Food and Chemical Toxicology 118 (2018) 131–153

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Review

Red meat and colon cancer: A review of mechanistic evidence for heme in the context of risk assessment methodology

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ARTICLE INFO

Keywords:
Red meat
Colon cancer
Risk assessment
Heme iron

ABSTRACT

On October 26, 2015, IARC published a summary of their findings regarding the association of cancer with consumption of red meat or processed meat (IARC 2015; The Lancet Oncology 2015). The Working Group concluded that there is limited evidence in human beings for carcinogenicity from the consumption of red meat and inadequate evidence in experimental animals for the carcinogenicity of consumption of red meat. Nevertheless, the working group concluded that there is strong mechanistic evidence by which ingestion of red meat can be linked to human colorectal cancer and assigned red meat to Group 2A "probably carcinogenic to humans". The Working Group cited supporting mechanistic evidence for multiple meat components, including those formed from meat processing, such as *N*-nitroso compounds (NOC) and heterocyclic aromatic amines, and the endogenous compound, heme iron. The mechanism of action for each of these components is different and so it is critical to evaluate the evidence for each component separately. Consequently, this review critically examined studies that investigated mechanistic evidence associated with heme iron to assess the weight of the evidence associating exposure to red meat with colorectal cancer. The evidence from *in vitro* studies utilized conditions that are not necessarily relevant for a normal dietary intake and thus do not provide sufficient evidence that heme exposure from typical red meat consumption would increase the risk of colon cancer. Animal studies utilized models that tested promotion of preneoplastic conditions utilizing diets low in calcium, high in fat combined with exaggerations of heme exposure that in many instances represented intakes that were orders of magnitude above normal dietary consumption of red meat. Finally, clinical evidence suggests that the type of NOC found after ingestion of red meat in humans consists mainly of nitrosyl iron and nitrosothiols, products that have profoundly different chemistries from certain N-nitroso species which have been shown to be tumorigenic through the formation of DNA adducts. In conclusion, the methodologies employed in current studies of heme have not provided sufficient documentation that the mechanisms studied would contribute to an increased risk of promotion of preneoplasia or colon cancer at usual dietary intakes of red meat in the context of a normal diet.

1. Introduction

On October 26, 2015, IARC published a summary of their findings regarding the assessment of the association of cancer with consumption red meat or processed meat (IARC, 2015; The Lancet Oncology, 2015). Data on the association of red meat consumption with colorectal cancer were available from 14 cohort studies. IARC concluded that chance, bias, and confounding could not be ruled out for the data on red meat consumption, since no clear association was seen in several of the high-quality studies and residual confounding from other diet and lifestyle risk is difficult to exclude. The Working Group concluded that: 1) there is limited evidence in human beings for the carcinogenicity of the consumption of red meat, 2) the strongest, but still limited, evidence for an association with eating red meat is for colorectal cancer, and 3) there is inadequate evidence in experimental animals for the carcinogenicity of consumption of red meat.

The Working Group cited supporting mechanistic evidence for multiple meat components, including those formed from meat processing, such as NOC and heterocyclic aromatic amines and the endogenous compound, heme iron, to evaluate an association between red meat intake and colorectal cancer. They concluded that studies support the role of heme iron from red meat in nitrosamine formation, genotoxicity and oxidative stress as mechanisms by which ingestion of red meat can be linked to human colorectal cancer. Based on this mechanistic evidence, the Working Group classified consumption of red meat as "probably carcinogenic to humans (Group 2A).

IARC, in the preamble to the Monographs, defines a cancer 'hazard' as an agent that is capable of causing cancer under some circumstances,

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https://doi.org/10.1016/j.fct.2018.04.048
Received 22 January 2018; Received in revised form 18 April 2018; Accepted 20 April 2018
Available online 22 April 2018

Abbreviations

8-Iso-PGF2α	8-*iso*-prostaglandin-F2α
8-oxo	8-hydroxy-2-deoxyguanosine
ACF	aberrant crypt foci
APC	adenomatous polyposis coli
ATNC	apparent total *N*-nitroso compounds
CRC	Colon rectal cancer
DHN-MA	1,4-dihydroxynonane mercapturic acid
DGAC	dietary guidelines advisory committee
DMPO	5,5-dimethyl-l-pyrroline-*N*-oxide
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DTPA	diethylenetriaminepentaacetic acid
EPR	electron paramagnetic resonance
HHE	4-hydroxyhexenal
HNE	4-hydroxynonenal
HPLC	high-performance liquid chromatography
IARC	international agency for research on cancer
IQ	2-aminion-3-methylimidazo [4,5-f] quinolone
KRAS	Kirsten ras
LAOOH	linoleic acid hydroperoxides
LOO·	lipid peroxyl radical
LOOH	lipid hydroperoxide
MDA	malondialdehyde
MDF	mucin-depleted foci
Min	multiple intestinal neoplasia
N-NO-IQ	^{14}C-2-nitrosoamino-3-methylimidazo[4,5-f]quinolone
NOC	*N*-nitroso compounds
Nrf2	nuclear factor (erythroid derived 2)-like 2
O^6MeG	O^6-methyldeoxyguanosine
OECD	The Organisation for Economic Co-operation and Development
PUFA	polyunsaturated fatty acid
satHNA	saturated 4-hydroxynonanoic acid
SSB	single strain breakage
TBARS	thiobarbituric acid reactive substances
t-BuOOH	*tert*-butylhydroperoxide
USDA	The United States Department of Agriculture
U.S. FDA	The United States Food and Drug Administration

while a cancer 'risk' is an estimate of the carcinogenic effects expected from exposure to a cancer hazard. The Monographs are an exercise in evaluating cancer hazards, despite the historical presence of the word 'risks' in the title. The distinction between hazard and risk is important, and the Monographs identify cancer hazards even when risks are very low at current exposure levels, because new uses or unforeseen exposures could engender risks that are significantly higher (IARC, 2006).

Because there is limited evidence of carcinogenicity in humans and insufficient evidence of carcinogenicity in animals, it is important to thoroughly examine mechanistic evidence cited by IARC as well as evidence from recent related publications on this topic. Therefore, studies that investigated the role of heme iron from red meat in nitrosamine formation, genotoxicity and oxidative stress, as mechanisms by which ingestion of red meat might be linked to human colorectal cancer, were critically reviewed. Pivotal to the assessment of the strength of the mechanistic evidence is an evaluation of the methodology employed in relevant studies as well as the consistency of the response across studies to determine the weight of the evidence. In addition, it is critical to appreciate the risk assessment process in order to evaluate the role of mechanistic data in identifying both potential for hazard and expression of risk under the conditions of a real world dietary exposure.

Risk assessments consists of hazard identification, followed by a dose-response or characterization, exposure assessment and, finally, risk characterization. When the full risk assessment process is not completed, there is the danger that hazard can be confused with risk. An identified hazard does not necessarily mean an identified risk. Hazard is defined as intrinsic toxicity whereas risk is the probability of manifesting that hazard under the conditions of the exposure (Kruger, 2016). As the first step in the risk assessment process, hazard assessment relies on the information gleaned from many sources including structure -toxicity analysis, *in vitro* testing, animal bioassays, and well conducted clinical trials. Hazard identification elucidates target organs, assesses the severity and reversibility of the intrinsic toxicity. Dose response allows the determination of the quantitative relationship between the dose and toxic effect and establishes a threshold for manifestation of that effect. Exposure assessment is critical as it describes the amount, intensity, frequency, duration, and route of exposure to the compound of interest. In the last step of a risk assessment, characterization integrates hazard identification, dose-response information and exposure assessment into an estimation of the adverse effects likely to occur in a specific population (Hayashi, 2009).

It is important to note that as part of the hazard assessment, it is critical to examine not only the results reported by the investigators, but the methodology, as different methodologies may not be appropriately used for extrapolation to human health risk assessments. The relevance of animal testing and the extrapolation of testing results to humans are the subject of continuing deliberation (Barlow et al., 2002). It is important to note that, for example, although carcinogenicity bioassays are intended to relate the relevance of certain tumor types and their causation to human risk, it is known that there are neoplasms that are rodent specific (Dybing et al., 2002) and may be induced by mechanisms that are not relevant to human risk assessment. In addition, for substances in the diet, there is continuing debate regarding the relevance of bioassays that use exposure to high doses of a single substance. Importantly, it has been suggested that it may be more appropriate to evaluate many food components as part of a whole food approach in which the chemical is tested in its usual food matrix rather than admixed to the diet (Barlow et al., 2002). Thus, in a hazard identification, study methodology is critical to interpreting the results.

The objective of this review is to identify those studies available in the public literature that explore the mechanisms of action whereby it has been suggested that heme could play a role in initiation or promotion of colorectal cancer. The methodology employed in these studies and the relevance of extrapolation from the results to human health risk assessment is presented.

2. Methods

2.1. Identification of literature

A search of the public literature referenced in PubMed over the last 20 years (1998 – present) was completed using the search terms colon cancer, colorectal cancer, *in vitro*, animal, clinical, red meat, iron, heme, hemin, lipid peroxidation, genotoxicity, nitrosamine and *N*-nitroso. The inclusion criteria applied were: (1) clinical trials, animal models or *in vitro* studies, (2) used red meat or heme or hemin as treatments, (3) were original research papers and (4) were in English. Exclusion criteria included: (1) review papers, editorials, book chapters, meeting abstracts, proceeding papers, or news items, (2) epidemiology studies, (3) studied processed meat or components of meat other than heme, heme iron or hemin (such as heterocyclic amines or nitrites). This was followed by a review of the references cited in the retrieved papers to identify relevant clinical, animal and *in vitro* studies that addressed the weight of the evidence available investigating proposed mechanisms of dietary heme ingestion on initiating or promoting colorectal cancer.

Proposed mechanisms included genotoxicity via catalytic formation of NOC and cytotoxicity due to lipid peroxidation end-products. Thirty-nine papers were identified.

2.2. Calculation of relative meat exposure based on experimental conditions

Heme iron content varies from 1.21 to 3.36 mg/100 g in raw beef (Pretorius et al., 2016; Pierre et al., 2004, and USDA nutrition database), thus, we used 2.63 mg heme iron/100 g cooked beef (Lombardi-Boccia et al., 2002) as a reference value for heme iron level in beef. In addition, 16 μmol/g of heme was used as a reference value for heme iron level in black pudding or blood sausage (Pierre et al., 2004). It is noted that black pudding contains 34 fold more heme than beef. Thus, the amount of heme, hemin, heme iron and hemoglobin per gram of beef is summarized in Table 1.

The calculation of red meat intake from studies reviewed is represented relative to the Dietary Guidelines Advisory Committee (DGAC, 2015) recommended daily intake of the meat group: 51 g/day/person.[1] In addition, the U.S. FDA default for daily food intake in the United States = 3000 g/day.[2] These values give perspective on authoritative guidance for consumer food and red meat intake. Although there are certainly consumers who exceed these guidelines, mean intakes tend to be within this order of magnitude. For example, a calculation of red meat intakes from 16 different countries in the EU shows mean intakes that range from 26 to 87 g/day/person (EFSA, 2015). Thus, for purposes of exposure assessment, the default of 51 g/day/person provides a realistic baseline by which to compare exposures utilized in experimental situations.

3. Results

3.1. Hypothesized mechanisms for the role of heme in colorectal cancer and use of these results in risk assessment

3.1.1. In vitro evidence

The mechanisms by which hemoglobin could contribute to DNA damage either directly or via the generation of lipid peroxide radicals or potentiation of nitric oxide-mediated nitrosation have been evaluated in *in vitro* studies (Table 2).

Glei et al. (2006) utilized a comet assay to explore the role of hemoglobin and hemin on production of DNA damage in human colon tumor cells (HT29 clone 19A) and primary colonocytes. The author hypothesized that unabsorbed dietary iron in the gut lumen may be available for Haber–Weiss and Fenton type reactions to yield genotoxic radicals from peroxide. Results indicated that while hemoglobin significantly induced DNA damage in primary cells at concentrations from 10 to 1000 μM, there was no dose response. Hemoglobin did not produce damage in HT29 cells in doses up to 500 μM. There was also no effect of hemin in either HT29 or primary cells at non-cytotoxic dose levels. Paradoxically, *in vitro* studies of iron uptake by cells showed a higher relative cellular uptake of iron from hemin compared to hemoglobin. The mechanism by which DNA damage is produced is hypothesized to be from the generation of hydroxyl radicals. A reduction in DNA damage was seen when cell suspensions were treated with a combination of hemoglobin (1000 μM) and the radical scavenger DMSO. Contradicting the role of iron in hydroxyl generation as the cause of the DNA damage reported in the study, however, the authors noted that a combination treatment of hemoglobin or hemin with H_2O_2 did not reveal any increase in DNA damage in HT29 cells.

Table 1
Amount of heme, hemin, heme iron and hemoglobin in beef and beef products.

	MW, g/mol	Amount in Beef[a]	Amount in Black Pudding or Blood Sausage
Heme	616	0.469,643 μmol/g, or 289 μg/g	16 μmol/g
Hemin	652	0.469,643 μmol/g, or 306 μg/g	16 μmol/g
Heme iron	56	0.469,643 μmol/g, or 26 μg/g	16 μmol/g
Hemoglobin	64,500	0.117,411 μmol/g, or 7,573 μg/g	4 μmol/g

[a] The level of heme-related compounds, which may not be naturally present in beef, is derived from the heme iron level.

Importantly, the levels of exposure used by Glei et al. (2006), 10–1000 μM hemin and hemoglobin, were based on the dosing regimen used in two previous studies, one in rats reported by Lund et al. (1998) and one in humans reported by Lund et al. (1999). Glei reported that gut lumen concentrations of water-soluble iron are normally 25 μM and indicated that in human feces this can rise to > 100 μM with an intraluminal pool reaching 350 μM after ferrous sulfate supplementation. Glei et al. (2006) suggests that these studies support the doses used in their study as representative of physiologic levels. A review of the studies upon which the dosing in the Glei study is based, however, demonstrates that the levels used do not represent physiologic levels and are in fact gross exaggerations of expected exposure. In the rat study reported by Lund and colleagues, intraluminal iron concentrations of around 25 μM were reported after a high iron diet; the amount of iron ingested was comparable to a human consuming approximately 9 kg of beef per day. In the human study reported by Lund and colleagues, fecal iron not gut lumen iron content of 300 μM was reported after volunteers consumed iron supplements. In addition, levels of iron supplementation used by Lund et al. (1999) represent a beef intake of approximately 730 g/day.

In another comet assay, the potential for DNA damage by production of reactive oxygen species produced by hemin was assessed in colonic epithelial cells using 0.1–1000 μM hemin (Ishikawa et al., 2010). In concentrations from 0.1 to 100 μM, significant increases in DNA damage were seen between 1 and 100 μM. The hypothesis for the study was that heme induces DNA damage via H_2O_2 produced by heme oxygenase. The study also evaluated the heme oxygenase inhibitor ZnPP and exogenous catalase in experiments using heme at a concentration of 1 μM. Addition of ZnPP and catalase both inhibited hemin induced DNA damage in a dose-dependent manner. Both ZnPP and catalase decreased IL-8 production induced by hemin. It is important to note, however, that conditions necessary for the generation of oxygen free radicals may not exist in the proximal colon to the extent found in *in vitro* systems because the *in vivo* intraluminal environment is predominantly anaerobic whereas atmospheric oxygen is available in the *in vitro* assay system. Therefore, the relevance of production of reactive oxygen species such as H_2O_2 in an *in vitro* system to the health consequences in an *in vivo* situation are unknown.

Lakshmi et al. (2005) evaluated the potential for endogenous N-nitrosation using a heterocyclic amine 2-amino-3-methylimidazo [4,5-f]quinoline (IQ) incubated with the nitric oxide donor spermine and H_2O_2 in the presence or absence of hemin. This *in vitro* system was intended to mimic concentrations of NO and H_2O_2 that occur during an inflammatory response. The authors, however, cite the basis for the experimental conditions on research that utilized *in vitro* models. Therefore, it is not possible to determine how or to what extent the conditions in the *in vitro* study reported by Lakshmi replicate a chronic inflammatory state in the human colon. A comparison of the concentration of IQ used in this *in vitro* study (10 μM) to the level of IQ

[1] 2015 Dietary Guidelines Advisory Committee (DGAC) recommended that food consumption at the 2000 calorie level contains meat at a level of 12.5 ounces per week, which is approximately 51 g/day. https://health.gov/dietaryguidelines/2015-scientific-report/06-chapter-1/d1-10.asp#table-d1-32. Accessed on May 24, 2017.

[2] http://www.fda.gov/Food/GuidanceRegulation/GuidanceDocumentsRegulatoryInformation/IngredientsAdditivesGRASPackaging/ucm081818.htm.

Table 2
In vitro studies.

Reference	Protocol and Treatment	Endpoints	Results
Angeli et al., 2011	Adenocarcinoma cell line (SW480 cells) were preincubated with 50 μM hemoglobin for 1 h or not before being incubated with LAOOH (0–200 μM) for 6,24, and 48 h.	The treated cells were analyzed for cytotoxicity, genotoxicity, production of reactive oxygen species, oxidative stress parameters, peroxide content, DNA damage, and DNA adduct.	LAOOH induced time- and dose-dependent cytotoxicity and lipid peroxidation, the effect of which was enhanced by the pretreatment of 50 μM hemoglobin. Hemoglobin alone did not induced DNA damage until reached to a level of 100 μM. DNA adducts were significantly increased in cells only when the cells were incubated with both hemoglobin and LAOOH.
Baradat et al., 2011	Mouse wild-type colon cells ($Apc^{+/+}$) and isogenic cells with a mutation on the APC gene ($Apc^{Min/+}$) were treated with radiolabeled HNE (5–40 μM) for 30 min or at 40 μM during 5–90 min.	HPLC analysis of cells for HNE content. LC-MS and NMR analysis for the level and type of HNE metabolites. Quantitative real time-PCR analysis for gene express of the enzyme involved in HNE-cysteine and GSH conjugates formation.	$Apc^{Min/+}$ cells metabolized significantly more HNE and faster than $Apc^{+/+}$ cells. Within 30 min, HNE at all tested levels (5–40 μM) resulted in increases in HNE-cysteine and decreases in saturated HNE (satHNE) in $Apc^{Min/+}$ cells than in $Apc^{+/+}$ cells. RT-PCR results showed that $Apc^{Min/+}$ cells had a significant increase in expression of HNE-cysteine transporters and HNE-GSH conjugation enzyme than $Apc^{+/+}$ cells, but HNE did not have any effect on these expressions. RT-PCR results showed that $Apc^{Min/+}$ cells had a significant increase in expression of different aldehyde dehydrogenases but not alkenal/one oxidoreductase than $Apc^{+/+}$ cells, whereas HNE did not have any effect on these expressions. Synthesized satHNE and satHNA were not cytotoxic to both $Apc^{Min/+}$ and $Apc^{+/+}$ cells.
Bastide et al., 2015	$Apc^{+/+}$ (derived fromC57BL/6 J mice) and $Apc^{-/+}$ (derived from C57BL/6 J $Apc^{Min/+}$ mice) colon epithelial cells were treated with fecal water from hemoglobin fed rats with or without aldehyde trapping, aldehydes (HNE, HHE, and MDA), and hemin.	The treated cell lines were analyzed for cytotoxicity, genotoxicity, and apoptosis.	Fecal water from hemoglobin-fed rats was more cytotoxic to $Apc^{+/+}$ than $Apc^{-/+}$. Fecal water treated with carbonyl compounds removal resulted in significantly less cytotoxicity in both cells. Both HNE (20 μmol/l) and hemin (100 μmol/L) lead cytotoxicity to the cell lines but the former had different effects on $Apc^{+/+}$ and $Apc^{-/+}$. HNE and HHE were more cytotoxic and more genotoxic to $Apc^{+/+}$ than $Apc^{-/+}$, but MDA was neither cytotoxic nor genotoxic in both cell lines.
Glei et al., 2006	Human colon tumor cells (HT29 clone 19A) and primary human colonocytes were incubated with bovine hemoglobin and hemin at 37 °C at different concentrations (1–1000 μM) for different periods of time.	Microscopic analysis of both HT 29 clone 19A and primary colon cells for DNA damage. Determination of both types of cells for cytotoxicity.	Hemoglobin significantly induced DNA damage of primary cells from 10 to 1000 μM, but did not induce the damage of HT29 cells until at a concentration of up to 500 μM. Hemin did not significantly induce DNA damage in neither HT29 cells nor colon cells until it reached to a level of 1000 μM. Both hemoglobin and hemin (250–1000 μM) significantly decreased the metabolic activity of primary and HT29 cells. Hemin started to significantly reduce the HT29 clone 19A cells number at a lower concentration than hemoglobin (10 vs 50 μM). The cytotoxicity induced by heme was not dependent on time of exposure.

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Table 2 (*continued*)

Reference	Protocol and Treatment	Endpoints	Results
Ishikawa et al., 2010	Human colonic epithelia Caco-2 cells exposed to different concentrations of hemin (1–10 μM) for 0–24 h in the absence and presence of various concentrations of a heme oxygenase inhibitor, zinc protoporphyrin (0.1–10 μM), or catalase (0.01–1 U/mL).	Analysis of Caco-2 cells for cell viability, DNA damage, cell proliferation, and indirect oxidative stress indicators (IL-8 m RNA expression and IL-8 production).	Hemin started to significantly affect Caco-2 cells' viability at 1000 μM and significantly induce DNA damage at concentrations of 1–100 μM. Hemin significantly increased the cell proliferation at levels of 0.1, 1, 10, and 100 μM but it was not dose-dependent. Addition of zinc protoporphyrin (0.1, 1, and 10 μM) or catalase (0.01, 0.1, and 1 U/mL) significantly reduced the DNA damage induced by 1 μM hemin and significantly inhibited the cell proliferation induced by 1 μM hemin. The oxidative stress (IL-8 mRNA expression) in Caco-2 cells was induced by 10 μM hemin from 1 to 6 h but not detectable at 10 h. The IL-8 production in Caco-2 cells was significantly increased by treating with 1, 10, or 100 μM but not 1000 μM hemin for 24 h. Addition of zinc protoporphyrin (1 or 10 μM) or catalase (0.1 or 1 μM) to Caco-2 cells treated with 10 μM hemin significantly reduced IL-8 production.
Lakshmi et al., 2005	A heterocyclic amine IQ (10 μM) was incubated with NO donor spermine NONOate (0–0.05 mM) in the presence of hemin (0–30 μM).	HPLC analysis for nitrosation of IQ to form N-NO-IQ	Addition of hemin and H_2O_2 significantly increased the formation of N-NO-IQ from IQ by NO. The kinetic study showed that IQ had a higher affinity to NO to form N-NO-IQ when hemin and H_2O_2 were present. NADH, ascorbic acid, catalase, and DMPO were found to inhibit the nitrosation of IQ in presence of hemin/H_2O_2.
Sawa et al., 1998	The assay mixture contained 0.2 mL of phosphate buffer (pH 7.4), 10 mM LOOH (t-BuOOH), 0.1 mg/mL hemoglobin, 180 mM DMPO, and 0.5 mM DTPA.	Detection of peroxyl radicals from LOOH by EPR spectroscopy and from edible oils by luminol-enhanced chemiluminescence assay.	EPR spectroscopy showed that addition of hemoglobin enhanced the production of LOO· radicals from t-BuOOH and oxidized linoleic acid. *However, the baseline intensity of each spectrum was different and the g value of the DMPO-LOO· adduct was not given.*
	The assay mixture contained 0.3 mL phosphate-buffered 0.15 M saline (pH 7.4), 50 μL of 10 mM DTPA, and 50 μL of ethanol-containing oil (3 mg/mL), and 50 μL of 0.1 mM luminol. Addition of 50 μL of hemoglobin (1 mg/mL) initiated the reaction.	SSB of supercoiled plasmid DNA was monitored when treated with LOOH and hemoglobin.	The chemiluminesence assay showed that LOO· radicals were produced from air-oxidized rapeseed and safflower oil in the presence of 0.1 mg/mL hemoglobin as a function of time and the generation speed was dependent on the oil refining methods. *It was not clear whether the same observation would be obtained in the absence of hemoglobin.*
	The assay mixture contained 2 μL of pUC19 plasmid DNA (50 μg/mL), 2 μL of 2 mM DTPA, 2 μL of LOOH, and 10 μL of 50 mM phosphate buffer (pH 7.4), with or without various inhibitors, with 2 μL of heme- or non-heme-iron, equivalent to a final concentration of 100 μM iron		Neither t-BuOOH nor hemin alone could induce SSB of the DNA, but their combination caused SSB. However, SSB was not significantly correlated with the LOO· production.
Surya et al., 2016	Normal wild-type and Apc-mutated colon epithelial cells treated with HNE or fecal water from the *in vivo* study that rats were fed hemoglobin and beef in Table 3.	Analysis of normal and Apc-mutated (preneoplastic) cells incubated with fecal water from both protocols for apoptosis, siRNA transfection, Nrf2 determination, and HNE adducts.	Fecal water from all rats resulted in apoptosis of both normal and preneoplastic cells, however, preneoplastic cells exhibited higher resistance than normal cells. The depletion of carbonyl compounds abolished the differential apoptosis in both normal and preneoplastic cells. Fecal water from hemoglobin- and beef-fed rats induced higher Nrf2 activity in preneoplastic cells than in normal cells, but the resin-treated fecal water did not induce activated Nrf2, which suggested that Nrf2 activation was associated with the carbonyl compounds in fecal water.

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Table 2 (*continued*)

Reference	Protocol and Treatment	Endpoints	Results
Van Hecke et al., 2014	4.5 g of uncured and nitrite-cured chicken, pork and beef were incubated for 5 min with 6 mL of saliva, for 2 h with 12 mL of gastric juice, and for 2 h with 2 mL of bicarbonate buffer (1 M, pH 8.0), 12 mL of duodenal juice, 6 mL of bile juice, and 22 mL human intestinal microbial ecosystem medium, and 22 mL a human fecal inoculum.	Digesta was analyzed for lipid oxidation products, MDA, HNE and other aldehydes, protein carbonyl compounds, and a NOC-specific DNA adduct O^6-carboxymethyl-guanine.	After digestion steps, beef resulted in higher production of lipid oxidation products than pork and chicken; nitrite-cured meat (pork and beef) resulted in lower production of lipid oxidations than uncured ones. The DNA adduct formation of colonic digestion was highly dependent on the individual bacterial inoculum used and not related to the heme concentration in the meat samples. Nitrite from cured meat samples had an anti-oxidative effect and did not increase formation of DNA adducts.

found in broiled meat (0.19 ng/g) (NTP, 1999) shows that the study was designed to evaluate the potential for nitrosation using an amount of IQ that is at least 5 orders of magnitude higher than the exposure a person would get from consuming a DGAC recommended daily intake of meat (51 g/day). Therefore, it is highly unlikely that this *in vitro* assay can be used to extrapolate to the *in vivo* condition.

Generation of lipid peroxyl radicals in the presence of various heme compounds and edible vegetable oils has been investigated as a mechanism by which heme could be associated with development of cancer. The two most prevalent reactive oxygen species that can damage lipids are the hydroxyl radical ($HO^{\cdot}$) and hydroperoxyl ($HO_2^{\cdot}$) and the primary products of lipid peroxidation are lipid hydroperoxides (LOOH). It is important to note, however, that the *in vivo* situation is more complex than the *in vitro* model. Response to membrane lipid peroxidation under specific cellular metabolic circumstances and repair capacities in the *in vivo* situation may result in promotion of cell survival or induction of cell death. Under physiological or low lipid peroxidation rates, cells stimulate their maintenance and survival through constitutive antioxidant defense systems or activation of signaling pathways that upregulate antioxidant proteins. Under conditions of high lipid peroxidation rates, the extent of oxidative damage overwhelms the repair capacity, and the cells induce apoptosis or necrosis programmed cell death (Ayala et al., 2014).

Sawa et al. (1998) used an *in vitro* model to explore the generation of lipid peroxyl radicals in the presence of various heme compounds and edible vegetable oils. Polyunsaturated fatty acids are susceptible to oxidation, which results in the formation of lipid hydroperoxides (LOOH); heme iron has been shown to catalyze the formation of lipid peroxyl radicals ($LOO\cdot$) from LOOH. The potency of different commercial vegetable oils to generate lipid peroxyl radicals in the presence of hemoglobin, however, varies greatly and is dependent on the purification steps used in the production of the oil. For example, in the presence of 100 μg/mL hemoglobin, safflower oil showed the strongest lipid peroxyl radical generating potential but unpurified virgin and extra virgin olive oils and sesame oils showed no apparent generation of lipid peroxyl radicals even in the presence of hemoglobin, with exposure to air at 37 °C for 30 days. Lipid peroxyl radical production increased upon air exposure in a time-dependent manner. The authors concluded that this *in vitro* system demonstrated that purification processes tended to remove the components in the oils that suppressed lipid peroxyl radical generation and/or lipid oxidation. Importantly, this study demonstrates variability in results may be introduced in experimental models due to differences in test articles used and therefore extrapolation from the results obtained in any one study to any conclusion about the generation of lipid peroxyl radicals in the presence of heme from red meat ingestion in an *in vivo* situation must proceed with caution.

Angeli et al. (2011) determined that linoleic acid hydroperoxide (LAOOH) produced a time and dose-dependent cytotoxicity (after 24 h and higher than 100 μM) in a human colon adenocarcinoma cell line which was increased after pretreatment with hemoglobin. Lipid peroxidation (MDA formation) was increased in a dose-dependent fashion (concentration higher than 50 μM) by Hb pretreatment. Specific DNA adducts were increased in cells exposed to Hb + LAOOH. No significant effect was seen on peroxide content. Hemoglobin was negative in the Comet assay at concentrations up to 250 μM. The results suggest that high levels of heme iron are needed to increase LAOOH induced genotoxicity which may be produced by the decomposition of LAOOH by heme iron to generate peroxyl alcoxyl radicals, $^1O2$, and aldehydes that could interact with DNA, generating promutagenic DNA lesions. The levels of heme and fat used, however, cannot be extrapolated to the *in vivo* situation.

An *in vitro* digestion model simulating mouth, stomach, duodenum and colon demonstrated that higher amounts of lipid oxidation products (such as MDA and HNE) are produced by sources of high heme-Fe compared to sources lower in heme-Fe (Van Hecke et al., 2014). DNA adduct formation, however, was not related to the heme concentration in the meat samples but was highly dependent on the individual bacterial inoculum used thus highlighting the difficulty of determining the relevance of these *in vitro* effects to the *in vivo* situation. Interestingly, this study also evaluated the effect of nitrite curing and found that it lowered lipid and protein oxidation.

Mutation of the Apc gene and loss of Apc function may be an early event in human colon rectal cancer (CRC) development and therefore an (ApcMin/+) cell line is frequently used to investigate the consequence of cytotoxic and genotoxic secondary lipid oxidation products, such as HNE that are formed as a result of the oxidative properties of heme. The use of this cell line provides information on the capacity of mutated cells to get rid of potentially harmful dietary secondary lipid oxidation products through coordinated biotransformation pathways thus providing them a possible survival advantage in a peroxidative environment. Baradat et al. (2011) demonstrated that Apc-mutated cells are more efficient than wild-type cells in metabolizing HNE into thiol conjugates and 4-hydroxynonanoic acid due to the higher expression of key biotransformation enzymes. These differential biotransformation capacities would explain the differences of susceptibility between normal and Apc-mutated cells regarding secondary lipid oxidation products. Using an Apc-mutated cell line, Bastide et al. (2015) demonstrated that fecal water from rats fed hemoglobin containing diets was more cytotoxic to nonmutated than premalignant cells. Resin trapping of carbonyl compounds from the fecal water from rats fed heme-based diets significantly decreased TBARS levels as well as cytotoxicity to both Apc+/+ and Apc-/+ cells. Only HNE, but not heme, had differential cytotoxic effects in normal vs preneoplastic cells that was similar to that observed with fecal water of rats fed heme. Lipid peroxidation end products, HNE and HHE, were more cytotoxic and genotoxic to normal Apc +/+ cells than to premalignant Apc −/+ cells; MDA had no effect.

Surya et al. (2016) reported that fecal water of both hemoglobin and red meat fed rats significantly increased apoptosis in normal cells compared to preneoplastic cells. Lipid peroxidation is postulated to play a role in this finding because depletion of lipid peroxidation derived

Table 3
In vivo studies.[a,b]

Reference	Protocol and Treatment	Equivalent Human Daily Meat Intake[4] (Relative to DGAC Recommended Level[1])	Endpoints	Results
Bastide et al. 2015	Protocol 1: 80 male 4-week-old F344 rats fed with control, heme-based (1% hemoglobin), heterocyclic amines (HCA)-based (50 µg/kg PhIP + MeIOx 25 µg/kg), or both and supplemented with drinking water with or without NOC; 0.17 $NaNO_2$ + 0.23 $NaNO_3$ g/L) for 100 days. After one week on the experimental diet, rats were intraperitoneal injected with azoxymethane (20 mg/kg body weight). Protocol 2: 35 male and female 4-week-old C57BL/6J $Apc^{Min/+}$ [5] and $Apc^{+/+}$ mice fed control or 2.5% hemoglobin diets for 49 days.	1% hemoglobin/diet, equivalent to 3,961 g beef (78) 2.5% hemoglobin/diet, equivalent to 9,904 g beef (194)	Protocol 1: Analysis of colon for MDF. Analysis of fecal water for levels of heme, TBARs, ATNCs, and cytotoxicity. Protocol 2: Analysis of small intestine and colon for tumor presence. Analysis of fecal water for TBARs. , and cytotoxicity. Analysis of fecal water in the small intestine for TBRS and cytotoxicity.	Protocol 1: Hemoglobin-based diets (1%) resulted in increased MDF and fecal TBARS, ATNC, and cytotoxicity. The major form of ATNC from heme-based diets is iron nitrosyl. Protocol 2: Hemoglobin diets (2.5%) significantly increased the small intestinal tumor load in premalignant $Apc^{Min/+}$ mice while not in the colon, but no neoplasia was induced in normal $Apc^{+/+}$ mice by hemoglobin diets. Heme-based diets significantly increased TBARs in fecal water and cytotoxicity of small intestine contents. Hemoglobin diets had no additional genotoxic effect in $Apc^{Min/+}$ mice.
Reference	**Protocol and Treatment**	**Equivalent Human Daily Meat Intake[4] (Relative to DGAC Recommended Level[1])**	**Endpoints**	**Results**
Chenni et al. 2013	4-week-old male F344 rats were used in both protocols. Protocol 1: Subjects (n=10) were fed control diets, control+1 g $NaNO_2$/L water, control + 0.63% hemoglobin, or control + 1 g $NaNO_2$/L water + 0.63% hemoglobin diets for 100 days. Protocol 2: Subjects (n=12, 10, 10, and 10) were fed with control diets, control + 0.17 g $NaNO_2$/L water + 0.23 g $NaNO_3$/L water, control + 1% hemoglobin, or control + 0.17 g $NaNO_2$/L water + 0.23 g $NaNO_3$/L water + 1% hemoglobin, for 100 days.	0.63% hemoglobin/diet, equivalent to 2,496 g beef (49) 1% hemoglobin/diet, equivalent to 3,961 g beef (78)	In both protocols, analysis of fecal water for TBARS, heme content, cytotoxicity on CMT93 cells, and ATNC. Analysis of urine for DHN-MA content.	In both protocols, hemoglobin diets resulted in increases of TBARS in fecal water and DHN-MA in urine. In protocol 2, hemoglobin diets resulted in an increase in cytotoxicity of fecal water. Hemoglobin diets resulted in an increase of ATNC in fecal water, which was mainly attributed to iron nitrosyl. Nitrite alone induced a significant increase in fecal ATNC, but not in the form of iron nitrosyl.
de Vogel et al. 2008	Two groups of eight-week-old male Wistar rats (n=16) were fed with control diets (40% energy as fat corresponding with 200 g of fat/kg diet, 20 mmol/kg of calcium) and control diets supplemented with 0.5 mmol hemin/kg, respectively, for 14 days.	0.5 mmol hemin/kg diet, equivalent to 3,194 g beef (63)	Analysis of fecal water for cytolytic activity. Analysis of colon tissues for mucosal proliferation, immunohistochemistry, apoptosis, and histopathology.	Hemin diet resulted in increase in fecal water cytolytic activity. Colon mucosal apoptosis of hemin-fed rats was reduced than that of control diet-fed rats. However, the reduced apoptosis of colonic epithelial cells was not correlated with the increased cell proliferation or hyperplasia of colonic crypts in heme-fed rats. Histological examination showed that rats fed with hemin diets had increased injury of both proximal and distal colons than control diets.

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Table 3 (*continued*)

Reference	Protocol and Treatment	Equivalent Human Daily Meat Intake[4] (Relative to DGAC Recommended Level[1])	Endpoints	Results
Gueraud et al. 2015	Female Fisher 344 rats (n=6/group) were fed five experimental diets containing low-calcium 0.8 g/kg 5% oil corn oil + 0.94 g/kg hemin, 5% Menhaden fish oil + 0.94 g/kg hemin, 5% safflower oil + 0.94 g/kg hemin, 5% Menhaden fish oil + 0.36 g/kg ferric citrate, or 5% safflower oil + 0.36 g/kg ferric citrate. All diets contained iron content of 80 mg/kg.	0.94 g hemin/kg diet, equivalent to 9,216 g beef (181)	Urine (24 hour sample) analyzed for markers of lipid peroxidation/ oxidative stress (MDA, DHN-MA, 8-iso-PGF2α, fatty acid, HNE-His protein adducts, myeloperoxidase, and C-reactive protein). Fecal water was analyzed for TBARS and cytotoxicity on immortalized epithelial colon cells and a mouse rectum carcinoma cell lines CMT93.	Oxidative biomarkers in the urine were highly dependent on the source of the lipids rather than heme in the diets. Oxidation product and cytotoxicity of fecal water were also highly dependent on the source of lipids rather than heme in the diets.
Ijssennagger et al. 2015	Four groups of eight-week-old male C57BL6/J mice (n=9/group) were fed with either a control diet (40 energy % fat, low calcium 30 μmol/g), A control diet supplemented with 0.5 μmol/g heme), a control diet with antibiotics treatment (1 g/L ampicillin, 1 g/L neomycin, and 0.5 g/L metronidazole), or a heme diet with antibiotics treatment for 14 days	0.5 μmol heme/g diet, equivalent to 3,194 g beef (63)	Fecal water was analyzed for TBARS, bile acids, and mucin. Colons were examined for Ki67-positive colonocytes. RNA from colon scrapings and DNA from fresh fecal pellet from the colon were analyzed.	Heme diets resulted in increased TBARs level and cytotoxicity of fecal water, the effect of which was compensated by the addition of antibiotics. Antibiotics treatment in the heme diet completely suppressed heme-induced hyperproliferation and hyperplasia to the level of non-heme treated-groups. Antibiotics blocked the heme-induced expression of cell cycle genes and the mucosal sensing of heme-induced luminal cytotoxicity. Antibiotics did not affect the heme-induced antioxidant response.
Reference	**Protocol and Treatment**	**Equivalent Human Daily Meat Intake[4] (Relative to DGAC Recommended Level[1])**	**Endpoints**	**Results**
Ijssennagger et al. 2011	Nine 8-week-old male C57BL6/J mice (n=9/group) were fed with control diets (40 en% fat, mainly palm oil, low calcium, 30 μmol/g) or this diet supplemented with 0.5 μmol heme/g diet for 14 days.	0.5 μmol heme/g diet, equivalent to 3,194 g beef (63)	Fecal water was analyzed for cytotoxicity. Colon mucosa was analyzed for cell proliferation, heme metabolism-related and stress-related genes, and gene expressions of downregulated and upregulated signaling molecules.	Mice fed with heme-supplemented diets had significantly higher level of cytotoxicity in fecal water and more intense colonic epithelial surface injury. Heme activated and upregulated oxidative stress-sensing transcription factors in surface epithelium. Heme downregulated epithelial expression of feedback inhibitors of cell proliferation.
Ijssennagger et al. 2012a (PlosOne-August)	Sv129 wild-type mice and corresponding PPARα knockout (KO) mice fed either a purified control diet (40 energy% fat and low calcium 30 μmol/g) or this diet supplemented with 0.5 μmol heme/g diet.	0.5 μmol heme/g diet, equivalent to 3,194 g beef (63)	Fecal water was analyzed for cytotoxicity. Colon mucosa was analyzed for cell proliferation, heme metabolism-related and stress-related genes, and gene expressions of downregulated and upregulated signaling molecules.	Heme-based diets increased luminal cytotoxicity, TBARS level, and mucosal injuries on both mice without significant differences between the knockout and the wild-type mice. The results showed that heme-induced hyperproliferation was not mediated by PPARα.
Ijssennagger et al. 2012b (PlosOne-Dec)	Male C57BL6/J mice (n=8/group) fed with a control diet (40% fat and low calcium (30 μmol/g)), or this diet supplemented with 0.5 μmol/g heme for 14 days.	0.5 μmol heme/g diet, equivalent to 3,194 g beef (63)	Fecal water was analyzed for cytotoxicity, epithelial exfoliation, and TBARS. Luminal colonic contents were analyzed for microbiome. Middle part of colons were examined for proliferating cells. Scraping of proximal and distal colons were analyzed for mucosal gene expression.	Dietary heme induced increases in fecal water, cytotoxicity, fecal host DNA, and fecal TBARS. Dietary heme resulted in an increase ratio of Gram-negative to Gram-positive bacteria, which was predominantly caused by increased abundance of the Gram-negative bacteria. Dietary heme induced colonic epithelia surface injury and hyperproliferation and hyperplasia. Heme did not change microbe-sensing pathways in colonic mucosa.

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Table 3 (*continued*)

Reference	Protocol and Treatment	Equivalent Human Daily Meat Intake[4] (Relative to DGAC Recommended Level[1])	Endpoints	Results
Ijssennagger et al. 2013	Protocol 1: Male C57B16/J mice were fed with purified control diets (40 en% fat [mainly palm oil], low calcium [30 μmol/g]) or control diets supplemented with 0.2 or 0.5 μmol heme/g diet for 14 days (n=8). Protocol 2: Mice were fed a heme diet (0.2 μmol heme/g diet) for 2, 4, 7, or 14 days (n=4 per time point).	0.2 and 0.5 μmol heme/g diet, equivalent to 1,278 g (25), and 3,194 g (63) beef, respectively.	In both protocols, analysis of fecal water for cytotoxicity and TBARS. Analysis of colon for cell proliferation and mucosal gene expression level.	Protocol 1: both heme-based diets induced hyperproliferation of colons in mice and increased fecal TBARS and cytotoxicity. Microarray assay showed that both 0.2 and 0.5 μmol heme/g diets modulated the expression of similar mucosal genes to a similar extent. Protocol 2: differentially expressed genes on day 2 and from day 4 were related to lipid metabolism, and neoplasia/cancer & proliferation, respectively. The gene expression results showed that mucosal response to luminal oxidative stress (on day 2) and cytotoxic stress (from day 4) coincided with the differential time course of the luminal stressors.
Martin et al. 2015	5-week-old male Fischer 344 rats were used in both protocols. Protocol 1: 32 male Fischer 344 rats randomly fed control diet or control diet supplemented with hemoglobin (2.5%) diets for 14 days. Protocol 2: 30 female Fischer 344 rats fed control, hemoglobin (2.5%), or hemin (0.094%) for 14 days. Low calcium is used at a concentration of 3.4 g/kg. Half of rats of each group treated with a daily gavage of 1 mL antibiotic mixture (4mg/mL kanamycin, 0.35 mg/mL gentamicin, 8500 U/mL colistin, 2.15 mg/mL metronidazole, and 0.45 mg/mL vancomycin) for 17 days.	2.5% hemoglobin/diet, equivalent to 9,904 g beef (194) 0.094% hemin/diet, equivalent to 9,216 g beef (181)	In both protocols, analysis of fecal water for TBARs. Histopathological analysis of colons from protocol 1 for cell proliferation and peroxidase activity.	In both protocols, hemoglobin and hemin diets resulted in increases of TBARs in fecal water and the administration of antibiotics lessened such increases. Protocol 1: Hemoglobin diets did not induce cell proliferation of colons; however, treatment of antibiotics resulted in significantly shorter colon crypts, less cell proliferation, and a larger cecum.
Reference	**Protocol and Treatment**	**Equivalent Human Daily Meat Intake[4] (Relative to DGAC Recommended Level[1])**	**Endpoints**	**Results**
Mirvish et al. 2008	Groups of male Swiss-Webster mice were fed with diets (semipurified diets by default) for 7 days (n=4/group). 1.0 g $NaNO_2$/L water, 2.0 g $NaNO_2$/L water, 1.0 g $NaNO_2$/L water + commercial diet, 2.0 g $NaNO_2$/L water + commercial diet, 125 mg $NaNO_2$/L water + 125 mg hemin/kg diet, 1.0 g hemin/kg diet, 12 g $NaNO_3$/L water, 1.0 g $NaNO_2$/L water + 23 g Na ascorbate/kg diet, 2.0 g $NaNO_2$/L water + 23 g Na ascorbate/kg diet, 23 g Na ascorbate/kg diet, 1.0 g $NaNO_2$/L water + 1.0 g ellagic acid/kg diet, 125 mg $NaNO_2$/L water + 5.0 g α-tocopherol/kg diet, 180 g hot dog/kg diet, 1.0 g $NaNO_2$/L water + 180 g hot dog/kg diet, 23 g Na ascorbate/kg diet + 180 g hot dog/kg diet, 1.0 g $NaNO_2$/L water + 0.40 g omeprazole/kg diet, 0.40 g omeprazole/kg diet, 0.40 g omeprazole/kg diet + 180 g hot dog/kg diet *Addition of 250 mg hemin/kg diet was mentioned in the results but it was not listed under various treatment in table 1.*	180 g hot dog/kg diet, equivalent to 540 g (11) hot dog; 125 mg and 1.0 g hemin/kg diet, equivalent to 1,225 g (24) and 9,804 g (192) beef	Analysis of fecal water for NOC and NOC precursor. Analysis of fecal water for nitrothiols in NOC and of hot dogs used in the study for nitrothiols.	Feeding hemin alone did no increase fecal NOC; feeding nitrite raised fecal NOC, the effect of which was enhanced by the addition of hemin; addition of omeprazole or ascorbate reduced the nitrite-induced fecal NOC increases by 65% and 42-56%, respectively. Nitrosothiols constituted 5-18% of the NOC in the mice and 15-21% in hot dogs.

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Table 3 (*continued*)

Reference	Protocol and Treatment	Equivalent Human Daily Meat Intake[4] (Relative to DGAC Recommended Level[1])	Endpoints	Results
Pierre et al. 2003	All F334 rats were injected with carcinogen azoxymethane to achieve ACF stage and 7 days later, they were fed different diets for 14 weeks. One group rats (n=22) were given control diets containing low-calcium (20 μmol/g) and 5% safflower oil; Three groups of rats (n=10, 10, and 20) were given control diets supplemented with 0.25, 0.5, and 1.5 μmol/g hemin, respectively ; Two groups of rats (n=10) were fed with control diets supplemented with 0.36 and 0.72 μmol/g hemoglobin; Three groups of rats (n=8) were fed with hemin (1.5 μmol/g) diets supplemented with calcium (230 μmol/g), antioxidant butylated hydroxyanisole and rutin (0.05% each), or olive oil (5%) instead of safflower oil; Two groups of rats (n=8) were fed with control diets supplemented with calcium (230 μmol/g) or olive oil (5%) instead of safflower oil.	0.25, 0.5, and 1.5 μmol hemin /g diet, equivalent to 1,597 (31), 3,194 (63), 9,582 (188) g beef 0.36 and 0.72 μmol hemoglobin /g diet, equivalent to 9,198 g (180) and 18,397 g (361) beef	All diets were analyzed for TBARs. Colons of all rats were examined for ACF and major ACF. Feces was analyzed for heme content. Fecal water was analyzed for TBARs, heme content, and cytotoxicity.	The diets supplemented with hemin at all tested levels reduced ACF while increased major ACF in the colon of rats; while hemoglobin diets increased ACF and decreased major ACF when compared to hemin diets. Addition of calcium, antioxidants, or olive oil to the hemin diet increased ACF and decreased major ACF as compared to hemin diet alone. All heme-containing diets resulted in significant increases of heme and TBARs in fecal water except the lowest hemin diets. Hemin diets rather than hemoglobin diets resulted in cytotoxicity of fecal water. Addition of calcium, antioxidants, or olive oil lessened the hemin-induced increases of TBARs and cytotoxicity in fecal water

Reference	Protocol and Treatment	Equivalent Human Daily Meat Intake[4] (Relative to DGAC Recommended Level[1])	Endpoints	Results
Pierre et al. 2004	Fisher 344 4-week-old female rats were treated with carcinogen azoxymethane and, 7 days later, were fed with control diet (n=20), chicken (600 g/kg, n=10), beef (600 g/kg, n=10), black pudding (600 g/kg, n=10), or hemoglobin (6.4 g/kg, n=10) diets, starting from day 14 for 100 days. All diet was low in calcium (0.8 g Ca/kg diet).	600 g chicken/kg diet, equivalent to 1,800 g chicken; 600 g beef/kg diet, equivalent to 1,800 g beef (35); 6.4 g hemoglobin/kg diet, equivalent to 2,535 g beef (50); 600 g black pudding/kg diet, equivalent to 61,323 g beef (1,202).	Analysis of colons for ACF and MDF. Analysis of fecal water for TBARs, heme content, and cytotoxicity on CMT93 cells and erythrocytes.	All experimental diets significantly increased ACF per colon from control diets, but only heme-based diets resulted in increases in MDF per colon, which was correlated with heme intake ($r=0.63$, $n=60$, $P<0.01$). Fecal water TBARS were significantly higher from heme-based diets than non-heme diets. Heme intake was significantly correlated with fecal water cytotoxicity on CMT93 cells, pH, and TBARS (All $r > 0.7$, all $P<0.01$). Only black pudding diets resulted in significant higher cytolytic activity on erythrocytes. MDF and ACF per colon were correlated with fecal TBARS, cytotoxicity on CMT93 cells and pH (all $r > 0.5$, all $P < 0.01$, $n=60$ rats).
Pierre et al. 2006	All rats were fed with diets low in calcium (2.6 g/kg). Protocol 1: Four groups of Fischer 344 female rats (n=5) were injected with carcinogen azoxymethane (20 mg/kg bw) and, 7 days later, they were fed with control, chicken (600 g/kg), beef (600 g/kg) and blood sausage diets (600 g/kg) with a heme level of not detectable, not detectable, 0.22 and 5.9 g/kg diet, respectively, for 100 days. Protocol 2: Four groups of rats (n=5) were fed the same diets in protocol 1 for 15 days.	600 g chicken/kg diet, equivalent to 1,800 g chicken; 600 g beef/kg diet, equivalent to 1,800 g beef (35); 600 g black pudding/kg diet, equivalent to 61,323 g beef (1,202).	Analysis of all diets for heme and HNE content. Protocol 1: Analysis of urine samples from Day 77 for DHN-MA and 8-Iso-PGF2α. Examination of colons for MDF and number of crypts per MDF. Protocol 2: Analysis daily urine samples for DHN-MA and 8-Iso-PGF2α.	In both protocols, DHN-MA excretion from rats fed beef or blood sausage was significantly increased over control diet; while only blood sausage diet resulted in higher urinary 8-Iso-PGF2α excretion. There was a significant correlation between heme intake and DHN-MA excretion. In protocol 1, beef and blood sausage fed rats had significantly more MDF than control rats. MDF promotion was correlated with urinary DHN-MA excretion ($r=0.654$, $P<0.001$) and dietary HNE ($r=0.577$, $P<0.001$).

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Table 3 (*continued*)

Reference	Protocol and Treatment	Equivalent Human Daily Meat Intake[4] (Relative to DGAC Recommended Level[1])	Endpoints	Results
Pierre et al. 2007	All Fischer 344 female rat were injected with carcinogen azoxymethane and 7 days later, they were fed with experimental diets. Protocol 1 Rats fed with control diet (20 μmol/g calcium, 5% safflower oil) and diets supplemented with 0.36 or 0.72 mmol/g hemoglobin. Protocol 2 Rats fed with control diet (20 μmol/g calcium, 5% safflower oil) and diets supplemented with 600 g/day chicken, beef, or blood sausage. Control diet was supplemented with ferric citrate to match the iron content of beef diet. Protocol 3 Rats fed with a control (20 μmol/g calcium, 5 % safflower oil) diet, a diet supplemented with 600 g beef/day, or a diet supplemented with 250 μmol/g calcium and 600 g beef/day. *Both protocols 1 and 2 were using 5% safflower oil in diets, but it was stated to be low-fat and high-fat diet, respectively.*	0.36, and 0.72 mmol hemoglobin/g diet, equivalent to 9,198,457 g (180,362) and 18,396,913 g (360,724) beef, respectively. 600 g chicken/kg diet, equivalent to 1,800 g chicken; 600 g beef/kg diet, equivalent to 1,800 g beef (35); 600 g blood sausage/kg diet, equivalent to 61,323 g beef (1,202)	In all protocols, fecal water was analyzed for TBARs and cytotoxicity on normal (Apc +/+) and premalignant (Apc -/+) colon epithelial cells and feces for HNE. In protocol 2, fecal water from control and beef group was assayed for cytotoxicity on both cells with or without vitamin E and sodium selenite. In protocol 3, fecal water from control group with HNE (from 10 to 250 μM) was analyzed for cytotoxicity on both cells. Caspase 3 activity and apoptosis of cells were analyzed in fecal water from all groups.	In protocol 1, fecal water from diets supplemented with hemoglobin resulted in increased level of TBARS and cytotoxicity on normal cells but not premalignant cells over non-heme diets. In protocol 2, fecal water from diets supplemented with beef or black pudding resulted in increased level of TBARS and cytotoxicity on normal cells but not premalignant cells over control or chicken diet and the addition of vitamin E and sodium selenite compensated such effect on the cytotoxicity. In protocol 3, fecal water from beef alone diet as compared to the control diet resulted in resulted in increased level of TBARS, cytotoxicity, caspase 3 activity, and apoptosis on the normal cells but not the premalignant cells, and the addition of calcium to the beef diet compensated such effects. HNE reduced the cellular viability of both cells but stronger effect was shown on normal cells.
Reference	**Protocol and Treatment**	**Equivalent Human Daily Meat Intake[4] (Relative to DGAC Recommended Level[1])**	**Endpoints**	**Results**
Pierre et al. 2008	All Fischer 344 female rat were injected with carcinogen 1,2-dimethylhydrazine and 7 days later, they were fed with 8 experimental diets for 100 days: Control, Control+ 2.7 g/kg calcium, 600 g/kg beef + 2.7 g/kg calcium, Control+ 33.8 g/kg calcium, 600 g/kg beef + 33.1 g/kg calcium, Control+ 2.7 g/kg calcium+ 50 g/kg olive oil, 600 g/kg beef + 2.1 g/kg calcium + 50 g/kg olive oil, Control+ 2.7 g/kg calcium + 0.5 g/kg rutin + 0.5 g/kg BHA, or 600 g/kg beef + 2.1 g/kg calcium + 0.5 g/kg rutin + 0.5 g/kg BHA All diets were balanced for protein (50%), fat (20%), and iron (110 mg/kg) by addition of casein, lard, and ferric citrate. *It is not clear how many rats were in each group.*	600 g beef/kg diet, equivalent to 1,800 g beef (35)	Analysis of colons for ACF and MDF. Analysis of fecal water for TBARS, heme, and cytotoxicity. Analysis of urine for DNA-MA excretion.	Beef diets induced promotion of ACF and MDF in colons, the effect of which was fully suppressed by addition of calcium. Beef intake resulted in significantly higher levels of heme and TABRS in fecal water. However, calcium normalized the beef-induced peroxidation to control level. Beef intake also induced cytotoxicity of fecal water on CMT93 cells, but the effect was fully inhibited by calcium rather than antioxidants and olive oil. Beef diets increased urinary DHN-MA excretion, the effect of which was significantly reduced by addition of antioxidants rather than calcium and olive oil.
Sawa et al. 1998	F344 female rats were administered 2 mg of *N*-nitroso-*N*-methylurea intrarectally and then fed with diets for 36 weeks: control, control + 12% safflower oil, control + 5% safflower oil + 3% hemoglobin, or 5% safflower oil + 3% hemoglobin with tea as drinking water.	3% (w/w) hemoglobin/ diet, equivalent to 11,884 g beef (233)	Identifying colon tumors of rats by H&E staining.	*N*-nitroso-*N*-methylurea-induced colon cancer was only enhanced significantly by the diet with fat and hemoglobin without tea as drinking water than other diets.

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Table 3 (*continued*)

Reference	Protocol and Treatment	Equivalent Human Daily Meat Intake[4] (Relative to DGAC Recommended Level[1])	Endpoints	Results
Sesink et al. 1999	Rats (n=8/group) fed with diets for 14 days: control, control + 1.3 μmol/g of hemin (ferriheme), control + 1.3 μmol/g protoporphyrin IX, control + 1.3 μmol/g ferric citrate, or control + 1.3 μmol/g bilirubin.	1.3 μmol hemin/g diet, equivalent to 8,304 g beef (163)	Analysis of fecal water for proliferation of colonic epithelial cells, cytotoxicity, and lipid peroxidation (TBARS). *Note: Fecal samples used for analysis were accumulatively from Day 12 to 14.*	Fecal water from the diets with heme resulted in significant higher proliferation and enhanced cytotoxicity on colonic epithelial cells. Fecal water from rats fed with heme had significant higher level of TBARs. However, oxygen measurement showed that cytotoxicity of fecal water induced by heme was not dependent on oxygen presence.
Sesink et al. 2001	Rats (n=8/group) fed with diets for 14 days: purified diets with 20 mmol calcium phosphate (low-calcium), purified diets with 20 mmol calcium phosphate + 1.3 mmol heme/kg diet (low-calcium), purified diets with 180 mmol calcium phosphate (high-calcium), or purified diets with 180 mmol calcium phosphate + 1.3 mmol heme/kg diet (high-calcium).	1.3 mmol heme/kg diet, equivalent to 8,304 g beef (163)	Analysis of colonic and ileal scrapings for proliferative activity. Analysis of fecal and ileal water for cytolytic activity.	Dietary heme increased the colonic epithelial proliferation in the low-calcium group but not in the high-calcium group. Dietary heme did not affect proliferation of the epithelial cells of the ileum on either low- or high-calcium diet. Cytolytic activity was only observed in the fecal water of rats fed the low calcium heme diet but neither control group nor high-calcium heme group. However, heme did not affect cytotoxicity of ileal contents on the low-calcium diet.
Sodring et al. 2015	Min/+ male and female mice (3-week-old) were fed with diets for 8 weeks: control (reduced calcium, 15 μmol/g, and no added vitamin D), control + 2.8 μmol/g of $NaNO_2$, control + 0.5 μmol/g of hemin, or control + 2.8 μmol/g of $NaNO_2$ + 0.5 μmol/g of hemin	0.5 μmol hemin/g diet, equivalent to 3,194 g beef (63)	Colon and small intestines were observed for lesions (flat ACF and tumors).	Supplementation of hemin had a suppressive effect on the lesions in the colons. Dietary nitrite had a suppressive effect on tumor growth in small intestine.

Reference	Protocol and Treatment	Equivalent Human Daily Meat Intake[4] (Relative to DGAC Recommended Level[1])	Endpoints	Results
Steppeler et al. 2016 (BMC cancer)	A/J Min/+ mice (3-week-old) were fed with diets for 8 weeks: no hemin+10% beef tallow, 0.5 μmol/g hemin+10% beef tallow, no hemin+40% beef tallow, or 0.6 μmol/g hemin+40% beef tallow.	0.5 and 0.6 μmol hemin /g diet, equivalent to 3,194 g (63), and 3,833 (75) g beef	Colons and small intestines of all mice were observed for colonic lesions flat ACF and tumors. Fecal water was analyzed for lipid peroxidation (TBARS)	Dietary hemin caused a significant decreases in the extent of lesions in colon, while dietary fat led to a significantly increased average tumor size in colon. Hemin did not influence the tumors in small intestines, while high dietary fat increased significantly tumor size and tumor load in small intestines. Both hemin and fat increased fecal water TBARS concentrations. However, fecal water TBARS was not correlated to colonic carcinogenesis. The association between TBARS and small intestine tumors was found to be dependent on the dietary fat level.
Surya et al. 2016	F344 male rats were injected with carcinogen azoxymethane. Control diet contained low calcium with addition of 5% (v/w) safflower oil. Protocol 1 Rats were fed with control diets, or hemoglobin (1%) diets for 100 days. Protocol 2 Rats fed with control diets or 50% control diet + 50% beef sirloin for 100 days. *in vivo*	1% hemoglobin, equivalent to 3,961 g beef (78) 50% raw beef sirloin, equivalent to 1,500 g beef (29)	Protocol 1 and 2: Analysis of fecal water for TBARs and free HNE.	Protocol 1 and 2: Hemoglobin and beef diets resulted in significant increases in TBARs of fecal water, but the treatment of carbonyl-trapping resin in fecal water caused a significant reduction in TBARs induced by hemoglobin and beef diets.

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Table 3 (*continued*)

Reference	Protocol and Treatment	Equivalent Human Daily Meat Intake[4] (Relative to DGAC Recommended Level[1])	Endpoints	Results
Winter et al. 2014	Protocol 1 60 mice (n=10/group) on control diets, control+ 5% resistant starch, control + 0.2 μmol/g hemin, or control + 5% resistant starch + 0.2 μmol/g hemin for 4 weeks Protocol 2 160 mice on the same diets described in protocol 1 for 18 months.	0.2 μmol hemin/g diet, equivalent to 1,278 g beef (25)	One centime of distal colon from protocol 1 and entire colon from protocol 2 were analyzed for tumors and all tumors were collected and processed for H&E staining for histological examination. Analysis of cells from distal colons from both protocols for apoptosis, proliferation, DNA adduct O^6MeG and level of oxidative adducts (8-oxo).	In both protocols, heme diets did not significantly affect the occurrence of neoplasm. Heme diets did not affect the apoptosis, cell proliferation, crypt height, O^6MeG and 8-oxo adducts in the distal colonic cells from both protocols except an increase in cell proliferation in protocol 1. In the comparison between protocol 1 and 2, longer time exposure of all diets resulted in increases in cell proliferation and 8-oxo adducts yet reductions in crypt height. However, longer time exposure of heme diets resulted in significantly reduction in apoptosis and increases in O^6MeG adduct.

[a] The value is estimated based on heme level in cooked beef (Table 1) and human intake of 3000 g diet per day; magnitude of intake relative to USDA level is in (X-fold).

[b] *Apc*$^{Min/+}$ mice is a genetic model of colorectal cancer.

carbonyl compounds (including aldehydes) significantly decreased the fecal water induced apoptosis. However, because the carbonyl resin traps not only aldehydes derived from lipid peroxidation but other carbonyl compounds such as ketones and endogenous aldehydes, it cannot be demonstrated that lipid peroxidation-derived aldehydes are solely responsible for the effects of fecal water towards cells. However, the TBARS concentration was significantly correlated with percentage of apoptotic nuclei. Nrf2 activation was noted in the normal and pre-neoplastic cells treated with fecal water of hemoglobin and red meat fed rats compared to fecal water from rats fed control diets. Fecal water activated Nrf2, known to be activated by aldehydes, in both normal and preneoplastic cell lines. Resistance to apoptosis induction in the pre-neoplastic cell line was suggested by the higher heme oxygenase 1 (HO1) expression (an Nrf2 target gene) in normal cells than pre-neoplastic cells following exposure to HNE and fecal water. Depletion of carbonyl compounds blocked the upregulation of HO1 expression.

3.1.2. *In vivo evidence*

In vivo models of red meat exposure and colon cancer promotion are summarized in Table 3. Many of the *in vivo* models evaluating an association among red meat exposure, potential for increased lipid peroxidation and neoplastic promotion utilized analysis of the composition of fecal water from heme fed rats (Sesink et al., 1999; Mirvish et al., 2008; Chenni et al., 2013; Guéraud et al., 2015; Pierre et al., 2007). It is important to remember that these studies did not evaluate exposure conditions that are representative of realistic human exposures to red meat; instead, the rats were given hemin or hemoglobin at levels that corresponded to 11–360,724 times the DGAC recommended total intake of meat. Sesink et al. (1999) noted that fecal water from rats fed a diet containing hemin for 14 days was more cytotoxic to erythrocytes *in vitro* than rats fed control diets. Mirvish et al. (2008) evaluated apparent total *N*-nitroso compounds (ATNC) in feces of mice given hemin in test diets for 7 days; incorporation exaggerated exposure to meat by 192 times the amount in a DGAC recommended diet. No increase in ATNC concentration was seen compared to control. An analysis of fecal water measured the level of thiobarbituric acid reactive substances (TBARS) as a marker for lipid peroxidation (Pierre et al., 2007). Fecal water from heme fed rats with increased levels of TBARS compared to non-heme fed rats had no effect on caspase activity but induced apoptosis in normal (Apc +/+) cells. The effect was abolished when calcium was added to the heme diet of the rats. Premalignant cells (Apc −/+) exhibited resistance to apoptosis induction (caspase 3 pathway) with exposure to the fecal water from heme fed rats. The authors noted a direct correlation between the cytotoxicity of the fecal water and the production of TBARS. Guéraud et al. (2015) fed rats experimental diet contained hemin at levels that exaggerated recommended meat intake by 54-fold along with various sources of lipid. End products of poly-unsaturated fatty acid (PUFA) peroxidation such as MDA, HNE, and isoprostanes (8-iso-PGF2α), which are widely used as systemic lipid oxidation/oxidative stress biomarkers, were measured in urine. Fecal water from these rats was tested for *in vitro* cytotoxicity. Results obtained demonstrated that MDA and the major urinary metabolite of HNE (the mercapturic acid of dihydroxynonane, DHN-MA) were highly dependent on the dietary factors tested, and analysis of these biomarkers of lipid peroxidation/oxidative stress in urine should be used with caution when dietary factors are not well controlled. Similarly, the TBARS content and cytotoxicity of the fecal water varied with the source of lipids used thus highlighting the importance of controlling dietary factors in these models.

In animal studies of cancer development, animal models have employed initiation with genotoxic chemicals followed by exposure to heme to evaluate promotion as well as models that did not utilize an initiating phase but evaluated various dietary interventions in addition to heme. Several papers reported the results of investigating the potential role of hemoglobin, hemin or red meat on the promotion of cancer, (Sawa et al., 1998; Pierre et al., 2003, 2004; 2006, 2007; 2008; Surya et al., 2016). In all models, it is critical to note that animals ingested meat or hemoglobin/hemin at levels that are a gross exaggeration (representative of 1.5 kg up to 18,000 kg per day) of possible chronic daily human intake of red meat. In addition, other dietary modifications such as high fat or low calcium were employed to provide optimal conditions for production of peroxyl radical species that can promote tumorigenesis. Sawa et al. (1998) administered *N*-nitroso-*N*-methylurea intrarectally to rats to initiate colon tumor development followed by inclusion of hemoglobin in high fat diets for 36 weeks. The hemoglobin levels were equivalent to a person consuming about 12 kg of red meat per day and inclusion of safflower oil at 5% of the diet would correspond to an intake of 150 g of oil per day for a person. As reference, USDA recommended intakes of oil range from approximately 30–50 g/day (USDA, 2015). Therefore, the increase in colon cancer seen in this study is not unexpected under the conditions of this bioassay. Introduction of a genotoxic insult along with a combination of hemoglobin and oil at exaggerated levels is expected to greatly increase lipid peroxyl radical formation.

Pierre et al. (2003, 2004, 2006, 2007, 2008) published a series of studies in rats initiated with various genotoxic carcinogens and given either hemin, hemoglobin or a form of beef. These studies demonstrated that high fat, low calcium diets supplemented with high levels of heme result in the production of lipid peroxidation products that can be measured in fecal water and urine. Promotion of ACF and MDF is noted. These effects are inhibited by the addition of adequate calcium in the diet. Pierre et al. (2003) utilized rats initiated with azoxymethane to produce ACF and subsequently, given dietary interventions. Diets utilized low calcium and 5% safflower oil in addition to hemin or hemoglobin at levels that ranged from the equivalent of a person ingesting 1.5–18 kg meat per day. Hemoglobin inclusion in the diet increased the number of ACF and fecal TBARS, but did not increase ACF size or fecal cytotoxicity. Although heme promoted ACF at levels associated with increased fecal water TBARS and cytotoxicity, dietary intervention by the addition of calcium, olive oil, and antioxidants inhibited these effects. Pierre et al. (2004) also used an azoxymethane initiated rat model but tested low calcium diets that incorporated beef, hemoglobin or black pudding. Beef, hemoglobin and black pudding provided what is equivalent to a person consuming 1.8, 2.5 or 61 kg meat per day, respectively. Rat colons were scored for ACF and MDF. Fecal water was assayed for lipoperoxides and cytotoxicity. As expected with a diet that is intentionally low in calcium and high in meat, promotion of MDF and ACF was noted. Measurement of TBARS in fecal water provided evidence that lipid peroxidation occurred. In a similar animal model, Pierre et al. (2006) reported urinary excretion of 8-iso-PGF2A and 1,4-dihydroxynonane mercapturic acid (DHN-MA), a urinary metabolite of 4-hydroxynonenal as a marker of lipid peroxidation, in an azoxymethane initiated rat model that utilized low calcium diets incorporating beef or black pudding. Beef and black pudding provided what is equivalent to a person consuming 1.8 or 61 kg meat per day, respectively. DHN-MA excretion increased in rats fed these high heme diets, and the excretion paralleled the number of preneoplastic lesions in azoxymethane initiated rats. Pierre et al. (2007) treated rats with azoxymethane to initiate preneoplastic cells and studied the effect of calcium on decreasing the lipid peroxidation the occurs when rats are given diets high in heme (meat intake in the diet was equivalent to a person consuming up to 61 kg meat per day) and fat. Fecal water from rats fed high heme diets contained more TBARS, a marker for lipid peroxidation and calcium supplementation decreased the formation of peroxides. Pierre et al. (2008) demonstrated that when using 1,2-dimethylhydrazine initiated rats given beef diets (fat added) equivalent to a person consuming 1.8 kg beef per day, there is an increase in the number of ACF and MDF associated with increased fecal water TBARS, cytotoxicity and urinary DHN-MA excretion. Calcium supplementation fully inhibited beef meat induced ACF and MDF promotion, and normalized fecal TBARS and cytotoxicity, but did not reduce urinary DHN-MA. An unexplained finding was that rats eating a high calcium control diet had more ACF and more MDF than rats given the low calcium control diet.

The pivotal role of calcium in preventing promotion of preneoplasia or neoplasia was noted by Sesink et al. (2001). This study demonstrated that *in vitro*, calcium phosphate precipitated heme and inhibited the heme-induced cytotoxicity. Subsequently, rats were fed diets, differing in heme (0 or 1.3 μmol/g) and calcium phosphate content only (20 or 180 μmol/g). In rats fed low calcium diets, dietary heme increased cytolytic activity of fecal water and the concentration of cations in feces when compared with controls. Colonic epithelial proliferation was increased compared with controls. This was accompanied by metabolism of the ingested heme and solubilization of heme compounds in the fecal water. A high calcium diet largely prevented this metabolism and solubilization. It also inhibited the heme-induced cytolytic activity of fecal water and increase in fecal cation concentration. Calcium inclusion in the diet prevented the heme-induced colonic epithelial hyperproliferation.

Several studies employed animal models that did not use genotoxic initiation (Winter et al., 2014 Steppeler et al., 2016; Sødring et al., 2015; de Vogel et al., 2008; Ijssennagger et al., 2012a, b, c; 2013, 2015; Bastide et al., 2015; Martin et al., 2015). Winter et al. (2014) evaluated the effect of short term and chronic (3 weeks and 18 months) high heme intake (equivalent to 1.3 kg meat per day in humans) in a high fat diet with or without the addition of resistant starch. Resistant starch is fermented in the colon; it produced an increased amount of SCFAs and reduced fecal pH. After 18 months, there were no differences between the high heme group and controls with respect to apoptotic cells per crypt, positive cells per crypt, crypt height, O^6MeG adducts or 8-oxo adducts. Although it appears heme lowered neoplasm incidence in the colon (0% in the heme group vs. 4.45% in the control group), there were no statistically significant differences in colon neoplasm incidence for any of the dietary interventions. Over time, dietary heme significantly lowered rates of apoptosis and produced an increase in O^6MeG DNA adduct accumulation in older mice compared to their younger counterparts compared to controls. All the changes observed had no influence on CRC risk when heme was studied at an exaggerated dietary amount with a western diet model of spontaneous CRC. The level of heme in a western diet even at exaggerated levels may not be sufficient by itself to initiate or promote CRC.

Three studies utilized the APC multiple intestinal neoplasia (Min/+) mouse (Mus musculus) model which is a widely used murine models for human familial adenomatous polyposis (FAP), characterized by the development of multiple adenomas in the colon (Steppeler et al., 2016; Sødring et al., 2015; Bastide et al., 2015). The Min mouse has a heterozygous truncation mutation at codon 850 of the tumor suppressor gene APC. This mutation is analogous to the mutation seen in the human APC gene, and results in the spontaneous formation of several neoplastic lesions in the mouse intestines. Steppeler et al. (2016) used the A/J Min/+ mouse model for Apc-driven colorectal cancer to investigate the effect of dietary heme combined with high or low dietary fat levels, on intestinal carcinogenesis. Animals were provided diet from weaning at 3 weeks to termination at 11 weeks. Dietary heme significantly reduced colonic carcinogenesis. The inhibitory effect was not dependent on the dietary fat level, and no association could be established between colonic carcinogenesis and the lipid oxidation rate measured as fecal TBARS. Small intestinal carcinogenesis was not affected by hemin. Results from this study indicate that dietary hemin inhibited colonic carcinogenesis and importantly, demonstrated that in this *in vivo* model, fecal TBARS concentration is not directly related to intestinal lesions.

Sødring et al. (2015) used the A/J Min/+ mouse model, providing a low calcium diet with added hemin (equivalent to 3 kg meat per day for a person) for eight weeks, from weaning at 3 weeks until 11 weeks at study termination. Dietary hemin decreased the number of colonic lesions (ACF) and tumors in the A/J Min/+ mouse.

Bastide et al. (2015) studied F344 rats diets for 100 days, C57BL/6 J $Apc^{Min/+}$ mice given diets for 49 days, $Apc^{+/+}$ mice given diets for 49 days and C57BL/6 J mice given diets for 14 days. Half the rats in each group were given drinking water with sodium nitrate and nitrite to produce NOC formation. Rats received a control diet or diet with hemoglobin, heterocyclic amines or both. Mice were given control diets or diets with hemoglobin. Safflower oil was supplemented in all the diets. Hemoglobin addition to diets for the rats was equivalent to a human ingesting 3.5 kg meat/day and the hemoglobin levels in the mice diets was equivalent to a human ingesting 9 kg meat/day. Heme iron increased the number of preneoplastic lesions (MDF), but dietary heterocyclic amines and NOC had no effect on preneoplastic lesions in rats. In $Apc^{Min/+}$ mice, hemoglobin significantly increased the intestinal tumor load but had no effect on the colon; this was associated with an increase in TBARS and cytotoxicity of fecal water. Giving the same hemoglobin diet to normal C57BL/6 J $Apc^{Min/+}$ mice did not induce neoplasia.

Martin et al. (2015) demonstrated the importance of the gut microflora composition on the effect of hemoglobin or hemin on lipid

Table 4
Clinical studies.

Reference	Protocol	Daily Meat Intake (Daily Use Level Relative to DCAC Recommended Level[1])	Endpoints	Results
Bingham et al., 2002	Randomized, cross-over:	Protocol 1: 60 g red meat (1.2), 600 g red meat (beef and pork) (11.8), and 600 g white meat.	Analysis of fecal samples for ATNC and nitrite.	For protocols 1 and 2, mean ATNC for groups given either 420 and 600 g red meat or 420 and 600 g white meat were combined for the comparison with low red meat intake group.
	Protocol 1: 7 males given protein diets over three 10-day periods.	Protocol 2: 60 g red meat (1.2), 420 g red meat (beef and pork) (8.2), and 420 g white meat.		*Data analysis presented is uninterpretable for protocols 1 and 2. ATNC levels for all groups were not presented. Nitrite levels were not presented.*
	Protocol 2: 5 males given protein diets over three 15-day periods.	Protocol 3: 60 g (1.2) and 120 g red meat (beef and pork) (2.4)		In protocol 3, there was no statistically significant difference in ATNC between the 60 and 120 g red meat-diet periods.
	Protocol 3: 9 males given protein diets over three 15-day periods. *No wash out period was specified.* *No non-meat control was included.*	In all protocols, energy content was equalized using glucose and dairy.		*A dose-response analysis incorporated results obtained from previous work (Hughes et al., 2001) and combined this with results from protocol 3. This is not an appropriate comparison because there is no comparison between baseline from these two studies.*
Cross et al., 2003	Randomized, cross-over:	Protocol 1: 60 g red meat (1.2), 420 g red meat (8.2), and vegetarian diet with equivalent protein to 420 g meat.	Analysis of fecal samples for ATNC and nitrite.	Both fecal ATNC and nitrite levels from 60 g red meat- and vegetarian-diets periods were not significantly different, but were significantly lower than those 420 g red meat-diet period.
	Protocol 1: Twelve males given protein diets over three 15-day periods.	Protocol 2: 60 g red meat (containing 9.9 mg/day iron) (1.2), 60 g red meat supplemented with 7.8 mg heme iron (as 50 g liver pate and 70 g blood sausage, equivalent to the iron content of 420 g red meat [17.7 mg iron/day]) (8.2), and 60 g red meat supplemented with 35 mg ferrous iron (1.2).	*Method cited was for analysis of ATNC in gastric juice; validation of this method for use in fecal samples was not provided.*	Protocol 2: Fecal ATNC from heme supplemented-diet period was significantly higher than low red meat- and inorganic iron supplemented-diet periods.
	Protocol 2: Nine males given protein diets over three 15-day periods. All diets were adjusted for energy needs of each subject with extra bread, low fat margarine, and marmalade.	*Note: the intended intake of red meat used in this study is not clear. The authors cite that 60 g red meat contained 9.9 mg iron; therefore, 420 red meat should contain 69.3 mg iron, however, the authors cite that this amount of red meat contained 17.7 mg iron. Thus, the calculation of red meat intake is not confirmed.*		There were no differences among periods in nitrite levels.

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Table 4 (*continued*)

Reference	Protocol	Daily Meat Intake (Daily Use Level Relative to DCAC Recommended Level[1])	Endpoints	Results
Cross et al., 2006	Randomized, cross-over: Protocol 1: 12 males given diets over three 15-day periods. Protocol 2: 9 males given diets over four 15-day periods. All diets are isocaloric and weighted to the nearest gram. *No wash out period was specified.*	Protocol 1: 60 g red meat (1.2), 420 g red meat (8.2), and vegetarian diet with equivalent protein to 420 g meat. Protocol 2: 60 g red meat containing 9.9 mg/day iron (1.2), 120 g red meat (2.4), 60 g red meat supplemented with 7.8 mg heme iron (from liver pate and blood sausage, match the iron content of 420 g red meat) (8.2), and 60 g red meat supplemented with 300 mg ferrous gluconate tablet (35 mg ferrous iron) (1.2). *Note: the intended intake of red meat used in this study is not clear. The authors cite that 60 g red meat contained 9.9 mg iron; therefore, 420 red meat should contain 69.3 mg iron, however, the authors cite that this amount of red meat contained 17.7 mg iron. Thus, the calculation of red meat intake is not confirmed.*	Analysis of fecal samples for ATNC. *Method cited was for analysis of ATNC in gastric juice; validation of this method for use in fecal homogenates was not provided.* Fecal water analyzed for genotoxicity under two incubation conditions with a human adeno-carcinoma cell line HT-29 cells: 1) 5 min at 4 °C and 2) 30 min at 37 °C.	Fecal water genotoxicity was not significantly correlated with neither its ATNC level nor the corresponding fecal homogenate ATNC level. ATNC levels in fecal water fractions were correlated with and significantly lower than those in fecal homogenates. However, no significant dietary effects were found on both levels. No significant dietary effect on the genotoxicity of fecal water was found.
Gilsing et al., 2013	Case-cohort study: 4026 subcohort members (aged 55–69 years) at baseline, 435 colon, and 140 rectal cancer patients during the 2.3rd to 7.3rd year of follow-up. A 150-item food-frequency questionnaire completed by participants.	Total fresh meat intake for subcohort, colorectal cancer cases, colon cancer case, and rectum cancer cases is 99.5 (2.0), 99.0 (1.9), 99.5 (2.0), 96.2 (1.9) g in average, respectively.	Analysis of DNA from tumor tissues, which were collected from incident colorectal cancer patients, for *APC*[a] and *KRAS* mutation and tumor protein 53 expression.	For all population, daily heme iron intake, fresh meat intake, and processed meat intake were not different among all groups of cases. In men, daily heme iron intake was highest in colon cancer cases and lowest in rectum cancer cases, but it is not clear whether this difference was statistically significant. In the colorectal cancer cases, 56% had overexpression of P53 in the cell nucleus, 33% had an activating *KRAS* mutation, and 36% had a truncating *APC* mutation. Heme iron intake was not associated with the risk of colorectal tumors with truncating *APC* mutation. Heme iron intake was significantly associated with colorectal tumors with activating *KRAS* gene mutations and P53 overexpression in the cell nucleus. Heme iron intake was not associated with the risk of colorectal tumors without G > T and G > A mutations in *KRAS*. *None of the tests for heterogeneity comparing the association with heme iron between molecular subgroups in any of the analyses was statistically significant.*

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Table 4 (*continued*)

Reference	Protocol	Daily Meat Intake (Daily Use Level Relative to DCAC Recommended Level[1])	Endpoints	Results
Hughes et al., 2001	Randomized, cross-over: Eight male subjects given protein diets over four 10-day periods. *No wash out period was specified.*	Subjects were given 0, 60 (1.2), 240 (4.7), and 420 g red meat (beef and pork) (8.2). Energy content was equalized using glucose and dairy.	Analysis of fecal samples for apparent total ATNC and nitrite.	Fecal ATNC and nitrite were significantly increased in the 240 g and 420 g red meat-diet periods compared to control and 60 g periods.
Joosen et al., 2009 [STUDY 1]	Randomized, cross-over: 12 subjects (6 male and 6 female) given red meat or vegetarian diets for 14 days. *No wash out period was included.*	Subjects fed with red meat 420 g (8.2) for males or 366 (7.2) g for female, and vegetarian diets without protein content. Fat content of the diets was kept constant by exchanging protein for carbohydrates. Energy requirements were estimated based on body weight and physical activity.	Analysis of fecal samples for total NOC, nitrosothiols, nitrosyl iron, other NOC and heme. Fecal water was assayed for genotoxicity.	Statistically significant increases were noted in heme, total NOC, nitrosothiols, nitrosyl iron, and other NOC levels/g fecal water in red meat groups compared to vegetarian group. Comet assay showed that red meat diets induced significantly more DNA strand breaks in fecal water than vegetarian diets, but no difference was noted between red meat and processed meat diets. *It is not clear whether NOC induced by red meat diets were significantly correlated to the DNA strand break in fecal water.*
Joosen et al., 2009 [STUDY 2]	Randomized, cross-over: 16 subjects (5 males and 11 females) given protein or vegetarian diets for at least 14 days. *No wash out period was included.*	Subjects fed with processed red meat (420 g (8.2) for males or 366 g (7.2) for females) diets and vegetarian diets. Fat content of the diets was kept constant by exchanging protein for carbohydrates. Energy requirements were estimated based on body weight and physical activity.	Fecal water was analyzed for heme and NOC* level and genotoxicity. **The units reported for heme and NOC levels are in µmol in Joosen et al. (2010), whereas in Joosen et al. (2009), units are in mmol. One of the manuscripts is in error.*	Fecal levels of NOC and heme and DNA strand breaks were significantly increased when fed processed red meat diets compared to when fed vegetarian diets. *It is not clear whether the NOC induced by processed red meat diets was significantly correlated to the DNA strand break.*
Joosen et al., 2010	Randomized, cross-over: 13 male and female subjects given protein diets over three 8-day periods. *No wash out period was included.* *No non-meat diet was used.*	Subjects were given high intake red meat (325 g (6.4) for males, 260 g (5.1) for females), high intake fish (375 g for males, 300 g for females), and meat plus fish (combined intake using half the amount of meat or fish given in the high meat or fish diets). Energy content was matched to each participant's estimated requirement using fat and carbohydrate.	Analysis of fecal samples for NOC* and heme. Fecal water was assayed for genotoxicity in the comet assay. **The units reported for heme and NOC levels are in µmol in Joosen et al. (2010), whereas in Joosen et al. (2009), units are in mmol. One of the manuscripts is in error.*	There was a statistically significant reduction in total NOC, nitrosothiols, nitrosyl iron, and other NOC in subjects when feeding fish only compared to the other two diet periods. No difference was seen between the meat and meat/fish diet periods. Excretion of heme was not significantly associated with nitrosyl iron excretion and nitrosyl iron accounted for 83–86% of the total NOC. There were no statistically significant effects of diets on DNA strand breaks.

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Table 4 (*continued*)

Reference	Protocol	Daily Meat Intake (Daily Use Level Relative to DCAC Recommended Level[1])	Endpoints	Results
Kuhnle et al., 2007	Randomized, cross-over: Protocol 1: 14 male and female subjects with ileostomy given protein diets over three 2-day periods. *The study was done previously and the ileal output was analyzed in current study.* Protocol 2: 12 healthy male and female subjects given high protein or vegetarian diets over two 3-day periods. *Wash out period was not specified.* Protocol 3 (*in vitro*): All diets used in protocol 2 were incubated under simulated gastric conditions.	Protocol 1: Subjects fed with 240 g (4.7) beef and meat free diets. Protocol 2: Subjects fed with isoenergetic diets of 420 g (8.2) red meat and vegetarian diets. Protocol 3: 420 g (8.2) red meat and vegetarian diets were combined (approximately 1 g from each sample) and suspended in 40 mL simulated gastric juice. $NaNO_2$ was added to achieve a final concentration of 150 μM to the diets and incubated in the dark at 37 °C.	Protocol 1: ileal output collected at least every 2 h and analyzed for heme, nitrosyl iron and nitrosothiols levels. Protocol 2: fecal samples collected and analyzed for heme, nitrosyl iron, and nitrosothiols levels. Protocol 3: *In vitro* stimulated stomach digestion of diets were analyzed for nitroso compounds, nitrite, and nitrate	Analysis of fecal homogenates and ileal output showed a statistically significant increases of heme, nitrosyl iron compounds, and nitrosothiols from dietary period fed with red meat diets than vegetarian diets. In ileal output, the levels of heme were significantly correlated with the nitrosyl iron level. In fecal water, there was a significant correlation among total heme, nitrosyl iron compounds, and nitrosothiols. Nitrosothiols were produced more than nitrosyl iron compounds
Lewin et al., 2006	Randomized, cross-over, each dietary period 15–21 days: Protocol 1: 8 of 12 male subjects given a vegetarian diet and a high red meat diet; Protocol 2: 6 females and 7 males given a vegetarian diet, a high red meat, high-fiber diet, or high red meat diet. All diets were isoenergetic and kept constant in fat (30% total energy) *Duration was 15 days and 15 to 21 days cited in the Abstract and Materials and Methods, respectively, for each dietary period; it was not clear how long each dietary period lasted.* *No wash out period was specified.*	Protocol 1: Subjects fed with a vegetarian diet and a 420 g (8.2) red meat diet. Protocol 2: Subjects fed with a vegetarian diet (30 g fiber), a 420 g (8.2) red meat (13 g fiber) diet, and a 420 g red meat (8.2) high-fiber diet (30 g fiber).	Analysis of fecal samples for ATNC. Detection of exfoliated cells positive for the O^6MeG adduct[b] from stools. Analysis of K-ras mutations for exfoliated cell DNA from stool samples obtained in protocol 1.	In protocol 1, no mutations were found in DNA isolates from collected exfoliated cells by K-ras assay. In both protocols, fecal ATNC from a high-red meat diet was significantly higher than those from a vegetarian diet. The percentage of exfoliated cells positive for O^6CMG was significantly higher when fed high-meat diets than vegetarian diets. In all 21 individuals from all three dietary periods, the levels of ATNC were positively correlated with percentage of exfoliated cells from fecal extracts positive for the O^6CMG adduct ($r = 0.56$, $P < 0.001$). However, in protocol 2, ATNC level from high-meat diets was not significantly different from the high-meat and high-fiber diets.

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Table 4 (*continued*)

Reference	Protocol	Daily Meat Intake (Daily Use Level Relative to DCAC Recommended Level[1])	Endpoints	Results
Lunn et al., 2007	Randomized, cross-over: Protocol 1: 14 male and female ileostomy subjects given protein or no meat diets for two days. Protocol 2: 13 male and female ileostomy subjects given protein or no meat diets with ascorbic acid or alpha-tocopherol for two days.	Protocol 1: Subjects fed with 240 g red meat (4.7), 240 g processed meat (4.7), and a no meat diet. Protocol 2: Subjects fed with 240 g red meat (4.7) with 1 g ascorbic acid or 400 IU alpha-tocopherol and 240 g processed meat (4.7) with 1 g ascorbic acid.	Analysis of ileal output for ATNC and nitrite.	Analysis for ileal output indicated a statistically significant increase in ATNC when feeding red meat and processed red meat diets compared to no meat diets. However, nitrite concentrations were only significant higher when feeding red meat diets than no meat diets. Ascorbic acid and alpha-tocopherol did not affect nitrosation in ileal output in terms of ATNC and nitrite levels.
Pierre et al., 2006	Randomized cross-over Eight volunteers were randomly assigned to each of 4 15-day dietary periods. Higher energy requirement than the 10 MJ baseline diet were given 1 MJ increments of white bread (50 g), low-fat spread (20 g), or marmalade (20 g). *No wash out period was included.*	Subjects were fed red meat diet of 60 g (9.9 mg iron) (1.2), 120 g (12.5 mg iron) (2.4), 60 g supplemented with 50 g liver pate (35) or 70 g blood sausage (48.5) (17.7 mg iron), and 60 g red meat (1.2) supplemented with ferrous gluconate to deliver, respectively, 55, 110, 80, 205 or 55 mg of heme	Urine analysis for 4-hydroxynonenal, DHN-MA and 8-Iso-PGF2α	Only DHN-MA from urine of subjects fed with 60 g/day red meat supplemented with 70 g/day blood sausage was significantly higher than other diets. Urinary 8-iso-PGF2A did not increase with higher meat containing diets.

[a] APC: Truncating APC mutations lead to the introduction of a stopcodon and result in a truncated and therefore inactive APC protein.

[b] O^6MeG (O^6-methyldeoxyguanosine) is a characteristic promutagenic and toxic adduct formed by many *N*-methyl-*N*-nitroso compounds that either spontaneously decompose or are metabolized to intermediates which are highly reactive methylating agents that react with nucleophilic centers on DNA bases (Lewin et al., 2006).

peroxidation. A rat model utilized addition of either hemoglobin (at an equivalent of 10 kg meat per day for a person) or hemin (at an equivalent of 9 kg meat per day for a person) in a low calcium diet. The suppression of microbiota by antibiotics was associated with a reduction of crypt height and proliferation and with a cecum enlargement, which are characteristics of germ-free rats. Rats given hemoglobin or hemin diets had increased fecal TBARS, which were suppressed by the antibiotic treatment. It is possible that gut microflora is involved in the modulation of lipoperoxidation associated with dietary heme. In a study to evaluate the role of microbiota in the compensatory proliferative response of colonocytes exposed to high fat, high heme and low calcium diets, a mouse model was employed (Ijssennagger et al., 2012b). Mice were given a high fat and low calcium diet supplemented with heme (equivalent to 3 kg meat per day for a human); results demonstrated an increase in cytotoxicity of fecal water and increased TBARS with heme supplementation. Increased epithelial cell proliferation as shown by Ki67-staining was seen in heme fed mice. This was associated with an increase in the ratio of Gram-negative to Gram-positive bacteria. Results suggested that the change in microbiota did not cause the observed hyperproliferation and hyperplasia via inflammation pathways. In a follow-up to these findings, a study reported by Ijssennagger et al. (2015) used a mouse model given either control diet that was high in fat and low in calcium or this diet supplemented with heme (equivalent to 3 kg meat per day for a human) and included a broad-spectrum antibiotic administered in drinking water during the time of intervention. Heme-induced hyperproliferation was shown to depend on the presence of the gut microbiota, because hyperproliferation was completely eliminated by antibiotics. Results suggested that gut microbiota is required for heme induced epithelial hyperproliferation and hyperplasia because of the capacity of sulfide-producing bacteria and mucin-degrading bacteria to reduce mucus barrier function.

Ijssennagger et al. (2012a, c) investigated cytotoxic stress markers in mice receiving a high fat, low calcium and high heme diet (0.2 μmol/g or 0.5 μmol/g diet, equivalent to either 1.3 or 3.1 kg meat per day for a person). The investigation reported by Ijssennagger et al. (2012c) showed that mice in the heme fed diet group were exhibiting had a significantly lower body weight compared to non heme fed controls; body weight loss is an important indicator of toxicity. These animals were compromised by the extreme dietary conditions and their condition compromises the results obtained or the ability to extrapolate them to the effects of physiologic levels of these nutrients. In Ijssennagger et al. (2012c), fecal water of heme-fed mice was significantly more cytotoxic than control fecal water and there was a significant increase in total number of cells per colonic crypt, number of Ki67-positive cells per crypt and labeling index. In a microarray analysis of the colon mucosa, heme downregulated some inhibitors of proliferation. Although upregulation of mRNAs for mitogenic signals was noted, this was not translated into increased protein levels. Ijssennagger et al. (2012a) hypothesized that because PPARa is proposed to protect against oxidative stress and lipid peroxidation, the absence of PPARa would lead to more surface injury and crypt hyperproliferation in the colon upon heme-feeding. Although Ijssennagger et al. (2012a) did not report body weight relative to non-heme fed controls, because the same dietary conditions were used as in their 2011 publication, it is likely that these mice were also exhibiting body weight reductions. Results showed that heme induced luminal cytotoxicity and lipid peroxidation and colonic hyperproliferation and hyperplasia occurred to the same extent in wild type and PPARa knock-out mice. PPARa does not mediate heme-induced hyperproliferation and implies that oxidative stress of surface cells is not the main determinant of heme-induced hyperproliferation and hyperplasia.

Ijssennagger et al. (2013) studied the impact of cytotoxic stress and/or oxidative stress on the colonic mucosa after 2, 4, 7 and 14 days of heme feeding. Mice received a high fat, low calcium and high heme diet (0.2 μmol/g or 0.5 μmol/g diet, equivalent to either 1.3 or 3.1 kg meat per day for a person) and oxidative and cytotoxic stress markers were measured in fecal water. TBARS were significantly higher in the heme-fed mice compared with the controls at day 2 of heme feeding and stayed higher than controls throughout the study. Cytotoxicity and increased proliferation of colonic epithelial cells only occurred after day 7 after feeding with differential expression of oncogenes and tumor suppressor genes in this model. It is important to note that while Ijssennagger and colleagues indicate that the mice are receiving a Westernized diet with intake of red meat that mimics an intake of 160 g per day, the calculation is flawed. Calcium levels in this diet were low to optimize the possibility of fat oxidation and fat intake was very high. Inclusion of palm oil at 40% of energy as fat corresponding with 200 g of fat/kg diet; this is equivalent to an intake of 600 g of oil per day for a person. As reference, USDA recommended intakes of oil range from approximately 30–50 g/day (USDA, 2015). Additionally, the calculation of red meat intake is based on the assertions that “an average human diet consists of about 400 g dry weight/day, 0.2 μmol heme/g diet corresponds to 80 μmol heme/day. As beef contains 0.5 μmol heme/g wet weight, this implies that 0.2 μmol heme/g used in this study mimics a daily intake of 160 g red meat, which is similar to the high intake of red meat in Westernized countries, observed in epidemiological studies.” There is no documentation or source citation to substantiate the use of 400 g dry weight/day as a food intake. In addition, although the presumed 400 g/day diet is on a dry matter basis, the heme level of 0.2 μmol/g diet used in current study is based on total matter. Therefore, the calculation of 80 μmol heme/day intake is not reliable and does not represent a daily intake of 160 beef. Therefore, this study did not mimic the Western diet but rather exaggerated the conditions of exposure to fat and meat while decreasing calcium intake.

A study in rats employing the same dietary conditions used by Ijssennagger et al. (2012a, c, 2013) showed that two weeks of high heme diets (0.5 μmol/g diet, equivalent to 3.1 kg meat per day for a person) resulted in increased injury of the surface epithelium, colonocyte proliferation and inhibition of mucosal apoptosis (de Vogel et al., 2008). Caspase-3 activity was lower in mucosa of heme-fed rats compared mucosa of control fed rats. In contrast to the results in mice, body weights were not different between heme-fed and control fed rats.

3.1.3. Clinical evidence

Clinical studies have examined the production and potential genotoxic consequence of ATNC formed with and without the ingestion of red meat (Table 4). It is important to note that levels of red meat ingestion in many of the studies far exceed recommended guidelines from DGAC for intake of protein from animal sources and thus are exaggerating exposure to heme. Additionally, there were some methodologic inconsistencies such as errors in dose calculations, errors in units reported for NOC and heme, lack of or unspecified wash-out periods, inconsistent reporting of dietary periods, no non-meat controls and lack of analytical method validation. These are noted in Table 4. As expected, these inconsistencies make interpretation of results difficult. In general, however, investigators demonstrated that high levels of red meat ingestion result in an increased level of ATNC in fecal samples and later showed that ingestion of heme alone (and not ferrous iron) was comparable to the effects of red meat (Hughes et al., 2001; Bingham et al., 2002; Cross et al., 2003, 2006; Lewin et al., 2006; Kuhnle et al., 2007; Lunn et al., 2007; Joosen et al., 2009, 2010). These studies strongly linked the presence of heme in the diet to the formation of nitroso compounds. However, many of these studies used hydrogen bromide to chemically denitrosate compounds in fecal matter, and did not differentiate between the N-nitrosamines that are mainly linked to colorectal cancer and other compounds, such as S-nitrosothiols, iron nitrosyls, and dinitrosyl iron complexes. The three major species that are present in ATNC that could be formed from acidified nitrite in the stomach are iron nitrosyls, formed from heme, S-nitrosothiols, formed from the S-nitrosation of dietary thiol groups (e.g., in cysteine and homocysteine), and N-nitrosamines, formed mainly from the

nitrosation of secondary amines (Hogg, 2007). These products have profoundly different chemistries, and while certain *N*-nitroso species, through the formation of DNA adducts, have been shown to be profoundly tumorigenic, the same is not true for *S*-nitrosothiols or iron-nitrosyl species (Hogg, 2002, 2007; Butler and Rhodes, 1997). Importantly, results from studies enumerating the type of ATNC found after ingestion of red meat indicate that it consists mainly of nitrosyl iron (83–86%) and nitrosothiols (Kuhnle et al., 2007; Joosen et al., 2010). It is interesting to note that Hogg (2007) speculated that the sequestration of the "nitrosating potential" of the diet as nitrosothiol or as nitrosyl iron may be a protective mechanism that would limit the formation of DNA alkylating agents. Additionally, investigators have speculated that since mutation of the Apc gene and loss of Apc function may be an early event in human CRC development, heme iron intake may play a role in the development of cancer in this subpopulation. Interestingly, however, in a clinical study reported by Gilsing et al. (2013), heme iron intake was not associated with the risk of colorectal tumors with truncating APC mutation.

In a randomized cross-over study of volunteers fed red meat diets at levels that are similar to or twice the DGAC recommended intake of meat per day, urinary excretion of DHN-MA, an end-product of lipid peroxidation was increased in the group with the highest meat intake by two-fold over the group with the lowest meat intake (Pierre et al., 2006). No change was noted in urinary excretion of 8-iso-PGF2α. Interestingly, the excretion of lipid peroxidation products in humans was much smaller than in the rat study reported in the same publication. The rat diets were high in heme and fat and low in calcium, optimizing the conditions for lipid peroxidation. The human volunteers had access to normal diet which contains components that have antioxidant properties.

4. Discussion

Red meat contains the iron-porphyrin pigment heme and is one of the largest sources of heme in the diet (Jeyakumar et al., 2017). IARC identified heme as one meat component that could theoretically be responsible for initiation or promotion of cancer mediated through the formation of NOC and/or by lipid oxidation. *In vitro* studies, animal studies and clinical trials have been conducted to explore these mechanisms of action. However, the relevance of the results from these studies in characterizing a human health of risk necessitates an understanding of the potential for expression of the hazard during the exposure. Thus, interpretation of results in the context of the exposures used in studies is critical. Exposure entails not only the amount of red meat to which people are typically exposed, but exposure to all the other components of the diet that can moderate expression of hazard. For example, most animal studies utilized unrealistic levels of exposure (up to 3 orders of magnitude greater than DGAC recommended human intake of meat) combined with methodologic perturbations and/or non-physiologic conditions such as low calcium and high fat, that were designed to optimize the conditions needed for free radical production. The interrelationship between diet, *in vivo* intraluminal chemistry and cell culture or fecal culture conditions is unknown.

Therefore, the relevance of the findings from many of the studies for a human health risk assessment is unknown because there are no data that bridge the level and duration of exposure used in these studies to the conditions of exposure that would occur in an *in vivo* situation. The effects seen are subject to dose-response, however, the threshold for response has not been established. Although *in vitro* models provide the optimal environment to demonstrate the ability of heme to produce lipid oxidation products, these models only demonstrate what occurs when the extent of oxidative damage overwhelms the repair capacity. It is not appropriate to extrapolate these results to the situation that occurs during exposure to more physiologic conditions that are part of a normal dietary pattern. Therefore, the mechanistic evidence provided from *in vitro* studies is specific to conditions that are not necessarily relevant for a normal dietary intake and thus do not provide sufficient evidence that typical red meat consumption would increase the risk of colon cancer.

Many of the *in vivo* models evaluating an association between increased lipid peroxidation due to heme exposure and neoplastic promotion utilized analysis of the composition of fecal water from heme fed rats (Sesink et al., 1999; Mirvish et al., 2008; Chenni et al., 2013; Guéraud et al., 2015; Pierre et al., 2007). It is important to remember that these studies did not evaluate exposure conditions that are representative of realistic human exposures to red meat; instead, the rats were given hemin or hemoglobin at levels that correspond to 11–360,724 times the DGAC recommended total intake of meat. Importantly, animal studies utilizing cancer initiation with genotoxic carcinogens tested tumor promotion by heme exposure in diets that were low in calcium and high in fat (Pierre et al., 2003, 2004, 2006, 2007, 2008). Although heme promoted preneoplastic lesions in these models at levels associated with increased fecal water TBARS and cytotoxicity, dietary intervention by the addition of calcium, olive oil, and antioxidants abolished these effects. Interestingly, animal models also demonstrated the importance of the microbiota in heme-induced hyperproliferation of colonic cells because hyperproliferation could be completely eliminated by antibiotics. Results suggested that gut microbiota plays a role in heme induced epithelial hyperproliferation and hyperplasia because of the capacity of sulfide-producing bacteria and mucin-degrading bacteria to reduce mucus barrier function (Ijssennagger et al., 2015). This raises an important consideration for the extrapolation of results in rodents to humans considering the profound differences in microbiota (Nguyen et al., 2015). Thus, exaggerations and perturbations in dietary exposure conditions combined with the inherent differences in gut microbiota make findings in the animal models examined impossible to extrapolate to the effects of a normal dietary intake of meat on risk of colon cancer.

Clinical studies subjected participants to intakes of red meat that typically far exceeded recommended guidelines from DGAC and thus exaggerated exposure to heme. Additionally, many studies contained methodologic inconsistencies that hindered interpretation of results. Importantly, although clinical studies strongly linked the presence of heme in the diet to the formation of NOC, most did not differentiate between the N-nitrosamines that are mainly linked to colorectal cancer and other compounds, such as S-nitrosothiols, iron nitrosyls, and dinitrosyl iron complexes which are not linked to cancer. Results from the few studies enumerating the type of ATNC found after ingestion of red meat indicate that it consists mainly of nitrosyl iron and nitrosothiols. Clinical studies have examined the production and potential genotoxic consequence of ATNC formed with and without the ingestion of red meat. Importantly, a critical finding from these studies was that the type of ATNC found after ingestion of red meat in humans consists mainly of nitrosyl iron (83–86%) and nitrosothiols (Kuhnle et al., 2007; Joosen et al., 2010). These products have profoundly different chemistries from certain *N*-nitroso species which have been shown to be tumorigenic through the formation of DNA adducts. The ATNC formed in humans after red meat ingestion are not associated with the formation of DNA adducts.

Results of the literature review reported here agree with a recently reported systematic review of the literature that examined availability of plausible mechanistic data linking red and processed meat consumption to colorectal cancer risk (Turner and Lloyd, 2017). Studies examined were designed to evaluate the role of heme iron or heterocyclic amines in relation to colon carcinogenesis. Turner and Lloyd found that studies utilized levels of meat or meat components well in excess of those found in human diets and most did not include potential biologically active protective compounds present in whole foods. They concluded that there is currently insufficient evidence to confirm a mechanistic link between the intake of red meat as part of a healthy dietary pattern and colorectal cancer risk.

In conclusion, review of the methodologies employed in studies

reviewed by IARC, as well as in more recent studies reported in the literature, have not provided sufficient evidence that heme would contribute to an increased risk of initiation or promotion of pre-neoplasia or colon cancer at usual dietary intakes of red meat in the context of a normal diet.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.fct.2018.04.048.

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