Effect of dietary meat and fish on endogenous nitrosation, inflammation and genotoxicity of faecal water

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N-3 polyunsaturated fatty acids have been associated with reduced colon tumorigenesis. However, their association with colorectal cancer incidence is not conclusive. We investigated the influence of isocaloric replacement of red meat with fatty fish on endogenous nitrosation, inflammation and genotoxicity of faecal water in apparently healthy human volunteers on controlled diets. Fourteen volunteers consumed a high red meat, a combined red meat/fish and a high fish diet for 8 days each. Faecal homogenates were analysed for haem, nitroso compounds (NOC) and calprotectin and associated supernatants for genotoxicity. Both faecal NOC and haem excretion decreased with more fish and less meat in the diet. Nitrosoyl iron (FeNO) was the main contributor to total NOC excretion, and may as such beneficially affect colorectal risk. It has been postulated that in an (apparently) healthy population a constant state of low-grade bowel inflammation exists, which is through beneficial effects on inflammation that fish or, more specifically, fish oil may reduce the risk of the disease. Fish oil is rich in the essential long chain n-3 PUFA α-linolenic acid (from plankton and algae), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Although α-linolenic acid can be converted in DHA and EPA, this is generally not efficient in humans leaving fish the main source of these fatty acids. High intakes of EPA and DHA may reduce inflammation by inhibiting the formation of inflammatory eicosanoids, cytokines and reactive oxygen species. Reactive oxygen and nitrogen species, which are produced and released as intermediates in normal metabolism but are elevated in inflammation and in response to several dietary factors, have the ability to cause several types of DNA damage, including DNA strand breaks, abasic sites and DNA adducts.

Red meat appears to increase the risk of CRC and the replacement of meat in the diet with fish could more effectively reduce CRC risk. Haem, abundantly present in red meat and less in fatty fish such as mackerel and salmon, facilitates the endogenous formation of mutagenic nitroso compounds (NOC) from dietary nitrite or nitrate. In addition, NOC can be formed endogenously by bacteria and activated macrophages via the nitric oxide pathway and may thus occur at sites of (low-grade) inflammation with an additional influence of dietary factors determining nitrosamine production by gut bacteria. A recent study shows that genotoxic concentrations of NOC are able to induce an inflammatory gene expression profile in the human colon adenocarcinoma cell line Caco2. We hypothesized that the isocaloric replacement of red meat with fatty fish would result in a decreased endogenous nitrosation and additionally investigated related risk markers of CRC, i.e. inflammation and genotoxicity of faecal water, in apparently healthy human volunteers on controlled diets.

Introduction

Animal studies show beneficial effects of n-3 polyunsaturated fatty acids (PUFA) on tumorigenesis. In contrast, epidemiological studies on fish consumption and colorectal cancer (CRC) risk appear to be inconsistent. A recent meta-analysis provides evidence that, among other lifestyle changes, moderation of red and processed meat consumption is likely to have a significant beneficial effect on CRC incidence, but there was no evidence to support recommendations on fish intake in relation to CRC risk. However, ‘fish consumption’ may include all types of fish or be limited to oil-rich fish and a possible confounding or modifying effect of meat consumption has not been investigated as yet.

Materials and methods

Subjects

Healthy males and females from Cambridgeshire were recruited through local advertisements. Participants had to be between 20 and 85 years of age, non-smokers, free from diabetes and bowel disease, not taking medication affecting the gut for at least 3 months prior to the study, not taking any nutritional supplements, not pregnant and not participating in another biochemical intervention study at the same time. Subjects completed a medical questionnaire before entering the study; only subjects in good health were included. All subjects received verbal and written information and signed a written consent form. The studies were approved by the Cambridge Local Research Ethics Committee. Fourteen volunteers (eight females, six males; 27 ± 7 years, body mass index 24.0 ± 5 kg/m²) were included. One volunteer could only complete two of three dietary periods (red meat and fish) for personal reasons; therefore, results for repeated measures are for n = 13 unless otherwise indicated.
study design

The studies had a randomized crossover design of three diets: a high red meat diet, a combined red meat and fish diet and a high fish diet. Each dietary period lasted eight days. Subjects lived in the volunteer suite of the MRC Dunn Human Nutrition Unit, where all food was provided and carefully controlled and all specimens could be collected and processed immediately. Subjects followed their normal routine but were only allowed to consume foods and drinks prepared by the diet technicians. Body weights were monitored to ensure a constant weight throughout. Faecal samples were collected and weighed daily and radio opaque marker capsules were taken throughout to check compliance and for measurement of the mean transit time (MTT) (20). Stools were collected on dry ice for the last four days of each dietary period (Days 5–8) for analysis of genotoxicity, NOC, haem and calprotectin. Blood samples were taken after each dietary period (Day 9).

Study diets

All diets were provided as similar menus on a 3-day rotating schedule. Isocaloric quantities of meat and fish (Table I) were given on the high red meat (males 325 g, females 260 g) and high fish diets (males 375 g, females 300 g), respectively; the combined meat and fish diet contained half the absolute amounts of both the red meat and the fish given in the other diets. Particularly, fatty fish types were chosen for the diets. All other food ingredients were kept the same over the three diets.

Energy and macronutrient composition of the diets (Table II) were calculated using DINNER (Data Into Nutrients for Epidemiological Research) (21). Energy requirements were estimated from body weight and physical activity using standard equations for basal metabolic rate and estimates of physical activity level (22). The energy intake of each participant was matched to estimated energy requirement with 1-MJ standardized increments (shortbread or a combination of white bread, butter and marmalade) added to 8 MJ/d (female) or 10 MJ/d (males) basal diets.

Meat was not overcooked to minimize the formation of heterocyclic amines. Purified water was given throughout for drinking and used for cooking and low nitrate vegetables were used to keep nitrate intake at constant low levels. Tea, coffee and an aspartame-based sweetener were provided in the suite and consumed freely, but subjects were asked to keep their intake constant during the study.

Dietary and faecal NOC and haem

Duplicates for each daily diet were prepared. All foods consumed on 1 day were prepared as normal, added together and diluted 1:2 in ultra pure water. The mixture was homogenized with a food processor, snap frozen on dry ice and stored at −20°C until analysis. Results are presented per gram of diet after correction for dilution.

For stool analysis, ~40 g of frozen stool was thawed, diluted 1:5 in ultrapure water and homogenized for 20 min in a stomacher (Colworth 3500, Seward Medical, London, UK). The faecal homogenates were snap frozen on dry ice and stored at −20°C until analysis. Results are presented per gram of faeces after correction for dilution.

NOC were analysed using a modification of the method previously used (18), using an Ecomedics CLD 88 Exhalyzer (Ecomedics, Duernern, Switzerland). Nitroso thiols (RSNO), nitrosyl iron (FeNO) and other NOC were analysed in separate analyses as described in detail in Joosen et al. (23).

Haem was analysed using the HemoQuant assay (24) as described previously (23). Plasma C-reactive protein (CRP) was analysed by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer’s protocol (ELISA kit from Invitrogen, Camarillo, CA).

Faecal calprotectin was analysed using a human calprotectin ELISA kit (Hycult biotechnology b.v., Uden, The Netherlands). Briefly, 500-μl extraction buffer (0.1 M Tris, 0.15 M NaCl, 0.1 M urea, 10 mM CaCl2, 0.1 M citric acid monohydrate, 5 g/l bovine serum albumin, 0.25 M dithioerol, pH 8.0) was added to 500 mg faecal homogenate. The suspension was vortex mixed and centrifuged at 3000 × g at 4°C for 1 h. The clear supernatant was analysed according to the manufacturer’s protocol. Results are expressed in nanopelera milliflare after correction for dilution (5 μL faecal homogenate, 2× extraction buffer) and in microgram per gram assuming a density of the faecal homogenates of 1 g/ml.

Table I. Meat and fish intakes (g; raw weights except for roast beef) on the three diets according to a 3-day rotating menu

<table>
<thead>
<tr>
<th></th>
<th>Days 1 and 3</th>
<th>Day 2</th>
<th>Male diet (g)</th>
<th>Female diet (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Red meat lunch</td>
<td>Roast beef</td>
<td>Roast beef</td>
<td>112.5</td>
<td>90</td>
</tr>
<tr>
<td>Dinner</td>
<td>Mince beef</td>
<td>Mince beef</td>
<td>212.5</td>
<td>170</td>
</tr>
<tr>
<td>½ Meat/fish lunch</td>
<td>Mackerel</td>
<td>Herringa</td>
<td>50</td>
<td>40</td>
</tr>
<tr>
<td>Dinner</td>
<td>Roast beef</td>
<td>Roast beef</td>
<td>56.3</td>
<td>45</td>
</tr>
<tr>
<td>Dinner</td>
<td>Salmon trout</td>
<td>Trout</td>
<td>137.5</td>
<td>110</td>
</tr>
<tr>
<td>Mince beef</td>
<td>Mince beef</td>
<td>Mince beef</td>
<td>106.3</td>
<td>85</td>
</tr>
<tr>
<td>Salmon trout</td>
<td>Trout</td>
<td>Trout</td>
<td>275</td>
<td>220</td>
</tr>
</tbody>
</table>

*aFrom tin, rinsed, no brine.

Table II. Mean nutrient intake (%energy between parentheses) on the three diets

<table>
<thead>
<tr>
<th></th>
<th>Red meat (%)</th>
<th>½ Meat/fish (%)</th>
<th>Fish (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy (MJ/d)</td>
<td>9</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td>Protein (g/d)</td>
<td>123 (23)</td>
<td>117 (22)</td>
<td>111 (20)</td>
</tr>
<tr>
<td>Fat (g/d)</td>
<td>79 (32)</td>
<td>85 (34)</td>
<td>91 (36)</td>
</tr>
<tr>
<td>SFA (g/d)</td>
<td>38 (16)</td>
<td>37 (15)</td>
<td>35 (14)</td>
</tr>
<tr>
<td>MUFA (g/d)</td>
<td>25 (10)</td>
<td>28 (11)</td>
<td>31 (12)</td>
</tr>
<tr>
<td>PUFAs (g/d)</td>
<td>6 (2)</td>
<td>10 (4)</td>
<td>14 (6)</td>
</tr>
<tr>
<td>n-3 PUFAs (g/d)</td>
<td>1 (0.4)</td>
<td>1 (0.4)</td>
<td>5 (2)</td>
</tr>
<tr>
<td>n-6 PUFAs (g/d)</td>
<td>5 (2)</td>
<td>5 (2)</td>
<td>5 (2)</td>
</tr>
<tr>
<td>Carbohydrates (g/d)</td>
<td>256 (48)</td>
<td>254 (47)</td>
<td>253 (47)</td>
</tr>
<tr>
<td>Fibre (g/d)</td>
<td>23</td>
<td>23</td>
<td>23</td>
</tr>
<tr>
<td>Iron (g/d)</td>
<td>17</td>
<td>15</td>
<td>13</td>
</tr>
<tr>
<td>Folate (µg/d)</td>
<td>278</td>
<td>272</td>
<td>262</td>
</tr>
<tr>
<td>Vitamin C (mg/d)</td>
<td>68</td>
<td>69</td>
<td>68</td>
</tr>
<tr>
<td>Vitamin D (µg/d)</td>
<td>2</td>
<td>14</td>
<td>25</td>
</tr>
</tbody>
</table>

MUFA, monounsaturated fatty acids; SFA, saturated fatty acids.

Results

MTT was calculated as a single value for the total 24-day study period as the 8-day individual dietary periods did not allow enough time for calculation per diet. MTT was 63 ± 18 h (n = 13, one volunteer no marker capsules for religious reasons). The maximum MTT was 103 h, slightly higher than the 96 h (day 1–4) adaptation period of each diet as faecal samples for analysis were pooled from Day 5 to 8. Results without inclusion of this volunteer were similar hence they were left in the analyses. All other MTT values were <88 h.
Dietary NOC and haem

Only male diets were analysed as they contained the largest amount of meat and fish. Total NOC could only be detected in the fish diets, but the amount was very low (1.4 μmol/d) and is likely to come from the tinned mackerel and herring. As expected from the choice of fish, haem intake on the fish diet (29 μmol/d) was less than half of that on the meat diet (85 μmol/d).

Faecal NOC and haem

The mean daily faecal weight, which was used to calculate daily excretion of haem and NOC, was not statistically different between dietary periods (P = 0.75). Both faecal NOC and haem excretion decreased with decreasing amounts of meat in the diet (Table III). FeNO was the main contributor to total NOC on all diets (Table III). The proportion of other NOC increased with more fish and less meat in the diet (P = 0.01), resulting in a non-statistically significant decrease in the proportion of FeNO on the fish diet (Table III). The excretion of haem was marginally positively associated with FeNO excretion on the ½ meat/fish diet (r = 0.56, P = 0.05) but showed no association with FeNO on the other diets (r = 0.25 and 0.27, P > 0.36). Total NOC concentrations showed no statistically significant association with faecal calprotectin on any of the diets (r = −0.37 to 0.06, P > 0.21).

Inflammatory markers

All plasma CRP values were within reference limits. Values for repeated measures analysis of all three diets were available for eight volunteers. Plasma CRP was 0.4 (0.4) mg/l on the red meat diet, 0.3 (0.5) mg/l on the ½ meat/fish diet and 0.4 (0.7) mg/l on the fish diet (P = 0.88). Faecal calprotectin on the red meat diet was 73.8 ± 43.7 ng/ml (0.074 μg/g), on the ½ meat/fish diet was 65.3 ± 32.1 ng/ml (0.065 μg/g) and on the fish diet was 70.1 ± 44.9 ng/ml (0.070 μg/g) (P = 0.54). There was no statistically significant association between plasma CRP and faecal calprotectin on any of the diets (r = −0.16 to 0.47, P > 0.14; red meat n = 12, ½ meat/fish n = 11, fish n = 13).

Faecal water genotoxicity

There was no statistically significant effect of diet on faecal water-induced DNA strand breaks, EndoIII- or FPG-sensitive sites in Caco2 cells (P > 0.36; Table IV). Faecal water-induced DNA strand breaks and oxidative damage showed no statistically significant associations with faecal total NOC (r = −0.44 to 0.10, P > 0.14) or calprotectin (r = −0.29 to 0.15, P > 0.38) concentrations on any of the diets.

Discussion

In this study, we found no evidence for differences in inflammatory markers and genotoxicity of faecal water on diets with varying amounts of red meat and fish, but endogenous nitrosation decreases with more fish and less meat in the diet. Our diets differed only in the amount of meat and fish and although nutrients in fish other than the n-3 PUFA, e.g. vitamin D, may contribute or be responsible for the response of any of our outcome variables, we have minimized the effects of possible confounders that may explain why epidemiological observations fail to show a consistent protective effect of fish consumption on CRC risk.

The intake of fatty fish in both the ½ meat/fish and the fish diets was considerably higher than intakes reported for Western countries (12) in order to maximize—if any—effects of the n-3 PUFA/fish. A lower risk of CRC seems to be more noticeable in studies where the highest and lowest categories of fish intake differed at least a factor seven for servings per month (8). With varying supplementation periods, experimental studies typically show a reduction in inflammatory markers at higher levels of fish oil supplementation providing >3 g/d EPA+DHA compared with no effect of lower levels of supplementation (13).

Longer term exposure does not seem to improve the effects, a 6-month intervention study increasing fish consumption, as either fatty fish or white fish, failed to show a beneficial effect on apoptotic and mitotic markers in the colon (26). Hence, our study, although we analysed cancer risk markers in stool rather than in colonic tissue, confirms that any effect of fish in a healthy population may be very small indeed and its efficacy in reducing CRC risk remains to be elucidated. On the other hand, fish consumption is inversely related to aggressive forms of prostate cancer in humans (27) and fish oil suppresses growth of colon cancer xenografts in nude mice (28), which suggests that fish (oil) may influence cancer progression, even through inhibition of inflammation, rather than protect against tumour initiation.

Calprotectin is a mainly neutrophil-derived protein, which is excreted in faeces. It is increased in inflammatory bowel disease, and has been related to colorectal cancer risk. Increased faecal calprotectin may indicate ongoing tissue damage, which could be a consequence of chronic inflammation and increased oxidative stress (29). Faecal calprotectin is also related to inflammatory bowel disease and to lower levels of fish and fish oil consumption (30). As faecal calprotectin is not increased on the fish diet, a reduction in faecal calprotectin on the fish diet may be a result of a low n-3 PUFA/fish ratio, rather than a reduction in faecal calprotectin on the fish diet.

In conclusion, the studies on inflammatory markers and genotoxicity do not support the hypothesis that higher fish consumption is associated with lower CRC risk. The results from this study also fail to support the hypothesis that higher fish consumption is associated with lower CRC risk.
disease, where bowel inflammation involves an acute phase reaction and migration of leukocytes to the bowel, but also colorectal neoplasia and gastrointestinal infection have been shown to increase faecal calprotectin (29,30). Faecal calprotectin is, to a lesser extent, increased in an asymptomatic population with a family history of inflammatory bowel disease (30) and concentrations showed an inverse association with fibre and vegetable intake in an apparently healthy population aged 50–70 years at average risk for CRC (31). In our study of younger volunteers (21–47 years), calprotectin concentrations were on average much lower [~0.07 versus ~29 µg/g; recommended cut-off for inflammation is 50 µg/g (32)] and were not influenced by the consumption of more fatty fish and less red meat.

When no or negligible amounts of haem are present in the diet, faecal haem excretion is ~60 nmol/g or ~0.02 mmol/d, depending on total faecal weight, and faecal NOC levels are 3–4 nmol/g (0.6–1.0 µmol/d) (23). The haem content of the fish diet (29 µmol/d) was approximately one-third of the red meat diet (85 µmol/d) resulting in relatively low faecal haem concentrations of about twice those observed previously on a vegetarian diet (23); values were intermediate on the ½ meat/fish diet. Faecal NOC concentrations showed a similar pattern, with a slight change in composition with changes in meat and fish content of the diet, and although no direct association could be shown on any of the diets, they seem to be mainly linked to the amount of haem given the large contribution of FeNO to total NOC. Fruit and vegetables contain bioactive compounds that are capable of inhibiting NOC formation, for example polyphenols (33); however, supplementation of a high red meat diet (420 g/d) with 400 g/d vegetables did not result in decreased faecal concentrations of apparent total NOC or faecal water genotoxicity (34). In a pilot experiment, enrichment of the same high red meat diet as used in this study with polyphenols by supplementation with onions (females 105 g, males 131.3 g), we did not find a difference in total NOC excretion or in NOC composition. This confirms the important contribution of haem to endogenous nitrosation (18).

The alkaline comet assay detects DNA strand breaks, abasic sites and sites where DNA repair is taking place, as well as creating additional strand breaks at sites of oxidatively damaged nucleobases by including DNA glycosylase enzymes in the assay (25). All of these are expected to be induced by exposure to NOC and reactive oxygen and nitrogen species; however, despite the lower faecal NOC concentrations with more fish instead of red meat in the diet, there was no clear effect on faecal water-induced DNA damage. We showed before that NOC concentrations in faecal water are relatively low compared with the concentration in faecal homogenates, but they tended to be positively related to altered purines on a high nitrite-preserved red meat diet (23). This was not the case on a vegetarian diet despite similar levels of FPG-sensitive sites and, unexpectedly, more DNA strand breaks than both the nitrite-preserved and fresh red meat diets (23) for which we have no explanation at present.

Still, little is known about the nature of faecal water or the effect of different types of preparation of the aqueous fraction of faeces (i.e. direct ultracentrifugation, dilution in PBS or ultrapure water or reconstitution of freeze-dried faeces), which may explain inconsistent results when investigating the effect of diet on DNA damage using the comet assay (34–37). We found that 300-µl faecal water from a 1:5 dilution of stool added to 300-µl Caco2 cell suspension (1:1 v/v) yielded a range of DNA strand breaks within the range of detection of the comet assay where DNA break frequency is linearly related to percentage DNA in the tail. As this concentration was within the linear part of the relation between faecal water concentration and DNA strand breaks and we did not observe a decrease in density of comets, we are confident there was no osmotic effect interfering with the genotoxicity.

In in vitro studies of haem and NOC genotoxicity, concentrations generally seem above those excreted in faeces. The highest excretion of total NOC in this study was 60 nmol/g (after correction for dilution), as expected on the high red meat diet. This converts into 60 µM assuming a density of 1 g/ml for the homogenized stool in which the NOC were analysed. With the exception of N-methyl-N’-nitro-N-nitrosoguanidine (1 µM), this is much lower than the concentrations of other individual NOC (1–100 mM) tested in vitro by Hebels et al. (19) or the concentrations of N-nitrosomorpholine (0–5 mM) tested in vitro by Robichova and Slamenova (38). With a similar calculation, the highest excretion of haem was 478 nmol/g or ~0.5 µM. Glei et al. (39) showed that a concentration of 10 µM of haemoglobin (corresponding to 40 µM haem) was genotoxic in primarily obtained colon cells, but no lower concentrations were analysed.

We are aware that any (beneficial) changes in response to an increased fatty fish consumption may have been underestimated in this study. To overcome the interindividual and intraindividual variation in faecal water and their possible effect on related genotoxicity, as well as day-to-day variation of faecal calprotectin (32), we pooled stool samples from the last 4 days of each 8-day dietary period. The change in NOC excretion is imminent with a change in (haem content of) diet; however, some diet-related factors that contribute to and are affected by the composition of faeces, e.g. the gut flora, may take more than 4 days to adapt. Also, we studied volunteers with no sign of inflammation, therefore results may only be generalized for similar populations and we cannot exclude the possibility that fatty fish may decrease faecal water genotoxicity in active gut inflammation.

In conclusion, in our healthy and relatively young study population, increasing fish intake and reducing the intake of red meat does not seem to have an effect on inflammation and faecal water-induced (oxidative) DNA damage; however, it does reduce the formation of mutagenic and potentially carcinogenic NOC.

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Conflict of interest statement: None declared.

References


