intervals  
108 (d 5, 62 and 77). Fecal mass was measured as the total over a 24 h period per two rats on d  
109 56, 61, 62, 76 and 77.

110

111 *Diets*

112 Experimental diets, as shown in Table I, were based on the diet fed to control rats (N=20 rats)  
113 consisting of a modified AIN-76 diet (22) prepared and formulated in a powdered form by  
114 the UPAE (INRA, Jouy-en-Josas, France). Dibasic calcium phosphate was included at a low  
115 concentration of 20 mmol/kg. Three meat diets given to three groups of rats (N=10  
116 rats/group), were formulated to contain varying concentrations of heme as hemoglobin or  
117 myoglobin by the addition of freeze-dried beef, chicken or black pudding at 60% (w/w) meat  
118 of the total diet by weight. The beef and chicken (skin-less) meat was obtained from UPAE.  
119 Meat was freeze-dried by LyoFal (Salon de Provence, France). The beef contained 0.6  $\mu$   
120 mol/g of heme while none was detected in the chicken diet (see the assay below). The low fat

121 black pudding (blood sausage) contained 16  $\mu\text{mol/g}$  of heme. It was specially made by  
122 Recape (Lanta, France) with 90% pork blood and 10% starch (w/w), and contained no  
123 potentially protective additives such as onion or milk. One group of rats (N=10) received a  
124 hemoglobin diet containing the same concentration of heme as the beef diet (0.36  $\mu\text{mol/g}$   
125 diet). This was achieved by adding powdered bovine hemoglobin (Sigma chemical,  
126 St.Quentin, France) to the control diet. All diets were balanced for protein (50%), fat (20%),  
127 calcium (20 mmol/kg) and iron (2.5 mmol/kg) by addition of casein, lard, calcium phosphate  
128 and ferric citrate. However, the black pudding diet could not be balanced for iron (17  
129 mmol/kg). The diets were made up twice a month and maintained at  $-20^{\circ}\text{C}$ , and TBARS  
130 assay showed no lipoperoxidation (data not shown).

131

### 132 ***ACF and MDF Assays***

133 All rats were killed by CO<sub>2</sub> asphyxiation in a random order on day 99 or 100. Coded colons  
134 were scored for ACF by Bird's procedure (23). ACF scoring was done in duplicate by two  
135 readers not knowing the rat treatment. Colons, after being scored for ACF, were stained with  
136 high iron diamine-Alcian blue procedure (HID-AB) to evaluate mucin production (20). MDF  
137 number, and number of crypts per MDF, were scored by a single reader, not knowing the rat  
138 treatment or the ACF results, under light microscope at x32 magnification. According to  
139 Caderni *et al.* , lesions were identified as MDF by the absence or very small production of  
140 mucins, and by at least two of the following criteria: multiplicity higher than 3 crypts,  
141 distortion of the lumen of the crypts, elevation of the lesion in comparison of normal mucosa  
142 (20). All lesions were photographed (Figure 1), and representative pictures were submitted  
143 by mail to Dr. Caderni for confirmation.

144

### 145 ***Preparation of Fecal Water, Assay of TBARS and Heme.***

146 For assay of TBARS, heme, and cytotoxic activity on CMT93, fecal water was prepared from  
147 24-h feces collected under each cage of two rats, as previously described (18), but black  
148 pudding samples were diluted twice more than other samples. For assay of cytolytic activity  
149 on erythrocytes, fecal water was prepared by Sesink's procedure and pH measured (16).  
150 TBARS were measured in fecal water according to Ohkawa *et al.* (24), exactly as previously  
151 described (18). Heme contents of freeze-dried feces and of fecal water were measured by  
152 fluorescence according to Van den Berg *et al.* (25) and to Sesink *et al.* (16), respectively, as  
153 already described (18).

154

#### 155 ***Cytolytic Assay of Fecal Water***

156 The cytotoxicity of fecal water was quantified by two methods, on erythrocytes, and on a cell  
157 line. First, the cytolytic activity of fecal water was quantified by potassium-release from  
158 erythrocytes as described by Govers *et al.* (26). Secondly, the cytotoxicity of fecal water  
159 obtained with a different method (see above) was also quantified by the MTT test on a cell  
160 line according to Bonneson *et al.* (27). Briefly, the cancerous mouse colonic epithelial cell  
161 line, CMT93 (ECAC), was seeded in 96-well microtiter plates ( $1.6 \times 10^4$  cells per well in 200  
162  $\mu\text{L}$  of medium) and at confluence the cells were treated for 24 h with the fecal water sample  
163 to be tested diluted in the culture medium at a concentration of 0.1% (v/v). Each fecal water  
164 sample was tested in 7 wells and 10 wells remained untreated to act as controls. One hundred  
165  $\mu\text{L}$  of MTT (9% in PBS) was added to each well. After 3 h of incubation at 37°C in the dark,  
166 100  $\mu\text{L}$  of a 10% SDS - 0.1 mol/L NaOH mixture was added. After 1 h of incubation in the  
167 dark, the absorbance of each well was read using a microplate reader at wavelength 570 nm  
168 for cytotoxicity and 690 nm for background.

169

170 *Statistical Analysis*

171 Results were analyzed using Systat 10 software for Windows, and reported as mean  $\pm$  SD.  
172 ACF scoring was done in duplicate. Values of ACF were considered firstly using two-way  
173 (groups and readers) analysis of variance (ANOVA). The (group x reader) interaction was  
174 never significant, and when total ANOVA was significant ( $p < 0.05$ ), pairwise differences  
175 between groups were analysed using the Fishers's least-significant-difference test. MDF  
176 values and all other data were considered using one-way ANOVA and groups were compared  
177 using the Fishers's least-significant-difference test. The Pearson coefficient was used to  
178 determine the correlation between ACF, MDF, heme intake and fecal values, and p values  
179 were calculated with Bonferroni correction for multiple comparisons. Because the black  
180 pudding diet contained a very high concentration of heme, heme values were log-transformed  
181 before statistical analysis.

182

183 **Results**

184

185 ***Weight gain and feed intake***

186 Beef-fed rats quickly became heavier than control rats, and the difference reached  
187 significance at d 30. Final body weight of beef-fed rats was  $210\pm 9$  g (cf. Table 2), higher than  
188 controls  $198\pm 12$  g ( $p<0.05$ ). Black pudding-fed rats had watery stools, a known effect of  
189 dietary heme, and they drank more water than controls ( $22\pm 1$  ml/d vs  $16\pm 0.5$  ml/d,  $p<0.001$ ).  
190 Furthermore, all groups had similar food intake, the mean value being  $8.4\pm 0.5$  g/d at day 75  
191 (full data not shown).

192

193 ***ACF data***

194 All meat-based diets (chicken, beef and black pudding) significantly increased the number of  
195 ACF ( $p<0.001$ , Figure 2A) and the number of aberrant crypts per colon ( $p<0.001$ , Table 2)  
196 after 100 d on the diets. Chicken and black pudding, but not beef, also increased the number  
197 of crypts per ACF ( $p<0.01$ , Table 2). Aberrant crypts and ACF promotion by black pudding  
198 diet was significantly more potent than promotion by chicken diet ( $p<0.05$ , Table 2). No  
199 significant difference was seen between rats given the beef diet and rats given the matching  
200 hemoglobin diet for aberrant crypts or ACF per colon, but the size of ACF was significant  
201 greater for haemoglobin group (Table 2).

202

203 ***MDF Data***

204 Beef and black pudding-fed rats had more MDF than control rats ( $p<0.01$ ), and promotion by  
205 black pudding was more potent than promotion by beef ( $p<0.05$ , Figure 2B). Chicken-based  
206 diet, which contains no heme, did not promote MDF (Figure 2B). The effects observed on the  
207 number of MDF were also observed on the number of mucin depleted crypts (Table 2). No

208 differences were observed between groups in the number of crypts per MDF. Last, no  
209 significant difference was observed between the beef and hemoglobin groups (table 2).

210

### 211 *Fecal heme, TBARS and Cytotoxicity*

212 The heme intake, and the fecal concentration of heme, matched the study design. As  
213 expected, no heme was detected in feces of control and chicken diet fed rats (Table 3). The  
214 analysis of fecal samples stored during previous meat study of the laboratory where diet  
215 containing too 60% beef meat but 130  $\mu\text{mol/g}$  calcium (13) yielded similar results: No heme  
216 was detected in feces of control and chicken diet fed rats, but  $1.7\pm 1.5$   $\mu\text{mol/g}$  in feces of beef  
217 meat fed rats. However, in the present study, the heme concentration was higher in the feces  
218 of hemoglobin-fed rats than in beef-fed rats (Table 3). This fits with the observation that less  
219 heme iron reaches the colon when it is supplied as red meat rather than in hemoglobin form  
220 (14). We measured the characteristics of fecal water because, according to studies on bile  
221 acids, the soluble fraction of colonic contents would interact more strongly with the mucosa  
222 than the insoluble fraction (28). As expected, the heme concentration in fecal water depended  
223 directly on the level of heme in the diet (Table 3), with, as noted above, a difference between  
224 meat and hemoglobin-fed rats. No heme was found in fecal waters stored during Parnaud's  
225 meat study, even in samples from rats given a 60% beef meat diet (13).

226

227 Heme can induce the formation of peroxy radicals in fats, which may be cytotoxic and  
228 cleave DNA *in vivo* (29). Lipid peroxidation was thus measured in fecal water by the TBARS  
229 assay. Lipid peroxidation was associated with heme concentration in fecal water (Table 3):  
230 The black pudding diet thus increased TBARS in the fecal water by 23-fold. The hemoglobin  
231 diet and beef diet increased TBARS by 2-4 fold (all  $p < 0.01$ ), but chicken diet did not change  
232 significantly fecal water TBARS, compared with control diet.

233 Furthermore, the fecal water of hemin-fed rats is cytotoxic, which would explain the hemin-  
234 induced increased proliferation (18). Cytotoxicity of fecal water was thus measured by two  
235 methods: lysis of erythrocytes, and toxicity on CMT93 cell in culture. The black pudding  
236 diet, a very high source of heme, enhanced erythrocytes cytolysis by more than 50-fold, and  
237 toxicity on CMT93 cells by 8-fold (both  $p < 0.001$ , Table 3). Beef and hemoglobin diets  
238 produced equivalent effects: no lytic activity on erythrocytes, but a four-fold increase in  
239 CMT93 cells toxicity ( $p < 0.001$ ). The cytotoxicity of fecal water from chicken-fed rats was  
240 not different from that of controls (Table 3). pH value of fecal waters was also measured. All  
241 meat-based diets increased the pH value, and fecal pH was higher when heme concentration  
242 was higher in the diet (Table 3). Taken together, these data suggest that, cytotoxicity, pH and  
243 lipoperoxides of faecal water follow heme intake and fecal heme. Indeed, significant  
244 correlations were seen between heme intake and fecal water cytotoxicity ( $r = 0.98$ ), pH  
245 ( $r = 0.86$ ) and TBARS ( $r = 0.73$ , all  $p < 0.01$ ,  $N = 30$  cages).

246 **Discussion**

247

248 This study is the first to show that meat can specifically promote colon carcinogenesis. In  
249 addition, the promoting effect was strong compared with other promoting agents (30), and  
250 clearly associated to heme concentration in meat. This study was done with a low calcium  
251 diet containing 60% meat and 5% easily oxidized oil. We used two putative pre-cancerous  
252 endpoints: the established ACF, and the recently described MDF. Heme in the diet led to  
253 ACF and MDF promotion in the colon. The no-heme chicken-based diet did not promote  
254 MDF, but increased the ACF number.

255

256 This study is, to our knowledge, the first non-Italian study to use a new carcinogenesis  
257 endpoint which was recently discovered by Caderni *et al.* (20). MDF may predict tumor  
258 outcome better than ACF, as shown in the studies of synbiotics, cholic acid and piroxicam  
259 (20,21). We found that MDF were quite easy to score, but we detected fewer MDF per  
260 control rat than did Caderni. This is likely the result of the carcinogen dose: azoxymethane  
261 was injected once instead of twice, and the resulting number of ACF was four times fewer  
262 here than in Caderni's study (72 vs. 298 ACF/colon).

263

264 That heme content in meat was responsible for promotion, at least in part, is supported by the  
265 following facts: (i) All tested meat diets promoted ACF formation, but this was significantly  
266 greater for the high heme diet, based on black pudding, than for the no-heme chicken diet  
267 (Figure 2). (ii) Only heme-containing diets promoted MDF formation, and the effect was  
268 dose dependent, since black pudding effect was significantly stronger than beef effect. MDF  
269 per colon correlated with heme intake ( $r = 0.63$ ,  $N=60$ ,  $p<0.01$ ). (iii) Beef and hemoglobin  
270 diets, which provided identical heme intake, promoted ACF and MDF equally (Table 3). This

271 meat study is thus consistent with our previous study, where ACF were promoted dose-  
272 dependently by graded doses of dietary heme (18). We think that previous rodents studies  
273 failed to show that red meat promotes carcinogenesis, because meat was included in a high-  
274 calcium diet. The standard AIN-76 diet contains 130 mmol/kg calcium, which is similar to  
275 the concentrations that inhibits heme-induced colonic proliferation (17) and heme-induced  
276 ACF promotion (18). Calcium precipitates heme in the gut lumen, and reduces heme  
277 concentration in fecal water (17,18). In Parnaud's study (13), the heme concentration was  
278 high in the feces of beef-fed rats, but not detectable in the fecal water (see results above). We  
279 suggest this be due to high dietary calcium, and it resulted in the lack of ACF promotion by  
280 beef-meat diet (13). However, the link between heme intake and ACF yield is not a direct  
281 one: black pudding provided a huge quantity of heme to the gut that was not mirrored  
282 linearly in the ACF outcome.

283  
284 The mechanism of heme promotion is not known, but might be linked to peroxidation,  
285 cytotoxicity and pH. In a previous study, we showed that pure heme and hemoglobin  
286 promote ACF formation, and strikingly induce lipoperoxidation and cytotoxicity of fecal  
287 water (18). Indeed, heme promotes the non-enzymatic peroxidation of polyunsaturated fatty  
288 acids (16,18,29). The lipid peroxy radicals (LOO•) generated from simultaneous fat and  
289 heme iron ingestion, and the resulting oxygen radicals, can cleave DNA or modify DNA  
290 bases, which could increase carcinogenesis (29). The beef-based diet contained 0.36 µmol/g  
291 heme. Its intake led to 19 µmol/L heme in fecal water, and a 2.5-fold increase in  
292 lipoperoxidation (Table 3). Similar TBARS values were seen in fecal water from beef-fed  
293 rats and, in our previous study (18), from hemoglobin-diet fed rats (138 and 187 mol/L MDA  
294 equivalents, respectively). In addition, red meat intake induced fecal cytotoxicity and  
295 increased pH value of fecal water (Table 3). Black-pudding contains 25 times more heme

326 than beef meat. Compared with beef, its consumption led to 60 times more heme in fecal  
327 water, seven times more TBARS, and a much higher cytotoxicity (Table 3). Fecal water from  
328 beef-fed rats or hemoglobin fed rats (18) did not induce cytolysis of erythrocytes, probably  
329 because heme intake was too low. In contrast, fecal water from black-pudding-fed rats  
330 strikingly induced erythrocyte cytolysis. Thus, it can be concluded that there was a dose-  
331 dependent effect of the heme concentration in the diet and in fecal water on the fecal  
332 lipoperoxidation, cytotoxicity and pH, and these values correlated significantly. In addition,  
333 MDF and ACF numbers per rat also correlated with these fecal values (all  $r > 0.5$ , all  $p < 0.01$ ,  
334  $N = 60$  rats, highest correlation,  $r = 0.65$  between number of MDF and cytotoxicity). These  
335 correlations suggest that fecal cytotoxicity, lipoperoxides and pH may explain heme  
336 promotion. That hemoglobin and meat diets, with same heme content as hemoglobin and  
337 myoglobin, produced the same effects also supports this idea (Table 3). Surprisingly, no ACF  
338 promotion was seen in a published study with a protocol very similar to this one (14). Fecal  
339 heme concentrations were similar in both studies, but fecal TBARS value was twice higher in  
340 this study. We suppose that lipoperoxidation was inhibited by *tert*-Butylhydroquinone in the  
341 AIN-93 diet used by Belobradjic (14). This chance observation supports the implication of  
342 heme-induced lipoperoxidation in heme-induced colon carcinogenesis.

313

314 The no-heme chicken-based diet surprisingly increased the ACF number and size (Table 2).  
315 ACF promotion was small yet significant, and MDF increase was not significant, which  
316 reduces the level of evidence that chicken contains a promoter. ACF data however suggest  
317 that the chicken meat used in this study may contain a promoter that is not heme, and remains  
318 to be explained. The prominent features of chicken diet were high arachidonic acid and  
319 niacin. Chicken diet contained 1 g/kg of arachidonic acid (calculated from ref.(31)) compared  
320 to 0.25 g/kg in other diets. Arachidonic acid has pro-tumorigenic properties, likely by

321 increasing prostaglandin synthesis (32). In addition, chicken diet contained 207 mg/kg of  
322 niacin, four times the 51 mg/kg found in control diet and twice the value in beef diet (assays  
323 done by LARA lab., Toulouse, France). Niacin can afford protection against carcinogenesis  
324 when added to a niacin-deficient diet (33), but high doses are toxic. Here, the high dose  
325 brought by the chicken-based diet would translate to 12 times the Recommended Dietary  
326 Allowances in humans (USA National Academy of Sciences). High niacin stimulates  
327 histamine release and prostaglandin synthesis, which might explain the ACF promotion (34).  
328 The intake of white meat is not associated with colorectal cancer risk in most  
329 epidemiological studies (1,2). In contrast, dietary heme iron intake is associated with an  
330 increased risk of proximal colon cancer (35). However, in a prospective cohort study of  
331 34198 Californian Adventists, the consumption of white meat, mostly chicken, was  
332 associated with a tripled risk of colorectal cancer (36).

333

334 In summary, this study shows for the first time a promoting effect of red meat on  
335 carcinogenesis. It corroborates epidemiological observations: high red meat intake is  
336 associated with increased colon cancer risk. In previous meat studies (3-13), the promoting  
337 effect of meat was hidden by dietary calcium, as shown here on Parnaud's study (13).  
338 Furthermore, MDF promotion was related to heme intake. Promotion was significantly  
339 greater for the high heme black pudding diet, than for the median-heme beef diet. This heme  
340 effect matches latest epidemiological data (35). The low-heme chicken diet did not promote  
341 MDF, but did increase ACF formation. For red meat diets, promotion was associated with  
342 high fecal water lipoperoxidation, cytolytic activity and increase of pH, which may explain  
343 the increased carcinogenesis.

344

345 **Acknowledgements:**

346 We thank Xavier Blanc (UPAE) for the preparation of experimental diets, Denise S.M.L.  
347 Termont for the assay of cytolytic activity of fecal water on erythrocytes, Florence Vallenari  
348 for the assay of heme in feces and fecal water of Parnaud *et al.* study, Marie-Claude Nicot for  
349 the assay of calcium and iron in meats, and Raymond Gazel and Florence Blas Y Estrada for  
350 the care of the animals.

351 **Table I:** Composition of diets (g/kg)

	<b>Control</b>	<b>Chicken</b>	<b>Beef</b>	<b>Black Pudding</b>	<b>Hemoglobin</b>
<b>Chicken</b>	0	600	0	0	0
<b>Beef</b>	0	0	600	0	0
<b>Black Pudding</b>	0	0	0	600	0
<b>Hemoglobin</b>	0	0	0	0	6.3
<b>Lard</b>	150	122	40	112	150
<b>Safflower Oil</b>	50	50	50	50	50
<b>Casein<sup>a</sup></b>	500	1.1	48.5	115	493.8
<b>Corn Starch</b>	60	60	60	5	60
<b>Sucrose</b>	139.5	68	102	20	139.5
<b>Cellulose</b>	50	50	50	50	50
<b>Methionine</b>	3	3	3	3	3
<b>Mineral mix<sup>b</sup></b>	35	35	35	35	35
<b>Vitamin mix<sup>b</sup></b>	10	10	10	10	10
<b>CaHPO<sub>4</sub>·2H<sub>2</sub>O</b>	2.7	1.4	1.6	1.8	2.7
<b>Ferric Citrate<sup>c</sup></b>	0.45	0.35	0	0	0.36

352 a. Low-calcium casein

353 b. AIN76 mix, but 500g/kg of dibasic calcium phosphate replaced by sucrose in mineral mix.

354 c. All diets contained 2.5 mmol/kg iron except the black pudding diet (17 mmol/kg). Iron  
355 concentration was measured in freeze-dried meat before preparing the diets: chicken: 37.5,  
356 beef: 172.6, and black pudding 1527 mg/kg. Other nutrients were balanced: 50% protein,  
357 20% fat, 18-25% carbohydrate, and 20 mmol/kg calcium (based on added components, no  
358 analysis was done on whole diets).

359 **Table 2:** Effect of meat-based diets on aberrant crypt foci and mucin-depleted foci in the colon of rats 107 d after the injection of  
 360 azoxymethane<sup>1</sup>.

361

Diets	Heme	Number	Final body	ACF			MDF		
	μmol/g diet	of rats	Weight (g)	ACF/colon	ACF crypts/colon	Crypts/ACF	MDF/colon	MDF crypts/colon	Crypts/MDF
Control	0.0	20	198±12 <sup>a</sup>	72±16 <sup>a</sup>	192±55 <sup>a</sup>	2.7±0.4 <sup>a</sup>	0.55±0.68 <sup>a</sup>	2.9±4.0 <sup>a</sup>	4.65±2.40 <sup>a</sup>
Chicken	0.0	10	199±10 <sup>a</sup>	91±18 <sup>b</sup>	267±65 <sup>b</sup>	2.9±0.4 <sup>b</sup>	1.20±0.63 <sup>a</sup>	6.0±3.9 <sup>a,b</sup>	4.92±1.64 <sup>a</sup>
Beef	0.36	10	210±9 <sup>b</sup>	100±13 <sup>b,c</sup>	280±49 <sup>b</sup>	2.8±0.2 <sup>a</sup>	1.90±1.37 <sup>b</sup>	8.5±6.9 <sup>b,c</sup>	4.23±1.15 <sup>a</sup>
Hemoglobin	0.36	10	196±11 <sup>a</sup>	93±24 <sup>b,c</sup>	285±78 <sup>b</sup>	3.1±0.5 <sup>b</sup>	2.40±1.50 <sup>b,c</sup>	11.5±9.0 <sup>c,d</sup>	4.60±1.93 <sup>a</sup>
Black Pudding	9.54	10	189±9 <sup>a</sup>	103±14 <sup>c,d</sup>	301±48 <sup>b</sup>	2.9±0.2 <sup>b</sup>	3.00±1.24 <sup>c</sup>	13.1±6.0 <sup>c,d</sup>	4.29±0.59 <sup>a</sup>

362 <sup>1</sup> Values are means ± SD. N= 10 rats/group, except 20 controls.

363 Means without a common letter differ, P<0.05

364

365 **Table 3:** Effect of meat-based diets on fecal values in rats, notably heme, lipoperoxides and cytotoxicity of fecal water<sup>1</sup>.

366

367

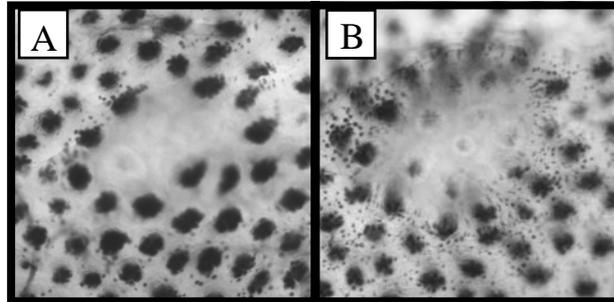
	Heme intake <sup>2</sup>	Dry fecal Mass	Heme in feces <sup>2</sup>	Heme in fecal water <sup>2</sup>	TBARS in fecal water MDA equivalents	pH of fecal water	Cytolytic activity on erythrocytes	Cytotoxicity on CMT93 cells
Diet	μmol/d	g/d	μmol/g	μmol/L	μmol/L	pH	% K release	% cells lysed
Control	0 <sup>a</sup>	0.50±0.11 <sup>a</sup>	0 <sup>a</sup>	0 <sup>a</sup>	40±15 <sup>a</sup>	7.85±0.03 <sup>a</sup>	1±2 <sup>a</sup>	12±12 <sup>a</sup>
Chicken	0 <sup>a</sup>	0.58±0.06 <sup>b,c</sup>	0 <sup>a</sup>	0 <sup>a</sup>	69±16 <sup>a</sup>	8.02±0.03 <sup>b</sup>	1±2 <sup>a</sup>	26±15 <sup>a</sup>
Beef	3.0±0.4 <sup>b</sup>	0.64±0.09 <sup>b,c</sup>	0.5±0.2 <sup>b</sup>	19±7 <sup>b</sup>	138±17 <sup>b</sup>	8.17±0.03 <sup>c</sup>	1±2 <sup>a</sup>	59±14 <sup>b</sup>
Hemoglobin	2.9±0.4 <sup>b</sup>	0.53±0.07 <sup>b</sup>	0.9±0.3 <sup>c</sup>	52±47 <sup>c</sup>	195±96 <sup>b</sup>	8.13 <sup>c</sup>	1±1	58±27 <sup>b</sup>
Black pudding	87.0±8.0 <sup>c</sup>	1.00±0.06 <sup>d</sup>	23.6±8.6 <sup>d</sup>	1097±484 <sup>d</sup>	975±229 <sup>c</sup>	8.30±0.06 <sup>d</sup>	73±36 <sup>b</sup>	88±03 <sup>c</sup>

368 1 Values are means ± SD. N= 5 cages per group, except 10 controls

369 2 ANOVA was done on log of data

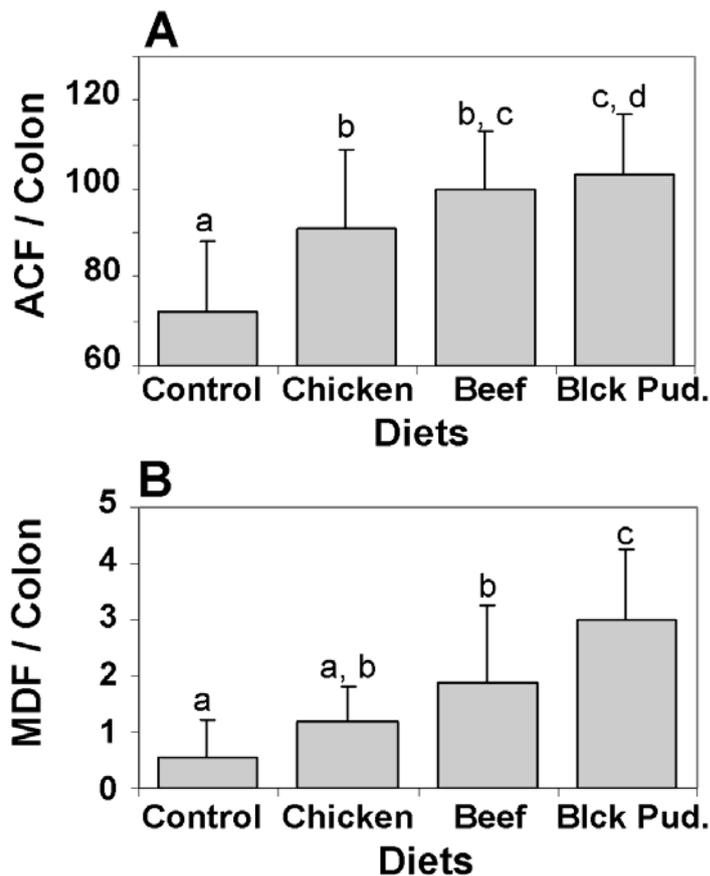
370 Means without a common letter differ, P<0.05

371 **Figure 1:** Formalin-fixed colon after HID-AB staining (original magnification, x32). A:  
 372 Identification of a MDF of five mucin-depleted crypts B: Identification of a MDF of eleven  
 373 mucin-depleted crypts.



374  
 375

376 **Figure 2:** Number of putative pre-cancerous lesions per rat colon after 100 d on the  
 377 experimental diets and one injection of azoxymethane. A: Number of aberrant crypt foci. B:  
 378 Number of mucin-depleted foci. Data are means  $\pm$  SD. N=10 rats/group, except 20 controls.  
 379 Means without a common letter differ,  $P < 0.05$



380

## References

385 1. WCRF & AICR (1997) Food, Nutrition and the prevention of cancer: a global  
386 perspective. Washington, DC: World Cancer Research Fund and American Institute for  
387 Cancer Research.

388 2. Norat, T., Lukanova, A., Ferrari, P. & Riboli, E. (2002) Meat consumption and  
389 colorectal cancer risk: dose-response meta-analysis of epidemiological studies. *Int. J. Cancer*  
390 98: 241-256.

391 3. McIntosh, G. H., Regester, G. O., Leleu, R. K., Royle, P. J. & Smithers, G. W.  
392 (1995) Dairy proteins protect against dimethylhydrazine-induced intestinal cancers in rats. *J.*  
393 *Nutr.* 125: 809-816.

394 4. Clinton SK, D. R., Anderson DB, Truex CR, Imrey PB, Visek WJ (1979) 1,2-  
395 dimethylhydrazine induced intestinal cancer in rats fed beef or soybean protein. *Nutr. reports*  
396 *Int.* 20: 335-342.

397 5. Nutter, R. L., Gridley, D. S., Kettering, J. D., Goude, A. G. & Slater, J. M.  
398 (1983) BALB/c mice fed milk or beef protein: differences in response to 1,2-  
399 dimethylhydrazine carcinogenesis. *J. Natl. Cancer Inst.* 71: 867-874.

400 6. Reddy, B. S., Narisawa, T. & Weisburger, J. H. (1976) Effect of a diet with  
401 high levels of protein and fat on colon carcinogenesis in F344 rats treated with 1,2-  
402 dimethylhydrazine. *J. Natl. Cancer Inst.* 57: 567-569.

403 7. Pence, B. C., Butler, M. J., Dunn, D. M., Miller, M. F., Zhao, C. & Landers,  
404 M. (1995) Non-promoting effects of lean beef in the rat colon carcinogenesis model.  
405 *Carcinogenesis* 16: 1157-1160.

406 8. Pence, B. C., Landers, M., Dunn, D. M., Shen, C. L. & Miller, M. F. (1998)  
407 Feeding of a well-cooked beef diet containing a high heterocyclic amine content enhances  
408 colon and stomach carcinogenesis in 1,2-dimethylhydrazine-treated rats. *Nutr. Cancer* 30:  
409 220-226.

410 9. Lai, C., Dunn, D. M., Miller, M. F. & Pence, B. C. (1997) Non-promoting  
411 effects of iron from beef in the rat colon carcinogenesis model. *Cancer Lett.* 112: 87-91.

412 10. Alink, G. M., Kuiper, H. A., Hollanders, V. M. H. & Koeman, J. H. (1993)  
413 Effect of heat processing and of vegetables and fruit in human diets on 1,2-  
414 dimethylhydrazine-induced colon carcinogenesis in rats. *Carcinogenesis* 14: 519-524.

415 11. Mutanen, M., Pajari, A. M. & Oikarinen, S. I. (2000) Beef induces and rye  
416 bran prevents the formation of intestinal polyps in *apc(min)* mice: relation to beta-catenin and  
417 PKC isozymes. *Carcinogenesis* 21: 1167-1173.

418 12. Kettunen, H. L., Kettunen, A. S. L. & Rautonen, N. E. (2003) Intestinal  
419 immune responses in wild-type and *apc(min/+)* mouse, a model for colon cancer. *Cancer Res.*  
420 63: 5136-5142.

421 13. Parnaud, G., Peiffer, G., Tache, S. & Corpet, D. E. (1998) Effect of meat  
422 (beef, chicken, and bacon) on rat colon carcinogenesis. *Nutr. Cancer* 32: 165-173.

423 14. Belobrajdic, D. P., Mcintosh, G. H. & Owens, J. A. (2003) Whey proteins  
424 protect more than red meat against azoxymethane induced ACF in wistar rats. *Cancer Lett.*  
425 198: 43-51.

426 15. Hakkak, R., Korourian, S., Ronis, M. J. J., Johnston, J. M. & Badger, T. M.  
427 (2001) Dietary whey protein protects against azoxymethane-induced colon tumors in male  
428 rats. *Cancer Epidemiol. Biomarkers Prev.* 10: 555-558.

429 16. Sesink, A. L. A., Termont, D. S. M. L., Kleibeuker, J. H. & Vandermeer, R.

430 (1999) Red meat and colon cancer: the cytotoxic and hyperproliferative effects of dietary  
431 heme. *Cancer Res.* 59: 5704-5709.

432 17. Sesink, A. L. A., Termont, D. S. M. L., Kleibeuker, J. H. & VanDerMeer, R.  
433 (2001) Red meat and colon cancer: dietary haem-induced colonic cytotoxicity and epithelial  
434 hyperproliferation are inhibited by calcium. *Carcinogenesis* 22: 1653-1659.

435 18. Pierre, F., Tache, S., Petit, C. R., Van der Meer, R. & Corpet, D. E. (2003)  
436 Meat and cancer: haemoglobin and haemin in a low-calcium diet promote colorectal  
437 carcinogenesis at the aberrant crypt stage in rats. *Carcinogenesis* 24: 1683-1690.

438 19. Corpet, D. E. & Tache, S. (2002) Most effective colon cancer  
439 chemopreventive agents in rats: a systematic review of aberrant crypt foci and tumor data,  
440 ranked by potency. *Nutr. Cancer* 43: 1-21.

441 20. Caderni, G., Femia, A. P., Giannini, A., Favuzza, A., Luceri, C., Salvadori, M.  
442 & Dolara, P. (2003) Identification of mucin-depleted foci in the unsectioned colon of  
443 azoxymethane-treated rats: correlation with carcinogenesis. *Cancer Res.* 63: 2388-2392.

444 21. Femia, A. P., Dolara, P. & Caderni, G. (2004) Mucin-depleted foci (MDF) in  
445 the colon of rats treated with azoxymethane (AOM) are useful biomarkers for colon  
446 carcinogenesis. *Carcinogenesis* 25: 277-281.

447 22. American Inst. Nutr. (1977) Report of the American Institute of Nutrition. Ad  
448 Hoc Committee on standards for nutritional studies. *J. Nutr.* 107: 1340-1348.

449 23. Bird, R. P. (1987) Observation and quantification of aberrant crypts in murine  
450 colon treated with a colon carcinogen: preliminary findings. *Cancer Lett.* 37: 147-151.

451 24. Ohkawa, H., Ohishi, N. & Yagi, K. (1979) Assay for lipid peroxides in animal  
452 tissues by thiobarbituric acid reaction. *Anal. Biochem.*, 95: 351-358.

453 25. Van den Berg, J. W., Koole-Lesuis, R., Edixhoven-Bosdijk, A. & Brouwers,  
454 N. (1988) Automating the quantification of heme in feces. *Clin. Chem.*, 34: 2125-2126.

455 26. Govers, M. J. A. P., Termont, D. S. M. L., Lapre, J. A., Kleibeuker, J. H.,  
456 Vonk, R. J. & Vandermeer, R. (1996) Calcium in milk products precipitates intestinal fatty  
457 acids and secondary bile acids and thus inhibits colonic cytotoxicity in humans. *Cancer Res.*  
458 56: 3270-3275.

459 27. Bonneson, C., Eggleston, I. M. & Hayes, J. D. (2001) Dietary indoles and  
460 isothiocyanates that are generated from cruciferous vegetables can both stimulate apoptosis  
461 and confer protection against DNA damage in human colon cell lines. *Cancer Res.* 61: 6120-  
462 6130.

463 28. Lapre, J. A. & Vandermeer, R. (1992) Diet-Induced Increase of Colonic Bile  
464 Acids Stimulates Lytic Activity of Fecal Water and Proliferation of Colonic Cells.  
465 *Carcinogenesis* 13: 41-44.

466 29. Sawa, T., Akaike, T., Kida, K., Fukushima, Y., Takagi, K. & Maeda, H.  
467 (1998) Lipid peroxy radicals from oxidized oils and heme-iron: implication of a high-fat diet  
468 in colon carcinogenesis. *Cancer Epidemiol. Biomarkers Prev.* 7: 1007-1012.

469 30. Corpet, D. E., Tache, S. & Pierre, F. (2002-2004) Colon cancer prevention:  
470 chemoprevention database. <http://www.inra.fr/reseau-nacre/sci-memb/corpet/indexan.html>  
471 July 1, 2004.

472 31. Li, D., Ng, A., Mann, N. J. & Sinclair, A. J. (1998) Contribution of meat fat to  
473 dietary arachidonic acid. *Lipids* 33: 437-440.

474 32. McEntee, M. F. & Whelan, J. (2002) Dietary polyunsaturated fatty acids and  
475 colorectal neoplasia. *Biomed. Pharmacotherapy* 56: 380-387.

476 33. Kirkland, J. B. (2003) Niacin and carcinogenesis. *Nutr Cancer* 46: 110-118.

477 34. Morrow, J. D., Awad, J. A., Oates, J. A. & Roberts, L. J. (1992) Identification  
478 of skin as a major site of prostaglandin D2 release following oral administration of niacin in

479 humans. *J. Invest. Dermatol.*, 98: 812-815.  
480 35. Lee, D. H., Anderson, K. E., Harnack, L. J., Folsom, A. R. & Jacobs, D. R., Jr.  
481 (2004) Heme iron, zinc, alcohol consumption, and colon cancer: Iowa Women's Health  
482 Study. *J. Natl. Cancer Inst.* 96: 403-407.  
483 36. Singh, P. N. & Fraser, G. E. (1998) Dietary risk factors for colon cancer in a  
484 low-risk population. *Amer. J. Epidemiol.* 148: 761-774.  
485