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1 **Beef meat-promotion of dimethylhydrazine-induced colorectal**  
2 **carcinogenesis biomarkers is suppressed by dietary calcium.**

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5

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9

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13

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15

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19

20 **Abbreviations:** ACF: aberrant crypt foci, MDF: mucin-depleted foci, TBARS: thiobarbituric  
21 acid reactive substances, DHN-MA: 1,4-dihydroxynonane mercapturic acid

## 22 **Abstract**

23

24 Red meat consumption is associated with increased risk of colorectal cancer. We have  
25 previously shown that haemin, haemoglobin and red meat promotes carcinogen-induced  
26 preneoplastic lesions: aberrant crypt foci (ACF) and mucin-depleted foci (MDF) in rats. We  
27 have also shown that dietary calcium, antioxidant mix and olive oil, inhibit haemin-induced  
28 ACF promotion, and normalize faecal lipoperoxides and cytotoxicity. Here we tested if these  
29 strategies are effective also against red meat promotion in dimethylhydrazine-induced rats.  
30 Three diets with 60% beef meat were supplemented with calcium phosphate (33 g/kg),  
31 antioxidant agents (rutin and BHA, 0.05% each), and olive oil (5%). ACF, MDF, faecal water  
32 cytotoxicity, thiobarbituric acid reactive substances (TBARS), and urinary 1,4-  
33 dihydroxynonane mercapturic acid (DHN-MA) were measured. Beef-meat diet increased the  
34 number of ACF (+30%) and MDF (+100%) ( $P < 0.001$ ), which confirms our previous  
35 findings. Promotion was associated with increased faecal water TBARS (x4) and cytotoxicity  
36 (x2), and urinary DHN-MA excretion (x15). Calcium fully inhibited beef meat-induced ACF  
37 and MDF promotion, and normalized faecal TBARS and cytotoxicity, but did not reduce  
38 urinary DHN-MA. Unexpectedly, high-calcium control diet-fed rats had more MDF and ACF  
39 in the colon than low-calcium control diet-fed rats. Antioxidant mix and olive oil did not  
40 normalize beef-meat promotion nor biochemical factors. The results confirm that haem causes  
41 promotion of colon carcinogenesis by red meat. They suggest that calcium can reduce  
42 colorectal cancer risk in meat-eaters. They support the concept that toxicity associated with  
43 the excess of a useful nutrient may be prevented by another nutrient.

44 **Introduction**

45 Red and processed meat consumption increases the risk of colorectal cancer, according  
46 to meta-analyses of epidemiological studies<sup>1, 2</sup>. Several explanations have been given for this  
47 increase, involving fat, heterocyclic amines, N-nitrosated compounds, and haem iron. Intake  
48 of dietary haem iron is associated with an increased risk of colon cancer among women of the  
49 Iowa Women's Health Study<sup>3</sup>. Intake of black pudding, a blood sausage high in haem iron, is  
50 associated with increased risk of colorectal cancer among women of the Swedish  
51 Mammography Cohort<sup>4</sup>.

52 Experimental animal studies support the hypothesis that haem iron promotes colorectal  
53 carcinogenesis<sup>5</sup>. We have shown that dietary haem, in the form of haemin, haemoglobin or  
54 red meat, promotes putative precancerous lesions, aberrant crypt foci (ACF) and mucin-  
55 depleted foci (MDF), in the colon of rats given a low-calcium diet<sup>6, 7</sup>. Haemin is a free haem  
56 ring stabilized by a chlorine atom, in contrast with chlorine-free protein-bound haem in meat  
57 myoglobin. This haem-induced promotion is associated with increased lipoperoxidation and  
58 cytotoxicity of faecal water, and linked to urinary DHN-MA excretion, a lipid peroxidation  
59 biomarker<sup>8</sup>. The addition of calcium, antioxidant mix or olive oil to the diet inhibits haemin-  
60 induced lipoperoxydation, cytotoxicity and promotion of carcinogenesis in rats<sup>6</sup>, but no  
61 demonstration has already been given that these dietary factors can inhibit promotion by red  
62 meat. We thus suggested, after van der Meer<sup>9</sup>, that diets high in calcium and oxidation-  
63 resistant fat could prevent promotion by red meat.

64 This study was designed to test the hypothesis that addition of calcium, antioxidant  
65 mix or olive oil to the diet can inhibit cancer-promoting effect of haem provided by beef  
66 meat.

## 67 **Materials and Methods**

### 68 **Animals and Diets**

69 Eighty Fischer 344 female rats were purchased at four weeks of age from Iffa Credo  
70 (St.Germain l'Arbresle, France). Animal care was in accordance with the guidelines of the  
71 European Council on animals used in experimental studies. The animal colony and staff got  
72 an official agreement #31-121 for in vivo rodents studies by French government. Rats were  
73 housed by pairs into stainless steel wire bottomed cages. The room was kept at a steady  
74 acclimatization to the animal colony and given control diet before being injected i.p. with the  
75 carcinogen 1,2 dimethylhydrazine (Sigma chemical, St.Quentin, France; 190 mg/kg body wt)  
76 in NaCl (9 g/L). Usually, several injections are given to rats. We reasoned that the first shot  
77 initiates carcinogenesis, and the following shots promote it, blurring diet-induced promotion.  
78 We thus chose a single-shot protocol, following Glauert<sup>10</sup>. Seven days later, rats were  
79 randomly allocated to eight groups of ten, and allowed free access to their respective diet for  
80 100 days. We chose to initiate all rats with the carcinogen, since the study was designed to  
81 show dietary promotion, and because a 2.5% haemoglobin diet does not initiate ACF in rats  
82 (Pierre and Corpet, unpublished results).

83 Eight experimental diets shown in Table 1 were based on the diet fed to control rats, a  
84 modified AIN-76 powdered diet<sup>11</sup> prepared and formulated in a powdered form by UPAE  
85 (INRA, Jouy-en-Josas, France). Dibasic calcium phosphate was included at a low  
86 concentration of 2.7 g/kg. Four beef meat diets were formulated to contain 60% meat (freeze-  
87 dried) w/w. Beef meat contained 0.6  $\mu\text{mol/g}$  of haem. We chose this meat level, higher than  
88 what most people eat, to be able to detect protection against promotion. The effect of calcium  
89 and of an antioxidant mix was tested in two groups of rats given a beef meat diet  
90 supplemented either with calcium phosphate (31 g/kg) or antioxidant mix (butylated  
91 hydroxyanisole and rutin, 0.05% each). Last, the effect of oil was investigated by replacing  
92 safflower oil by extra-virgin olive oil (5%, Puget, France) in a beef meat diet. Because  
93 calcium, antioxidant mix and olive oil might be protective even in the absence of meat, three  
94 other groups received beef-free control diet, supplemented with calcium phosphate,  
95 antioxidant mix or olive oil. As shown on Table 1, all diets were balanced for protein (50%),  
96 fat (20%) and iron (110 mg/kg) by addition of casein, lard, and ferric citrate. The diets were  
97 made up every 14 days and maintained at  $-20^{\circ}\text{C}$  to reduce lipoperoxidation.

98

99 **ACF Assay**

100 All 80 rats were killed by CO<sub>2</sub> asphyxiation in a random order at day 99 or 100. Colons were  
101 excised from rats immediately post mortem, flushed with cold Krebs solution (Sigma  
102 chemical, St.Quentin, France), opened longitudinally and fixed flat between two sheets of  
103 filter paper in 10% formalin (Sigma chemical, St.Quentin, France). Eighty colons picked up  
104 in random order were stained for 6 min in a 0.05% filtered solution of methylene blue<sup>12</sup>.  
105 Number of ACF per colon, and number of crypts in each ACF, were counted under light  
106 microscope at x40 magnification in duplicate by two readers, blinded for the origin of the  
107 colon. Data from the two readers were pooled.

108

109 **MDF Assay**

110 MDF may predict colon carcinogenesis better than ACF, since Apc mutations are present in  
111 MDF with a frequency similar to that of tumours<sup>13</sup>. Colons, after being scored for ACF, were  
112 stained with high iron diamine-Alcian blue procedure (HID-AB) to evaluate mucin  
113 production<sup>14</sup>. Briefly, colons were rinsed in distilled water and left overnight in freshly  
114 prepared HID solution (50 mL of distilled water with 120 mg N-N'-dimethyl-m-phenylene  
115 diamine, 20 mg N-N'-dimethyl-p-phenylene diamine, and 1.4 mL of 60% ferric chloride).  
116 After rinsing, colons were counterstained in 1% alcian blue solution for 30 min. MDF  
117 number, and number of crypts per MDF, were scored blindly under light microscope at x40  
118 magnification by a single reader.

119

120 **Preparation of Faecal Water**

121 Faecal pellets were collected under each cage of two rats for 24 h, thus leading to five  
122 samples per group. Freeze-dried faeces were used to calculate dry faecal mass and to prepare  
123 faecal water by adding 1 mL of sterilized water to 0.3 g of faeces. Samples were then  
124 incubated at 37°C for one hour, stirring thoroughly every 20 min, followed by centrifugation  
125 at 20 000g for 10 min. The aqueous phase was re-centrifuged at the same speed and duration  
126 and the subsequent supernatant (faecal water) collected and conserved at -20°C until use.

127

128 **TBARS and Haem Assay**

129 Thiobarbituric acid reactive substances (TBARS) were measured in faecal water according to  
130 Ohkawa et al.<sup>15</sup>, exactly as previously described<sup>7</sup>. Haem contents of freeze-dried faeces and  
131 of faecal water were measured by fluorescence according to Van den Berg et al.<sup>16</sup> and Sesink  
132 et al.<sup>5</sup>, respectively, as already described<sup>7</sup>.

133

#### 134 **Cytotoxicity Assay of Faecal Water**

135 Cytotoxicity of faecal water was quantified on a cell line according to Bonneson et al. <sup>17</sup>, and  
136 as previously described <sup>7</sup>. Briefly, cancerous mouse colonic epithelial cell line, CMT93  
137 (ECAC), was seeded in 96-well microtiter plates ( $1.6 \times 10^4$  cells per well in 200  $\mu$ L of  
138 medium) and treated for 24 h with faecal water sample diluted at 10% (v/v) in the culture  
139 medium. Cytotoxicity of each faecal water was quantified by the 3-(4,5-dimethylthiazol-2-yl)-  
140 2,5 diphenyl tetrazolium bromide (MTT) test.

141

#### 142 ***Urinary DHN-MA assay***

143 Each rat was placed alone in a metabolic cage, for two days during the fifth week of  
144 experimental diet. The 24-hour urine was collected under each cage of one rat, thus leading to  
145 ten samples per group. DHN-MA assay was done by competitive enzyme immunoassay (EIA)  
146 as previously described <sup>18</sup>, using DHN-MA-linked acetylcholinesterase enzyme. Each urine  
147 sample was assayed in duplicate.

148

#### 149 **Statistical Analysis**

150 Results were analyzed using Systat 10 software for Windows, and reported as mean  $\pm$  SD.  
151 Values were considered firstly using one-way analysis of variance (ANOVA). If a significant  
152 difference was found between groups ( $p < 0.05$ ) then each experimental group was compared  
153 to the control group using the Fishers's least-significant-difference test (ACF and MDF data),  
154 and the Dunnett multiple comparison test (all other data).

## 155 **Results**

### 156 **Weight and food intake**

157 Final body weight of rats was  $198\pm 3$ g, without significant differences between groups at the  
158 end of the experimentation. Food intake was the same in all groups of rats (data not shown).

159

### 160 **ACF and MDF data**

161 Beef meat diet doubled the number of MDF per colon compared to control rats, and increased  
162 the number of ACF per colon (both  $p<0.01$ , Fig. 1). Number of crypts per lesions also was  
163 enhanced by beef meat diet (Table 2).

164 Calcium fully suppressed beef meat-induced promotion of ACF and MDF (Fig. 1), but did not  
165 reduce the mean number of crypts per lesions (Table 2). Antioxidant mix and olive oil  
166 significantly reduced meat-induced promotion of MDF number, but did not normalize it to  
167 control values (Table 2). In contrast, antioxidant mix and olive oil did not interfere  
168 significantly with beef meat-induced promotion of ACF number (Table 2). Thus, only  
169 calcium supplementation fully suppressed the beef-induced promotion of ACF and MDF.

170 Supplementation of no-meat control diet with antioxidant mix or olive oil did not modify  
171 ACF or MDF incidence (Table 2), as previously observed <sup>6</sup>. In contrast, surprisingly, high-  
172 calcium control diet-fed rats had more MDF and ACF in the colon than low-calcium control  
173 diet-fed rats, with more crypts per lesion ( $p<0.01$ , Table 2).

174

### 175 **Faecal haem, TBARS and Cytotoxicity**

176 Haem intake and faecal haem values matched study design: as expected, little haem was  
177 detected in faeces of control rats, in contrast with beef meat-fed rats (Table 3). We also  
178 measured haem concentrations in faecal water because, according to bile acids studies, the  
179 soluble fraction of colonic contents would interact more strongly with the mucosa than the  
180 insoluble fraction. As expected, haem concentration in faecal water depended directly on  
181 haem level in beef-based diets (Table 3). However, rats fed the beef meat high-calcium diet  
182 had little haem in faecal water, as previously observed <sup>6</sup>.

183 Haem can induce the formation of peroxy radicals in fats, which may be cytotoxic and cleave  
184 DNA *in vivo*. Lipid peroxidation was thus measured in faecal water by TBARS assay. Lipid  
185 peroxidation correlated with haem concentration in faecal water (Table 3,  $r=0.95$ ,  $p=0.0002$ ).  
186 Calcium almost normalized beef-induced lipid peroxidation to control level (66 and 33  $\mu$ M  
187 MDA equivalent, respectively), but antioxidant mix or olive oil did not reduce peroxide



188 values (130 and 148  $\mu\text{M}$  MDA equivalent, similar to 141  $\mu\text{M}$  in beef meat-fed rats).  
189 Furthermore, as already seen, faecal water of beef meat-fed rats was cytotoxic to CMT93  
190 cells. Calcium fully inhibited beef meat-induced cytotoxicity of faecal water, but antioxidant  
191 mix and olive oil did not.

192

### 193 **Urinary DHN-MA Excretion**

194 Table 3 shows that beef meat-based diet increased urinary DHN-MA excretion by 15-fold  
195 compared with control diet ( $P<0.001$ ). Calcium and olive oil did not reduce DHN-MA  
196 excretion in beef meat-fed rats, while antioxidant mix gave a slight decrease, though  
197 statistically significant.

## 198 **Discussion**

199           These data confirm that a red meat diet promotes colon carcinogenesis in rats. They  
200 also establish that red meat promotion can be suppressed by dietary calcium. In addition  
201 faecal water cytotoxicity and lipoperoxides level were linked with haem-induced promotion.

202           Red meat promoted ACF and MDF in the colon of carcinogen-induced rats, and meat  
203 promotion was associated with faecal water haem, cytotoxicity and TBARS, in line with our  
204 previous study <sup>7</sup>. The mechanism of haem promotion is not known, and may be linked to the  
205 stimulation of the endogenous production of N-nitroso compounds <sup>19, 20</sup>. Alternatively, we  
206 suggest promotion is linked to peroxidation and cytotoxicity. We have explored the effect of  
207 faecal water from beef-fed rats on normal (Apc +/+) and premalignant colonic cells (Apc  
208 Min/+). Results show that Apc mutated cells survive haem-induced faecal lipoperoxides,  
209 notably 4-hydroxynonenal (HNE) that is toxic to normal cells. Selection of mutated cells by  
210 cytotoxic lipoperoxides may explain haem-promotion of colon carcinogenesis <sup>21</sup>. Beef diet  
211 reduces caspase-3 activity in the rat colonic mucosa, thus decreasing apoptosis induction <sup>22</sup>. In  
212 addition, loss of APC induces histone deacetylase 2: it is overexpressed in polyps of APC-  
213 deficient mice, and it prevents apoptosis of colonic cells <sup>23, 24</sup>. Furthermore, HNE alters  
214 histone acetylation <sup>25</sup>. Therefore, the modification of histone acetylation by meat-induced  
215 HNE may reduce apoptosis induction and explain the selection of Apc-mutated cells. This  
216 speculation on meat-promotion mechanism is supported by the present study: ACF and MDF  
217 promotion in beef meat-fed rats was associated with high faecal lipoperoxides and  
218 cytotoxicity, and, as discussed below, dietary changes that normalized faecal lipoperoxides  
219 and cytotoxicity also suppressed beef meat-promotion.

220           Calcium added on top of beef-meat diet normalized number of ACF and MDF at the  
221 same low level as control group, a protection already observed against haemin-induced  
222 promotion<sup>6</sup>. In faeces from rats given high-calcium beef meat diet, haem concentration was  
223 high (similar to beef meat-fed rats), but faecal water haem level was low (similar to no-meat  
224 control rats) (Table 3). Calcium phosphate precipitates haem in the gut, and little haem  
225 remains available to induce lipoperoxidation, as previously showed with haemin <sup>6, 9</sup>. Faecal  
226 water thus contained little lipid peroxides and showed little cytotoxic activity (Table 3). The  
227 trapping of haem by calcium abolished carcinogenesis promotion. This confirms that haem  
228 is the cause of red meat promotion. The results also suggest that calcium can reduce colorectal  
229 cancer risk in meat-eaters. It might explain discrepancies between clinical trials with calcium  
230 supplements, since results were not analyzed based on meat consumption by volunteers. This

231 could be looked for in a prospective cohort study, to know if the protective effect of calcium  
232 against red meat promotion can also be seen in human beings. Similarly, an epidemiological  
233 study shows that chlorophyll from vegetables attenuates the elevated risk of colon cancer  
234 associated with red meat intake <sup>26</sup>.

235 No protection against beef meat promotion was afforded by the antioxidant mix or by  
236 olive oil (Table 2). These supplements did not normalize faecal values of haem lipoperoxides  
237 and cytotoxicity either (Table 3). These results conflicts with a previous study in which the  
238 antioxidant mix or olive oil had been effective against haemin-induced promotion<sup>6</sup>. This  
239 conflict suggests that dietary haemin is not a suitable model for red meat to test preventive  
240 strategies in rodents. We speculate that antioxidant molecules added to the diet can directly  
241 prevent the effect of haemin powder on dietary oil<sup>6</sup>. Here, in contrast, protein-bound haem in  
242 red meat might have been protected from the antioxidant effect. Furthermore, since faecal  
243 water lipoperoxides and cytotoxicity are associated with haem-induced carcinogenesis, here  
244 and in previous studies <sup>6, 7</sup>, we suggest they can be used as short term biomarkers to screen  
245 preventive strategies against red meat-induced promotion of colon cancer.

246 An unexpected result here was that rats eating a high-calcium control diet had more  
247 ACF and more MDF than low-calcium control diet-fed control rats. This promotion by  
248 calcium phosphate in a high-fat context was not seen in a low-fat context, in our previous  
249 haemin study <sup>6</sup>. It is generally agreed that calcium reduces the risk of colorectal cancer <sup>27</sup>, and  
250 a meta-analysis study shows that calcium modestly decreases tumour incidence in rats <sup>28</sup>.  
251 However, in 14 studies out of 32, a non-significant tumour incidence increase was seen in  
252 calcium-fed rats. The meta-analysis also shows that some calcium salts are more protective  
253 than others, and that calcium phosphate affords no protection <sup>28</sup>. Calcium phosphate (which  
254 we used here) sometimes promotes ACF or tumours <sup>29, 30</sup>. For instance Bull et al. showed that  
255 calcium phosphate promotes colon tumourigenesis in two genetic contexts (Sprague-Dawley  
256 and Fisher 344 rats). In both types of rat the promotion was more important in 30%-fat diet-  
257 fed rats than in 3%-fat diet-fed rats <sup>30</sup>. Phosphate, not calcium, might be the promoting  
258 nutrient, an issue discussed by Bruce<sup>31</sup>. To conclude, promotion by calcium phosphate in a  
259 high-fat context was unexpected, but has already been reported.

260 Another surprise here is that all beef meat diets increased urinary DHN-MA excretion  
261 more than ten-fold. We have recently proposed urinary DHN-MA as a noninvasive biomarker  
262 of haem iron-induced promotion of carcinogenesis. Indeed, DHN-MA excretion increases  
263 dramatically in rats given high-haem diets, and the excretion parallels ACF and MDF

264 numbers in azoxymethane initiated rats<sup>8</sup>. However, in the present study, the addition of  
265 calcium to the meat diet did not reduce DHN-MA excretion while it normalized ACF and  
266 MDF promotion. DHN-MA is the major urinary metabolite of HNE, a lipid peroxidation  
267 product. DHN-MA excretion reflects endogenous HNE formation in the body, together with  
268 formation in the diet<sup>8</sup>, the latter being probably quantitatively the most important<sup>32</sup>, while  
269 preneoplastic lesions such as ACF and MDF may be due to lipid peroxidation occurring  
270 locally in the colon lumen. One can hypothesize that calcium blocks haem in the intestine<sup>9</sup> but  
271 not in the diet, because of its powdered form, so the overall DHN-MA excretion is not  
272 significantly reduced by the addition of calcium although HNE formation is reduced in the  
273 colon lumen. Indeed, in a previous work, we have shown that HNE was found three times less  
274 in the faeces of rats fed beef + calcium diet as compared to beef diet<sup>21</sup>. It thus seems that  
275 DHN-MA is a biomarker of exposure to a promoting dose of haem in the diet, but DHN-MA  
276 is not a risk factor. However, measurement of HNE in faeces, which can reflect HNE in colon  
277 lumen, could be an indicator of the colon cancer risk associated with diets.

278 In conclusion, this study shows for the first time calcium prevention of red meat  
279 promotion of colon carcinogenesis. This study supports the concept that toxicity associated  
280 with the excess of a useful nutrient like haeminic iron may be prevented by another nutrient  
281 like calcium. Yoghurt or cheese can be eaten after red meat, provided portions are small  
282 enough to avoid fat overload. We found hardly any example of similar antidote effect, except  
283 may be the lactulose prevention of protein-induced hepatic encephalopathy<sup>33</sup>. This calcium  
284 preventive effect should be looked for in a cohort study by crossing haem and calcium intake  
285 with adenoma or cancer risk. Furthermore, different haem forms do not have the same  
286 promoting potency and cannot be antagonized by identical agents, since haemin effect is not  
287 identical to red meat effect. Last, faecal water lipoperoxidation and cytotoxicity may be  
288 responsible, at least in part, of haem-induced promotion of colon carcinogenesis.

289

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**Table 1:** Composition of diets (g/kg)

	<b>Control</b> (low calcium)	<b>Beef</b> (low calcium)	<b>Control +</b> <b>Calcium</b>	<b>Beef +</b> <b>Calcium</b>	<b>Control +</b> <b>Olive Oil</b>	<b>Beef +</b> <b>Olive Oil</b>	<b>Control +</b> <b>Antiox.</b>	<b>Beef +</b> <b>Antiox.</b>
<b>Beef</b>	0	600	0	600	0	600	0	600
<b>Lard</b>	160	0	160	0	160	0	160	0
<b>Safflower Oil</b>	50	50	50	50	0	0	50	50
<b>Olive Oil</b>	0	0	0	0	50	50	0	0
<b>Casein <sup>a</sup></b>	500	59.7	500	59.7	500	59.7	500	59.7
<b>Corn Starch</b>	60	60	60	60	60	60	60	60
<b>Sucrose</b>	127.5	128.6	96.5	97.5	127.5	128.6	126.5	127.6
<b>Cellulose</b>	50	50	50	50	50	50	50	50
<b>Methionine</b>	3	3	3	3	3	3	3	3
<b>Mineral mix <sup>b</sup></b>	35	35	35	35	35	35	35	35
<b>Vitamin mix <sup>b</sup></b>	10	10	10	10	10	10	10	10
<b>Choline bitartrate</b>	2	2	2	2	2	2	2	2
<b>CaHPO<sub>4</sub>·2H<sub>2</sub>O</b>	2.7	2.1	33.8	33.1	2.7	2.1	2.7	2.1
<b>Rutin+BHA <sup>d</sup></b>	0	0	0	0	0	0	0.5 + 0.5	0.5 + 0.5
<b>Ferric Citrate <sup>c</sup></b>	0.27	0	0.27	0	0.27	0	0.27	0

a. Low-calcium casein

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

c. All diets contained 110 mg/kg iron. Iron concentration was measured in freeze-dried beef before preparing the diets. Other nutrients were balanced: 50% protein, 21% fat, 20-24% carbohydrate, (based on added components, no analysis was done on whole diets).

d. BHA, butylated hydroxyanisole



**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 dimethylhydrazine<sup>1</sup>.

Diets <sup>2</sup>	Haem	ACF						MDF					
	μmol/g diet	ACF/colon		AC/colon		Crypts/ACF		MDF/colon		MDC/colon		Crypts/MDF	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Control	0	105 <sup>a</sup>	24	245 <sup>a</sup>	52	2.3 <sup>a</sup>	0.2	3.5 <sup>a</sup>	2.0	18.2 <sup>a</sup>	15.3	4.6 <sup>a</sup>	1.7
Beef	0.4	137 <sup>b</sup>	26	347 <sup>b</sup>	55	2.6 <sup>b</sup>	0.2	7.4 <sup>b</sup>	2.0	40.7 <sup>b</sup>	18.9	5.3 <sup>a</sup>	1.6
Beef + Calcium	0.4	106 <sup>a</sup>	24	265 <sup>a</sup>	74	2.5 <sup>b</sup>	0.2	3.4 <sup>a</sup>	1.8	24.3 <sup>a</sup>	12.6	7.8 <sup>b</sup>	3.1
Beef + Antiox.	0.4	125 <sup>b</sup>	20	299 <sup>c</sup>	60	2.4 <sup>a</sup>	0.2	5.3 <sup>c</sup>	1.2	22.5 <sup>a</sup>	5.3	4.3 <sup>a</sup>	0.7
Beef + Olive Oil	0.4	127 <sup>b</sup>	22	300 <sup>c</sup>	40	2.4 <sup>a</sup>	0.3	5.6 <sup>c</sup>	1.1	22.4 <sup>a</sup>	9.5	3.9 <sup>a</sup>	1.4
Con. + Calcium	0	130 <sup>b</sup>	22	365 <sup>b</sup>	71	2.8 <sup>c</sup>	0.2	7.6 <sup>b</sup>	3.0	58.2 <sup>c</sup>	27.5	7.6 <sup>b</sup>	2.4
Con. + Antiox.	0	104 <sup>a</sup>	25	258 <sup>a</sup>	71	2.5 <sup>b</sup>	0.2	3.8 <sup>a</sup>	2.5	15.6 <sup>a</sup>	13.0	4.0 <sup>a</sup>	1.2
Con. + Olive Oil	0	107 <sup>a</sup>	22	243 <sup>a</sup>	48	2.3 <sup>a</sup>	0.2	3.2 <sup>a</sup>	1.3	14.7 <sup>a</sup>	8.8	4.4 <sup>a</sup>	1.4

<sup>1</sup> Values are means and SD, N = 10 rats /dietary group. Mean values within a column with unlike superscript letters were significantly different ( $P < 0.05$ )

<sup>2</sup> Diets were based on a low calcium formula, and were balanced for iron (110mg/kg). Each group had ten rats. See Table 1 for precise composition.

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

Diets <sup>2</sup>	TBARS in faecal													
	Haem		Dry faecal		Haem in		Haem in		water		Cytotoxicity on		Urinary	
	intake	SD	Mass	SD	faeces	SD	faecal water	SD	MDA	SD	CMT93 cells	SD	DHN-MA	SD
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean %	SD	Mean	SD
	μmol/d		g/d		μmol/g		μM		μM		cells lysed		ng/d	
Control	0 <sup>a</sup>		0.49 <sup>a</sup>	0.12	0.56 <sup>a</sup>	0.65	7 <sup>a</sup>	6	33 <sup>a</sup>	9	35 <sup>a</sup>	8	58.2 <sup>a</sup>	7.5
Beef	3.8 <sup>b</sup>	0.3	0.68 <sup>b</sup>	0.05	2.94 <sup>b</sup>	1.01	49 <sup>b</sup>	7	141 <sup>b,c,d</sup>	16	63 <sup>b</sup>	12	899.4 <sup>b</sup>	244.7
Beef + Calcium	3.8 <sup>b</sup>	0.3	0.99 <sup>c</sup>	0.14	2.63 <sup>b</sup>	1.28	9 <sup>a</sup>	4	66 <sup>e</sup>	7	26 <sup>a,c</sup>	15	858.3 <sup>b</sup>	262.3
Beef + Antiox.	3.6 <sup>b</sup>	0.2	0.56 <sup>a</sup>	0.07	3.20 <sup>b</sup>	1.80	67 <sup>b</sup>	17	130 <sup>d</sup>	35	65 <sup>b</sup>	10	630.5 <sup>c</sup>	169.8
Beef + Olive Oil	3.6 <sup>b</sup>	0.3	0.55 <sup>a</sup>	0.11	3.11 <sup>b</sup>	0.70	64 <sup>b</sup>	21	148 <sup>c</sup>	16	66 <sup>b</sup>	9	747.1 <sup>b,c</sup>	232.8
Con. + Calcium	0 <sup>a</sup>		0.79 <sup>b</sup>	0.10	0.53 <sup>a</sup>	0.51	6 <sup>a</sup>	1	22 <sup>a</sup>	8	16 <sup>c</sup>	6	36.4 <sup>a</sup>	6.5
Con. + Antiox.	0 <sup>a</sup>		0.58 <sup>a</sup>	0.06	0.39 <sup>a</sup>	0.35	9 <sup>a</sup>	4	43 <sup>a</sup>	21	32 <sup>a</sup>	19	40.6 <sup>a</sup>	2.4
Con. + Olive Oil	0 <sup>a</sup>		0.56 <sup>a</sup>	0.07	0.52 <sup>a</sup>	0.43	7 <sup>a</sup>	5	39 <sup>a</sup>	14	50 <sup>b</sup>	12	55.6 <sup>a</sup>	23.4

<sup>1</sup> Values are means and SD. Faecal values, N= 5 cages /group. Urine values, N=10 rats /group. Mean values within a column with unlike superscript letters were significantly different ( $P<0.05$ )

<sup>2</sup> Diets were based on a low calcium formula, and were balanced for iron (110mg/kg). Each group had ten rats. See Table 1 for precise composition.

**Figure 1:** Effect of beef meat, and high-calcium beef meat, diets on putative precancerous lesions per rat colon 100 d after the injection of dimethylhydrazine. Top panel, **A:** Number of aberrant crypt foci (ACF). Bottom panel, **B:** Number of mucin-depleted foci (MDF). Values are means  $\pm$  SD, N = 10. Means without a common letter differ,  $P < 0.05$ .

