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Red meat intake-induced increases in fecal water genotoxicity correlate with pro-carcinogenic gene expression changes in the human colon

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ABSTRACT

Red meat consumption is associated with an increased colorectal cancer (CRC) risk, which may be due to an increased endogenous formation of genotoxic N-nitroso compounds (NOCs). To assess the impact of red meat consumption on potential risk factors of CRC, we investigated the effect of a 7-day dietary red meat intervention in human subjects on endogenous NOC formation and fecal water genotoxicity in relation to genome-wide transcriptomic changes induced in colonic tissue. The intervention showed no effect on fecal NOC excretion but fecal water genotoxicity significantly increased in response to red meat intake. Colonic inflammation caused by inflammatory bowel disease, which has been suggested to stimulate endogenous nitrosation, did not influence fecal NOC excretion or fecal water genotoxicity. Transcriptomic analyses revealed that genes significantly correlating with the increase in fecal water genotoxicity were involved in biological pathways indicative of genotoxic effects, including modifications in DNA damage repair, cell cycle, and apoptosis pathways. Moreover, WNT signaling and nucleosome remodeling pathways were modulated which are implicated in human CRC development. We conclude that the gene expression changes identified in this study corroborate the genotoxic potential of diets high in red meat and point towards a potentially increased CRC risk in humans.

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1. Introduction

Consumption of red meat is associated with a higher risk of colorectal cancer (CRC) and this may be a result of an increased genotoxic stress in the colon since diets high in red meat content have been found to increase fecal water genotoxicity (Pearson et al., 2009). Aside from the formation of food preparation-related heterocyclic amines (HCAs) and polycyclic aromatic hydrocarbons (PAHs), it has also been proposed that endogenously formed N-nitroso compounds (NOCs) are responsible for the link between red meat consumption and CRC risk (Bingham et al., 1996; Cross

and Sinha, 2004; Wakabayashi et al., 1992) since red meat is known to stimulate NOC formation in the colon (Kuhnle and Bingham, 2007). Meat in general is a source of NOC precursors in the form of amines and amides and heme protein present in red meat is thought to catalyze endogenous nitrosation (Cross et al., 2003; Haorah et al., 2001). In addition, meat products, and especially processed meat products, already contain pre-formed NOCs (Ozel et al., 2010). As most NOCs have mutagenic and genotoxic properties, which explain their carcinogenic effect in test animals (Lijinsky, 1992; Rao et al., 1984), they may also contribute to CRC development in humans. Moreover, gene expression changes associated with NOC exposure could play a part in the carcinogenic process. Indeed, following NOC exposure *in vitro*, transcriptomic modifications in important developmental signaling pathways have been found that may influence their carcinogenic effects (Hebels et al., 2009, 2010).

We have previously investigated the possible role of NOCs in human CRC development by studying gene expression modifications associated with exposure to these compounds in the inflamed human colon (Hebels et al., 2011). There are indications that

Abbreviations: ATNC, apparent total nitroso compounds; CRC, colorectal cancer; FDR, false discovery rate; Fpg, formamidopyrimidine-DNA glycosylase; GEO, Gene Expression Omnibus; HCAs, heterocyclic amines; IBD, inflammatory bowel disease; IBS, irritable bowel syndrome; NOCs, N-nitroso compounds; PAHs, polycyclic aromatic hydrocarbons; TM, tail moment.

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inflammation stimulates endogenous nitrosation which makes the inflamed colon an interesting model to study the effect of NOCs in relation to CRC (de Kok et al., 2005; Mirvish et al., 2003). Although we were unable to detect this stimulatory effect in our previous study by comparing patients with inflammatory bowel disease (IBD) with irritable bowel syndrome (IBS) patients who served as controls, we did find transcriptomic changes in colonic tissue in relation with fecal NOC levels that may contribute to pre-carcinogenic events induced in the colon.

The relation between red meat consumption and increased levels of nitrosation makes a dietary red meat intervention study suitable as a model for investigating NOC exposure-associated gene expression changes in the human colon. Furthermore, an interaction between inflammation as a result of IBD and red meat consumption may hypothetically result in an even higher rate of fecal nitrosation. In the present study, we therefore investigated the impact of dietary red meat intake on fecal NOC formation and fecal water genotoxicity in relation to transcriptomic changes induced in colonic tissue obtained from a subset of subjects from our previous study (Hebels et al., 2011). Gene expression changes identified in this study may be helpful in explaining the role of red meat consumption in human CRC.

2. Materials and methods

2.1. Subjects, study design, and food diary analysis

The study population was recruited in the Netherlands at the Department of Gastroenterology in the Orbis Medical Center in Sittard and the Maastricht University Medical Center and consists of a subset of 12 subjects who participated in our previous study (Hebels et al., 2011). The difference between these 12 subjects and the remaining subjects in our previous study is that these 12 subjects also volunteered to participate in a dietary red meat intervention. Six of these subjects were diagnosed with IBD (5 male, 1 female, mean age 51.2 ± 14.5 , range 31–71 years) and the other six with IBS (3 male, 3 female, mean age 55.0 ± 15.0 , range 25–65 years), thus allowing for a comparison of the effect of a diet high in red meat between IBD subjects and inflammation-free IBS control subjects. The IBD patients were only considered eligible when they had a history of ulcerative colitis with a moderate exacerbation of the inflammatory state at the time of inclusion as based on an anamnesis and the scheduled colonoscopy. Crohn's disease patients were excluded from participation as well as patients who had to be admitted to hospital due to severity of the disease or had a history of colorectal adenomas. An exacerbation of inflammation was characterized by increased intestinal cramps, increased incidence of fecal blood, and changes in stool consistency or frequency of bowel movements. Vomiting or severe diarrhea did not occur in the participating subjects. Only IBS patients proven free (as evaluated by colonoscopy) from colorectal disease (including inflammation) and other gastrointestinal disorders were included as controls. None of the subjects suffered from obesity.

After enrollment, food diaries were filled out on three consecutive days, after which subjects collected a first feces sample. This was immediately frozen (-20°C) upon collection. During a colonoscopic examination, following the collection of the first feces sample, six biopsies were taken from mucosal tissue in visually non-inflamed regions of the colon (in most cases from the sigmoid and descending colon) which were immediately frozen in liquid nitrogen and stored at -80°C until use. The intervention week started immediately after the first colonoscopy exam and involved a high red meat diet consisting of 300 g of red meat/day for 7 days. Only beef products were consumed, which included round steak, entrecôte, veal schnitzel, veal cutlet, and veal escalope. Participants were instructed to pan-fry the meat medium, regularly flip it, and avoid charring, as to minimize HCA and PAH formation, and were not allowed to eat any other meats (including meat toppings, fish and other seafood) during the intervention week. Food diaries were filled out daily during this week. Three days before and during the intervention period subjects followed their normal dietary habits, with a few modifications. To keep nitrate intake between subjects similar, participants were asked to use low-nitrate mineral water when preparing food or drinks and to avoid vegetables with high nitrate concentrations, such as spinach, lettuce, and celery. A high fruit consumption, especially fruit rich in vitamin C, and use of vitamin supplements also had to be avoided as this has been reported to inhibit endogenous nitrosation (Vermeer et al., 1999).

After the intervention, a second colonoscopy was performed and six biopsies were taken at the same location as in the first exam. Subjects collected a second feces sample on the last intervention day prior to the colonoscopy which was also immediately frozen (-20°C) upon collection. The first colonoscopy was performed as part of either surveillance or suspected gastrointestinal disorders, while the second colonoscopy was performed solely for the purpose of this study. Standard med-

ication of all IBD patients during participation consisted of the immunosuppressor 5-aminosalicylic acid. Use of additional anti-inflammatory medication in IBD patients to treat the exacerbated colon inflammation was postponed until the end of the study.

Subjects were instructed to record their daily dietary intake during the study using standardized food diaries. Daily diaries consisted of a list of several dozens of frequently consumed food items and additional room to write down items not on the list. For each food item, the amount consumed (standard portions: number of units, glasses, cups) was recorded, as well as the cooking method if applicable (e.g. frying, boiling, etc.). Food diaries were processed to calculate the average daily amounts of energy and nutrients using the software program "Eetmeter" designed by the Netherlands Nutrition Center (Netherlands Nutrition Centre [Stichting Voedingcentrum Nederland], 2010).

This study was approved by the Medical Ethical Committees Atrium Orbis Zuyd and Clinical Trial Center Maastricht (Registration number: NL13359.096.06) and written informed consent was obtained from the participants prior to the start of the study.

2.2. Fecal apparent total N-nitroso compound determination

Apparent total N-nitroso compounds (ATNC), as a measure for NOCs, were analyzed in feces as described previously (Kuhnle et al., 2007), using an Ecomedics CLD 88 Exhalyzer (Ecomedics, Duernten, Switzerland). In short, 100 mg fecal material was diluted 1:5 in ultrapure water and homogenized for 20 min. Thereafter, 500 μl of a 5% (wt/vol) sulfamic acid solution was added to remove nitrite and samples were injected into a purge vessel kept at 60°C and filled with a standard triiodide reagent (38 mg I_2 was added to a solution of 108 mg KI in 1 ml water; to this mixture, 13.5 ml glacial acetic acid was added) to determine ATNC. Results are presented as nmol ATNC/g feces.

2.3. Fecal water alkaline single-cell gel electrophoresis (comet assay)

Fecal water samples were prepared from fecal material collected from patients who participated in the red meat intervention. After manual homogenization of the fecal material for 2 min, samples were ultracentrifuged at $50,000g$ for 2 h at 10°C . The supernatant fecal water was aliquoted and stored at -20°C until use. The human colon adenocarcinoma cell line Caco-2 was used to test fecal water genotoxicity in the standard and formamidopyrimidine-DNA glycosylase (Fpg) comet assay. Fpg cuts the DNA strand specifically at oxidized purines and thus creates more strand breaks which represent oxidative DNA damage. Caco-2 cells were cultured in DMEM (Sigma-Aldrich, Zwijndrecht, The Netherlands) supplemented with 1% (v/v) nonessential amino acids, 1% Na-pyruvate, 1% penicillin/streptomycin, and 10% (v/v) heat-inactivated fetal calf serum, all purchased from Gibco BRL (Breda, The Netherlands). Cell cultures were incubated at 37°C in a humidified incubator containing 5% CO_2 . For fecal water exposures, cells were harvested by trypsinization and resuspended in growth medium containing 10% fecal water followed by a 30 min incubation at 37°C . After incubation, cells were centrifuged (300g, 5 min), washed once in phosphate buffered saline, and placed on ice. The standard and Fpg alkaline comet assays were subsequently performed in triplicate as described by Singh et al. (1988) and Pflaum et al. (1997) with minor modifications. Comets were visualized using a Zeiss Axioskop fluorescence microscope (at $200\times$ magnification). Randomly, 50 cells were analyzed using the Comet assay III software (Perceptive Instruments, Haverhill, UK). DNA damage was expressed as tail moment (TM, the product of tail DNA content and mean tail migration distance). In each experiment, control cells from a batch of frozen H_2O_2 exposed Caco-2 cells (100 μM , 30 min, frozen at -80°C in freeze medium containing 10% DMSO) were co-electrophoresed and scored along with the fecal water-exposed cells to compensate for any inter-electrophoresis variation.

2.4. Microarray hybridization and data analysis

From each subject, three biopsies were separately dissolved in QIAzol[®] (Qiagen, Venlo, The Netherlands) using a tissue disruptor, and subsequently pooled. RNA was isolated according to the manufacturer's protocol (average RNA integrity number: 7.1 ± 1.0). Microarray hybridization was performed as described previously with some modifications (Hebels et al., 2009). In short, dye-labeled cRNA (Cy3) was synthesized following the one-color labeling protocol supplied by the manufacturer (Agilent Technologies, Amstelveen, The Netherlands). Dye incorporation rates were used to hybridize equal amounts of Cy3-labeled samples (i.e. 20 pmol Cy3) to Agilent 4x44K Whole Human Genome microarrays. Slides were subsequently washed and dried. After scanning the microarray slides (wavelength 532 nm, laser power 100%, photo multiplier tube gain saturation tolerance 0.02%), bad and empty spots were flagged using the GenePix Pro software (version 6.0, Molecular Devices, Sunnyvale, CA). Quality control was performed in the statistical software environment R (version 2.10.1, The R Foundation for Statistical Computing, Vienna, Austria). Quantile normalization and subsequent data processing was performed in ArrayTrack (version 3.4, NCTRI, Jefferson, AR). Log2 transformed spot intensities were used for further analyses.

ArrayTrack and Microsoft Excel were used to find genes significantly modulated as a result of the red meat intervention groups by combining a log ratio (after/before) ranking (absolute log₂ ratio > 0.5) with a non-stringent *p*-value cut-off (paired *t*-test, *p* < 0.05), as suggested by Shi et al. (2006). The Benjamini–Hochberg false discovery rate (FDR) at this *p*-value was <15%. Further filtering was performed by selecting only gene pairs (before paired with after) that were present in at least eight out of 12 subjects. Genes were subsequently imported in MetaCore™ (GeneGo, San Diego, CA) to identify the involvement of differentially expressed genes in specific cellular GeneGO pathways. Pathways with a *p* < 0.05 were considered significantly modulated.

For Spearman's rank correlation analyses, gene expression data from all 12 subjects were correlated with genotoxicity levels (TMs from standard and Fpg comet assays). Only genes present in at least 70% of subjects were used without further pre-selection. Prior to correlation analysis, missing values were imputed in GenePattern (version 3.1, <http://www.broad.mit.edu/cancer/software/genepattern/>) by finding the *k* nearest neighbors (*k* was set to 15), using a Euclidean metric, and imputing the missing elements by averaging the (non-missing) elements of its neighbors. The online Gene Expression Profile Analysis Suite (version 4.0, <http://gepas.bioinfo.cipf.es/>) was used to perform correlation analyses and significantly correlating genes (*p* < 0.05) with a minimum correlation coefficient of 0.4 were subsequently further analyzed in MetaCore. The Benjamini–Hochberg FDR at this *p*-value was <6%.

For all pathways identified in MetaCore the Benjamini–Hochberg FDR was also calculated and the associated *q*-values are reported.

The gene expression data discussed in this publication have been deposited in NCBI's (National Center for Biotechnology Information) Gene Expression Omnibus (GEO) and are available through GEO Series accession number GSE25220 (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE25220>).

2.5. Statistical analysis of non-microarray data

Results are presented as mean ± SD. Normality of data sets was tested using the Kolmogorov–Smirnov test. Potential differences between groups were assessed using the Student's *t*-test (for equal or unequal variances, as based on Levene's test), paired *t*-test or Wilcoxon signed-rank test depending on the normality of data and presence of paired samples.

3. Results

3.1. Dietary intake during red meat intervention

The results of the food diary analysis of nutrients and food groups are presented in Table 1 and are consistent with the significantly increased intake of red meat (*p* < 0.0001). The average daily dietary intake of protein, animal protein, potassium, and zinc was significantly increased during the red meat intervention as compared to before (*p* < 0.05 or <0.01). White meat intake was significantly decreased during the intervention as a result of the dietary restrictions (*p* < 0.05). All other dietary components or food groups were not significantly influenced by the intervention.

3.2. Fecal ATNC levels

Fecal material of subjects completing the red meat intervention was first analyzed for ATNC content. IBD patient and IBS control groups were initially analyzed separately for covering the possibility of detecting an additional effect of inflammation on endogenous nitrosation during the intervention period. However, neither the IBS nor the IBD patients displayed a significant difference in fecal ATNC levels after the intervention (paired *t*-test) and there were no differences between IBS and IBD patients before or after the red meat intervention period (Student's *t*-test) (Fig. 1), nor was there a significant correlation with calprotectin levels, a marker for inflammation severity, measured in our previous study (results not shown) (Hebels et al., 2011). One IBS subject displayed a strong increase in fecal ATNC content, but this could not be linked to an unusual increase or decrease of a nutrient or food group as compared to the other subjects. Combining all subjects also showed no significant effect of the intervention on fecal ATNC levels and there was no significant correlation with the reported red meat intake (results not shown).

3.3. Fecal water genotoxicity

Although endogenous NOC formation was not significantly affected by the red meat diet, we investigated *in vitro* fecal water genotoxicity of all subjects before and after the red meat intervention as a broader marker of exposure and which is known to respond to increased red meat intake. Fecal water genotoxicity reflects the balance between all genotoxic fecal water constituents that induce DNA damage and those with potential protective properties. Individual fecal water genotoxicity levels are presented in Fig. 2. In 10 out of 12 subjects fecal water genotoxicity appeared to be increased after the meat intervention, while the remaining two subjects did not respond to meat intake. Similar to fecal ATNC levels, there was no significant difference in genotoxicity level between IBD patients and IBS controls before or after the intervention (Student's *t*-test) and genotoxicity also did not significantly correlate with calprotectin levels measured in our previous study (results not shown) (Hebels et al., 2011). Therefore both groups were combined to determine the effect of the red meat intervention (Table 2). Since inter-individual variability in fecal water genotoxicity was high, the Wilcoxon signed-rank test was used. Averaged at group level, the intervention resulted in a significant increase in genotoxicity of more than a factor 2 in both the standard and the Fpg comet assay (*p* < 0.05). Incubation with Fpg enzyme led to a significant increase in DNA strand breaks compared to the standard comet assay both before and after the intervention (*p* < 0.01), indicating the presence of oxidized purines. As can be expected from the absence of an increase in fecal ATNC after the intervention, genotoxicity levels did not significantly correlate with fecal ATNC levels.

3.4. Gene expression modifications associated with red meat intervention

Since fecal ATNC and genotoxicity levels were not different between IBS and IBD subjects, for subsequent transcriptomic analyses both groups were combined to find red meat intervention-related gene expression modifications. To analyze whether the intervention influenced the expression of genes in these subjects, modulated genes were identified as having an absolute log₂ ratio >0.5 and a paired *t*-test *p* < 0.05. The complete list of modulated genes is presented in Suppl. Table 1. At this *p*-value the FDR was below 15%. As shown by the heatmap in Suppl. Fig. 1, all significantly modulated genes show a similar behavior (i.e. up or down regulation) for the majority of subjects. Significantly modulated genes were subsequently analyzed in MetaCore. A small number of GeneGO pathways were found to be significantly modified by meat consumption (Table 3). Pathways were involved in several processes, including cytoskeleton remodeling, development, and immune response, but the percentages of involved genes for each pathway are low, indicating only small effects. An overview of the significantly modulated genes in each pathway and the pathway maps are presented in Suppl. Table 2 and Suppl. Figs. 2A–I, respectively. The pathway maps contain directional indicators (up or down regulation) for every significantly modulated gene in the pathway to allow for an assessment of the net biological effect on the pathway.

3.5. Fecal water genotoxicity-associated gene expression modifications

By specifically looking at gene expression changes associated with the overall increase in fecal water genotoxicity as a phenotypic marker of effect, relevant genes may be identified that are otherwise missed when comparing the before and after measurements with each other. To investigate the transcriptomic response linked to the increase in fecal water genotoxicity we performed a

Table 1Reported average daily dietary intake before and during the red meat intervention for all subjects ($n = 12$).

Dietary intake (average/day)	Before intervention		During intervention	
	Mean $\pm$ SD	Median [range]	Mean $\pm$ SD	Median [range]
Energy (kcal)	2072 $\pm$ 596	2147 [1215.3–3095]	2028 $\pm$ 509	1919 [1268.9–2964.3]
Protein (g)	80.0 $\pm$ 24.7	81 [42–120.7]	121 $\pm$ 19.2 ^b	117 [98–161.3]
Animal protein (g)	32.0 $\pm$ 24.9	23.8 [8–90]	69.6 $\pm$ 11.2 ^b	72.3 [41.4–80.1]
Total fat (g)	85.7 $\pm$ 37.8	86.7 [32.7–145.3]	73.8 $\pm$ 23.6	67 [39.6–115.9]
Saturated fat (g)	29.9 $\pm$ 12.3	30.4 [10.7–51]	29.7 $\pm$ 8.2	29 [16.7–39.4]
Cholesterol (mg)	226 $\pm$ 144	174 [103.3–525.7]	274 $\pm$ 101	265 [135–481.7]
Carbohydrates (g)	199 $\pm$ 57.3	200 [123–283.3]	196 $\pm$ 70.5	208 [84.9–291.9]
Fibers (g)	19.2 $\pm$ 6.2	17.4 [13–31.3]	19.9 $\pm$ 7.2	19.2 [10.1–30.4]
Alcohol (g)	19.4 $\pm$ 15.5	11.9 [0–48]	14.5 $\pm$ 13.4	12.4 [0–43.7]
Water (g)	1579 $\pm$ 358	1551 [1065.3–2037]	1671 $\pm$ 467	1768 [1008.6–2306.6]
Sodium (mg)	3305 $\pm$ 928	3032 [1897.7–4736.7]	2991 $\pm$ 751	2977 [2004.9–4445.3]
Potassium (mg)	2702 $\pm$ 596	2799 [1781.3–3690.7]	3408 $\pm$ 771 ^c	3456 [2395.9–4669.6]
Calcium (mg)	632 $\pm$ 241	650 [299.7–896]	762 $\pm$ 283	718 [428.7–1246.9]
Magnesium (mg)	281 $\pm$ 68.8	271 [177.3–398]	303 $\pm$ 90.7	295 [191.3–442.1]
Iron (mg)	11.3 $\pm$ 3.6	11.1 [6.5–17.9]	12.9 $\pm$ 4.0	12.1 [8.2–19.9]
Selenium (μ g)	52.2 $\pm$ 42.3	35.2 [18.3–159.3]	47.3 $\pm$ 15.6	48.5 [21.7–73.3]
Zinc (mg)	8.6 $\pm$ 2.2	8.8 [5–11.6]	13.7 $\pm$ 2.5 ^b	13.3 [9.2–17.2]
Folic acid (μ g)	142 $\pm$ 31.4	149 [90–190.7]	152 $\pm$ 35.4	156 [103–207.1]
Vitamin A (μ g)	881 $\pm$ 320	817 [418.7–1565.7]	694 $\pm$ 194	668 [383.4–1065.6]
Vitamin B1 (mg)	1.1 $\pm$ 0.3	1.15 [0.6–1.4]	1.4 $\pm$ 0.5	1.45 [0.6–1.9]
Vitamin B2 (mg)	1.1 $\pm$ 0.4	0.95 [0.5–1.7]	1.1 $\pm$ 0.4	1.05 [0.6–1.9]
Vitamin B3 (mg)	18.5 $\pm$ 7.7	17.35 [9–31]	23.2 $\pm$ 6.2	24.95 [11.9–29.6]
Vitamin B6 (mg)	1.6 $\pm$ 0.5	1.65 [0.9–2.6]	1.7 $\pm$ 0.4	1.6 [1.2–2.3]
Vitamin B12 (μ g)	4.3 $\pm$ 4.0	2.95 [0.6–13.6]	5.6 $\pm$ 1.9	4.9 [3.8–9.3]
Vitamin C (mg)	34.7 $\pm$ 15.8	29.5 [22–73.7]	49.5 $\pm$ 16.6	47 [27.4–74.4]
Vitamin D (μ g)	5.9 $\pm$ 4.4	4.8 [1.2–16.4]	4.0 $\pm$ 1.3	4.6 [2.2–5.8]
Vitamin E (mg)	15.9 $\pm$ 11.4	13.7 [3.1–36.7]	13.3 $\pm$ 9.3	11.3 [3.2–30.7]
Food groups				
Red meat (g)	88.6 $\pm$ 53.4	70 [25–186.67]	298 $\pm$ 42.0 ^a	303 [195.71–361.43]
White meat/fish (g)	85.6 $\pm$ 109	44.2 [0–350]	5.3 $\pm$ 8.3 ^d	0 [0–25]
Fruit and vegetables (g)	144 $\pm$ 96.1	143 [0–307.5]	160 $\pm$ 81.3	147 [21.43–294.29]
Dairy products (g)	137 $\pm$ 108	123 [36–412]	198 $\pm$ 159	113 [61–550.86]
Bread and cereals (g)	326 $\pm$ 84.5	339 [176.67–463.67]	333 $\pm$ 87.7	350 [147.86–435.71]

Significant differences determined by paired t -test analysis are indicated with: ^{a,b,c}higher after the intervention compared to before at respectively $p < 0.0001$, $p < 0.01$, and $p < 0.05$; ^dlower after the intervention compared to before at $p < 0.05$.

Fig. 1. Fecal apparent total N -nitroso compounds (ATNC) levels in IBD (black lines) and IBS patients (gray lines) before and after the red meat intervention. No significant differences were detected between before and after measurements or between IBS and IBD subjects.

Spearman's rank correlation analysis between TM levels and log2 gene expression intensities. Two separate correlation analyses were performed, using TMs from the standard and the Fpg comet assay. The complete list of significantly correlating genes ($p < 0.05$) is presented in Suppl. Table 3. At this p -value the FDR was below 6%. Significantly correlating genes with a minimum correlation coefficient of 0.4 from both analyses were subsequently analyzed in MetaCore for their involvement in GeneGO pathways and the two lists of pathways resulting from this analysis were combined, the results of which are presented in Table 4. An

overview of the significantly correlating genes in each pathway and the pathway maps are presented in Suppl. Table 4 and Suppl. Figs. 3A–S, respectively. The pathway maps contain directional indicators (positive or negative correlation) for every significantly correlated gene in the pathway to allow for an assessment of the net biological effect on the pathway. Pathways were found to be involved in a wide range of cellular processes. Pathways controlling apoptosis and survival, cell cycle regulation, and DNA damage repair were significantly associated with levels of fecal genotoxicity. Pathways involved in closely related processes like cytoskeleton remodeling, cell adhesion, and proteolysis were also found. Development pathways associated with genotoxicity levels included the strongest affected pathway in the list, the WNT signaling pathway of which more than 50% of genes were correlated with genotoxicity levels and which had the lowest FDR. The other development pathways were involved in signaling pathways linked with cytoskeleton reorganization. Another strongly associated process was regulation of lipid metabolism, which contains pathways implicated in cholesterol biosynthesis.

4. Discussion

The underlying mechanism of the correlation between red meat consumption and cancer of the colon and rectum has been investigated for many years and endogenous formation of NOCs has been suggested to play a role in this association. Although red meat intake-induced increases in fecal NOC levels, as measured by ATNC, have been demonstrated in several studies (Bingham et al., 2002; Hughes et al., 2001; Kuhnle et al., 2007), we were unable to find

Fig. 2. Total mean and per subject fecal water genotoxicity level of IBS and IBD subjects before and after the red meat intervention week using the standard and formamidopyrimidine–DNA glycosylase (Fpg) comet assay. Values were corrected for blank levels. Genotoxicity is presented as tail moment (TM). Total $n = 12$. Significance is indicated by * ($p < 0.05$) and ** ($p < 0.01$).

Table 2

Fecal water genotoxicity level of all subjects before and after the red meat intervention week ($n = 12$) using the standard and Fpg comet assay.

	Standard comet assay genotoxicity ^a		Fpg comet assay genotoxicity ^a	
	Mean $\pm$ SD	Median [range]	Mean $\pm$ SD	Median [range]
Before intervention	9.6 $\pm$ 13.8	5.5 [0.2–50.1]	17.6 $\pm$ 20.7 ^b	12.6 [0.3–62.1]
After intervention	16.7 $\pm$ 18.2 ^c	12.4 [0.1–55.2]	25.5 $\pm$ 21.4 ^{b,c}	21.9 [1.5–61.0]
Ratio after/before	2.6 $\pm$ 1.6	2.2 [0.44–5.63]	2.4 $\pm$ 1.4 ^d	2.5 [0.91–4.88]

Measurements were corrected for blank levels.

^a Genotoxicity is quantified as the average tail moment (TM) of the 12 subjects.

^b TM significantly higher with Fpg ($p < 0.01$) based on Wilcoxon signed-rank test.

^c TM significantly higher after the intervention ($p < 0.05$) based on Wilcoxon signed-rank test.

^d Mean ratio minus outlier subject A.

Table 3

GeneGO pathways significantly modulated in subjects following a 7-day red meat intervention ($n = 12$).

Cellular process	Pathways involved	% genes ^a	p -value
Atherosclerosis	Role of ZNF202 in regulation of expression of genes involved in Atherosclerosis	25	0.020 ^d
Cytoskeleton remodeling	Fibronectin-binding integrins in cell motility	17	0.042 ^d
Development	Notch-induced EMT	29	0.000 ^b
	Notch signaling pathway	13	0.002 ^c
	NOTCH1-mediated pathway for NF-KB activity modulation	12	0.010 ^d
Immune response	CD16 signaling in NK cells	19	0.030 ^d
	Murine NKG2D signaling	18	0.038 ^d
Transcription	Assembly of RNA Polymerase II pre-initiation complex on TATA-less promoters	14	0.026 ^d
Transport	Intracellular cholesterol transport	13	0.036 ^d

More details on the significantly modulated genes in each pathway and the pathway map images can be found in the [Supplementary data Excel file](#).

Abbreviations: CD16, Fc fragment of IgG low affinity IIIa receptor; EMT, epithelial-mesenchymal transition; NK, natural killer; NKG2D, killer cell lectin-like receptor subfamily K member 1; NOTCH, Notch homolog translocation-associated (Drosophila); ZNF202, zinc finger protein 202.

^a Percentage of significantly correlated genes compared to the total number of genes in the pathway.

^b q -value ≤ 0.02 .

^c q -value ≤ 0.18 .

^d q -value ≤ 0.49 .

an increase following a red meat intervention in this study (Fig. 1). The presence of inflammation in the colon, which was previously found to stimulate endogenous nitrosation (de Kok et al., 2005), in combination with the red meat diet also did not increase ATNC formation in a subgroup of subjects with IBD. Although participating IBD subjects displayed an exacerbation of their inflammatory status, the severity was generally moderate and did not cause vomiting or heavy diarrhea. In fact, severity may not have been sufficient to cause increased nitrosation (Hebels et al., 2011). As shown by the food diary analysis (Table 1), red meat intake, animal protein and total protein were significantly increased after the intervention, as was potassium and zinc intake, which can all be ascribed to the increase in meat consumption (Netherlands

Nutrition Centre [Stichting Voedingscentrum Nederland], 2010). The significant decrease in white meat consumption is a result of the dietary restrictions and cannot explain the absence of an increase in nitrosation. However, in contrast to other red meat diet intervention studies, food intake was not controlled in this study apart from white meat, nitrate/nitrite, and anti-oxidant vitamin restricting modifications (Bingham et al., 2002; Cross et al., 2006; Hughes et al., 2001). In addition, although the switch from their normal everyday diets to a high red meat diet did constitute a significant increase in the subjects' meat intake, this may not have been sufficient. Although a previous study found significant increases in endogenous nitrosation following a diet with only 240 g of red meat per day, the duration of that diet was 10 days

Table 4
GeneGO pathways significantly associated with genotoxicity levels as found by MetaCore analysis of significantly correlating genes in subjects participating in a red meat intervention ($n = 12$).

Cellular process	Pathways involved	% genes ^a	p-value
Apoptosis and survival	Regulation of apoptosis by mitochondrial proteins	21	0.017 ^c
Carbohydrate metabolism	Pentose phosphate pathway	50	0.001 ^b
	Glycolysis and gluconeogenesis	33	0.019 ^d
Cell adhesion	Integrin-mediated cell adhesion and migration	33	0.018 ^c
	Histamine H1 receptor signaling in the interruption of cell barrier integrity	24	0.018 ^c
	Role of tetraspanins in the integrin-mediated cell adhesion	23	0.022 ^d
Cell cycle	The metaphase checkpoint	20	0.015 ^c
Cytoskeleton remodeling	Role of PKA in cytoskeleton reorganisation	39	0.007 ^c
	Fibronectin-binding integrins in cell motility	29	0.007 ^c
Development	WNT signaling pathway. Part 1. Degradation of beta-catenin in the absence of WNT signaling	54	0.001 ^b
	Growth hormone signaling via STATs and PLC/IP3	22	0.014 ^c
	Slit-Robo signaling	30	0.027 ^d
DNA damage	Role of SUMO in p53 regulation	47	0.002 ^b
Neurophysiological process	Thyroliberin in cell hyperpolarization and excitability	33	0.035 ^d
Proteolysis	Putative SUMO-1 pathway	38	0.003 ^b
	Putative ubiquitin pathway	27	0.023 ^d
Regulation of lipid metabolism	Cholesterol Biosynthesis	40	0.003 ^b
	Niacin–HDL metabolism	43	0.012 ^c
Transcription	Sin3 and NuRD in transcription regulation	31	0.003 ^b

More details on the significantly correlating genes in each pathway and the pathway map images can be found in the [Supplementary data Excel file](#).

Abbreviations: PKA, protein kinase A; WNT, wingless-type MMTV integration site family member; STAT, signal transducer and activator of transcription; PLC, phospholipase C; IP3, inositol trisphosphate; SUMO, small ubiquitin-like modifier; HDL, high-density lipoprotein; Sin3, paired amphipathic helix protein; NuRD, nucleosome-remodeling and histone deacetylation.

^a Percentage of significantly correlated genes compared to the total number of genes in the pathway.

^b q -value ≤ 0.22 .

^c q -value ≤ 0.59 .

^d q -value ≤ 0.71 .

and it was compared to a control diet with 0 or 60 g of red meat per day (Hughes et al., 2001), whereas the average pre-intervention intake of red meat in this study was somewhat higher (86 g) and more variable. Although the number of subjects in our study was limited, previous red meat diet intervention studies used similar numbers and found significant differences (Bingham et al., 2002; Cross et al., 2006; Hughes et al., 2001). Although we did not find any significant differences between IBD and IBS subjects with regard to ATNC formation, it is still possible that the disease status introduces heterogeneity in the combined analysis that requires a larger study population. The age range of subjects in this study was comparable to other studies (Bingham et al., 2002), but differences in gender may have influenced endogenous nitrosation since previous red meat intervention studies have focused exclusively on male subjects. These factors could have introduced more variation, making it difficult to establish effects of the red meat intervention on ATNC levels.

Despite the absence of an increase in NOC formation after the red meat intervention, fecal water genotoxicity was significantly higher after the intervention (Fig. 2 and Table 2). Red meat consumption is not only associated with a more than twofold increase in fecal water genotoxicity, but oxidative stress also appears to be of relevance since both before and after the intervention, implementation of Fpg in the comet assay demonstrated significantly increased oxidative DNA damage levels. Although NOCs are capable of generating oxidative stress (Hebels et al., 2010), it is very unlikely that fecal water NOC concentrations are capable of inducing damage at levels observed in previous *in vitro* experiments where NOC-induced genotoxicity in Caco-2 cells was observed at millimolar concentrations which do not occur *in vivo* (Hebels et al., 2009). It has indeed been shown before that fecal water genotoxicity is independent of endogenous NOC formation (Cross et al., 2006), and therefore DNA damage induced as a result of an increased meat intake does not appear to be related to the presence of NOCs. Although HCAs and PAHs, which are formed during the cooking of meat, also pose a genotoxic and carcinogenic risk (Bjeldanes et al., 1982; Cross et al., 2010; Felton et al., 1994;

Jägerstad and Skog, 2005; Salmon et al., 2006), they are not likely to cause the increase in fecal genotoxicity levels as seen here either. Participants were given meat cooking instructions that are known to minimize PAH and reduce HCA formation (Jägerstad and Skog, 2005; Knize and Felton, 2005) and cooking methods did not differ from the pre-intervention period. Moreover, fecal HCA and PAH concentrations are not expected to be high enough to induce the level of DNA damage found in the comet assay as demonstrated by the low level of HCA and PAH-induced DNA adducts in human colon tissue (Astrup et al., 1978; Moonen et al., 2005). A small contribution of HCAs to the observed genotoxicity levels can, however, not be ruled out completely. Heme protein on the other hand, which is present in red meat in high levels and associated with an increased CRC risk (Balder et al., 2006; Cross et al., 2010; Lee et al., 2004; Sesink et al., 1999), has been shown to induce genotoxicity as measured by comet assay at concentrations found in the gut lumen (Glei et al., 2006). Apart from the reported heme-stimulated NOC formation (Cross et al., 2003), which was not observed in our study, heme may act as a catalyst of oxidative stress, which may also explain the increase in oxidative DNA damage in the Fpg comet assay. Heme-catalyzed oxidations can damage lipids, proteins, and DNA and a major pathway involves lipid peroxidation resulting in the formation of lipid alkoxyl radicals and heme oxyradicals which can initiate further oxidations (Glei et al., 2006; Tappel, 2007).

Fecal water genotoxicity has previously been related to colon carcinogenesis in animals (Klinder et al., 2004), and the results found here support such a relation in humans as well. Red meat intake-stimulated fecal water genotoxicity may result in a higher level of DNA damage in human colonic epithelium and induce the carcinogenic process. To study the effects of the red meat intervention in more detail with emphasis on possible pro-carcinogenic modifications, we subsequently addressed differences in the transcriptomic response as a result of the intervention, with a particular focus on fecal water genotoxicity. Genes differentially regulated as a result of the red meat intervention were first investigated and found to be involved in a small number of pathways, but the

percentage of involved genes was relatively low (Table 3). Although the three Notch signaling pathways are interesting in light of their role in gut development and homeostasis in relation to the proliferative potential of intestinal adenomas and adenocarcinomas (Bolos et al., 2007), modifications in these pathways are too limited to accurately assess their biological relevance regarding red meat intake and CRC risk. Effects on the other identified pathways suffer from the same limitation (see also Suppl. Table 2 and Suppl. Figs. 2A–I) and display a relatively large FDR, which prompted us to analyze genes specifically associated with fecal water genotoxicity since this is more directly related with a potential CRC risk. Significantly correlating genes found in this analysis not only were involved in a much larger number of pathways, the percentage of involved genes per pathway was also considerably higher (Table 4). Several of the identified pathways can be related to an increased genotoxic stress. The DNA damage pathway involved in p53 regulation, for example, would theoretically lead to breakdown of the p53 protein since the MDM2 and ubiquitin genes were both positively correlated with fecal water genotoxicity in this pathway (Suppl. Table 4 and Suppl. Fig. 3M). MDM2 and ubiquitin work together in targeting p53 for degradation (Lakin and Jackson, 1999) and this is an indication that, as a result of increased DNA damage, high levels of p53 protein are present. The two proteolysis pathways involving ubiquitin and SUMO-1 serve as an additional indicator that levels of p53 are being regulated since both are intimately involved in modifying p53 stability (see also Suppl. Figs. 3O and P) (Watson and Irwin, 2006). Because of p53's central role in the regulation of apoptosis and cell cycle progression (Levine, 1997), it is not surprising that pathways controlling these processes were also found. The identified cell cycle pathway is involved in the metaphase checkpoint while the modulated genes in the apoptosis pathway suggest an inhibition of the apoptotic process, since several pro-apoptotic genes were lowered in expression (Suppl. Table 4 and Suppl. Figs. 3A and G). This is an indication that after the intervention the balance between growth arrest (to repair DNA damage) and apoptosis is still favored towards the former in the colon of these subjects. The modified pathways belonging to the cellular processes of cell adhesion and cytoskeleton remodeling all appear to be interrelated since they share a large number of significant genes. The overall theoretical effect that can be deduced from these pathways points towards promotion of stress fiber formation (Suppl. Table 4 and Suppl. Figs. 3D–F, H and I). Stress fibers are formed in response to genotoxic stressors through activation of the small GTPase RhoA, and play a role in delaying cell death which is in agreement with the anti-apoptotic response found in the apoptosis pathway (Guerra et al., 2008). The expression of RhoA was indeed increased in this pathway as fecal water genotoxicity increased (Suppl. Table 4). Stress fiber formation is also supported by modifications in the Slit-Robo pathway, which is involved in cytoskeleton reorganization and displays a large degree of overlap with the cytoskeleton remodeling and cell adhesion pathways (Suppl. Fig. 3K).

One of the most interesting pathways found to be related to fecal water genotoxicity is the strongly modified WNT signaling pathway which is involved in epithelial proliferation and differentiation. Aberrant activation of WNT signaling represents a major oncogenic process in the development of many epithelial cancers, including CRC (Bienz and Clevers, 2000; Fearon and Vogelstein, 1990; Segditsas and Tomlinson, 2006). Activation of WNT signaling results in β -catenin accumulation which subsequently forms a complex with the transcription factor TCF/LEF resulting in the activation of oncogenic target genes. Our results indicate that expression of the TCF/LEF transcription factor in this pathway increases with the level of fecal water genotoxicity (Suppl. Table 4 and Suppl. Fig. 3L). This constitutes a carcinogenic risk and could be an important discovery in explaining the red meat intake-associated cancer

risk in humans. Moreover, since the WNT signaling pathway is also targeted by p53 (Lee et al., 2010), our observation of pathways involving p53 regulation suggests a close link between fecal water genotoxicity, DNA damage control by p53, and WNT signaling. We have also previously found modifications in WNT signaling in Caco-2 cells following exposure to an oxidative environment (Briede et al., 2010), suggesting that oxidative mechanisms may be responsible for the effects seen here. Interestingly, Brookes et al. (2008) have demonstrated that iron increases WNT signaling. Since iron plays an essential role in mediating the oxidative genotoxicity associated with heme exposure, this is another indication that heme protein plays a role in the effects observed here. In this line, modifications in genes controlling epithelial proliferation and differentiation have been discovered in rats following an increase in dietary heme intake (van der Meer-van Kraaij et al., 2005). It could be hypothesized that the gene expression response associated with red meat intake-induced fecal water genotoxicity is attributable to the heme content of red meat and this would be a worthwhile topic for future research. The transcriptomic modifications in the WNT pathway in particular are interesting targets for validation in novel studies focusing on the relation between red meat intake or oxidative stress and the development of CRC.

Another interesting discovery is the Sin3 and NuRD transcription regulation pathway. Sin3 and NuRD are nucleosome remodeling and histone deacetylase complexes that control transcriptional repression (Ahringer, 2000; Feng and Zhang, 2003) and several genes that form part of these complexes, including histone deacetylase 2, were positively correlated with fecal water genotoxicity levels (Suppl. Table 4 and Suppl. Fig. 3S). NuRD complex-induced gene silencing has also been demonstrated in colon cancer cells (Zhu et al., 2004; Zuo et al., 2009). These modifications point towards an epigenetic mechanism by which red meat could influence important molecular pathways.

The remaining pathways are suggestive of modifications in carbohydrate and cholesterol metabolism, but it is not clear what their relevance could be in relation to fecal water genotoxicity.

In summary, we observed a red meat intake-induced increase in fecal water genotoxicity which was not related to fecal NOC content nor influenced by inflammation in the colon. We propose that heme protein is a likely candidate for this increase, especially since fecal water-induced oxidative damage was higher following the intervention. Furthermore, we have identified a large number of pathway modifications related to red meat intake-induced fecal water genotoxicity some of which could contribute to carcinogenic transformations in the human colon. The gene expression changes identified in this study corroborate the genotoxic potential of a diet high in red meat and point towards a possible risk for CRC development in humans.

Conflict of Interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.fct.2011.10.038.

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