The effect of haem in red and processed meat on the endogenous formation of N-nitroso compounds in the upper gastrointestinal tract

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Red and processed meat (PM) consumption increases the risk of large bowel cancer and it has been demonstrated that haem in red meat (RM) stimulates the endogenous production of N-nitroso compounds (NOCs) within the human intestine. To investigate whether N-nitrosation occurs in the upper gastrointestinal tract, 27 ileostomists were fed diets containing no meat, or 240 g RM or 240 g PM in a randomly assigned crossover intervention design carried out in a volunteer suite. Endogenous NOC were assessed as apparent total N-nitroso compounds (ATNC) in the ileostomy output. ATNC concentration in the diets was 22 µg ATNC/kg (RM) and 37 µg ATNC/kg (PM), and 9 µg ATNC/kg in the no meat diet. Levels significantly increased to 1175 µg ATNC/kg SEM = 226 µg ATNC/kg following the RM (P = 0.001) and 1832 µg ATNC/kg (SEM = 294 µg ATNC/kg) following PM (P < 0.001) compared to the no meat diet (283 µg ATNC/kg, SEM = 74 µg ATNC/kg). ATNC concentrations in the ileal output were equivalent to those measured in faeces in similarly designed feeding studies. Supplementation with either 1 g ascorbic acid or 400 IU α-tocopherol had no effect on the concentration of ATNC detected in the ileal output. In in vitro experiments, N-nitrosomorpholine (NMor) was formed in the presence of nitrosated haemoglobin, at pH 6.8 but not in the absence of nitrosated haemoglobin. These findings demonstrate that haem may facilitate the formation of NOC in the absence of colonic flora in the upper human gastrointestinal tract.

Introduction

Epidemiological studies have consistently demonstrated that red and processed meat (PM) consumption is associated with an increased risk of colorectal cancer (1). One suggested mechanism underlying this relationship is that haem in red, but not white meat, stimulates the endogenous formation of NOCs2, as assessed by apparent total NOCs (ATNC) in faeces (2–4). Amines, amides and amino acids present in the gastrointestinal tract are readily nitrosated and in human volunteers, there is a dose–response to increased red meat (RM) consumption (5). Faecal ATNC levels increase when a low meat diet is supplemented with haem; an effect not seen with inorganic iron supplementation or when other sources of animal protein (fish and white meat) or vegetable protein are consumed (3,4,6). The majority of NOCs investigated have been shown to be carcinogens (7).

Feeding studies in rodents have demonstrated a significant (P < 0.02) 2- to 3-fold increase in faecal ATNC following a diet containing beef, a 4- to 5-fold increase when hot dogs were fed and a significant (P < 0.0001) increase with bacon (8–10). Supplementation of the drinking water of conventional flora rats with nitrate significantly increased faecal ATNC detected in the colons, with a highly significant positive correlation (r = 0.95, P < 0.01) between the level of ATNC in the colon and the amount of nitrate ingested. In germ free rats, nitrate did not stimulate endogenous ATNC formation (11). However, little is known regarding the bacterial species involved in endogenous nitrosation.

Although, these studies with germ free rats demonstrated that the colonic flora was involved in the metabolism of nitrate and ATNC formation, the effect of haem or RM was not investigated in these in vivo studies. Furthermore, in vitro studies have shown that haem and myoglobin are readily nitrosated and can then act as nitrosating agents (12–14). Thus, nitrosation in the absence of the colonic flora may occur, and this might not be confined to the large intestine. We have therefore investigated the in vivo endogenous formation of ATNC by haem in RM in humans whose colon and associated flora had been removed. The effect of PM and of supplementation with ascorbic acid and α-tocopherol on the endogenous formation of ATNC was also studied. Ascorbic acid and α-tocopherol are known inhibitors of nitrosation in vitro (15). In addition, the formation of the nitrosated amino acid N-nitrosomorpholine (NMor) was investigated in vitro under conditions representative of those in the stomach and small intestine in the presence of haem.

Materials and methods

Participants

Volunteer ileostomists were recruited by advertisement in the UK Ileostomy Association newsletter. There were over 300 replies, of whom the majority had had their colon removed for ulcerative colitis, and were not taking antibiotics. Of the eligible subjects, 27 (18 male and 9 female; aged 40–85 years) were randomly selected and asked to take part in the study. Ethical permission was granted by Cambridgeshire Local Research Ethics Committee (LREC 02/153 and LREC 03/174).
Dietary protocol
On recruitment to the study, the ileostomists were accommodated for 7 days in the metabolic suite of the Dunn Human Nutrition Unit where all food was provided and carefully controlled and all specimens collected and processed immediately. A randomized crossover design was used to avoid time effects.

In the first protocol, 14 ileostomists received a 240 g RM diet (beef, topside, roasted until well done) and 240 g PM diet (80 g corned beef and a no meat diet for two days in a randomized order of presentation. The subjects acted as their own controls as each received all three dietary periods. All the meat was consumed as ‘breakfast’ with two slices of white toast, condiments (mustard or relish) and a glass of apple juice or herbal tea. For the no meat diet, breakfast consisted of a bowl of cereal with milk and toast with jam. No tea or coffee was consumed with the breakfast. Breakfast was completed by 11 am. After 11 am, food was provided from a set diet that was repeated on the second day of the dietary period. Volunteers could consume as much as or little of this food as they required. Diets were prepared without pigs and skins to avoid stoma blockage. Diets were prepared from food purchased from the same batch and stored for later use during the study in order to minimize day-to-day variation. Meat was not overcooked to reduce the formation of heterocyclic amines. To minimize nitrate intake, deionised water was used for cooking and drinking and low nitrate vegetables, such as carrots, green beans and peas were included in the diets.

Duplicates of the meals breakfasts were taken and snap frozen and analysed for the ATNC content of the diet. The ATNC contents of the test breakfasts were: RM diet = 22 µg ATNC/kg, PM diet = 37 µg ATNC/kg, control diet = 9 µg ATNC/kg. Nitrite concentrations were 10 µg NO2/kg, 318 µg NO2/kg and 190 µg NO2/kg, respectively.

In protocol two, a further 13 volunteers (9 male and 4 female) were fed RM diets supplemented with commercially available 1 g ascorbic acid or 400 IU vitamin C tablets. The PM diet was supplemented with 1 g ascorbic acid. Dietary periods lasted for 2 days during which time the meat was consumed as a breakfast meal before 11 am as in protocol 1. The supplement was consumed concurrently with the meat breakfast. If the volunteer chose to eat the breakfast in two sittings during the morning, the supplement was split in half to ensure that some was consumed with each ‘portion’ of meat. Additionally, the protocol included a single day of a RM, PM and control no meat diet period totalling a 9 day investigative study. The order of presentation of the supplement and meat breakfast were randomly assigned. As in the previous study, after 11 am, food was provided for the volunteers from set menus that could be consumed ad libitum.

Sample collection and processing
Volunteers were asked to collect all ileal output at least every 2 h during the daytime. Samples were collected overnight when possible. The sample was passed within 14 days of the sample being passed, the sample was weighed and stored at −80°C freezer where it was thawed and stored until processing.

Within 14 days of the sample being passed, the sample was weighed and thawed. The sample was homogenized in a stomacher machine (Colworth 3500, Seward) for 5 min. The homogenate was aliquoted into glass universal tubes and stored at −20°C until analysis. Samples were stored for ~2 weeks before they were transported on dry ice to Pollock and Pool Ltd, Reading, UK for ATNC determination (16).

ATNC analysis
A total of 50 µl of the sample to be analysed was injected directly into a refluxing mixture of ethyl acetate and hydrogen bromide. A further portion of each sample was pre-treated with sulphamic acid to remove any excess nitrite, thereby eliminating artefactual nitrosamine formation. After reaction for ~5 min, 50 µl was injected into the refluxing mixture. N-nitroso dipropylamine (160 ng) was injected into the system after the analysis of each sample as an internal standard to allow quantification of the—NNO group. The nitric oxide released as a result of denitrosation of the sample was directed into a thermal energy analyser (Thermal Electron Corp., Waltham, MA, USA) in a stream of nitrogen gas where the amount of ATNC in the sample was quantified. The method detects ATNCs because nitric acid, nitrosyl-haemoglobin and thionitrates are also denitrosated under these conditions. Results are expressed as the concentration of the common unit of structure, NNO, as µg/kg.

Microflora composition of the ileal output
Molecular microflora quantification (FISH) and typing (DGGE) methods were used to determine if increased excretion of ATNC correlated with specific changes in the ileal microbiota. For DGGE analysis, bacterial genomic DNA was extracted from the ileal content thawed on ice by the bead beating method. This method efficiently lyzes most bacterial cells and appears to have little bias (17). A 457 bp fragment from the V6 to V8 region of the bacterial 16S rDNA gene was amplified with primers U968-GC (18). DGGE was performed on an 8% (w/vol) polyacrylamide gel with a denaturing gradient ranging from 40 at the top to 50% at the bottom (100% denaturing conditions were defined as 7 M urea and 40% formamide). After electrophoresis (16 h, 65 V and 60°C), the gels were stained with SYBER Green (Novex, San Diego, CA, USA) and scanned/analysed with Quantity One and Diversity Database software (Bio-Rad, Hercules, CA, USA).

For FISH analysis, aliquots (0.5 ml) of homogenized intestinal contents were added to phosphate-buffered saline (PBS) (4.5 ml), and the samples were prepared for FISH analysis as described previously (19). Hybridization was performed by applying aliquots (10 µl) of appropriate dilutions of the fixed bacterial preparations to gelatin-coated microscopic slides, fixing the specimens to the slides with 95% ethanol, and hybridizing with 10 ng/µl of the appropriate probe, using the conditions described previously (19–21). The following five probes were used: (i) Bac303 for Bacteroides and Prevotella (22), (ii) Bio164 for bifidobacteria (23), (iii) LAB158 for lactic acid bacteria (24) and (iv) EC1532 for Escherichia coli (25). We also hybridized with Erec482 a probe against eubacteria, clostridia and ruminococci belonging to Clostridium cluster XIVa (19), however, we were unable to get sufficient signal with this Cy3 labelled probe. These five probes yield a high coverage of the microbiota in faecal samples. Slides were mounted with Vectashield containing DAPI (Vector Laboratories, Burlingame, CA, USA). Fluorescent cells were enumerated by counting at 100x magnification a minimum of six fields of view per sample. The cells were counted directly with a Zeiss axioscope 40 epifluorescence-equipped microscope (Zeiss, Germany), or by capturing images and counting cells using ImageJ software (NIH, Bethesda, MD, USA).

Statistical methods
Results were analysed using SPSS 11 for Mac OSX and Microsoft Excel:Mac vX. Data were log transformed. Results are presented as mean and SEM. Mean ATNC concentrations for each dietary period were compared using a one-way repeated measures ANOVA. Specific effects of diet were detected using post hoc comparisons (paired Student’s t-test with Bonferroni correction for multiple comparisons). Statistically significant associations were those where the two-tailed probability was less than 0.05, Bonferroni corrected to 0.0017 for paired Student’s t-tests.

From previous repeat analyses on subjects on high (420 g) meat diets, the within-person SD was 56 µg/day, and setting α as 0.05 and β as 0.2, the study had sufficient power to detect 17 and 18 µg differences in ATNC between study periods with 14 and 13 subjects, respectively.

In vitro formation of NMor
The effect of bovine haemoglobin (250 mg) on the nitrosation of a model compound, morpholine, was investigated after incubation with 0.3 mg NaNO2 in 50 ml of a solution simulating the acidic conditions (pH 2) in the stomach (0.03 M NaCl and 7 mM HCI) for 1 h in a shaking water bath at 37°C. The pH was then adjusted to pH 6.8 with 0.2 M NaOH and 50 µl morpholine was added and incubated for a further 6 h in a shaking water bath at 37°C. A total of 50 µl of the sample was injected into a GC column (10% carbowax 20 M column on chromosorb WH 80/100 mesh) in a stream of nitrogen gas. The separated fractions of NMor were directed into a TEA where they were detected as described above. Results are compared with incubations in which no haemoglobin was present and those where 0.2 g ascorbic acid was added to the reaction flask.

Results
Ileostomy volunteer study I—unsupplemented diets
An average of 48 samples per subject was produced over the duration of the first study; in total, 673 samples were collected. Due to cost limitations, not all of these samples could be analysed. The ATNC content of all the samples collected throughout a 24 h period on all three diets was determined for two of the volunteers. These results are shown in Table I. The ATNC peak appeared between 6 and 9 h after the breakfasts were consumed which then returned to baseline levels overnight. It was thus decided that samples produced on one randomly selected day of each dietary period between midday and 8 pm would be selected for the study.
analysis and the peak ATNC concentration for each diet would be compared.

In the 13 individuals studied in protocol 1, the amount of ileal output produced in a 24 h period was not affected by the study diets ($P = 0.35$). An average of 638.9 ± SEM 61.7 g, 709.4 ± 67.2 g and 684.5 ± 52.3 g of ileal output was produced on the control, PM and RM diets respectively. The length of time that an individual had had the ileostomy was not a significant predictor of the ATNC content of the ileal output ($r = 0.057, P = 0.612$).

The concentration of ATNC measured in ileal output from the no meat diet were 283 ± 74 mg ATNC/kg; ATNC concentration was elevated following consumption of the RM diet (mean = 1175 ± 226 µg, ATNC/kg) and the PM diet (mean = 1832 ± 294 µg ATNC/kg).

A one-way repeated measures ANOVA comparing the peak ATNC concentration in the ileal output showed a significant, very large effect of diet (Wilks’ Lambda = 0.082, $F (2,12) = 67, P < 0.0005$, partial eta squared = 0.918).

Ileostomy volunteer study II—supplemented diets

As in protocol 1, there was a significant increase in the ATNC concentration of the ileal output collected after the red and PM diets compared to that after the no meat diet. These results are shown in Table III. However, when the RM diet was supplemented with 1 g ascorbic acid or 400 IU $\alpha$-tocopherol there was no inhibition of endogenous nitrosation (RM 1795 ± 282 µg ATNC/kg; RM + ascorbic acid mean = 2039 ± 361 µg ATNC/kg; RM + $\alpha$-tocopherol mean = 1969 ± 473 µg/kg; PM mean = 2457 ± 441 µg ATNC/kg; PM + ascorbic acid mean = 2330 ± 372 µg ATNC/kg; control mean = 476 ± 45 µg ATNC/kg). The nitrite concentration did not reach the level required for significance ($P = 0.066$).

### Table I. ATNC concentration (µg ATNC/kg ileal output) in samples collected over a 24 h period for two volunteers (D, N)

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>Control diet</th>
<th>PM diet</th>
<th>RM diet</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µg ATNC/kg</td>
<td>µg ATNC/kg</td>
<td>µg ATNC/kg</td>
</tr>
<tr>
<td>00:00–06:00</td>
<td>182</td>
<td>250</td>
<td>D</td>
</tr>
<tr>
<td>06:00–08:00</td>
<td>172</td>
<td>218</td>
<td>N</td>
</tr>
<tr>
<td>08:00–10:00</td>
<td>149</td>
<td>196</td>
<td>D</td>
</tr>
<tr>
<td>10:00–12:00</td>
<td>90</td>
<td>780</td>
<td>D</td>
</tr>
<tr>
<td>12:00–14:00</td>
<td>79</td>
<td>137; 116*</td>
<td>N</td>
</tr>
<tr>
<td>14:00–16:00</td>
<td>1830; 2240*</td>
<td>511</td>
<td>D</td>
</tr>
<tr>
<td>16:00–18:00</td>
<td>2790</td>
<td>1650</td>
<td>N</td>
</tr>
<tr>
<td>18:00–20:00</td>
<td>274</td>
<td>4690</td>
<td>D</td>
</tr>
<tr>
<td>20:00–22:00</td>
<td>47</td>
<td>107</td>
<td>N</td>
</tr>
<tr>
<td>22:00–00:00</td>
<td>143</td>
<td>182</td>
<td>N</td>
</tr>
</tbody>
</table>

*Two samples collected over this time period.

### Table II. Ileal output, and ATNC and nitrite concentration in ileal output (mean ± SEM) in response to diets; data from 14 volunteers

<table>
<thead>
<tr>
<th></th>
<th>µg Ileal output/24 h (range)</th>
<th>µg ATNC/kg</th>
<th>Nitrite concentration (µg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control diet</td>
<td>639 ± 62 (233–1004)</td>
<td>283 ± 74</td>
<td>355 ± 209</td>
</tr>
<tr>
<td>PM diet</td>
<td>709 ± 67 (449–1145)</td>
<td>1832 ± 294*</td>
<td>845 ± 283</td>
</tr>
<tr>
<td>RM diet</td>
<td>684 ± 52 (239–962)</td>
<td>1175 ± 226**</td>
<td>831 ± 396***</td>
</tr>
</tbody>
</table>

*t-test versus control diet, $P < 0.0005$.

**t-test versus control diet, $P = 0.001$.

***t-test versus control diet, $P = 0.005$.

### Table III. ATNC concentration (µg ATNC/kg ileal output) in samples collected over a 24 h period for two volunteers (D, N)

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>Control diet</th>
<th>PM diet</th>
<th>RM diet</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µg ATNC/kg</td>
<td>µg ATNC/kg</td>
<td>µg ATNC/kg</td>
</tr>
<tr>
<td>00:00–06:00</td>
<td>182</td>
<td>250</td>
<td>D</td>
</tr>
<tr>
<td>06:00–08:00</td>
<td>172</td>
<td>218</td>
<td>N</td>
</tr>
<tr>
<td>08:00–10:00</td>
<td>149</td>
<td>196</td>
<td>D</td>
</tr>
<tr>
<td>10:00–12:00</td>
<td>90</td>
<td>780</td>
<td>D</td>
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<td>12:00–14:00</td>
<td>79</td>
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<td>1830; 2240*</td>
<td>511</td>
<td>D</td>
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<tr>
<td>16:00–18:00</td>
<td>2790</td>
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<td>N</td>
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</tr>
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<td>22:00–00:00</td>
<td>143</td>
<td>182</td>
<td>N</td>
</tr>
</tbody>
</table>

*Two samples collected over this time period.

### Fig. 1. Individual changes in ATNC concentration of ileal output in response to the control, PM (A) and RM (B) diets. Each line represents results from a volunteer.
Bacterial species present

The total numbers of bacteria detected in the ileal output by FISH were orders of magnitude lower and the proportions of bacteria hybridizing to the probes differed when compared to faecal samples of healthy humans (26). Table IV shows that the proportions of lactic acid bacteria and E. coli were significantly higher than those detected in normal faecal samples. Intake of PM resulted in a significant decrease in the mean proportions of bifidobacteria but not lactic acid bacteria. No DGGE bands, representing different 16S rDNA molecules associated with bacterial species/groups were associated with any of the dietary regimen. The DGGE analysis showed less microbiota diversity in the ileal output as compared to faeces. We detected an average of only 6 distinct molecular species/profile, which compares to more than 15 bands in normal faeces (26).

In vitro effect of haem

In the absence of haemoglobin in acidic then neutral conditions, no nitrosated morpholine was detected after a 6 h incubation with sodium nitrite at pH 6.8. However when haemoglobin was present, 777 ± 146 ng NMor from five repeat experiments was formed equivalent to 3.9 ng/g Hb. On the addition of 0.2 g ascorbic acid, the yield of NMor was reduced by 72% to 0.7 ng/g haemoglobin, in contrast to the effect observed in the feeding study. The in vitro effect of α-tocopherol was not investigated.

Discussion

Ileostomists were investigated in this study because, in the small bowel, the extent of bacterial colonisation is greatly reduced compared with the large bowel. Bacterial counts suggest there are up to $10^5$ colony-forming units (c.f.u.) per ml compared to $10^{12}$–$10^{13}$ c.f.u./ml in the colon. The variation in the species present was also reduced (27). Our failure to detect differences in specific DGGE bands on diets that resulted in significantly different ileal ATNC levels and the low numbers of bacteria in the ileal output compared with that present in faeces using the same method (26) confirm that bacterial colonisation of the terminal ileum in the volunteers studied here was minimal.

Calmes et al. (28) demonstrated that bacterial flora commonly present in the large bowel are capable of N-nitrosation, and work by Massey and colleagues showed the need for a bacterial flora in the colon in order for nitrosation to occur in rats (11). However, the mean ileal ATNC output determined in this study of humans was 182 µg/day, which is similar to the mean faecal ATNC output of 159 µg/day measured in a similar study of eight volunteers consuming a diet containing 240 g RM (5). NOCs may not therefore be formed entirely by a nitrosation reaction brought about by microbes sited in the large bowel (28). However, as in previous studies of levels of ATNC in faecal samples, there was a striking 10-fold variation in the individual responses to the test diets. This has previously been attributed to variation in microbial flora which would depend on the nature and number of the micro-organisms colonising that region (3, 28). However, we were unable to detect differences in the microbial flora, apart from a reduction in Bifidobacteria bacteria, which are generally thought of as beneficial commensal microbes with PM intake. This observation does not exclude the possibility that changes in the presence/absence of specific bacteria/bacterial groups can occur after longer periods of dietary intervention.

In the in vitro work, in neutral conditions simulating those in the small intestine, a small amount (mean = 3.1 ± 0.6 ng/g haemoglobin) of a known carcinogen, NMor was formed in the presence of nitrosated haemoglobin. This reaction proceeded without the further addition of a nitrosating agent. When nitrosated haemoglobin was not present, morpholine was not nitrosated by sodium nitrite at pH 6.8. These findings suggest that nitrosated haemoglobin or myoglobin, the major haemoprotein in RM, can facilitate the formation of NOC in vivo. As white meat, such as chicken contains only 0.3–0.4 mg per gram compared with 6–10 mg in beef (29), this would explain why red but not white meat stimulates endogenous NOC formation.

In vivo, supplementation with either ascorbic acid or vitamin E did not inhibit endogenous nitrosation. This is in contrast to other in vitro studies which have reported inhibitory effects on endogenous NOC formation, although

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>No meat Mean (N) SEM</th>
<th>RM Mean (N) SEM</th>
<th>PM Mean (N) SEM</th>
<th>P-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteroides</td>
<td>2.55 (12) 1.62</td>
<td>3.28 (13) 1.47</td>
<td>1.08 (11) 0.90</td>
<td>0.24</td>
</tr>
<tr>
<td>Bifidobacteria</td>
<td>9.58 (11) 3.23</td>
<td>9.02 (11) 2.73</td>
<td>3.55 (9) 1.04</td>
<td>0.05</td>
</tr>
<tr>
<td>E. coli</td>
<td>4.64 (12) 1.44</td>
<td>3.95 (13) 0.87</td>
<td>4.28 (11) 1.10</td>
<td>0.96</td>
</tr>
<tr>
<td>Lactic acid</td>
<td>4.69 (9) 2.96</td>
<td>3.18 (11) 1.78</td>
<td>1.07 (11) 0.54</td>
<td>0.14</td>
</tr>
</tbody>
</table>

*P-value for comparison of interventions to baseline diet using a generalizing estimating equations model, clustering on subject.
these studies were carried out in the absence of haem (30,31). We are unable to explain the absence of effect in inhibiting nitrosation of vitamin E or C in this in vivo study. Our previous in vivo work has also failed to show inhibition of endogenous ATNC formation by vegetables, which are good sources of these and other antioxidants, in the presence of haem from RM (6).

The in vitro experiments demonstrated that acidified then neutralized haem enhances the nitrosation of the amine morpholine under neutral conditions. Nitrosyl-haemoglobin, formed in acidic conditions equivalent to those in the stomach, appeared to be acting as a nitrosating agent at pH 6.8. Haem could therefore be responsible for the increase in endogenous ATNC in the ileostomy output in the presence of minimal bacterial flora. As we have established that the majority of endogenously formed ATNC in faeces after eating RM is nitrosyl-haemoglobin (G. Kuhnle et al., manuscript in preparation), it is possible that there is a common mechanism accounting for the increase in ATNC and consequent production of promutagenic nitrosated amines and amino acids in both the upper and lower gastrointestinal tract. Nitrosation by haem might be the underlying mechanism accounting for the fact that red and PM consumption is associated with increased risk of both gastric and colon cancer (1,32).

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References


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