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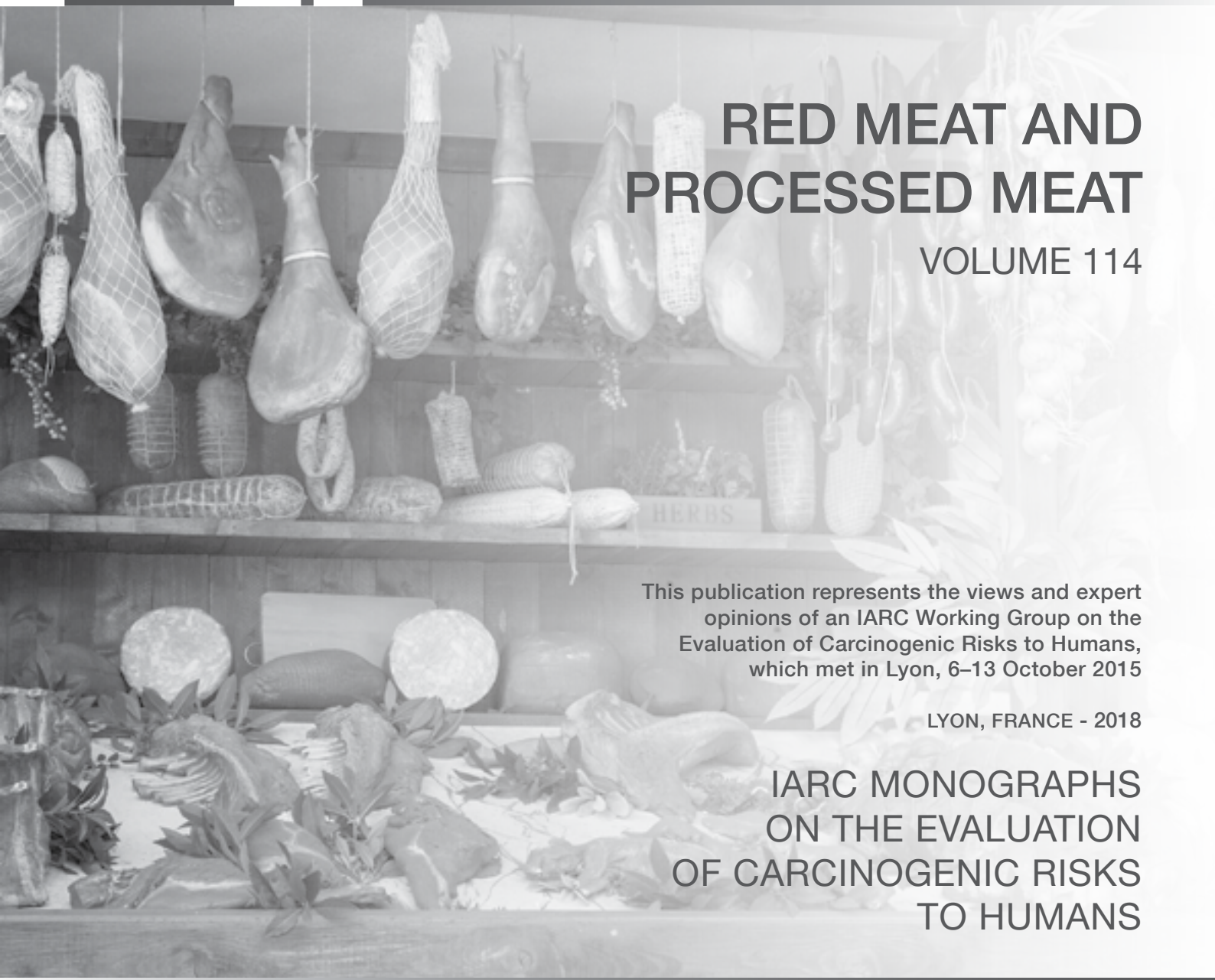


RED MEAT AND PROCESSED MEAT

VOLUME 114

This publication represents the views and expert opinions of an IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, which met in Lyon, 6–13 October 2015

LYON, FRANCE - 2018

IARC MONOGRAPHS ON THE EVALUATION OF CARCINOGENIC RISKS TO HUMANS

International Agency for Research on Cancer

World Health Organization

4. MECHANISTIC AND OTHER RELEVANT DATA

4.1 Digestion and metabolism

The composition of red meat and processed meat, as well as their potential contaminants, is described in detail in Section 1 of this *Monograph*. Red meat and processed meat are sources of high-quality protein, fat in highly variable amounts, and a range of micronutrients. The impact of the digestion of protein and fat, and the modifications that these macronutrients may undergo in the processing of meat, is addressed in this section. The specific components of red meat and processed meat, including haem iron, lipid oxidation products, heterocyclic aromatic amines (HAAs), polycyclic aromatic hydrocarbons (PAHs), and *N*-nitroso compounds (NOCs), that are potentially involved in carcinogenesis are discussed in Section 4.5.

After the hydrolytic breakdown of dietary proteins by the activity of proteases, and the absorption of the resultant amino acids and dipeptides in the proximal gut, fermentation of excess proteins may yield toxic compounds. The amount of protein that enters the colon depends on the protein content of the ingested food and the protein digestibility (Windey et al., 2012). Digestibility of dairy and animal proteins exceeds 90%, and is generally higher than the digestibility of plant proteins (70–90%). Storage and processing of meat before consumption may alter its protein digestibility. Cooking of beef affected bovine myofibrillar protein susceptibility to proteases in vitro, with increased or decreased rates depending on the nature of the proteases, and the time and temperature parameters (Santé-Lhoutellier et al., 2008). Similarly, Bax et al. (2012) reported that ageing and mincing had little impact on the in vitro digestion of pig muscle proteins, but heat treatment had temperature-dependent effects. At 70 °C, the proteins underwent denaturation, enhancing the speed of pepsin digestion by increasing enzyme accessibility to protein cleavage sites. At above 100 °C, the proteins underwent oxidation-related aggregation, slowing the speed of pepsin digestion, but improving overall meat protein digestibility. In a study of miniature pigs fed meat from a calf, the true ileal protein digestibility averaged 95%, and was not affected by cooking temperature or by the level of meat intake (Bax et al., 2013). Chemical oxidation of pig myofibrillar proteins has been shown to reduce protein digestibility in vitro (Santé-Lhoutellier et al., 2007). Overall, the impact of thermal denaturation and oxidation of meat proteins during processing and storage on their digestibility, as well as the formation of carcinogenic compounds during digestion, is not well known.

On a normal mixed diet, the amount of protein rather than the source determines the quantity that reaches the colon (Silvester & Cummings, 1995). Hence, high-meat, low-fibre diets may stimulate protein fermentation in the colon, producing short- and branched-chain

fatty acids, ammonia, phenolic and indolic compounds, and hydrogen sulfide (O'Keefe, 2008). Bacterial proteases and peptidases are more active when pH is neutral to alkaline. In the proximal colon, pH is more acidic due to the production of short-chain fatty acids, primarily from carbohydrate fermentation, but also from reductive deamination of many amino acids. In more distal parts of the colon, pH is higher and protein fermentation becomes more prominent. In relation to meat intake, ammonia and hydrogen sulfide are the most critical compounds because of their known toxicity (Attene-Ramos et al., 2007; Windey et al., 2012). Meat is rich in sulfur-containing amino acids, possibly leading to higher hydrogen sulfide concentrations in the colon. However, hydrogen sulfide in the gut originates from both the fermentation of sulfur-containing amino acids and dietary sulfate.

A diet high in red meat or processed meat may contain high levels of fat. The digestion of food lipids consists of a series of enzyme-catalysed steps resulting in absorbable components, whereby the release of bile from the gallbladder is essential. It has been suggested that dietary fat promotes the development of cancer of the colorectum (Boyle et al., 1985; Reddy, 1992). Several mechanisms have been postulated to explain this association, including the stimulating effect of high-fat intake on the secretion of secondary bile acids in the gut; this proposed mechanism has received the most attention. These bile acids may promote tumour formation by acting as aggressive surfactants on the mucosa, thus increasing cell loss and proliferation (Bruce, 1987; Owen, 1997; Bernstein et al., 2005). Other proposed mechanisms for the promoting role of dietary fat include an increase in the amount of free fatty acids in the colonic lumen, which may damage the colonic epithelium and induce cell proliferation, and an augmented risk for obesity (Calle & Kaaks, 2004). Dietary fat intake is also associated with peroxidation of unsaturated fatty acids (see Section 4.5.2).

[The Working Group noted that the digestion of red meat and processed meat provides energy and supplies essential nutrients, such as amino acids, iron, other minerals (including zinc), long-chain fatty acids, and various vitamins. At the same time, the digestion of protein and fat yields intrinsically toxic compounds. However, protein and fat are also present in dairy, fish, poultry, and other food products (Demeyer et al., 2015).]

4.2 Mechanisms of carcinogenesis

This section summarizes the evidence for the key characteristics of carcinogens (Smith et al., 2016), concerning whether red and processed meat intake is genotoxic, induces epigenetic effects, induces oxidative stress, and alters cell proliferation, cell death, and nutrient supply. Other mechanistic effects of red and processed meat intake, including whether it induces chronic inflammation and modulates receptor-mediated effects, are also addressed. Potential indirect mediators and studies of hemin and hemin chloride are summarized. Within each topic, studies are presented according to species (human and experimental systems) and test system (in vivo and in vitro), and red meat and processed meat studies are presented separately.

4.2.1 Genetic and related effects

Red meat and processed meat have been tested in studies of DNA damage, gene mutation, chromosomal damage, and epigenetic end-points. These studies are summarized in Table 4.1 to Table 4.6.

(a) Exposed humans

(i) DNA damage and DNA adducts

See Table 4.1

Regarding studies of red meat, Lewin et al. (2006) conducted a randomized crossover study in 21 human subjects fed diets that were high in red meat (420 g/day), vegetarian, or high in

Table 4.1 Genetic and related effects of red meat or processed meat in exposed humans

Tissue or body fluid	End-point	Test	Exposure	Response, significance	Reference
Colon	DNA adducts	O^6-CMG (IHC using polyclonal antibodies)	High–red meat (420 g), vegetarian, or high–red meat, high-fibre diets for 15 days (randomized crossover study) (n = 21)	+ $P < 0.0001$	Lewin et al. (2006)
Rectum	DNA adducts	O^6-MeG adducts (IHC using monoclonal antibodies)	Red meat (300 g/day) for 4 weeks (randomized crossover study) (n = 23)	+ $P < 0.01$	Le Leu et al. (2015)
Breast	DNA adducts	PhIP-DNA adducts (IHC using polyclonal antibodies)	Well-done meat consumption (assessed via questionnaire) in women (n = 49) undergoing reduction mammoplasty;	–	Zhu et al. (2003)
Breast	DNA adducts	^{32}P-postlabelling	Meat and HAA intake (assessed via FFQ) in women undergoing reduction mammoplasty (n = 44)	+ $P < 0.05$	Rohrmann et al. (2009a)
Urine	DNA adducts	8-OHdG	Barbecued pork (15 or 30 g/kg bw) (n = 13)	+ $P < 0.05$	Chien & Yeh (2010)
Colorectal carcinoma	Mutation	K-*RAS* mutation	Red meat consumption (assessed via FFQ) in colorectal cancer patients (n = 43)	–	O'Brien et al. (2000)
Colorectal carcinoma	Mutation	K-*RAS* mutation	Red meat consumption in NLCS cancer patients (n = 608)	–	Brink et al. (2005)
Colon	Mutation	K-*RAS* mutation	High–red meat (420 g), vegetarian, or high–red meat, high-fibre diets for 15 days (randomized crossover study) (n = 21)	–	Lewin et al. (2006)
Colorectal carcinoma	Mutation	*TP53* mutation	Colorectal cancer patients (n = 185) divided according to red meat consumption assessed via FFQ	+ $P = 0.01$	Park et al. (2010)
Colorectal adenoma	Mutation	*APC* mutation	Red meat consumption (assessed via FFQ) in cases with colorectal adenoma (n = 184) vs controls (n = 259)	(+)	Diergaarde et al. (2003)
Colorectal carcinoma	Mutation	*APC* mutation	Processed meat consumption (assessed via FFQ) in colorectal cancer patients (n = 185)	+ $P = 0.04$	Gay et al. (2012)

+, positive; –, negative; 8-OHdG, 8-hydroxy-2′-deoxyguanosine; FFQ, food frequency questionnaire; HAA, heterocyclic aromatic amine; IHC, immunohistochemistry; NLCS, Netherlands Cohort Study; O^6-CMG, O^6-carboxymethyl guanine; O^6-MeG, O^6-methylguanine; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine

red meat and fibre for 15 days. Compared with vegetarian diet, red meat intake significantly increased levels of O^6-carboxymethyl guanine (O^6-CMG), a DNA adduct putatively related to NOCs, in exfoliated colon cells. This adduct was detected by immunohistochemistry using polyclonal antiobodies. [The Working Group noted the lack of specificity of this method for this particular adduct).]

Increased levels of O^6-methylguanine (O^6-MeG), also a DNA adduct putatively related to NOCs, were shown by immunohistochemistry using monoclonal antibodies in rectal biopsies of human volunteers after an intake period of 4 weeks that was high in red meat (300 g/day) in a randomized crossover study (Le Leu et al., 2015).

No statistically significant associations were found between dietary intake of well-done meat assessed by questionnaire and DNA adducts putatively related to 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) in normal breast tissue (Zhu et al., 2003). The study included 106 women newly diagnosed with cancer of the breast, of which 49 women underwent reduction mammoplasty. PhiP-DNA adducts were assessed by immunohistochemistry using polyclonal antibodies. [The Working Group noted the lack of specificity of this method for these adducts. The type of meat was not specified.]

Fried meat [not specified] intake, assessed by food frequency questionnaire (FFQ), was significantly correlated with the presence of bulky, non-specific DNA adducts (^{32}P-postlabelling analysis) in the breast tissue of 44 women undergoing reduction mammoplasty (Rohrmann et al., 2009a).

Chien & Yeh (2010) showed that barbecued pork meat exposure increased oxidative DNA lesions in urine. They gave one meal of barbecued pork meat (reported as 15 or 30 g/kg bw) to eight or five volunteers, respectively. Statistically significant increases in urinary 8-hydroxy-2′-deoxyguanosine (8-OHdG) were observed 2 or 3 days after barbecued pork meat consumption. A correlation was found between PAH metabolites and 8-OHdG in urine (Chien & Yeh, 2010). [The Working Group noted the very high reported intake level of pork meat and of the borderline significance of the results.]

Regarding processed meat, the study by Rohrmann et al. (2009a) previously mentioned reported a significant correlation of intake (assessed by FFQ) with the presence of bulky, non-specific DNA adducts (^{32}P-postlabelling analysis) in the breast tissue of 44 women undergoing reduction mammoplasty.

(ii) Gene mutation

See Table 4.1

Regarding red meat, no association was found between K-*RAS* mutation frequency and meat consumption, assessed by FFQ, in colorectal cancer samples from 43 patients (O' Brien et al., 2000). Similarly, there was no association between tumours with K-*RAS* mutations and meat consumption in a large cohort study of 448 patients with cancer of the colon and 160 patients with cancer of the rectum from the Netherlands Cohort Study (NLCS) (Brink et al., 2005).

In the randomized crossover study by Lewin et al. (2006) previously mentioned, no K-*RAS* mutations were present in the exfoliated colon cells of volunteers fed diets that were high in red meat (420 g/day), vegetarian, or high in red meat and fibre for 15 days.

A positive association between haem iron intake and risk of cancer of the colorectum harbouring G→A transitions in K-*RAS* and *APC* genes, and TP53 overexpression was found in a prospective study (Gilsing et al., 2013). In the European Prospective Investigation into Cancer and Nutrition (EPIC) study in Norfolk, England, *TP53* mutations in cancer of the colorectum were examined in relation to dietary and lifestyle factors (Park et al., 2010). Higher daily total meat and red meat intake (assessed by FFQ) was significantly associated with harbouring *TP53* mutations in cancer of the colorectum.

In a case–control study in the Netherlands, Diergaarde et al. (2003) evaluated the association between dietary factors and *APC* mutations in sporadic colon carcinomas (184 cases, 259 controls). Direct sequencing was used to screen the mutation cluster region of *APC* in the colon tumours. Red meat intake appeared to be associated with *APC* mutated tumours: the odds ratios (ORs) for the association between the two highest tertiles (58–87 g/day and ≥ 86 g/day) of red meat intake and *APC* mutations were 1.5 (95% confidence interval, CI, 0.7–3.0) and 1.7 (95% CI, 0.8–3.6), respectively.

Regarding processed meat, analyses of *APC* mutations and *APC* promoter 1A methylation were performed on 185 archival colorectal cancer samples from participants of the EPIC-Norfolk study, with the aim of relating these to a 7-day dietary and lifestyle data collected prospectively (Gay et al., 2012). Truncating *APC* mutations and *APC* promoter 1A methylation were identified in 43% and 23% of colorectal cancer samples analysed, respectively. Cases with *APC* mutations or *APC* promoter 1A methylation consumed significantly higher levels of processed meat and iron from red meat and red meat products. In a logistic regression model adjusted for age, sex, and cigarette smoking status, each 19 g/day (one standard deviation, SD) increment increase in processed meat consumption was associated with *APC* mutations with GC→AT transitions (OR, 1.68; 95% CI, 1.03–2.75).

(iii) Faecal water genotoxicity

See Table 4.2

Rieger et al. (1999) first reported that a diet high in fat and meat increased faecal water genotoxicity (tested with comet assay in HT-29 cell cultures) in seven healthy volunteers over a period of 12 days. Compared with a diet rich in vegetables and poor in fat and meat, a diet rich in fat (total energy intake, 50%), meat, and sugar, and poor in vegetables and free of wholemeal products [no exact composition was given], significantly increased faecal water genotoxicity. [type of meat was not specified].

Faecal water genotoxicity (tested with comet assay in HT-29 cell cultures) from two randomized controlled studies of red meat (60 or 420 g/day), a vegetarian diet, or haem iron supplements for 15 days in volunteers (n = 21) was evaluated by Cross et al. (2006). Diet had no effect on faecal water genotoxicity (i.e. red meat had no effect). This study was performed under the same conditions as those described by Rieger et al. (1999), but did not confirm those results.

Hughes et al. (2002) studied the effect of vegetables, tea, or soy on faecal water genotoxicity (tested with comet assay in Caco-2 cell cultures) in 11 volunteers fed a high–red meat (420 g/day) diet for 15 days. Low to moderate levels of genotoxicity were observed. [The Working Group noted that the study did not contain a control group consuming a low–red meat diet.]

Faecal water from volunteers (n = 12) fed a red meat (420 g/day, males; 366 g/day, females) or vegetarian diet (Joosen et al., 2009) was tested for genotoxicity by comet assay in Caco-2 cells. Surprisingly, the vegetarian diet produced more DNA strand breaks than the red meat diet. No effect of diet was found in a similar study by the same authors (Joosen et al., 2010) assessing faecal water genotoxicity in volunteers (n = 13) fed a red meat versus fish diet.

More recently, Hebels et al. (2012) showed increased faecal water genotoxicity in a heterogeneous group of inflammatory bowel disease/irritable bowel syndrome patients (n = 12) after 7 days of high–red meat intake (300 g/day), compared with the results obtained before the intervention. In 10 of the subjects, faecal water genotoxicity significantly increased with red meat intake (tested with both standard comet assay and the formamidopyrimidine procedure to measure oxidative damage in Caco-2 cells). Microarray analyses in colon biopsies indicated significant modulation of various signalling

Table 4.2 DNA damage induction by human faecal water following meat consumption

Tissue, cell line	End-point	Test	Results	Exposure[a]	Comments	Reference
HT-29	DNA strand breaks	Comet assay	+	High-fat (125.8 g) and high-meat diet (51.9 g) for 12 days ($n = 7$)	Type of meat not defined	Rieger et al. (1999)
HT-29	DNA strand breaks	Comet assay	–	Red meat (60 or 420 g/day), vegetarian, or haem iron supplemented diet for 15 days ($n = 21$)		Cross et al. (2006)
Caco-2	DNA strand breaks	Comet assay	–	High–red meat (420 g/day) diet with vegetables, tea, or soy for 15 days ($n = 11$)	No control group consuming a low–red meat diet	Hughes et al. (2002)
Caco-2	DNA strand breaks	Comet assay	–	Red meat ($n = 12$) or processed meat ($n = 16$) (males, 420 g/day; females, 366 g/day) or vegetarian diet for 10 days	Vegetarian diet increased genotoxicity of faecal water ($P < 0.05$)	Joosen et al. (2009)
Caco-2	DNA strand breaks	Comet assay	–	Red meat (males, 325 g/day; females, 260 g/day) or fish diet for 3 days ($n = 13$)		Joosen et al. (2010)
Caco-2	DNA strand breaks	Comet assay	+	High–red meat (300 g/day) diet for 7 days in IBD/IBS patients ($n = 12$)		Hebels et al. (2012)

[a] Diet consumed before faecal water collection

+, positive; –, negative; IBD, inflammatory bowel disease; IBS, irritable bowel syndrome

pathways (e.g., cytoskeleton remodelling, development, and immune response) (Hebels et al., 2012).

Regarding processed meat, the study by Joosen et al. (2009) previously described found that faecal water from subjects on the vegetarian diet compared with that from subjects on the processed meat diet produced more DNA strand breaks, as assessed by comet assay in Caco-2 cells.

(iv) Mutagenic activity in urine

See Table 4.3

The first report of mutagenic activity in the urine of subjects after ingestion of meat was published in 1982 (Baker et al. 1982). Fried pork (150 g) was given to five subjects, and bacterial mutagenicity of urine was determined. Peaks in urinary mutagenicity (in *Salmonella typhimurium* strains TA98 and TA1538 with S9) were detected 2–4 hours after ingestion.

Dolara et al. (1984) reported a modest increase in mutagenic activity in the urine of subjects fed pork fried in a pan at 200 °C. Mutagenic activity (in *S. typhimurium* strain TA1538 with S9) was present in 3 of 13 samples analysed and was much lower than that reported by Baker et al. (1982). Hayatsu et al. (1985) also documented mutagenicity (*S. typhimurium* strain TA98 with S9) in the urine of three volunteers 1.5 hours after consumption of fried ground beef.

Doolittle et al. (1989) studied the effects of different cooking methods on mutagenicity. As a small part of the study, the urinary mutagenicity of different cooking procedures was compared in 12 subjects (6 males, 6 females). Fried meat increased urinary mutagenicity (*S. typhimurium* strains TA98 and TA100 with S9) compared with boiled or baked meat.

Gabbani et al. (1998) determined urinary mutagenicity 24 hours after ingestion of two pan-fried hamburgers (2 × 100 g) in 32 volunteers. *GSTM1* and *NAT2* genotypes were also evaluated. Urinary mutagenicity was tested in *S. typhimurium* strains TA98 and YG1024, with the latter overexpressing *O*-acetyltransferase. Mutagenicity (a doubling over the spontaneous revertant number) was seen in the YG1024 strain (in 23 of 32 samples), but not in the TA98 strain. Furthermore, *NAT2* slow acetylators had higher urinary mutagenicity. Similar results (i.e. increased mutagenic activity after a pan-fried hamburger meal) were shown by Pavanello et al. (2002) in a larger group of subjects (n = 50).

Peters et al. (2004) studied urinary mutagenesis in a group of 60 volunteers who consumed red meat cooked at 100 °C for 7 days followed by red meat cooked at 250 °C for an additional 7 days. Both unhydrolysed and acid-hydrolysed urine samples, containing unmetabolized mutagens and both metabolized and unmetabolized mutagens, respectively, were tested in *S. typhimurium* strain YG1024. Unhydrolysed and hydrolysed urine samples were 22 and 131 times more mutagenic, respectively, in subjects who consumed red meat cooked at 250 °C compared with those who consumed red meat cooked at 100 °C.

Shaughnessy et al. (2011) reported increased mutagenic activity (in *S. typhimurium* strains TA98 and YG1024) in the hydrolysed urine and faeces of subjects (n = 8) who consumed red and processed meat cooked at a high temperature of 250 °C (11 minutes/side) for a period of 2 weeks.

Regarding processed meat, the previously mentioned study by Baker et al. (1982) reported increased urinary mutagenicity (in *S. typhimurium* strains TA98 and TA1538 with S9) in five subjects fed fried bacon (150 g). Similarly, Dolara et al. (1984) reported a modest increase in mutagenic activity in the urine of subjects consuming pan-fried bacon.

(v) Epigenetics

Regarding red meat, microRNA expression in the rectal mucosa of volunteers consuming a high–red meat diet, with or without supplementation with butyrylated high-amylose maize starch (HAMSB), was evaluated by Humphreys

Table 4.3 Bacterial mutagenic activity of human urine following meat consumption

Salmonella typhimurium strain	Results		Exposure	Comments	Reference
	Without metabolic activation	With metabolic activation			
TA98 and TA1538	NT	+	Fried pork or bacon (150 g), (n = 5)		Baker et al. (1982)
TA1538	NT	+	Fried pork or bacon (2 g/kg bw), (n = 7)	Modest effect	Dolara et al. (1984)
TA98	–	+	Fried ground beef (130 g), (n = 3)		Hayatsu et al. (1985)
TA98 and TA100	–	+	Meat and food cooked by different methods (n = 12)	Fried meat increased mutagenicity compared with boiled or baked meat	Doolittle et al. (1989)
TA98	NT	–	Two hamburgers (2 × 100 g) fried to taste (n = 32)		Gabbani et al. (1998)
YG1024	NT	+	Two hamburgers (2 × 100 g) fried to taste (n = 32)	23/32 urine samples were mutagenic; higher mutagenicity in *NAT2* slow acetylators	Gabbani et al. (1998)
YG1024	NT	+	Two hamburgers (2 × 100 g) fried to taste (n = 50)		Pavanello et al. (2002)
YG1024	NT	+	Meat cooked at 100 °C for 7 days followed by meat cooked at 250 °C for 7 days (n = 60)	No effect with meat cooked at 100 °C	Peters et al. (2004)
TA98 and YG1024	NT	+	Red and processed meat cooked at 100 °C or 250 °C (2 weeks at each cooking temperature in a crossover design), (n = 8)	No effect with meat cooked at 100 °C	Shaughnessy et al. (2011)

+, positive; –, negative; NT, not tested

et al. (2014). Volunteers received the high–red meat diet for 4 weeks, with washout periods and different orders of treatments. HAMSB significantly lowered a cluster of microRNAs (miR17–92, designated oncomir-1) associated with carcinogenesis. This effect was attributed more to a decrease of these microRNAs by HAMSB than an increase of these microRNAs by red meat.

Tarallo et al. (2014) showed an association between microRNA (miR-92a) levels in the plasma of healthy individuals and consumption of processed meat and other dietary factors (Tarallo et al., 2014).

(b) Human cells in vitro

See Table 4.4

The basic fraction of a beef extract did not induce chromosomal aberrations in human lymphocyte cultures (irrespective of the presence of S9) (Aeschbacher & Ruch, 1989). A small but statistically significant increase in sister-chromatid exchange was seen in the presence of S9.

No study of processed meat in human cells in vitro was available to the Working Group.

Table 4.4 Genetic and related effects of meat extract in human cells in vitro

Tissue, cell line	End-point	Test	Results		Exposure	Comments	Reference
			Without metabolic activation	With metabolic activation			
Human lymphocytes	Chromosomal damage	Chromosomal aberrations	–	–	Beef extract (200 mg/mL)		Aeschbacher & Ruch (1989)
Human lymphocytes	Chromosomal damage	Sister-chromatid exchange	–	±	Beef extract (200 mg/mL)	Small but statistically significant increase	Aeschbacher & Ruch (1989)

+, positive; –, negative; ±, small magnitude of effect

(c) Non-human mammals in vivo

See Table 4.5

(i) DNA damage

In studies of DNA adducts after red meat consumption, Winter et al. (2011) quantified O^6-MeG adducts by immunohistochemistry in CBJ57 mouse colonocytes after mice were fed different diets (containing 15% or 30% protein as casein or red meat, 30% protein with high-amylose maize starch) for 4 weeks. O^6-MeG and *para*-cresol, a protein metabolite with reported genotoxic activity, significantly increased ($P < 0.02$) with consumption of red meat compared with casein. O^6-MeG adducts were present at the apex of the crypts. Starch attenuated the increase in DNA adduct levels.

DNA damage in colonocytes (assessed by comet assay) was measured in Sprague-Dawley rats fed diets containing 25% cooked lean red meat (300 g/kg diet) or casein (15% or 25%), with or without high-amylose maize starch for 4 weeks (Toden et al., 2006). When starch was absent from the diet, red meat caused a significant increase in DNA damage (26%) compared with casein ($P < 0.05$). When starch was present in the diet, the red meat effect was not significant. The same authors later fed rats diets of 15%, 25%, or 35% cooked beef or chicken, with or without high-amylose maize starch (Toden et al., 2007). DNA single- and double-strand breaks (assessed by comet assay in colonocytes) were significantly higher in the groups fed high levels of both meats compared with those fed low levels of meat. Red meat was more active than chicken, and starch prevented the damage. Apoptotic cells were also increased by red meat (see Section 4.2.3).

The effect of red meat on colonocyte DNA damage was also studied in pigs (Belobrajdic et al., 2012). Ten male animals (Large White strain) were fed diets containing 300 g/kg of cooked red meat or the same diet supplemented with arabinoxylans (arabinoxylan-rich fraction from wheat) for 4 weeks. The comet assay was performed on colonocytes, together with additional determinations (short-chain-fatty-acids (SCFA), phenol, cresol in the feces, bacterial profile). There was a significant decrease in DNA damage in the diet supplemented with arabinoxylans.

Regarding processed meat, 7-methyldeoxyguanosine levels were measured by immuno-slot-blot assay in the colonic DNA of Swiss mice fed hot dogs containing beef or pork (18% of the diet) for 7 days. The levels of this non-mutagenic adduct were similar in control ($n = 5$) and treated mice ($n = 4$) (Mirvish et al., 2002).

Table 4.5 Genetic and related effects of red meat or processed meat in non-human mammals in vivo

Species, strain, sex	Tissue	End-point	Test	Results	Dose (LED or HID)	Route, duration, dosing regimen	Comments	Reference
Mouse, CBJ57, male	Colon	DNA adducts	O^6-MeG (IHC)	+	Diets of 15% or 30% protein as red meat or casein, or 30% protein with starch	Oral, 4 wk	Starch inhibited the increase in DNA strand breaks with red meat	Winter et al. (2011)
Mouse, Swiss albino, male and female	Colon	DNA adducts	7-MedG (immuno-slot-blot assay)	–	Hot dogs with beef and pork (18% of diet)	Oral, 7 days		Mirvish et al. (2002)
Rat, Sprague-Dawley, male	Colon	DNA strand breaks	Comet assay	+	Red meat (25%) or casein (15% or 25%) diet, with or without starch	Oral, 4 wk	Starch inhibited the increase in DNA strand breaks with red meat	Toden et al. (2006)
Rat, Sprague-Dawley, male	Colon	DNA strand breaks	Comet assay	+	Red meat or chicken (15%, 25%, 35%) diet, with or without starch	Oral, 4 wk	Red meat more active than chicken; inhibitory effect of starch	Toden et al. (2007)
Pig, Large White, male	Colon	DNA strand breaks	Comet assay	±	Cooked red meat (300 g/kg bw), with or without arabinoxylans	Oral, 2 meals/day, 4 wk	Significantly lower DNA strand breaks with arabinoxylans; no control diet (without red meat)	Belobrajdic et al. (2012)
Mouse, Swiss albino, male	Urine	Reverse mutation	*Salmonella typhimurium* TA98	–	Beef extract	Oral or intraperitoneal		Dolara et al. (1980)
Mouse, Swiss albino, male	Host-mediated assay	Reverse mutation	*Salmonella typhimurium* TA98	–	Beef extract	Oral or intraperitoneal		Dolara et al. (1980)
Mouse, NMRI, male	Host-mediated assay	Reverse mutation	*Salmonella typhimurium* TA98	±	Pan-fried sausage extract (500 mg/kg bw)	Intraperitoneal	Low mutagenicity with Aroclor pretreatment	Gocke et al. (1982)

Table 4.5 (continued)

Species, strain, sex	Tissue	End-point	Test	Results	Dose (LED or HID)	Route, duration, dosing regimen	Comments	Reference
Mouse, C57BL, female	Whole body	Mutation	Mouse spot test	–	Pan-fried sausage extract (500 mg/kg bw)	Intraperitoneal, gestation period	Only one dose tested	Gocke et al. (1982)
Mouse, NMRI, male and female	Bone marrow	Chromosomal damage	Micronuclei	–	Pan-fried sausage extract (1000 mg/kg bw)	Intraperitoneal		Gocke et al. (1982)
Rat, Sprague-Dawley	Caecal water	Chromosomal damage in cultured WIL2-NS cells	Micronuclei	+	Barbecued beef (in a high fat, low fibre, low calcium diet) or casein (in a low fat, high fibre and high calcium diet)	Oral, 15 days		Benassi et al. (2007)

+, positive; –, negative; +/–, small magnitude of effect; 7-MedG, 7-methyldeoxyguanosine; HID, highest ineffective dose; IHC, immunohistochemistry; LED, lowest effective dose; O^6-MeG, O^6-methyl-2-deoxyguanosine; wk, week

(ii) Gene mutation

Dolara et al. (1980) reported no increase in the mutagenicity of the urine after administration of a beef extract to Swiss albino mice. Similarly, the beef extract did not have a mutagenic effect as assessed by intrasanguine host-mediated assay.

Regarding processed meat, the mutagenic activity detected by intrasanguine host-mediated assay in NMRI mice given an extract of pan-fried sausage was very low (Gocke et al., 1982). An extract of pan-fried sausage (500 mg/kg bw) fed to pregnant female C57Bl mice did not increase the frequency of coat-coloured spots in the mouse spot test (Gocke et al., 1982).

(iii) Chromosomal aberrations

A basic extract of pan-fried sausage (up to 1000 mg/kg bw) did not increase the frequency of micronucleated erythrocytes in mouse bone marrow (Gocke et al., 1982).

The caecal water from Sprague-Dawley rats fed a high fat, low fibre, and low calcium diet containing barbecued beef as the protein source (equivalent to 17% of the total diet) for 2 weeks significantly increased all the parameters assessed – micronuclei, nucleoplasmic bridges, and nuclear buds –in the WIL2-NS human B-lymphoblastoid cell line. Control rats were fed 17% casein as the protein source in a diet low in fat, and high in fibre and calcium (Benassi et al., 2007).

(d) Non-mammalian experimental systems

See Table 4.6

(i) Drosophila

No study of red meat in *Drosophila* was available to the Working Group. Regarding processed meat, an extract of pan-fried sausage did not increase the frequency of sex-linked recessive lethals in *Drosophila* (Gocke et al., 1982).

(ii) Bacteria

In red meat studies in vitro, extracts of the charred surface of broiled beef meat and fish, together with smoke produced from the broiling of fish, were first demonstrated to be mutagenic by Sugimura and colleagues Nagao et al. (1977). The charred parts of medium-broiled beef were suspended in dimethyl sulfoxide and tested using the Ames test. The dimethyl sulfoxide extract of the charred meat was mutagenic in *S. typhimurium* strain TA98 with S9 prepared from the liver of rats treated with polychlorinated biphenyl (PCB). This mutagenic activity was much higher than that anticipated from the benzo[*a*]pyrene (BaP) content of the cooked food.

Commoner et al. (1978) reported that cooked red meat was highly mutagenic (in *S. typhimurium* strain TA1538). Hamburger meat cooked rare, medium, or well done was extracted with methylene chloride, dried, and dissolved in dimethyl sulfoxide. Mutagenic activity was dependent on the presence of S9. The mutagens formed did not belong to the class of BaP or protein and amino acid pyrolysis products. Mutagens were produced during common cooking procedures, including the use of electrically heated hot plates.

A sharp rise in the frequency of mutations in *S. typhimurium* strain TA1538 with S9 was detected when beef was boiled for different periods of time and reached temperatures between 140 °C and 180 °C. The mutagenic activity of the beef (hamburger) cooked under different conditions was limited to the surface layer; uncooked meat or microwave-cooked meat did not produce mutagenic activity (Dolara et al., 1979).

Similarly, Pariza et al. (1979) demonstrated that the mutagenic activity of pan-fried hamburger meat was dependent on cooking time and temperature. Mutagenic activity (in *S. typhimurium* strain TA1538) was not detected in uncooked hamburger or hamburger pan-fried at 143 °C. In contrast, hamburger pan-fried at 191 °C or 210 °C for up to 10 minutes generated

Table 4.6 Genetic and related effects of red meat or processed meat in non-mammalian experimental systems

Species, strain	End-point	Test	Results		Dose (LED or HID)	Comments	Reference
			Without metabolic activation	With metabolic activation			
Drosophila melanogaster Berlin K (wildtype) and Basc tester strain	Mutation	Sex-linked recessive lethal mutations	–	NA	Extract of pan-fried sausage (0.1 µg/fly)		Gocke et al. (1982)
Salmonella typhimurium TA98	Mutation	Reverse mutation	–	+	Extracts of the charred surface of broiled beef meat and fish (12 mg/plate)		Nagao et al. (1977)
Salmonella typhimurium TA1538	Mutation	Reverse mutation	–	+	Beef extract (0.1 g dry weight/plate) and red meat (5 g dry weight/plate) cooked under normal conditions		Commoner et al. (1978)
Salmonella typhimurium TA1538	Mutation	Reverse mutation	–	+	Extracts of boiled beef, or hamburger cooked at different times and temperatures (5 g dry weight/plate)	Negative results with uncooked or microwave-cooked meat	Dolara et al. (1979)
Salmonella typhimurium TA1538	Mutation	Reverse mutation	–	+	Extract of hamburger cooked at different times and temperatures (10 g/plate)	Negative results with uncooked meat, or meat cooked at 143 °C	Pariza et al. (1979)
Salmonella typhimurium TA98	Mutation	Reverse mutation	NT	+	Extract of meat cooked at 100 °C or 250 °C (0.06–1.25 g/eq per plate)	No effect at 100 °C	Peters et al. (2004)
Salmonella typhimurium TA1538	Mutation	Reverse mutation	–	+	Beef extract (50 mg dry weight/plate)		Dolara et al. (1980)
Salmonella typhimurium TA98, TA1538	Mutation	Reverse mutation	–	+	Fried sausage extract (100 µg/plate)		Gocke et al. (1982)

+, positive; –, negative; HID, highest ineffective dose; LED, lowest effective dose; NA, not applicable; NT, not tested

considerable mutagenic activity. Higher mutagenic activity was observed when S9 was from Aroclor 1254–treated rats.

Another study with *S. typhimurium* strain TA98 showed meat cooked only at a high temperature of 250 °C had mutagenic activity (Peters et al., 2004).

Dolara et al. (1980) reported that the mutagenic activity of a beef extract required the presence of S9 fractions from the liver of rats treated with PCB or 3-methylcholanthrene, but no induction was necessary when the liver came from Swiss albino mice. CD-1 mice had intermediate activation capabilities, which increased after the addition of 0.75% butylhydroxyanisole to their diet. S9 from the liver of human donors had low-activation capabilities.

Regarding processed meat, an extract of pan-fried sausage was mutagenic in *S. typhimurium* strains TA1538 and TA98 in the presence of S9 mix (Gocke et al., 1982).

4.2.2 Oxidative stress

(a) Humans

(i) Red meat

In a randomized crossover study, Pierre et al. (2006) measured the excretion of 1,4-dihydroxynonane mercapturic acid (DHN-MA), the major urinary metabolite of 4-hydroxynonenal (4-HNE). Eight volunteers were fed different diets providing 55, 55, 80, 205, and 110 mg/day of haem for 15 days: red meat (60 g/day) baseline diet, red meat (60 g/day) with non-haem iron diet, red meat (60 g/day) with haem iron in the form of liver pâté diet, red meat (60 g/day) with haem iron in the form of blood sausage diet, or red meat (120 g/day) diet. The blood sausage diet increased urinary DHN-MA by about two-fold ($P < 0.001$), but mean urinary 8-iso-prostaglandin $F_{2\alpha}$ was similar in all groups (Pierre et al., 2006).

In contrast, Hodgson et al. reported no elevation in oxidative stress markers with consumption of lean red meat (Hodgson et al., 2007). Sixty participants were randomized to maintain their usual diet for 8 weeks or to partially replace carbohydrate-rich foods with 200 g/day of lean red meat. In terms of the mean between-group difference in comparison to the control diet, the red meat diet increased iron intake (3.2 mg/day; 95% CI, 1.1–5.4), lowered urinary F_2-isoprostane excretion (–137 pmol/mmol of creatinine; 95% CI, –264 to –9), and did not change plasma F_2-isoprostanes (–12 pmol/L; 95% CI, –122 to 100) or serum γ-glutamyltransferase (–0.8 U/L; 95% CI, –3.2 to 1.5).

Montonen et al. (2013) reported an association between red meat intake and a blood oxidative stress marker. In 2198 participants selected from the EPIC-Potsdam study, higher consumption of red meat was significantly associated with higher levels of γ-glutamyltransferase, even after adjustment for potential confounding factors related to body mass index (BMI), waist circumference, lifestyle, and diet (Montonen et al., 2013).

Lam et al. (2014) identified several genes involved in oxidative stress that were differentially expressed in patients with lung adenocarcinoma who consumed more red meat. Genome-wide expression (HG-U133A) was measured in the tumour tissue and non-involved lung tissue of 64 patients with adenocarcinoma. Gene expression of 232 annotated genes in the tumour tissue significantly distinguished patients who consumed above or below the median intake of fresh red meat. Several genes were involved in lipid metabolism (e.g. *NCR1*, *TNF*, *UCP3*) and oxidative stress (e.g. *TPO*, *SGK2*, *MTHFR*) (Lam et al., 2014).

(ii) Processed meat

As previously noted, Pierre et al. (2006) reported that a blood sausage diet significantly increased DHN-MA by about two-fold. In a later study, Pierre et al. (2013) showed that cured meat intake increased lipid peroxidation and nitroso compounds in human stool. In a single-blind,

crossover, randomized controlled trial, 18 volunteers first followed a low-meat diet for a one week control period and were then fed the following diets for four days each, in a random order: cooked, cured pork shoulder meat (similar to air-exposed picnic ham, 180 g/day); cooked, cured pork shoulder meat with a calcium carbonate capsule (1 g/day of calcium); and cooked, cured pork shoulder meat with α-tocopherol (0.05%). Thiobarbituric acid reactive substances (TBARS) and apparent total *N*-nitroso compounds (ATNC) increased in the faecal water of volunteers given ham compared with control periods. Calcium carbonate normalized both biomarkers, whereas α-tocopherol normalized only lipid peroxidation in the faeces of volunteers (Pierre et al., 2013).

Belinova et al. (2014) showed that consumption of "cooked-pork seasoned meat" was accompanied by increased oxidative stress marker levels in diabetic patients. In a randomized crossover study, 50 type 2 diabetic patients and 50 healthy subjects underwent two 3-hour meal tolerance tests. The acute effects of a processed hamburger meat meal (150 g/meal) were compared with those of a vegan meal (235 g/meal). During the postprandial phase, consumption of the hamburger meat meal was associated with a significant increase in TBARS in the diabetic patients, but not in the healthy subjects, compared with the consumption of the vegan meal. However, superoxide dismutase activity in the healthy subjects was significantly increased after the vegan meal compared with the hamburger meat meal. In the diabetic patients, plasma concentrations of superoxide dismutase, reduced glutathione, or ascorbic acid did not change during the postprandial phase for either meal (Belinova et al., 2014).

No data concerning direct evaluation of red meat or processed meat in human cells in vitro were available to the Working Group.

(b) Rodents

(i) Red meat

Pierre et al. repeatedly showed that diets containing red meat or haemoglobin significantly increased lipid peroxides in faecal water (TBARS) and urinary DHN-MA, a metabolite of the lipid oxidation product 4-HNE, in rats. In a seminal study by Pierre et al. (2004), groups of carcinogen-initiated rats were given one of three low-calcium, meat-based diets containing 60% freeze-dried meat products: raw chicken (low haem), beef (medium haem), or blood sausage (high haem). Two additional groups of rats were given a non-haem control diet supplemented with ferric citrate or a haem control diet supplemented with haemoglobin to match the iron and haem concentrations of the beef diet, respectively. The blood sausage diet increased TBARS in faecal water by 23-fold. The haemoglobin and beef diets increased TBARS in faecal water by two- to four-fold (all $P < 0.01$), but the chicken diet did not affect TBARS in faecal water compared with the control diets (Pierre et al., 2004). A recent carcinogenesis study confirmed that only diets containing haemoglobin increased faecal and urinary oxidation biomarkers ($P < 0.001$), independent of dietary HAAs or nitrates and nitrites, and resulting faecal ATNC (Bastide et al., 2015). Other rat studies by the same researchers on beef meat, haemoglobin, or hemin chloride (see Section 4.2.6 for hemin chloride studies) confirmed that dietary haem induced faecal and urinary lipid peroxides (Pierre et al., 2003, 2006, 2008; Guéraud et al., 2015). Additionally, dietary calcium phosphate (31 g/kg) normalized faecal TBARS induced by beef consumption (Pierre et al., 2008). In contrast, dietary antioxidant agents (rutin and butylated hydroxyanisole, 0.05% each) and olive oil (5%) did not reduce faecal TBARS (Pierre et al., 2008).

(ii) Processed meat

Several studies showed that cured meat intake increased lipid peroxidation and nitroso compound (ATNC) formation in rat stool (Pierre et al., 2010, 2013; Santarelli et al., 2010, 2013). For instance, Santarelli et al. (2013) reported increased urinary DHN-MA in rats fed nine different types of purchased cured meats, including hot dogs, sausages, raw and cooked ham, and pâté. Fermented, raw, dry sausages induced 1.8 times more TBARS in faecal water than hot dogs, but only hot dogs promoted preneoplastic lesions in the colon (see Section 4.3). Thus, no association was found between the occurrence of preneoplastic lesions and the biomarkers of lipid oxidation (Santarelli et al., 2013).

No data from non-human mammalian in vitro studies of red meat or processed meat and oxidative stress were available to the Working Group.

4.2.3 Alteration of cell proliferation and cell death

(a) Humans

Regarding red meat, Le Leu et al. (2015) reported an increase in epithelial proliferation in the rectal biopsies of 23 volunteers given cooked lean red meat (300 g/day) for 4 weeks. Proliferating cell nuclear antigen (PCNA) staining revealed a 38% increase in positive cells per crypt ($P < 0.001$). Caderni et al. (1999) observed that subjects who reported consuming a diet low in red meat had decreased colorectal mucosa proliferation. The labelling index in the upper part of the crypt was increased in subjects at high risk of cancer of the colon. In a study of 69 subjects who previously underwent surgery for at least two sporadic colon adenomas, dietary habit information was collected by FFQ, and proliferation was measured by [^{3}H]thymidine incorporation into colorectal biopsies. Subjects with low–red meat consumption showed decreased proliferation in the upper part of the crypt (mean ± SD: 2.4 ± 2.1, 5.3 ± 4.6, and 5.9 ± 4.8 for low, middle, and high consumption, respectively; $P < 0.01$) (Caderni et al., 1999).

Humphreys et al. (2014) reported decreased expression of *CDKN1A*, an inhibitor of cell proliferation, and increased cell proliferation in the rectal cells of volunteers fed a high–red meat diet, with or without supplementation with HAMSB.

In contrast, O'Brien et al. (2000) observed no correlation between red meat consumption and rectal crypt cell proliferation. Crypt cell proliferation was significantly higher in the normal mucosa of patients with left-sided colorectal carcinoma than in that of healthy controls. Meat consumption was assessed by FFQ, and crypt cell proliferation was determined using rectal biopsies obtained before surgery (O'Brien et al., 2000).

Regarding processed meat, Pierre et al. detected no effect of cured meat intake (180 g/day of a model ham for 4 days) in 18 volunteers on faecal water cytotoxicity in two cell lines, nor on genotoxicity (measured by γ-H2AX induction) (Pierre et al., 2013).

No data concerning direct examination of red meat or processed meat on human cells in vitro were available to the Working Group. As described in Section 4.2.6, contrasting effects of hemin chloride on the proliferation of human colon cancer cells were shown in vitro.

(b) Rodents

Regarding red meat, apoptosis (determined by halo assay) increased in a dose-dependent manner in colonocytes isolated from rats fed diets containing 15%, 25%, or 35% cooked beef or chicken for 4 weeks (Toden et al., 2007). In contrast, lean beef meat was without effect on proliferation or apoptosis in the colon in mice fed a standard American Institute of Nutrition (AIN)-76 diet with 15% or 30% protein as casein or cooked, dried lean beef meat for 4 weeks

(Winter et al., 2011). Neither the amount nor the type of protein had an effect on cell proliferation (Ki-67), cell mass (crypt height), or rate of apoptosis (terminal deoxynucleotidyl transferase dUTP nick end labelling, TUNEL, assay)

Khil & Gallaher (2004) showed no proliferative effect of dietary beef protein or beef tallow in 1,2-dimethylhydrazine (DMH)-initiated rats given either casein or beef as the protein source and soybean oil or tallow as the fat source in a 2 × 2 factorial design for 9 weeks. However, there was a significantly greater apoptotic labelling index in the distal colonic mucosa of rats fed the beef tallow compared with the soybean oil (Khil & Gallaher, 2004).

Yang et al. (2002) showed that a beef-based diet (24% freeze-dried beef meat vs casein control) reduced caspase-3 activity and neutral ceramidase activity in the colonic mucosa, but had no effect on sphingomyelinase activity in the colonic mucosa.

Pierre et al. repeatedly showed that diets containing red meat or haemoglobin significantly increased faecal water cytotoxicity. In a seminal study, groups of carcinogen-initiated rats were given one of three low-calcium, meat-based diets containing 60% freeze-dried meat products: raw chicken (low haem), beef (medium haem), or blood sausage (high haem). Two additional groups of rats were given a non-haem control diet supplemented with ferric citrate or a haem control diet supplemented with haemoglobin to match the iron and haem concentrations of the beef diet, respectively. The haem control diet was supplemented with haemoglobin to match the haem concentration of the beef diet. The blood sausage diet enhanced erythrocyte cytolysis by more than 50-fold and CMT93 cell toxicity by eight-fold compared with the non-haem control diet. The haemoglobin and beef diets increased CMT93 cell toxicity by four-fold compared with the non-haem control diet; the chicken diet did not increase CMT93 cell toxicity. A correlation was seen between haem intake and faecal water cytotoxicity (r = 0.98), which was correlated with carcinogenesis promotion (r = 0.65; all $P < 0.01$) (Pierre et al., 2004). Other studies of dietary beef, haemoglobin, or hemin chloride have confirmed that dietary haem can induce faecal water cytotoxicity (Pierre et al., 2003, 2008; Guéraud et al., 2015).

In in vitro studies, faecal water of rats given a diet with red meat or haemoglobin was more cytotoxic to the wild type $Apc^{+/+}$ murine cells than to premalignant $Apc^{-/+}$ murine cells (Pierre et al., 2007; Bastide et al., 2015). Trapping of aldehydes from the faecal water of haem-fed rats reduced peroxides by 95% and cytotoxicity by 75%.

Regarding processed meat, several studies by a single research group showed that cured meat intake in rats can increase faecal water cytotoxicity. For instance, faecal water cytotoxicity increased three-fold in rats given a diet with 55% freeze-dried cooked ham for 100 days (Pierre et al., 2010). Santarelli et al. (2010) tested the effect on faecal water cytotoxicity of 16 types of cooked ham diets fed for 2 weeks to rats, with dark or light muscle colour (a proxy for haem level), low or high processing temperature, added nitrite or none, and plastic anaerobic packaging or none, in a 2 × 2 × 2 × 2 design. Faecal water cytotoxicity depended mostly on processing temperature, with cooked ham being more cytotoxic than raw ham, and nitrite, with nitrite being more cytotoxic than no nitrite. [The Working Group noted that both red meat and processed meat were cytotoxic.]

4.2.4 Other mechanisms of carcinogenesis

(a) Chronic inflammation

(i) Humans

Regarding red meat, four observational studies in humans (Azadbakht & Esmaillzadeh, 2009; Montonen et al., 2013; Viscogliosi et al., 2013; Ley et al., 2014) lent little or no support to the hypothesis that red meat intake is directly associated with inflammation markers. Three

intervention studies in volunteers found no effect of red meat intake on inflammation markers (Hodgson et al., 2007; Joosen et al., 2010; Maduro et al., 2013).

Regarding processed meat, two studies were identified. In a nested case–control study of 656 women with type 2 diabetes and 694 healthy women from the Nurses' Health Study (NHS), Schulze et al. (2005) observed that a dietary pattern including processed meat was strongly related to inflammatory markers. This dietary pattern was high in sugar-sweetened soft drinks, refined grains, diet soft drinks, and processed meat, but low in wine, coffee, cruciferous vegetables, and yellow vegetables. Among the inflammatory markers examined, interleukin-6, C-reactive protein, and E-selectin were correlated with processed meat intake. Scores were adjusted for age and BMI, as well as for six other possible confounders (Schulze et al., 2005).

Spehlmann et al. (2012) reported an association between processed meat intake and inflammatory bowel disease in twins. In German monozygotic and dizygotic twins, where at least one sibling had inflammatory bowel disease (n = 512), a high consumption of processed meat, including sausage, was one of the variables most significantly associated with Crohn disease or ulcerative colitis. Likewise, differences in consumption of red meat were also detected in all discordant twin and non-twin Crohn disease groups (Spehlmann et al., 2012).

The hypothesis associating *N*-glycolylneuraminic acid (Neu5Gc) and chronic inflammation is discussed in Section 4.5.7 (Samraj et al., 2015).

No data from human in vitro studies of red meat or processed meat and inflammation were available to the Working Group.

(ii) Rodents

Regarding studies of red meat, mice fed grain-finished beef for 2 weeks showed enhanced prostaglandin E2 from peritoneal macrophages after inflammatory stimulation. The release of prostaglandin E2 was lowest with diets of range-fed beef, range-fed bison, and elk, and highest in mice fed grain-finished beef ($P < 0.05$). Prostacyclin release was highest in mice fed elk, intermediate in mice fed feedlot-finished beef or bison, and significantly decreased in mice fed range-fed bison, range-fed beef, or chicken (Broughton et al., 2011). [The Working Group noted that the study design did not include a no-meat control group. Thus, the comparison was done between types of meat, but the effect of meat per se was not assessed.]

Studies that reported on the effect of dietary haem on inflammation markers are described in Section 4.2.6 and Section 4.5.1.

(b) Modulation of receptor-mediated effects (hormones)

Regarding red meat, three observational human studies were found suggesting that red meat intake may be associated with slightly unfavourable insulin-like growth factor-1, sex hormone–binding globulin, or fasting insulin profiles. The associations (expressed as mean or median change across categories of red meat intake) were usually weak, and were often not confirmed by more than one study. In addition, sometimes the trend over categories lost statistical significance when BMI was included in the model (Allen et al., 2000; Brinkman et al., 2010; Ley et al., 2014).

No data from in vitro human studies, or from experimental systems, on hormones or receptor-mediated effects were available to the Working Group.

(c) Telomere length

O'Callaghan et al. (2012) showed that telomere length in colonocytes in Sprague-Dawley rats decreased in proportion to the level of red meat (15%, 25%, and 35% for 4 weeks) in their diet. High-amylose starch attenuated the effect of red meat.

4.2.5 Other relevant data and potential indirect mediators

(a) Dysregulation of the gut microbiota

Diet is a key factor in determining the composition of the human gut microbiota (Graf et al., 2015). A role for microbiota in the development of cancer has been described (Louis et al., 2014; Garrett, 2015), acting via various mechanisms. Bacterial metabolites such as hydrogen sulfide, secondary bile acids, polyamines, and reactive oxygen species (ROS) may provoke inflammation and affect carcinogenesis, while other metabolites such as acetate, propionate, and butyrate may exert protective activities (O'Keefe, 2008). Pathogenic bacteria, in particular, exert proinflammatory effects and might thus increase carcinogenesis (Louis et al., 2014). Specifically, several studies have reported a positive association between the gram-positive *Streptococcus gallolyticus* (previously named *Streptococcus bovis*) and cancer of the colorectum (Ellmerich et al., 2000; Tjalsma et al., 2006; Abdulamir et al., 2011).

As discussed in Section 4.5.2(b), Martin et al. (2015) observed reduced faecal TBARS when haemoglobin-fed rats were treated with a cocktail of antibiotics. Some *Lactobacillus* strains reportedly exert antioxidant behaviour by preventing the Fenton reaction (Sun et al., 2010), while other bacterial species such as *Enterococcus faecalis* can stimulate extracellular superoxide (Huycke & Moore, 2002). Although the lower faecal TBARS in antibiotic-treated rats in the study by Martin et al. (2015) could be the result of a diminished or altered colonic microbiome, this reduction could be attributed to the direct antioxidant or pro-oxidant effects of the applied antibiotics. For example, metronidazole, which was among the antibiotics administered by Martin et al. (2015), has been described to scavenge ROS in a cell-free environment (Narayanan et al., 2007) and to have an antioxidant effect in colonic tissues (Pélissier et al., 2007).

[The Working Group noted that no direct data on the dysregulation of the gut microbiota by red meat or processed meat were available.]

(b) Type 2 diabetes

A link between high–processed meat intake and diabetes has been hypothesized, and epidemiological meta-analyses have observed a positive association between diabetes and a variety of cancers, including cancer of the liver (El Serag et al., 2006), pancreas (Ben et al., 2011), endometrium (Zhang et al., 2013), colorectum (Larsson et al., 2005), and bladder (Larsson et al., 2006). Various mechanisms have been proposed, such as increased oxidative stress (Ihara et al., 1999; Ceriello & Motz, 2004). Hua et al. (2001) reported lower insulin sensitivity in healthy meat eaters compared with lacto-ovo-vegetarians. Lowering the iron content of the body by phlebotomy improved insulin sensitivity in the meat eaters. The development of insulin resistance increases circulating levels of insulin, triglycerides, and non-esterified fatty acids, which may stimulate colon cell proliferation (Bruce et al., 2000). Other possible mechanisms include the formation of NOCs, advanced glycation end products (AGEPs), trimethylamine *N*-oxide, branched amino acids, endocrine disruptor chemicals, and inflammation (Azadbakht & Esmaillzadeh, 2009; Tong et al., 2009; Kim et al., 2015). [The Working Group noted that a high–red meat and/or high–processed meat consumption may have an indirect stimulating effect on carcinogenesis by contributing to an increased BMI, which has also been linked to insulin resistance and an increased risk of diabetes.]

4.2.6 Studies of hemin and hemin chloride

In many rodent studies not previously mentioned, a model haem molecule was added to the diet as a surrogate for red meat: hemin chloride. This molecule is a protoporphyrin IX containing a ferric iron ion (haem B) stabilized

with a chloride ligand (Deo et al., 2015). It is not present in human diets, is not soluble in water, and quickly induces oxidation of polyunsaturated oils.

(a) Hemin and proliferation

The Van der Meer group repeatedly showed that dietary hemin chloride increased colonic epithelial proliferation and faecal water cytotoxicity in rats (e.g. Sesink et al., 1999, 2000, 2001). For instance, colonic epithelial proliferation increased in rats fed a purified diet supplemented with 1.3 μmol/g of hemin for 14 days. The faecal water of haem-fed rats contained approximately three-fold higher levels of faecal TBARS and was highly cytotoxic compared with that of control rats (Sesink et al., 1999). Cytotoxicity and proliferation were independent of dietary fat content, but were suppressed by dietary calcium phosphate and by dietary chlorophyll both of which bind physically to hemin (Sesink et al., 2000, 2001; de Vogel et al., 2005).

Winter et al. (2014) also showed that dietary hemin chloride increased proliferation in the short term and inhibited apoptosis in the long term in mice fed a high-fat, low-calcium control diet or a high-fat, low-calcium diet with hemin chloride (0.013%). Changes from 1 to 18 months showed increased cell proliferation ($P < 0.01$) in all groups, but only hemin chloride–fed mice showed reduced apoptosis ($P < 0.01$) (Winter et al., 2014).

(b) Hemin and inflammation

Several studies investigated the effect of dietary hemin on inflammation markers, showing various effects on myeloperoxidase in the gut mucosa (decreased, increased, or no change). In mice, dietary hemin exacerbated colitis induced by trinitrobenzene sulfonic acid, but decreased myeloperoxidase activity (Schepens et al., 2011). In mice fed a "Western-type" diet with 40% fat (mainly palm oil) and low calcium (30 μmol/g) for 14 days, dietary hemin resulted in a ruffled intestinal epithelium, which was attributed to luminal necrosis. However, there was no indication of local inflammation: no infiltration of neutrophils or macrophages in the lamina propria, no change in the expression of inflammation markers for macrophages (CD14, CD68, CD11b, and F4/80) and for neutrophils (myeloperoxidase, lactoferrin, neutrophil elastase, and EMR4), and no effect on mucins or on gene expression of secreted MUC2 (IJssennagger et al., 2012b). Finally, in rats given a high-fat safflower oil diet, dietary hemin chloride significantly increased colonic myeloperoxidase activity (Guéraud et al., 2015).

(c) Hemin in vitro

Hemin chloride was a potent growth factor in iron-depleted human colon cancer HT-29 cells, but it showed dose-dependent cytotoxic effects on the same cell line (Klenow et al., 2009). It had hyperproliferative effects on Caco-2 cells mediated by haem oxidase and hydrogen peroxide, which was shown using the inhibitors zinc protoporphyrin and catalase (Ishikawa et al., 2010).

[The Working Group noted that dietary hemin chloride markedly increased faecal water cytotoxicity and proliferation of the colonic epithelium in rats and mice. However, the relevance to red meat intake was unclear since the hyperproliferative effect was not reproduced with natural haemoprotein or meat.]

4.3 Precancerous lesions

4.3.1 Precancerous colorectal lesions

(a) Humans

(i) Red meat

Several cohort and case–control studies examined the association between red meat consumption and risk of colorectal adenomas. Of the cohort studies, all showed a positive, but not

statistically significant, association between red meat and risk of adenomas (Nagata et al., 2001; Chan et al., 2005a; Wu et al., 2006; Rohrmann et al., 2009b; Tantamango et al., 2011; Ferrucci et al., 2012). However, in a meta-analysis of these studies, the overall association was statistically significant: per 100 g/day increase in intake of red meat, the relative risk (RR) increased by 20% (95% CI, 1.06–1.36) (Aune et al., 2013). The meta-analysis of 10 case–control studies also yielded a positive association (OR, 1.34; 95% CI, 1.12–1.59). Several sensitivity analyses examined potential confounders, also addressed in Section 2.1.5 of this *Monograph*. These did not appreciably change the risk estimates, such that the associations of the meta-analysis were still statistically significant (Aune et al., 2013). As they are more likely to progress to adenocarcinomas than smaller adenomas, large adenomas were specifically evaluated in some studies, including the EPIC-Heidelberg study (OR, 1.98; 95% CI, 1.09–3.58; top vs bottom quintile) (Rohrmann et al., 2009b) and the Health Professionals Follow-Up Study (HPFS) (OR, 1.95; 95% CI, 0.97–3.91; top vs bottom quintile) (Wu et al., 2006). In single studies, differences in the adenoma characteristics and/or types of red meat were sometimes noted. For example, in the EPIC-Heidelberg study, a high intake of red meat and processed meat (combined) was related to an increased risk of colon adenomas (OR, 1.53; 95% CI, 1.01–2.30) and large adenomas (as noted above), but there was no statistically significant association with adenomas at all sites or small adenomas (Rohrmann et al., 2009b). Ferrucci et al. (2012) did not observe an association between red meat consumption and all types of adenomas. However, they found a statistically significant association between grilled meat (OR, 1.56; 95% CI, 1.04–2.36; top vs bottom quartile), and also well-done or very well-done meat (OR, 1.59; 95% CI, 1.05–2.43), and risk of rectal adenomas. No association was found between these meat types and colon adenomas.

The meta-analysis by Aune et al. (2013) also examined the effects of meat intake by type, and reported statistically significant positive associations between beef and pork intake and risk of adenoma (Aune et al., 2013). A meta-analysis of case–control studies reported a statistically significant increased risk of colorectal adenoma with beef consumption (meta-RR, 1.56; 95% CI, 1.15–2.10; I^2, 49.9%) (Carr et al., 2016).

(ii) Processed meat

Fewer studies examined the association between processed meat consumption and risk of colorectal adenomas. In the Prostate, Lung, Colorectal and Ovarian (PLCO) Cancer Screening Trial, a non-statistically significant increased risk of colorectal adenomas was observed with high–processed meat consumption (OR, 1.23; 95% CI, 0.99–1.52; top vs bottom quartile) (Ferrucci et al., 2012). In the HPFS, an association was found between high–processed meat consumption and risk of distal colon adenomas (OR, 1.52; 95% CI, 1.12–2.08; top vs bottom quintile), which was stronger than that found for red meat (OR, 1.18; 95% CI, 0.87–1.62; top vs bottom quintile) (Wu et al., 2006). A combined analysis of these two studies revealed a 45% increase in the risk of colorectal adenoma (95% CI, 1.10–1.90) per 50 g increase in consumption of processed meat per day (Aune et al., 2013). The only study that examined adenoma recurrence did not find any statistically significant association between processed meat consumption and risk of recurrence for any adenoma types, advanced adenomas, or multiple adenomas (Mathew et al., 2004). Overall, case–control studies showed positive associations between intake of processed meat and colorectal adenomas, but only a minority of these findings were statistically significant. For example, a 30% increased risk of adenoma (95% CI, 1.1–1.5) was observed in the Tennessee Colorectal Polyp Study (TCPS) with 1881 cases (Fu et al., 2011), but no increased risk of adenoma was found in

the PLCO trial with 3696 cases (OR, 1.04; 95% CI, 0.90–1.19) (Sinha et al., 2005b). A meta-analysis of eight case–control studies found a positive association that did not reach statistical significance (OR, 1.23; 95% CI, 0.99–1.52) (Aune et al., 2013).

Several studies reported on specific types of processed meats, including bacon and sausage. Higher intake of bacon and sausage was associated with an increased risk of colorectal adenoma (OR, 1.14; 95% CI, 1.00–1.30) in the PLCO trial (Sinha et al., 2005b). Similarly, in the TCPS, several different types of processed meats (e.g. hot dogs, sausage, or bacon) were associated with an increased risk of adenoma (Fu et al., 2011).

[The Working Group noted that the epidemiological studies supported a positive association between red meat and processed meat consumption and risk of colorectal adenomas. However, results differed with respect to the type of meat, as well as the site, size, number, and histology of the adenomas.]

(b) Experimental systems

(i) Red meat

Parnaud et al. (1998) first studied the effect of meat (beef, chicken, and bacon) on aberrant crypt foci (ACF). Azoxymethane-induced F344 female rats were randomized to 10 different AIN-76–based experimental diets, all high in calcium. Five diets were adjusted to include 14% fat and 23% protein (standard levels), and five other diets were adjusted to include 28% fat and 40% protein (high levels). Fat and protein were supplied by either lard and casein, olive oil and casein, beef, chicken with skin, or bacon. The meat diets contained 30% or 60% freeze-dried, fried meat. The rats were fed ad libitum for 100 days, and ACF multiplicity (the number of crypts forming each focus) was assessed as a parameter related to tumour promotion. ACF multiplicity was similar among the rats, except for the bacon-fed rats.

The same investigators studied the effects of various meats on ACF, using diets high in calcium (Parnaud et al., 2000). Rats fed a diet containing beef, pork, or chicken meat had a lower concentration of faecal NOCs than those fed the control diet ($P < 0.01$). In the promotion experiment, unprocessed, cooked meat–based diets did not change the number or multiplicity of ACF compared with the control diet.

Hypothesizing that a high level of calcium in the diet may mask the potential carcinogenicity of red meat, subsequent studies administered meat to azoxymethane-induced F344 rats fed a low-calcium diet (0.8%; mimicking the "Western-type" diet). Accordingly, Pierre et al. (2004) formulated the meat-based diets to contain varying concentrations of haem with the addition of raw chicken (low haem), beef (medium haem), or black pudding (blood sausage, high haem). Chicken, beef, and black pudding were administered at 60% of the diet, thus providing a higher intake of protein than the standard nutritional intake of protein (20% of the diet) for rats. Only diets with haem significantly promoted mucin-depleted foci (MDF) formation ($P < 0.01$), but all meat diets promoted ACF formation. MDF promotion was greater with the high-haem black pudding diet than with the medium-haem beef diet. MDF promotion was also correlated with increased lipid peroxides in faecal water, measured by TBARS, and cytotoxicity in erythrocytes and the mouse epithelial cell line CMT93 ($r = 0.65$; $P < 0.01$).

The same group of researchers tested whether calcium and various antioxidants would reduce the promotion of preneoplastic lesions in DMH-induced F344 rats fed red meat (Pierre et al., 2008). Three diets with 60% beef meat were supplemented with calcium phosphate (31 g/kg diet), antioxidant agents (rutin and butylated hydroxyanisole, 0.05% each), and olive oil (5%). The beef meat diet significantly increased the number of ACF (+30%) and MDF (+100%). These results were associated with increased faecal

water TBARS (4-fold) and cytotoxicity in CMT93 cells (2-fold), and urinary DHN-MA excretion (15-fold). Calcium fully inhibited beef meat–induced ACF and MDF promotion, and normalized faecal TBARS and cytotoxicity; however, it did not reduce urinary DHN-MA. The antioxidant mix and olive oil did not normalize beef meat promotion or lipid peroxides.

The effects of red meat and whey protein on azoxymethane-induced ACF were studied by Belobrajdic et al. (2003). Wistar rats were fed red meat (barbecued kangaroo muscle meat) or whey protein concentrate to provide 8%, 16%, and 32% protein by body weight in a modified AIN-93 diet with low fibre, low calcium (0.1%), and high polyunsaturated fat. The 32% whey protein group had significantly fewer ACF in the proximal colon than the 16% and 32% red meat groups ($P < 0.05$). No effect of the diets was observed in the distal colon.

Khil & Gallaher (2004) examined the effects of individual red meat components (beef protein and tallow) on DMH-induced ACF and colon apoptosis and proliferation. DMH-induced Sprague-Dawley rats were fed either casein or beef protein as the protein source, and either soybean oil or tallow as the fat source, for 9 weeks in an AIN-93 standard diet. Rats fed tallow had fewer ACF (only determined in a portion of the distal colon) and significantly higher apoptosis compared with those fed soybean oil. In addition, faecal bile acid concentrations were significantly lower in rats fed tallow than in those fed soybean oil. There were no significant differences in mucosal cell proliferation.

[The Working Group noted that red meat given to carcinogen-initiated animals promoted the growth of preneoplastic lesions in the colon, and that this effect could be modified by factors such as calcium and antioxidants.]

(ii) Processed meat

In the study by Parnaud et al. (1998) previously mentioned, a significant reduction in ACF multiplicity was observed in bacon-fed, carcinogen (azoxymethane)-initiated rats compared with control rats when calcium levels in the diet were high.

Parnaud et al. (2000) also assessed the effect of a high-fat, high-calcium, bacon-based diet on ACF number and multiplicity in the colon of F344 rats (Parnaud et al., 2000). As previously mentioned, other meats tested were pork, chicken, and beef. The faeces of the rats fed the bacon-based diets contained 10–20 times more NOCs than the faeces of the rats fed the casein-based control diet ($P < 0.0001$). No ACF were detected in the colon of uninitiated, bacon-fed rats. The number of large ACF per rat and ACF multiplicity were consistently reduced by 12% and 20% in rats fed a 30% or 60% high-fat, bacon-based diet and by 17% in rats fed a 30% low-fat, bacon-based diet (all $P < 0.01$). [The Working Group noted the lack of effect of dietary bacon on rat colon carcinogenesis in the context of a high-calcium diet.]

Using diets containing low levels of calcium (0.8 g/kg diet), Pierre et al. (2010) showed that a freeze-dried, cooked, cured ham diet fed for 100 days to DMH-induced F344 rats significantly increased the number of MDF in the colon. Promotion was associated with cytotoxicity and lipid peroxidation. In a short-term study (14 days) by the same authors, the cytotoxicity (tested in CMT93 cell lines) and lipid peroxidation (TBARS) of faecal water, and the urinary marker of lipid peroxidation (DHN-MA), increased dramatically in ham- and hemin-fed rats; however, this effect was not observed in rats fed the haemoglobin diet or the sodium chloride (NaCl), nitrite, phosphate diet.

This group also demonstrated that experimental cured meat diets (dark cooked pork meat with nitrite, oxidized; dark cooked meat with

nitrite, anaerobic; dark cooked meat, oxidized; dark raw meat, anaerobic; with dark meat obtained from supraspinatus and infraspinatus pig muscle, which contained 15–17 mg of haem per 100 g) fed to DMH-induced rats for 100 days significantly increased the number of ACF per colon compared with the no-meat control diet (Santarelli et al., 2010).

In another study, DMH-induced rats were fed a diet containing hot dogs or saucisson (fermented, raw, dry sausage) (40% and 50% on a dry basis) for 100 days (Santarelli et al., 2013). The hot dog diet significantly increased the number of MDF per colon. The saucisson diet increased the number of MDF per colon, but the increase lacked statistical significance compared with the no-meat control diet. The addition of calcium carbonate (150 µmol/g) to the hot dog diet decreased the number of MDF per colon and faecal ATNC compared with the hot dog diet without calcium carbonate.

In DMH-induced F344 rats, the addition of calcium or α-tocopherol to a diet containing cured pork meat (47% meat diet for 100 days) also significantly reduced the number of MDF per colon, but the number of ACF was not affected (Pierre et al., 2013).

[The Working Group noted that results from a single laboratory showed three different kinds of processed meat given to carcinogen-induced animals promoted the growth of preneoplastic lesions in the colon.]

(iii) Haem and other components of red and processed meat

Pierre et al. (2003) showed that haemoglobin or hemin, the ferric porphyrin component of haemoproteins, promoted ACF in azoxymethane-induced rats when dietary calcium was low. This result suggested that myoglobin, the haemoprotein present in red meat, could also promote cancer of the colon when dietary calcium is low.

Santarelli et al. (2010) further evaluated 4 of 16 diets containing cured meat that modified biomarkers of haem-induced carcinogenesis promotion (faecal and urinary fat oxidation and cytotoxicity) in a short-term (14-day) study. The diets differed in muscle colour (a proxy for haem level), processing temperature, nitrite, and packaging. In DMH-induced rats fed for 100 days, only the cooked, nitrite-treated and oxidized, high-haem meat diets significantly increased faecal levels of apparent total *N*-nitroso compounds (ATNC) and the number of MDF per colon compared with the no-meat control diet. Specifically, the cooked, nitrite-treated and oxidized, high-haem meat diets increased the number of MDF compared with the cooked, non–nitrite-treated meat diet and with the non-oxidized, high-haem meat diet; faecal ATNC levels were 5–15 times higher in the cooked nitrite-treated and oxidized high-haem meat diets than in the other diet groups, but lipid oxidation products (TBARS) in faecal water and urinary DHN-MA were lower in these groups than in the other selected meat diet groups.

In DMH-induced F344 rats, various biomarkers (TBARS in faecal water and cytotoxicity of faecal water in CMT93 cell lines, ATNC in faeces and urinary DHN-MA) were all significantly reduced by the addition of calcium to a diet containing cured pork meat (47% meat diet for 100 days), while α-tocopherol decreased only the concentration of haem in faecal water and DHN-MA in urine (Pierre et al., 2013). Within the same report, Pierre et al. (2013) also showed that the consumption of cured meat increased ATNC and lipid peroxidation (TBARS) in the faeces of human volunteers (both $P < 0.05$). Calcium normalized both biomarkers in the human faeces, whereas α-tocopherol only decreased lipid peroxidation in the human faeces (all $P < 0.05$).

Bastide et al. (2015) investigated the role of various components present in red meat, including haem iron, HAAs, and endogenous NOCs, in causing promotion of cancer of the colon. The relative contribution of haem iron (1% of the diet), HAAs (PhIP and MeIQx, 50 + 25 µg/kg

diet), and NOCs (induced by sodium nitrite and sodium nitrate, 0.17 + 0.23 g/L drinking-water) was determined in chemically (azoxymethane) induced rats and in *Min* mice (fed a 2.5% haemoglobin diet). Haem iron increased the number of preneoplastic lesions (MDF) in rats, but dietary HAAs and NOCs had no effect. Dietary haemoglobin increased tumour load in the small intestine of the *Min* mice ($Apc^{Min/+}$) (control diet, 67 ± 39 mm^2; 2.5% haemoglobin diet, 114 ± 47 mm^2; $P = 0.004$). In vitro, faecal water from rats given dietary haemoglobin was rich in aldehydes and was cytotoxic to normal cells ($Apc^{+/+}$), but not to *Apc*-deficient cell lines ($Apc^{-/+}$). The aldehydes 4-HNE and 4-hydroxyhexenal were more toxic to normal cells than mutated cells, and were only genotoxic to normal cells. Genotoxicity (measured by γ-H2AX for DNA double-strand breaks) was also observed in the small intestine of *Min* mice given haemoglobin.

[The Working Group noted that these studies, coming from a single laboratory, highlighted the contribution of haem iron in the promotion of preneoplastic lesions by red meat. One study also suggested that in cured meat–fed rats, the driving mechanism of promotion was due to NOCs, and not to lipid peroxidation products.]

4.3.2 Other precancerous lesions in exposed humans

(a) Barrett oesophagus

Barrett oesophagus is defined as the replacement of oesophageal squamous epithelium with metaplastic columnar epithelium. Four epidemiological studies, three case–control studies and one cohort study, examined whether the consumption of meat is related to risk of Barrett oesophagus (Kubo et al., 2009; O'Doherty et al., 2011; Jiao et al., 2013; Keszei et al., 2013). Only the USA case–control study observed a statistically significant association between total meat consumption and risk of Barrett oesophagus (multivariate-adjusted OR, 1.91; 95% CI, 1.07–3.38; top vs bottom tertile) (Jiao et al., 2013), but none of the other three studies observed this same risk (Kubo et al., 2009; O'Doherty et al., 2011; Keszei et al., 2013). However, the USA case–control study by Jiao et al. (2013) did not differentiate between the consumption of red, white, and processed meat. In another USA case–control study, there was no association between total meat, well-done meat, or barbecued meat consumption and risk of Barrett oesophagus (Kubo et al., 2009), but an analysis of the same case–control study reported a positive association between a "Western-type" dietary pattern, which is characterized by a high intake of red and processed meat, and risk of Barrett oesophagus (Kubo et al., 2008).

(b) Gastric intestinal metaplasia

Gastric intestinal metaplasia is considered a precursor lesion of cancer of the stomach (Correa et al., 1975). Based on four studies (Nomura et al., 1982; Stemmermann et al., 1990; Fay et al., 1994; Chen et al., 2004), a meta-analysis reported a combined odds ratio of 1.68 (95% CI, 0.98–2.90) for the association between salted/salty meat and intestinal metaplasia, but the heterogeneity between studies was large (I^2, 55.4%), which may have been due to their use of different definitions of foods (e.g. all processed meat, cured meat, or bacon only), types of dietary assessment methods, or subgroups of the population (some studies were conducted only among men) (Dias-Neto et al., 2010).

4.4 Cancer susceptibility

4.4.1 Genetic polymorphisms

(a) Humans

(i) Red meat and certain meat components

Several studies have suggested an increased risk of cancer of the colorectum in individuals with *NAT2* rapid acetylator status (individuals

with two "rapid" alleles), assessed by phenotyping or genotyping. However, meta-analyses of the literature on *NAT2* acetylator status (rapid/intermediate vs slow genotype or phenotype) have typically not confirmed such a main effect association (Brockton et al., 2000; Liu et al., 2012; Zhang et al., 2012). This is also true of other cancers, such as those of the lung (Cui et al., 2011), stomach (Zhong et al., 2010), and breast (Ochs-Balcom et al., 2007; Ambrosone et al., 2008), as well as non-Hodgkin lymphoma (Gibson et al., 2013). Unfortunately, adding to the difficulty of interpreting these data, only a few studies, and no meta-analysis or pooled analysis, have reported risk estimates specifically for rapid acetylators– the subset expected to be at the greatest risk. Instead, grouping intermediate with rapid acetylators has been the norm, especially for populations in which the latter phenotype is relatively rare (e.g. Europeans). The inconsistent results for *NAT2* in cancer of the colorectum are in sharp contrast to those for *NAT2* in cancer of the bladder; the slow *NAT2* acetylator status has consistently been associated with an increased risk of cancer of the bladder (except for benzidine (Rothman et al., 1996)), due to the ability of NAT2 to detoxify arylamines, as shown in a meta-analysis and found in a genome-wide association study (Marcus et al., 2000; Figueroa et al., 2014).

A smaller number of studies have explored associations between polymorphisms in other genes involved in the metabolism of HAAs and PAHs (e.g. *CYP1B1*, *GSTM1*, *GSTT1*, *SULT1A1*, *UGT2B17*) and cancer risk. The results of these studies have also been inconsistent or have not been replicated (Andersen & Vogel, 2015). Genome-wide association studies have recently shown that common (i.e. allele frequency > 5%) genetic variants have only a small effect on cancer risk. Importantly, few of the risk variants identified in cancer genome-wide association studies have been in metabolic genes, suggesting that stratification of exposure and very large samples sizes are needed to identify such associations. Indeed, it can be expected that polymorphisms in xenobiotic-metabolizing enzymes (XMEs) involved in carcinogen activation or detoxification would only affect cancer risk when there is a high, biologically sufficient level of exposure to a carcinogen. Thus, it is likely important to consider both the exposure and the genetic variants to detect an association with cancer risk.

Studies that have examined the combined effects of exposure (e.g. red meat, well-done meat, or HAA intake) and metabolic genotypes or phenotypes have mainly focused on cancer of the colorectum and its precursor, adenomatous polyps. Interactions were suggested between intake of red meat, well-done meat, or HAAs and *NAT2* acetylator status (Welfare et al., 1997; Kampman et al., 1999; Chan et al., 2005b; Lilla et al., 2006; Nöthlings et al., 2009; Voutsinas et al., 2013), *NAT1* (Ishibe et al., 2002; Gilsing et al., 2012), *AHR* (Wang et al., 2011), *CYP1B1* (Cotterchio et al., 2008; Wang et al., 2011), *CYP1A1* (Turner et al., 2004, Little et al., 2006; Goode et al., 2007), *CYP2E1* (Morita et al., 2009), *EPHX1* (Cortessis et al., 2001; Ulrich et al., 2001; Goode et al., 2007), *NQO1* (Turner et al., 2004), *SULT1A1* (Cotterchio et al., 2008; Barbir et al., 2012), and *UGTs* (Butler et al., 2005; Girard et al., 2008), as well as with a combination of metabolic genes (*CYP1A2*, *CYP2E1*, *CYP1B1*, and *CYP2C9)* (Küry et al., 2007) and with a polygenic risk score based on variants in *AHR*, *CYP1A2*, *CYP1B1*, *NAT2*, *SULT1A1*, *UGT1A7*, *GSTM1*, and *GSTT1* (Fu et al., 2012) on the risk of colorectal neoplasia. A meta-analysis of three cohort studies (1404 cases, 2186 controls) (Chen et al., 1998, Chan et al., 2005b, Nöthlings et al., 2009) on the modifying effect of *NAT2* on the association between red meat and cancer of the colorectum suggested an interaction between *NAT2* status and meat intake. High–red meat intake or preference for browned meat was not associated with an increased risk of cancer of the colorectum in carriers of the slow *NAT2* phenotype. In

contrast, *NAT2* fast acetylators with high-meat intake were at increased risk (OR, 1.25; 95% CI, 0.92–2.01) compared with *NAT2* slow acetylators with low-meat intake ($P_{interaction}$ = 0.07) (Andersen et al., 2013). However, other studies, some with large sample sizes, failed to replicate this interaction between red meat intake and *NAT2* acetylator status on risk of cancer of the colorectum or adenoma (Barrett et al., 2003; Murtaugh et al., 2004; Sørensen et al., 2008; Ananthakrishnan et al., 2015; Budhathoki et al., 2015). Of note, the recent pooled analysis conducted by the Genetics and Epidemiology of Colorectal Cancer Consortium (GECCO) (8290 cases, 9115 controls) found no interaction between red meat or processed meat intake and *NAT2* acetylator status in case–control and cohort studies (separately and overall) (Ananthakrishnan et al., 2015). High–red meat intake was similarly associated with cancer of the colorectum in subjects with the rapid/intermediate *NAT2* genotype (OR, 1.38; 95% CI, 1.20–1.59) and in subjects with the slow *NAT2* genotype (OR, 1.43; 95% CI, 1.28–1.61; $P_{interaction}$ = 0.9).

Only four studies of adenoma and/or cancer of the colorectum have considered NAT2 jointly with CYP1A2 activity, which, as previously mentioned, shows high inter-individual variability and may account for individual differences in susceptibility to HAAs. Two of the four studies were case–control studies, and both found that rapid NAT2 activity combined with rapid CYP1A2 activity was a risk factor for colorectal neoplasia or cancer of the colorectum in individuals who ate well-done meat (Lang et al., 1994; Le Marchand et al., 2001). However, in one of the case–control studies, this association was limited to smokers (Le Marchand et al., 2001), which is consistent with the inducing effect of smoking on CYP1A2. In the third study, only NAT2 activity, and not CYP1A2 activity, showed an interaction with HAA intake on the risk of adenoma (Voutsinas et al., 2013). The fourth study failed to observe any modifying effect of NAT2 or CYP1A2 activity, also measured by caffeine phenotyping, on the relationship between HAAs and adenoma (Ishibe et al., 2002).

Fewer studies have examined the interaction between meat intake and genetic polymorphisms on the risk of other cancer sites. However, multiple reports have focused on cancer of the breast and *NAT2* (Ambrosone et al., 1998; Gertig et al., 1999; Deitz et al., 2000; Delfino et al., 2000; Mignone et al., 2009; Lee et al., 2013), *CYP1A2* (Lee et al., 2013), *GSTM1* (Zheng et al., 2002), and *SULT1A1* (Lee et al., 2012). Similar to the literature on cancer of the colorectum, publications on cancer of the breast have been inconsistent.

Other cancer-related mechanisms, such as DNA repair, have been explored in studies of cancer, genetic variation, and meat intake. For example, a variant in *MGMT*, a gene involved in the repair of DNA damage caused by alkylating agents, including NOCs from the diet, was found to interact with both red and processed meat intake on the risk of cancer of the colorectum (Loh et al., 2010). Variants in the nucleotide excision repair enzyme gene, xeroderma pigmentosum group D (*XPD*), have also been found to increase the risk of cancer of the colorectum when combined with a high intake of heavily browned red meat (Joshi et al., 2009).

Thus, a large number of studies have evaluated the role of genetic polymorphisms in an attempt to clarify the association between cancer susceptibility and red meat consumption. Historically, these studies have focused on suspected mechanisms, and mainly on genes involved in the metabolism of carcinogens present in cooked red meat. The results of these candidate gene studies have mostly been inconsistent. Many were underpowered and had multiple testing, publication, and reporting biases. Inconsistencies in the gene–meat interaction studies may also have resulted from differences in the comprehensiveness of the dietary assessments or the lack of consideration for cytochrome P450 (CYP) enzyme inducers (e.g. smoking). The strongest

evidence provided by these studies supported an interaction between *NAT2*, red meat intake, and risk of cancer of the colorectum. However, as previously noted, meta-analyses and pooled analyses (Brockton et al., 2000; Liu et al., 2012; Zhang et al., 2012; Ananthakrishnan et al., 2015) have failed to confirm a main effect or modifying effect of *NAT2* or other genes on cancer of the colorectum or other cancers. Insufficient focus has been given to the group expected to be at the highest risk – those with two rapid alleles. Data are lacking for populations in which this genotype is common, and that consume significant amounts of well-done meat and have high rates of cancer of the colorectum (e.g. Japan and Republic of Korea).

In recent years, genome-wide association studies have identified several cancer susceptibility loci, each with a relatively small effect on risk. Statistical methods have been developed to analyse interactions between diet and variants across the entire genome. These analyses, with the currently available sample sizes, have rarely replicated results from candidate gene studies and have not identified interactions with red meat intake (Jiao et al., 2012; Kantor et al., 2014). However, larger sample sizes are needed to detect modest or weak interactions.

(ii) Processed meat

Processed meat has not always been examined separately from red meat in studies of genetic polymorphisms. A population-based case–control study in Hawaii, USA (Le Marchand et al., 2002a) found an increased risk of cancer of the colorectum in individuals who consumed a high amount of red meat or processed meat and who carried a variant in *CYP2E1* that had been shown to alter enzyme activity (Lucas et al., 1995; McCarver et al., 1998; Le Marchand et al., 1999). This association was more pronounced for cancer of the rectum and was observed in individuals who consumed salted/dried fish and oriental pickled vegetables, both food sources of NOCs. An association with the same *CYP2E1* variant and cancer of the stomach was also observed (Nishimoto et al., 2000; Chen et al., 2004).

Finally, a genome-wide search for diet–gene interactions identified an interaction between processed meat intake and a variant (rs4143094) on 10p14 (near *GATA3*) on the risk of cancer of the colorectum (Figueiredo et al., 2014). Although the mechanism was unclear, *GATA3* was involved in cell maturation, proliferation arrest, and survival. Loss, or silencing, of expression of *GATA* genes has been described in colorectal tumours.

[The Working Group noted that few studies have explored the role of genetic susceptibility as a potential modifier of the association between processed meat and cancer. These studies have typically been small, and have not allowed for any conclusions to be drawn.]

(b) Experimental systems

Two studies by the same research group showed that mice humanized for CYP1A2 are more susceptible to HAAs (e.g. PhIP) than wild-type mice (Cheung et al., 2011; Li et al., 2012). [The Working Group noted that the doses used in these studies were greater than human exposure levels, and the relative levels of h*CYP1A2* expression may have exceeded the range in humans.]

4.4.2 Microflora

Evidence is also available concerning individual differences in intestinal microflora profiles that may affect the carcinogenic effect of red meat. In rodents, the gut microbiota has been shown to facilitate haem-induced hyperproliferation by opening the mucous barrier (Ijssennagger et al., 2015). Similarly, it has been suggested that intestinal microflora play an important role in the bioactivation of HAAs. Kassie et al. (2004) inoculated intestinal flora collected from either vegetarians or meat eaters into germ-free rats. The rats were fed a diet mimicking the donors' diets, in terms of the origin of the protein and

fat (animal or plant). After oral administration of 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ), DNA damage in both colon and liver cells, as determined by comet assay, was significantly lower in animals harbouring the flora from vegetarians than in those harbouring the flora of the meat eaters.

The human intestinal microbiota has been shown to selectively convert PhIP to a major metabolite, 7-hydroxy-5-methyl-3-phenyl-6,7,-8,9,-tetrahydropyridol[3′,2′:4,5]imidazo[1,2-*a*]pyrimidin-5-ium chloride (PhIP-M1) (Vanhaecke et al., 2008a). PhIP-M1 was found to cause cell division arrest and induce DNA strand breaks in the human epithelial intestinal colon carcinoma Caco-2 cell line (Vanhaecke et al., 2008b), suggesting that the ability of the colon microbiota to bioactivate PhIP may affect the risk of cancer of the colorectum. [The Working Group could not make any conclusions regarding effect modification due to the microbiota.]

4.5 Meat components potentially involved in carcinogenesis

4.5.1 Haem iron

(a) Iron intake and digestion

One of the defining characteristics of red meat is its haem iron content. Two types of iron occur in foods: haem iron (organic) and non-haem iron (inorganic) (Fonseca-Nunes et al., 2014). Haem, which is made up of an iron atom surrounded by a porphyrin ring, is included in haemoglobin and myoglobin, and is involved in supplying oxygen to the body's tissues (Bastide et al., 2011). The redness of meat is mainly determined by its concentration of myoglobin, with the oxidation state and sixth ligand of iron determining the specific colour of meat. Haem iron in nitrite-cured meat is mostly nitrosylated (Demeyer et al., 2015).

Iron overload has many adverse health effects, irrespective of the iron source. While the human body maintains homeostatic control of this trace element, the absorption of haem iron in the small intestine is less regulated and more efficient (15–40%) than the absorption of non-haem iron (Layrisse et al., 1969; Carpenter & Mahoney, 1992; Hooda et al., 2014). Red meat is the largest dietary source of haem iron. Non-haem iron, which is present in both animal and vegetable sources, accounts for the majority of total dietary iron intake and has a wide range of absorption (1–40%). The absorption of non-haem iron is influenced by the body's iron stores, hypoxia, and erythropoietic activity, as well as by the intake of vitamin C, calcium, and haem iron (Layrisse et al., 1969; Carpenter & Mahoney, 1992; Fonseca-Nunes et al., 2014).

Haem in meat may undergo modifications during processing and digestion. Depending on the time and temperature, myoglobin is denatured after cooking, and the haem moiety is liberated. Haem iron can also be converted, to varying degrees, into non-haem iron by heat treatment (Kristensen & Purslow, 2001; Purchas et al., 2006). Purchas et al. (2006) showed an overall loss of iron from cooking of beef, together with a marked shift from soluble haem and non-haem iron to their insoluble forms. However, after simulated stomach and duodenal digestion, solubility was regained to a significant extent. Kristensen & Purslow (2001) reported that NaCl, widely used in meat processing, increased the haem:non-haem ratio in cooked meat by preventing the haem molecule from liberating iron, whereas calcium ions had a negative effect on the haem:non-haem ratio during cooking of meat. Thus, the type of processing and the cooking conditions affected the content and solubility of haem and free iron in meat, determining the absorption of iron in the proximal gut, and thus the amount that entered the distal gut.

(b) Mechanisms of carcinogenesis

Possible mechanisms by which haem iron may promote colon carcinogenesis include its catalytic effect on the formation of NOCs and on the oxidation of polyunsaturated fats. A third potential mechanism involves its direct effect on colon cells (Bastide et al., 2011; Corpet, 2011; Fonseca-Nunes et al., 2014).

A first possible mechanism of tumour promotion by haem iron is related to NOC formation by *N*-nitrosation of amines and amides by bacterial decarboxylation of amino acids from meat in the presence of a nitrosating agent. A controlled feeding study showed that high–red meat consumption is associated with greater excretion of ATNC (Cross et al., 2003). ATNC is a collective term that encompasses nitrosyl iron, *S*-nitrosothiols, nitrosamines, and nitrosamides. In another controlled feeding study, the endogenous production of NOCs was further enhanced when the diet contained haem iron from blood sausage compared with red or white meat (Cross et al., 2003; Hammerling et al., 2015). The main ATNC in the faeces of study participants fed a red meat diet was nitrosyl haem, but in those fed cured meat, NOCs predominated (Joosen et al., 2009; Corpet, 2011). Similarly, in animal studies, diets containing red and processed meat increased faecal NOCs (Mirvish et al., 2003; Demeyer et al., 2015). Some NOCs are carcinogenic compounds, inducing multisite tumours in animals (Lijinsky, 1992).

Several mechanisms have been suggested to explain the effect of haem on faecal ATNC content. One hypothesis concerns the combined action of haem and free thiols on NOC formation. Nitrosothiols are readily formed under the acidic conditions of the stomach, a process that is promoted by haem, and may release nitric oxide (NO) once they are exposed to the alkaline and reductive conditions of the small and large intestine, thereby stimulating the nitrosylation of haem iron. Nitrosyl haem is an NO donor and can act as a nitrosating agent in the lower gut (Kuhnle et al., 2007). Although an increase in ATNC after consumption of red and processed meat has been demonstrated, the potential carcinogenicity of the NOCs formed in the gut is unclear (Demeyer et al., 2015); this is addressed further in Section 4.5.5. A second hypothesis that has been proposed is that changes in the microbiota may be related to NOC production (Ijssennagger et al., 2012, 2013, 2015). Ijssennagger et al. (2012) showed a distinctive shift in the colonic microbial composition of mice fed a Westernized diet (40% fat) supplemented with 0.5 µmol/g of haem iron compared with mice fed the same diet without haem iron. After 2 weeks, the colonic contents of the mice given haem iron contained higher amounts of Bacteroidetes (gram-negative) and lower amounts of Firmicutes (gram-positive) than those not given haem iron supplementation. After the haem iron supplementation, Ijssennagger et al. (2012) also observed an increase in the nitrate-reducing capacity of the colonic microflora, while the sulfate-reducing capacity was unchanged. This increase by haem iron in the nitrate-reducing capacity might be important, as considerable inter-individual variation was observed in the ability of different individual porcine and human microbiota to form NOCs and NOC-specific DNA adducts (Engemann et al., 2013; Vanden Bussche et al., 2014). Similarly, Van Hecke et al. (2014b) showed that haem iron had a stimulating effect on O^6-CMG production during in vitro fermentation of meat.

A second possible mechanism of tumour promotion by haem iron involves its ability to catalyse the oxidation of polyunsaturated fats (Corpet, 2011). The formation of lipid oxidation products is discussed in Section 4.5.2. Tumour promotion was found to be associated with increased urinary excretion of DHN-MA, a fat peroxidation biomarker, in rats after intake of haem (Pierre et al., 2006). An increase in this biomarker was also observed in humans

consuming blood sausage, which is high in haem (Pierre et al., 2006).

A third possible mechanism of tumour promotion by haem iron involves a direct effect of haem or one of its metabolites on colon cells. In vitro studies by Glei et al. (2002) showed that, when haemoglobin was added to a culture medium, it was taken up by human colon cells and participated in the induction of oxidative DNA damage such as DNA breaks and oxidised bases. As reported in Section 4.2.6, the Van der Meer group (e.g. Sesink et al., 1999) showed that supplementing a diet with hemin chloride, which is not present in food, increased epithelial proliferation and enhanced apoptosis in the colonic mucosa, and induced cytotoxicity in faecal water. Cytotoxicity-induced stress, rather than oxidative stress of surface cells, was the determinant of hemin-induced hyperproliferation.

(c) Epidemiological studies

Methods for estimating haem intake in epidemiological studies are varied. The information on haem iron concentrations in meats was sparse, partially due to the lack of appropriate analytical methods, and the variable concentrations across the range of meat types (e.g. beef, chicken, or pork), cuts of meats from the same animal, and methods of preparation (Kongkachuichai et al., 2002; Lombardi-Boccia et al., 2002; Cross et al., 2012). Two methods for estimating haem iron were to use 40% of total iron from meat (Lee et al., 2005) or to use meat-specific proportions (65% for beef; 39% for pork, ham, bacon, pork-based luncheon meats, and veal; and 26% for chicken and fish) (Balder et al., 2006). Recently, a haem iron database and complementary FFQs were developed to estimate haem iron intake from meats prepared by different cooking methods to a range of doneness levels (Cross et al., 2012) for use in etiological studies.

The inconclusive data for an association between haem iron intake and a variety of cancers may be partially explained by inconsistencies in the methods used to measure haem iron intake. Haem iron was positively associated with colorectal adenomas in two cohort studies: the PLCO trial (Ferrucci et al., 2012) and the National Institutes of Health – American Association of Retired Persons (NIH-AARP) Diet and Health Study (Cross et al., 2010). In a meta-analysis of five prospective studies, the summary relative risk for cancer of the colon was 1.18 (95% CI, 1.06–1.32) for those in the highest versus lowest category of haem iron intake (Bastide et al., 2011). In two other meta-analyses of eight studies each, the summary relative risks for cancer of the colorectum were 1.14 (95% CI, 1.04–1.24) for the highest versus the lowest category of haem iron intake (Qiao & Feng, 2013) and 1.08 (95% CI, 1.00–1.17) for an increase of 1 mg/day in the intake of haem iron (Fonseca-Nunes et al., 2014). Although these analyses were suggestive of a significant but modest increased risk, the measurement of haem iron intake differed in each of the studies included.

In the NIH-AARP study, individuals in the highest category of haem iron intake were at increased risk of cancer of the lung (Tasevska et al., 2009) and prostate (Sinha et al., 2009), as well as chronic liver disease mortality (Freedman et al., 2010), but not hepatocellular carcinoma (hazard ratio, HR, 0.95; 95% CI, 0.68 –1.32; top vs bottom quintile) (Freedman et al., 2010), non-Hodgkin lymphoma (Daniel et al., 2012b), or cancer of the breast (Kabat et al., 2010); haem iron intake was also not associated with cancer of the breast in the PLCO trial (Ferrucci et al., 2009). In a meta-analysis of four studies, the summary relative risk for cancer of the breast was 1.03 (95% CI, 0.97–1.09) (Fonseca-Nunes et al., 2014), and the summary relative risk for cancer of the lung was 1.12 (95% CI, 0.98–1.29) for an increase of 1 mg/day in the intake of haem iron (Fonseca-Nunes et al., 2014). Results were heterogeneous for cancer of the stomach (Fonseca-Nunes et al.,

2014) and oesophagus (Cross et al., 2011a; Steffen et al., 2012).

Regarding specific gene mutations in colorectal tumours, haem iron intake was positively associated with an increased risk of colorectal tumours with P53 overexpression, but not colorectal tumours without P53 overexpression in the NLCS (Gilsing et al., 2013). Haem iron intake was associated with an increased risk of colorectal tumours harbouring G→A transitions in *K-RAS* and *APC*, and overexpression of TP53 (Gilsing et al., 2013).

4.5.2 Lipid oxidation products

(a) Lipid oxidation in meat

The oxidation of unsaturated fatty acids in meat results in the formation of lipid oxidation products, which are in part cytotoxic and genotoxic (Kanner, 2007; Guéraud et al., 2010). Polyunsaturated fatty acids are especially sensitive to oxidation, which proceeds via a free radical chain reaction involving initiation, propagation, and termination steps. Transition metals, especially iron, catalyse this reaction, in the presence of oxygen, producing unstable ROS, including superoxide, hydrogen peroxide, and hydroxyl radicals. The ROS initiates a chain of oxidative reactions, generating lipoperoxyl radicals and lipid hydroperoxides. Lipid hydroperoxides may decompose to several low-molecular-mass break-down products, such as aldehydes and hydroxyalkenals, or condense to polymers. The main lipid peroxidation by-products are malondialdehyde (MDA) and 4-HNE (Marnett, 2000; Fig. 4.1); both of these lipid oxidation end products are risk factors to human health (Kanner, 2007; Bastide et al., 2011). MDA is most abundant and can reach 300 μM or more in meat products (Kanner, 2007). It is also toxic and binds to DNA and proteins, or undergoes further oxidation to more reactive epoxy derivatives that can be mutagenic in bacterial, mammalian, and human cells (Basu & Marnett, 1983; Esterbauer, 1993; Guéraud et al., 2010; Bastide et al., 2011). In foods, MDA is bound mainly to the lysine residues of proteins, from which it is released in the course of digestion, as N-α-acetyl-ϵ-(2-propenal) lysine (Piche et al., 1988).

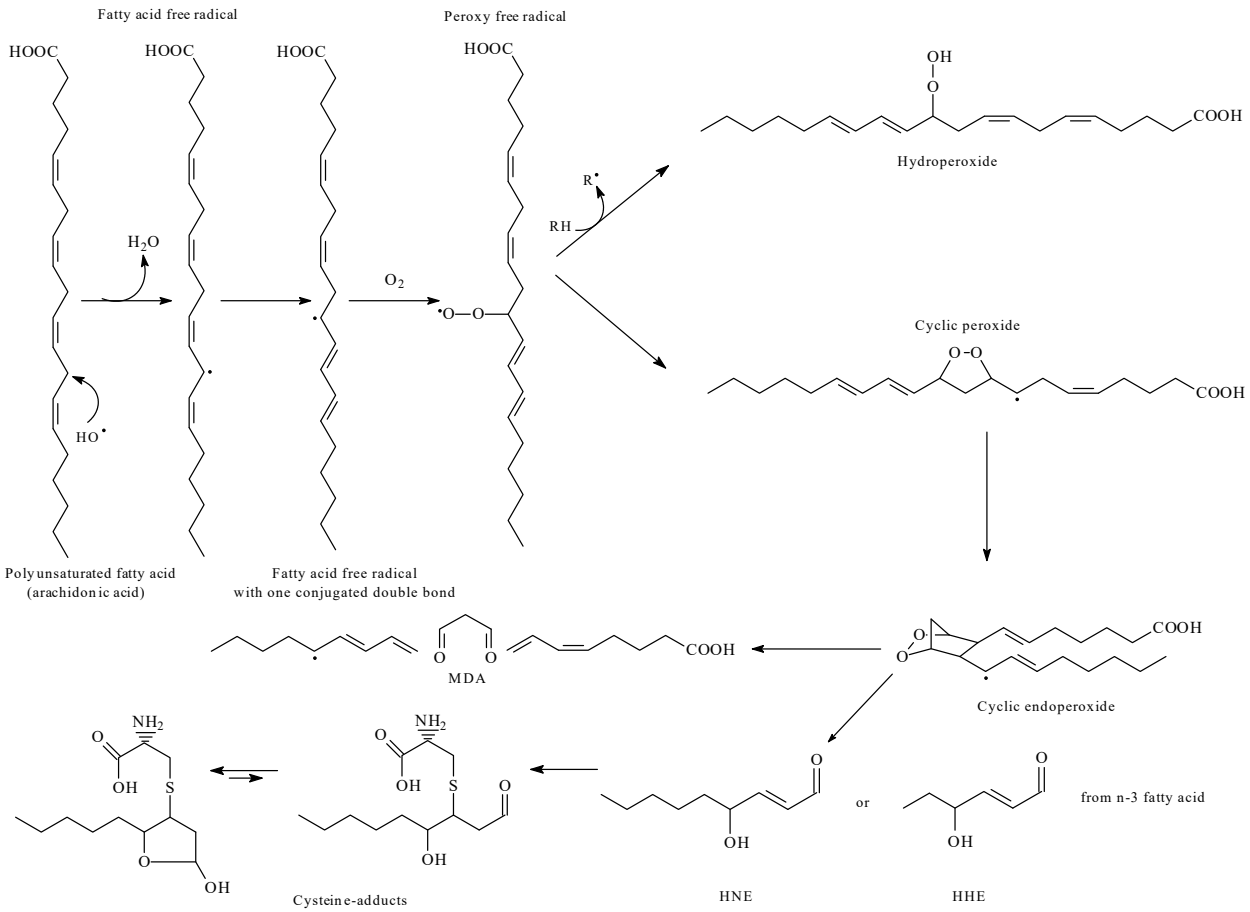
The fat fraction of meat also contains cholesterol, and it is known that dietary fatty acids and dietary cholesterol are co-oxidized (Kanner, 2007). Cholesterol oxidation via lipid free radicals results in the formation of many oxidation by-products, such as oxycholesterol, which has cytotoxic, pro-oxidant, and proinflammatory activities (Lemaire-Ewing et al., 2005). The amount of oxycholesterol in cholesterol-rich food products, including precooked meat and poultry, can reach 10–100 μM (Kanner, 2007).

The degree of lipid oxidation during the manufacturing of processed meat and storage of fresh and processed meat before consumption depends on many factors, such as the iron and polyunsaturated fatty acid content; the presence of endogenous or added antioxidants, and other additives; and the processing and storage conditions (Morrissey et al., 1998). When good storage and processing practices are followed, the levels of lipid oxidation products in meat at the time of consumption are low. Oxidation of myoglobin and other proteins also occurs, which can interfere with lipid oxidation (Faustman et al., 2010).

(b) Lipid oxidation during digestion of meat

The composition of meat and the conditions prevailing in the different compartments of the digestive tract, including interactions with other foods, determine the extent of formation of lipid oxidation products during the digestion of meat. Saliva in the mouth, acidic gastric juice in the stomach, emulsifying pancreatic and bile juice in the small intestine, and anaerobic fermentation by the microbiota in the large intestine all influence lipid oxidation in the gut. Kanner & Lapidot (2001) showed that lipid oxidation in heated muscle tissue was enhanced in the stomach due to the low pH and dissolved oxygen.

Fig. 4.1 Generic scheme of polyunsaturated fatty acid peroxidation



HHE, 4-hydroxy-2E-nonenal; HNE, 4-hydroxy-2E-hexenal; MDA, malondialdehyde

Extensive evidence is available indicating that haem iron in red and processed meat is a key factor in promoting lipid oxidation (Carlsen et al., 2005). Feeding rats heated red turkey cutlets, which are high in haem, increased lipid hydroperoxides and MDA in the stomach (Gorelik et al., 2008). As previously noted, urinary excretion of DHN-MA was increased in rats fed haem and in humans fed blood sausage (Pierre et al., 2006). Plasma MDA concentrations were higher in rats fed beef versus chicken (Toden et al., 2010). Higher MDA, 4-HNE, and hexanal concentrations resulted from in vitro duodenal and colonic digests of beef compared with pork, followed by chicken (Van Hecke et al., 2014a).

A large proportion of ingested haem iron reaches the colon (Pierre et al., 2008), and could thus stimulate oxidation reactions in the colonic contents. However, lower MDA, 4-HNE, and hexanal concentrations resulted from in vitro colonic compared with duodenal digests (Van Hecke et al., 2014a, b; Vanden Bussche et al., 2014). This could be due to the anaerobic conditions in the colon, degradation or metabolism into other compounds. The colonic microbial composition likely also has an influence on

oxidation processes (Huycke & Moore, 2002; Sun et al., 2010; Martin et al., 2015).

The high-fat content of many processed meats is likely to result in an increased production of lipid oxidation products. In vitro digestion of a heated pork product containing 5% or 20% pork lard resulted in a higher production of 4-HNE and hexanal compared with heated lean pork containing 1% fat (Van Hecke et al., 2014b). The fatty acid profile is also important. Urinary MDA and DHN-MA in rats increased when haem iron was combined with fish oil (high in n-3 fatty acids) and safflower oil (high in n-6 fatty acids), but not with hydrogenated coconut oil (98% saturated fatty acids) (Guéraud et al., 2015). Similarly, levels of hepatic 4-HNE-histidine protein adducts were higher when haem iron was combined with safflower oil compared with hydrogenated coconut oil.

During the heating of meat, the content of free Fe^{2+} increases through destruction of the haem-porphyrin moiety, oxymyoglobin releases oxygen with production of hydrogen peroxide, and antioxidant enzymes (e.g. glutathione peroxidase) are inactivated (Kanner, 1994). This stimulates the Fenton reaction, and thus the formation of lipid oxidation products. Rats consuming cooked meat products had increased faecal TBARS and urinary DHN-MA compared with rats consuming raw meats (Santarelli et al., 2010). Similarly, heating compared with not heating a pork product increased the formation of MDA, 4-HNE, and hexanal before and during digestion (Van Hecke et al., 2015).

Nitrite salt is widely used as a curing agent in meat processing. Nitrite has antioxidant properties in processed meat. The formed nitric oxide myoglobin, nitric oxide ferrous complexes, and *S*-nitrosocysteine have antioxidant properties, and nitric oxide inhibits the Fenton reaction (Kanner, 1994). In acidic conditions, such as in the stomach, nitrous acid generates dinitrogen trioxide and water, which is in equilibrium with nitric oxide (NO) and nitrogen dioxide (NO_2) (Honikel, 2008). The balance between NO and ROS has been described as a determinant of the effect of nitrite on oxidant reactions whereby a 1:1 ratio of NO to ROS enhances lipid peroxidation, whereas an excess of NO inhibits oxidation (Darley-Usmar et al., 1995). A study using rats showed that addition of nitrite to meat products reduced TBARS in faecal water (Santarelli et al., 2010). Chenni et al. (2013) found that intake of nitrite through drinking-water (1 g/L) reduced haem-induced lipid peroxidation in the colon of rats by 25%. During in vitro digestion of different nitrite-cured meat products, the formation of lipid oxidation products was markedly inhibited (Van Hecke et al., 2014a, b, 2015). However, this inhibition was less efficient when the fat content of the diet was high (20% fat), and absent when the meat products were subjected to intense heating. The intensely heated meat products, in which nitrite was less efficient at preventing oxidant reactions, contained less residual nitrite.

(c) Absorption, distribution, metabolism, and excretion

A vast amount of literature is available concerning the biotransformation of lipid oxidation products. With respect to toxic aldehydes, the Working Group refers to extensive reviews of Esterbauer et al. (1991) and of Guéraud et al. (2010), and of Poli et al. (2008) for 4-HNE specifically. Most lipid peroxidation–derived aldehydes such as 4-HNE can travel across membranes by passive diffusion. Metabolism occurs in most cells and tissues, and is rapid and complete. As a first and major step is conjugation with glutathione by Michael addition, which may be considered a detoxification reaction, facilitating urinary excretion. Other modifications of the aldehyde function may also occur (e.g. reduction into an alcohol or oxidation into an acid). The liver and the kidneys are the organs primarily involved in the elimination of 4-HNE. DHN-MA appears to be the major urinary metabolite of 4-HNE. MDA is metabolized to carbon dioxide and water via

transformation into acetaldehyde, but it is also found unmodified in urine and plasma (Guéraud et al., 2010).

(d) Mechanisms of carcinogenesis

Due to their chemical reactivity, aldehyde breakdown products of lipid oxidation can covalently modify nucleic acids, proteins, and lipids (Guéraud et al., 2010). They also serve as biomarkers of oxidative stress, and are important in cell signalling in both pathological and physiological conditions, mainly in cell cycle regulation. 4-HNE is able to exert cytotoxic, mutagenic, and genotoxic effects. Similarly, MDA has mutagenic and genotoxic properties (Esterbauer, 1993; Guéraud et al., 2010).

After meat consumption, the levels of lipid peroxidation products and their adducts or metabolites increase. For instance, lipid hydroperoxides and MDA accumulation increased more than twofold in the stomach contents of rats fed red turkey cutlets and after pyloric ligation. Postprandial plasma MDA levels increased significantly by 50%. The addition of red wine polyphenols altered these outcomes (Gorelik et al., 2008). In a human study by Brown et al. (1995), urinary MDA increased from 2.1 to 23.1 μmol/day with the consumption of high quantities of cooked meat over a 7-day period. After consumption of red meat by rats and humans, excretion of both MDA and DHN-MA increased in the urine (Pierre et al., 2006, 2008).

4.5.3 Heterocyclic aromatic amines

The following discussion is restricted to studies involving exposure to HAAs as a result of the consumption of red meat or processed meat, together with studies that reported findings directly relevant to the issue of whether such exposure may account for any risk of cancer. The weight accorded to such data depended, among many other considerations, on current knowledge of the carcinogenicity of individual HAAs and of HAAs as a class. See *IARC Monographs* Volume 56 (IARC, 1993).

Meats cooked at a high temperature contain HAAs (see Section 1.2.3 and Fig. 1.2). HAAs are pyrolysis by-products formed from the reaction between creatine or creatinine found in muscle meats, amino acids, and sugars (Wakabayashi et al., 1992; Sugimura et al., 2004). HAA formation increases with the temperature and duration of cooking, and depends on the type of meat and cooking method (Cross & Sinha, 2004). More than 20 individual HAAs have been identified. After meat consumption of a fried beef meal by human subjects, 24-hour hydrolysed urine contained 2–8.5% PhIP and 13–32% MeIQx of the ingested dose (Reistad et al., 1997).

Most HAAs are potent bacterial mutagens, based on the Ames *S. typhimurium* test (Ames et al., 1973; Felton et al., 2007). In 1993, the Working Group concluded that HAAs are *possibly or probably carcinogenic to humans*, including IQ (*Group 2A*), MeIQ (*Group 2B*), MeIQx (*Group 2B*), and PhIP (*Group 2B*) (IARC, 1993).

(a) Mechanisms of carcinogenesis

Most HAAs are not mutagenic or carcinogenic in their parent form. HAAs acquire the capacity to form DNA adducts and potentially cause DNA damage only after metabolic activation. HAAs undergo rapid and extensive metabolism by phase I and II XMEs (Alexander et al., 1995; Turesky & Le Marchand, 2011), which can lead to either bioactivation or detoxification of the HAAs, as discussed in Section 4.4.

HAA–DNA adduct formation is considered a biomarker for the mutagenic and carcinogenic potential of these xenobiotic compounds (Cheng et al., 2006). Many HAAs have been shown to form DNA adducts in both in vitro and in vivo experiments (Cheng et al., 2006; Turesky & Le Marchand, 2011). The major reaction of the *N*-hydroxy-HAA derivatives with DNA occurs at deoxyguanosine (dG) to produce dG-C8-HAA adducts, where bond formation occurs between

the C8 atom of dG and the activated exocyclic amine group of the HAA (Schut & Snyderwine, 1999; Turesky & Vouros, 2004). For IQ and MeIQx, DNA adducts also form at the N^2 group of dG and the C5 atom of the heterocyclic ring structures (Turesky & Vouros, 2004; Turesky & Le Marchand, 2011). While the amount of dG-N^2 adducts formed is small relative to the dG-C8 isomers, the dG-N^2 adducts can persist in vivo (Turesky & Vouros, 2004; Turesky & Le Marchand, 2011).

In addition to DNA adduct formation, HAAs may exhibit other carcinogenic mechanisms. For example, PhIP may also possess estrogenic activity at very low doses (10^{-9} to 10^{-11} M), which can invoke a mitogenic response (Lauber et al., 2004). PhIP at doses as low as 10^{-11} M had direct effects on a rat pituitary lactotroph model, and induced cell proliferation and the secretion of prolactin. These PhIP-induced effects were suppressed by an estrogen receptor inhibitor. Such hormone-like activities of PhIP provide mechanistic plausibility for carcinogenicity in the breast (Lauber & Gooderham, 2007).

Considerable interspecies differences have been found in the carcinogenicity, mutagenicity and metabolism of HAAs (Hengstler et al., 1999). Carcinogenicity studies have been performed in rats, mice, and monkeys (Ohgaki et al., 1985; Adamson et al., 1990; Hengstler et al., 1999). In rodents, long-term feeding of HAAs induced tumours of the oral cavity, liver, stomach, colon, pancreas, and prostate gland in males and mammary gland in females (Turesky & Le Marchand, 2011). IQ was shown to be a potent hepatocarcinogen in cynomolgus monkeys, but MeIQx failed to induce hepatocellular carcinoma after a 5-year dosing period (Hengstler et al., 1999). Species differences in mutagenicity were most pronounced for MeIQx in *S. typhimurium* strain TA98 (Ames test) using liver microsomes from cynomolgus monkeys, rats, and humans. Higher mutation rates occurred with human and rat, than with cynomolgus monkey microsomes. DNA adduct levels were highest in male rats, followed by female rats, and were much lower in cynomolgus monkeys after an oral MeIQx dose. Species differences in the bioactivation of PhIP were also observed among in human, rat, and mouse hepatic microsomes, with those of human origin having the highest capacity to catalyse the initial activation step to *N*-hydroxy-PhIP (Hengstler et al., 1999).

The total dose required to induce tumours formation varied for each HAA, was species-dependent, and could range from 0.1 to 64.6 mg/kg per day in rodents (Turesky & Le Marchand, 2011). Doses of HAAs used in the animal feeding studies exceeded by several orders of magnitude the levels of HAAs found in the human diet (Stavric, 1994). However, several HAA–DNA adducts have been detected in human tissue (Turesky & Le Marchand, 2011). The results reported by Garner et al. (1999) suggest that humans metabolize HAAs differently compared with rats. After low-dose oral administration of MeIQx and PhIP, humans developed higher DNA adduct formation in colonic tissue compared with rats. Similarly, Mauthe et al. (1999) showed that low-dose MeIQx formed DNA adducts in the human colon. This implied that the human colon may be more sensitive to this compound than the mouse or rat colon. Using accelerator mass spectrometry, a tool for measuring isotopes with attomolar sensitivity, Turteltaub et al. (1999) showed that protein and DNA adduct levels in rodents were dose-dependent. The adduct levels in human tissue and blood were generally greater than those in rodents administered equivalent doses. Furthermore, the metabolite profiles for both MeIQx and PhIP differed substantially between humans and rodents, with more *N*-hydroxylation (bioactivation) and less ring oxidation (detoxification) in humans. There are also important differences between humans and rats in CYP activity and regioselectivity of HAA oxidation, which can affect the toxicological

properties of these compounds (Turesky, 2007; Turesky & Le Marchand, 2011).

(b) Epidemiological studies

Estimating HAA exposure in epidemiological studies has been difficult due to the variability of these compounds across the range of meat types, cooking methods and doneness levels. Moreover, there is a lack of gold-standard biomarkers to validate the questionnaires. Surrogate measures of HAA intake, such as cooking methods, meat doneness, and surface browning, have been used to investigate the etiological association between these mutagens and cancer risk. In addition, questionnaires with detailed cooking and doneness information have been linked to an HAA database to estimate individual HAA intake in the USA (Sinha, 2002; Sinha et al., 2005c), Sweden (Augustsson et al., 1997), and Germany (Rohrmann et al., 2009b). The HAA database was created by measuring levels of HAAs in a variety of meats cooked by different high-temperature methods to a range of doneness levels (rare, medium, well done, and very well done) (Sinha et al., 1995, 1998a, b). For example, while grilled, well-done chicken contains high levels of HAAs, roasted chicken contains very low levels of HAAs.

Urinary HAA biomarkers are good indicators of short-term intake, but such one-time measures cannot be used to estimate an individual's usual exposure level (Cross & Sinha, 2004; Turesky & Le Marchand, 2011; Busquets et al., 2013). Adducts in DNA, haemoglobin, and serum albumin have also been evaluated, but their utility in epidemiological studies at the present time is unclear (Turesky & Le Marchand, 2011). There is enthusiasm for using HAA levels in hair as a long-term measure of exposure, but the use of this measure in epidemiological studies is still being evaluated (Kobayashi et al., 2007; Turesky & Le Marchand, 2011; Kataoka et al., 2013; Iwasaki et al., 2014) (see also Section 1.4.2).

Putative DNA adducts of several HAAs have been detected in human tissues by non-specific ^{32}P-postlabelling (Totsuka et al., 1996) or immuno-histochemistry methods (Zhu et al., 2003; Tang et al., 2007), and studies have reported on the analysis of presumed PhIP–DNA adducts after acid hydrolysis of DNA in human lymphocytes or colon DNA samples (Friesen et al., 1994; Magagnotti et al., 2003). However, few studies have unambiguously identified and quantified intact HAA–DNA adducts in human biospecimens by specific tandem mass spectrometry–based methods (Gu et al., 2012).

Using detailed meat cooking questions and linkage to the HAA database, case–control and prospective studies have evaluated the association between HAA intake and cancer risk (Alaejos et al., 2008; Zheng & Lee, 2009; Abid et al., 2014). The results have been mixed, depending on the cancer site and the study population. Results considered here are from large prospective cohort studies. Both MeIQx and DiMeIQx were positively associated with cancer of the colorectum in the NIH-AARP study (Cross et al., 2010), but not with colorectal adenoma incidence in the PLCO trial (Ferrucci et al., 2012). In contrast, MeIQx was associated with colon adenomas in a cohort of men from the USA (Wu et al., 2006). PhIP intake has been linked to colorectal adenomas in the PLCO trial (Ferrucci et al., 2012) and in the EPIC-Heidelberg cohort study (Rohrmann et al., 2009b), but not to cancer of the colorectum in the NIH-AARP study (Cross et al., 2010). PhIP, MeIQx, and DiMeIQx were not associated with cancer of the colorectum in the Multiethnic Cohort Study (Ollberding et al., 2012).

Cancer of the prostate was not associated with PhIP, MeIQx, or DiMeIQx in the EPIC-Heidelberg study, the NIH-AARP study, or the Agricultural Health Study (AHS) (Koutros et al., 2008; Sinha et al., 2009; Sander et al., 2011). In contrast, in the HPFS, intake of PhIP from red meat was associated with advanced cancer of the prostate (Rohrmann et al., 2015). In the PLCO trial, PhIP, but not MeIQx or DiMeIQx,

was associated with risk of cancer of the prostate (Cross et al., 2005).

In various prospective studies, none of the HAAs considered were associated with cancer of the breast (Ferrucci et al., 2009; Kabat et al., 2009; Wu et al., 2010). Although DiMeIQx was linked to cancers of the gastric cardia (Cross et al., 2011) and pancreas (Stolzenberg-Solomon et al., 2007; Anderson et al., 2012), no association was found with cancers of the lung (Tasevska et al., 2009, 2011) or liver (Freedman et al., 2010). Similarly, no association between MeIQx intake and cancer of the liver was seen (Freedman et al., 2010). However, MeIQx intake was linked to cancers of the lung (Tasevska et al., 2009) and pancreas (Anderson et al., 2012) in the NIH-AARP study and PLCO trial, respectively. In the NIH-AARP study, both MeIQx and DiMeIQx were associated with a decreased risk of chronic lymphocytic leukaemia and small lymphocytic lymphoma (Daniel et al., 2012b). PhIP was linked to an increased risk of renal cell carcinoma (Daniel et al., 2012a), but not to cancers of the lung (Tasevska et al., 2009, 2011), bladder (Ferrucci et al., 2010b), pancreas (Stolzenberg-Solomon et al., 2007), or liver (Freedman et al., 2010).

(c) HAAs and inter-individual genetic susceptibility

As HAAs can be activated or detoxified by phase I and phase II metabolic reactions, various studies have evaluated single-nucleotide polymorphisms in the genes encoding XMEs. Results were mixed for interactions between XME polymorphisms and HAA consumption for colorectal adenomas or carcinomas (Ishibe et al., 2002; Le Marchand et al., 2002b; Chan et al., 2005b; Lilla et al., 2006; Girard et al., 2008; Shin et al., 2008; Yeh et al., 2009; Ferrucci et al., 2010a; Wang et al., 2011; Fu et al., 2012; Gilsing et al., 2012; Voutsinas et al., 2013). Some studies evaluated XMEs and HAAs for cancer of the breast (Lee et al., 2013), prostate (Nowell et al., 2004), and bladder (Lin et al., 2012). Many of these studies had a small number of cases with inadequate power or examined only a small set of single-nucleotide polymorphisms from a limited number of candidate genes. As the balance of activating and detoxifying enzymes is thought to influence carcinogen metabolism, comprehensive studies including numerous markers across multiple genes involved in xenobiotic metabolism are essential for studying this complex association. Furthermore, the inconsistencies in the data may have resulted partly from the inability of most studies to estimate specific HAAs, due to a lack of information regarding cooking technique and doneness level, or appropriate availability of biomarkers.

4.5.4 Polycyclic aromatic hydrocarbons

The following discussion is restricted to studies involving exposure to PAHs as a result of the consumption of red meat or processed meat, together with studies that reported findings directly relevant to the issue of whether such exposure may account for any risk of cancer. The weight accorded to such data depended, among many other considerations, on current knowledge of the carcinogenicity of individual PAHs and of PAHs as a class. See *IARC Monographs* Volume 92 (on PAHs) (IARC, 2010) and Volume 100F (on BaP) (IARC, 2012a).

The carcinogenicity of PAHs, specifically BaP (e.g. from active smoking, inhaling second-hand tobacco smoke, or working in coal- and tar-based industries) has prompted scrutiny of other circumstances of PAH exposure (e.g. from air pollution and dietary intake). In non-smoking, non–occupationally exposed populations, diet is frequently the major source of exposure to PAHs (IARC, 2010). Dietary intake of PAHs is often assessed by reference to levels of BaP, which is recognized as a good marker of PAH exposure. When fed to mice, BaP caused multiple tumour types, particularly in the upper gastrointestinal tract (IARC, 2010). PAHs can be formed during

the curing and processing of meat, and can be generated during cooking through pyrolysis of fat, particularly if the meat is charred or burned (Phillips, 1999). See Section 1 for further discussion on PAH levels and PAH occurrence in different meat preparations.

(a) Mechanisms of carcinogenesis

The carcinogenic mechanisms of PAHs are extensively reviewed in *IARC Monographs* Volume 92 and Volume 100F, and include activation and detoxification by phase I and II XMEs.

In a study of 114 subjects (48 women, 66 men), Cocco et al. (2007) reported that frequent intake of grilled meat was a predictor of urinary 1-hydroxypyrene levels of 0.50 μg/g creatine or greater. In the study previously described in Section 4.2 by Chien & Yeh (2010), consumption of barbecued meat (with higher PAH content) resulted in a significant correlation between urinary 8-OHdG concentrations, and 1-hydroxypyrene and 3-hydroxy-BaP concentrations.

A case–control study reported higher PAH–DNA adduct levels in colorectal adenoma cases (median, 1.4 adducts per 10^8 nucleotides) than in polyp-free controls (median, 1.2 adducts per 10^8 nucleotides; $P = 0.02$) (Gunter et al., 2007). The DNA adduct levels were measured by chemiluminescence immunoassay (using an antiserum elicited against DNA modified with (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene, which recognizes several PAHs bound to human DNA). Rothman et al. (1990, 1993) found PAHs in urine and DNA adducts in white blood cells. These data support that PAHs are absorbed from the consumption of grilled meat and have a genotoxic effect.

(b) Epidemiological studies

Dietary intake of PAHs, irrespective of the dietary source, has been examined in relation to a range of tumour types, including colorectal adenoma (Sinha et al., 2005a), cancer of the breast (Rundle et al., 2000; Jeffy et al., 2002; Mordukhovich et al., 2010), cancer of the stomach (Liao et al., 2014), and renal cell carcinoma (Daniel et al., 2011). Some positive associations were reported, specifically in relation to colorectal adenoma.

Based on the BaP intake of participants, determined using the Computerized Heterocyclic Amines Resource for Research in Epidemiology of Disease (CHARRED) developed by the National Cancer Institute (NCI), various studies have evaluated PAHs. While studying the association between meat intake and cancer of the colon, a case–control study in North Carolina, USA (Butler et al., 2003), determined levels of BaP intake. Associations with BaP intake, stratified by race, were imprecise, but stronger effects were seen among African Americans than among white Americans. In a large prospective study of meat consumption and risk of cancer of the colorectum, BaP intake was not associated with cancer of the colorectum (Cross et al., 2010). In a study of screening-detected colorectal adenoma (Sinha et al., 2005b) evaluating dietary intake of PAHs, an increased risk of adenoma of the descending colon and sigmoid colon was observed with BaP intake. exposure to BaP from meat consumption was not associated with a risk of cancer of the colorectum in an investigation of the postulated association between high consumption of meat and colorectal carcinoma in a case–control study in Western Australia (Tabatabaei et al., 2010).

No association between PAHs and cancer of the breast was found in a large population-based case–control study that evaluated dietary intake of PAHs from cooked meat, determined by self-administered Block FFQs (Steck et al., 2007). However, this same study did find an association with intake of BaP from meat in postmenopausal women whose tumours were positive for both the estrogen receptor and progesterone receptor.

Daniel et al. (2011) undertook a case–control study that examined exposure to PAHs from meat intake in 1192 newly diagnosed renal cell

carcinoma patients and 1175 controls. Risk of malignancy increased with intake of BaP. Risk of renal cell carcinoma was more than two-fold higher in African Americans and current smokers.

In a population-based case–control study, Girard et al. (2008) investigated whether cancer of the colon was associated with genetic variations in *UGT1A1* and *UGT1A9*. The *UGT1A1*–53 and –3156 genotypes significantly modified the association between dietary BaP and cancer of the colon. The strongest association between dietary BaP exposure was observed in those with less than 7.7 ng/day of BaP exposure and low-activity genotypes. These data support the hypothesis that UDP-glucuronosyltransferases (UGTs) modify the association between meat-derived PAH exposure and cancer of the colon.

4.5.5 N-*Nitroso compounds*

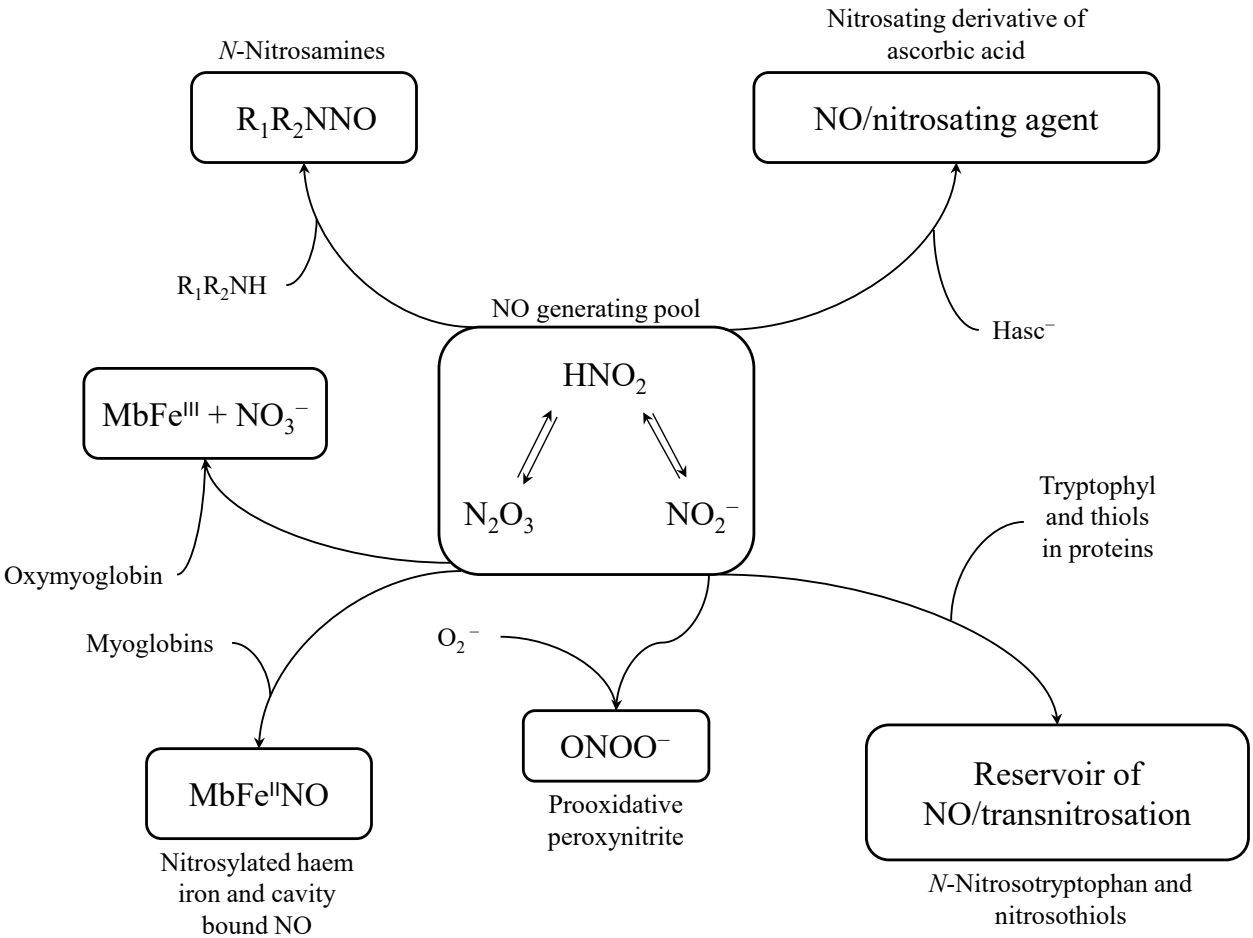
The following discussion is restricted to studies involving exposure to NOCs as a result of the consumption of red meat or processed meat, together with studies that reported findings directly relevant to the issue of whether such exposure may contribute to any risk of cancer. The weight accorded to such data depended, among many other considerations, on current knowledge of the carcinogenicity of individual NOCs and of NOCs as a class. See *IARC Monographs* Volume 89 (IARC, 2007) and Volume 100E (IARC, 2012b) for NOCs, and Volume 94 (IARC, 2010) for ingested nitrate and nitrite.

NOCs can be produced during processing, storage, and preparation of foods. They are formed by the reaction of secondary amines (R_1NHR_2) and *N*-alkylamides ($R_1NH{\cdot}CO{\cdot}R_2$) with nitrite in food or in the acidic environment of the stomach (Honikel, 2008). Metabolism of nitrosamines or spontaneous breakdown of nitrosamides can give rise to reactive alkylating intermediates, which can be identified by their reaction with DNA and other macromolecules. Nitrosation of primary amino acids, including glycine and methionine, may also give rise to alkylating intermediates (Issenberg 1976; Mirvish, 1995). NOCs are genotoxic carcinogens associated with particular mutational signatures (Rao, 2013).

The general term NOC covers all substances with *N*-nitroso groups, including *N*-nitrosamines and *N*-nitrosamides. However, the analytical method generally used to analyse NOCs in digestion does not differentiate between *N*-nitrosamines and other compounds such as *S*-nitrosothiols, *O*-nitroso compounds, and iron nitrosyls (Kuhnle & Bingham, 2007). Given this lack of specificity, the term ATNC has been used to describe the substances measured by this technique. Nitrosyl haem and nitrosothiols have been identified as major constituents of both faecal and ileal ATNC, and the formation of these compounds increases significantly after consumption of a diet rich in red meat. Nitrosothiols are readily formed under the acidic conditions of the stomach, a process that is promoted by haem. Haem becomes easily nitrosylated under the anaerobic and reductive conditions of the lower gut to form nitrosyl haem, which is an NO donor and can act as a nitrosating agent. In turn, nitrosothiols can act as NO donors and nitrosating species. Thus, the combined actions of haem and free thiol groups can promote the endogenous formation of NOCs (Kuhnle et al., 2007).

Nitrite used in meat processing is involved in many reactions with myoglobin, proteins, and lipids (Honikel, 2008; Skibsted, 2011; Demeyer et al., 2015; Fig. 4.2). When combined with myoglobin, these reactions result in nitrosomyoglobin in cured meat and nitrosyl haemochromogen after cooking, which are responsible for the characteristic colour of cured meats. Residual nitrite in cured meat proteins may be important as a "hidden NO-generating pool", a source of nitric oxide for numerous reactions during the storage and cooking of cured meats (Skibsted, 2011).

Fig. 4.2 Nitric oxide formed from nitrite during meat curing can participate in numerous reactions modifying proteins and pigments



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The occurrence of nitrate, nitrite, and NOCs in meat is discussed in Section 1. Mirvish et al. (2002) reported that NOC and NOC precursor levels in hot dogs were about 10 and 4 times higher, respectively, than those in fresh meat. The NOC precursors were considered of greater relevance to carcinogenicity, as they are more stable and approximately 1000 times more abundant than NOCs. The main NOC precursors identified were *N*-glycosyl amino acids and peptides. Dich et al. (1996) described dietary intake of nitrate, nitrite, and *N*-nitrosodimethylamine (NDMA) in 5304 men and 4750 women who participated in the Finnish Mobile Clinic Health Examination Survey in 1967–1972. Dietary nitrite was mainly provided by meat products (specified as cured meats, cooked sausage, and salami), contributing about 95% of the total intake. The mean daily intake of NDMA was calculated to be 0.052 μg, approximately half of which was derived from meat products.

(a) Absorption, distribution, metabolism, and excretion

Few studies have reported on the absorption, distribution, metabolism, and excretion of NOCs after meat consumption. Human saliva contains nitrate and nitrite due to enterosalivary circulation (Mirvish et al., 2000). Since rats convert a low amount of salivary nitrate into nitrite, it has been argued that the rat may not be a good model for humans (Cockburn et al., 2013). Therefore, Chenni et al. (2013) tested the relevance of this enterosalivary cycle by giving haem iron–fed rats drinking-water containing sodium nitrite, mimicking human salivary nitrite levels. They observed increased faecal ATNC. Phillips et al. (1975) showed that NDMA is absorbed in the rat stomach and the small intestine. Zhou et al. (2014) also reported increased urinary ATNC in rats fed sodium nitrite and/or hot dogs. In a rat model, Santarelli et al. (2010) showed that a combination of nitrite curing, cooking, and oxidation of red meat increased faecal ATNC. In addition, rats fed a diet containing commercially purchased hot dogs or fermented, raw, dry sausages had increased faecal ATNC compared with those fed a control diet without meat (Santarelli et al., 2013). Intake of sodium nitrite (0.17 g/L) and sodium nitrate (0.23 g/L) through drinking-water increased faecal ATNC in rats on a 1% haemoglobin diet (Chenni et al., 2013).

Several mechanisms have been proposed to explain the effect of red meat on the formation of NOCs. Lunn et al. (2007) observed no difference in ATNC levels in the ileal output of ileostomists and in the faecal output of healthy subjects consuming large amounts of red meat. In contrast to the stomach contents, which consisted only of nitrosothiols, nitrosyl iron was present in higher concentrations than nitrosothiols in ileal and faecal samples, with no difference in ATNC composition between both sample types (Kuhnle et al., 2007). Thus nitrosothiols formed in the acidic stomach may release NO once they are exposed to the alkaline and reductive conditions of the small and large intestine, thereby stimulating the nitrosylation of haem iron. However, the consequences of the formation of these products are unclear (Hogg, 2007). On the one hand, nitrosyl haem and nitrosothiols could act as nitrosating agents and promote the formation of NOCs in the intestinal epithelium (Kuhnle et al., 2007). On the other hand, nitrosothiols and nitrosyl iron may act as a protective mechanism by capturing NO and facilitating its excretion, thereby limiting the formation of DNA alkylating agents.

In a series of human intervention studies, Bingham and colleagues demonstrated a dose–response increase in faecal excretion of ATNC with red meat intake. This was not observed with vegetable proteins, white meat, or an Fe^{2+} supplement, but mimicked by a haem iron supplement (provided by blood sausage) (Bingham et al., 1996; Hughes et al., 2001; Bingham et al., 2002; Cross et al., 2003). Holtrop et al. (2012) conducted three dietary trials in obese men consuming body weight maintenance or weight loss diets, and measured NOCs in faecal samples. The meat-based weight loss diets increased levels of faecal NOCs ($P < 0.001$). Red meat intake was positively correlated with the faecal log NOC concentrations ($r = 0.60$; $P < 0.001$).

The genotoxic effects of faecal ATNC were investigated using different comet assay protocols in individuals consuming high levels of red meat (Cross et al., 2006a). The inter-individual effects were variable, and diet, mean transit time, and weight had no effect on faecal water genotoxicity; see also Lewin et al. (2006), as discussed in Section 4.2. Rats treated with the *N*-nitrosopeptide *N*-acetyl-*N*′-prolyl-*N*′-nitrosoglycine showed the presence of O^6-CMG in the intact small intestine. This was also observed in HT-29 cells treated with diazoacetate (Lewin et al., 2006). Since the analysis of ATNC includes both toxic and non-toxic compounds, the quantification of O^6-CMG might offer a more specific insight into the formation of genotoxic NOCs.

Although Lunn et al. (2007) and Kuhnle et al. (2007) observed no difference between ileal and faecal ATNC and its individual components, suggesting no influence of the colonic microbiota on NOC formation, other research has suggested a major facilitating role of the gut microbiota. Using a pig caecum model containing nitrate and amines/amides, Engemann et al. (2013) showed large inter-individual variation in porcine microbiota to form *N*-nitrosamines *N*-nitrosomorpholine (NMOR) and *N*-nitrosopyrrolidine (NPYR), and *N*-nitrosamides *N*-methyl-*N*-nitrosourea and *N*-ethyl-*N*-nitrosourea. Moreover, a clear increase in NOCs was observed in time, with the microbiota responsible for the reduction of nitrate to nitrite. In accordance, Vanden Bussche et al. (2014) found considerable inter-individual variation in human microbiota for the in vitro formation of the NOC-specific DNA adduct O^6-CMG during fermentation of white and red meat. This large inter-individual variation was also acknowledged by Van Hecke et al. (2014a, b, 2015). However, in vitro formation of O^6-CMG was stimulated by a higher haem iron content, a higher fat content, and more intense heating conditions, while nitrite curing was not perceived to be of influence, despite the large impact of the applied microbiota (Van Hecke et al., 2014a, b, 2015).

A broad body of evidence indicates that the formation of NOCs may be inhibited by agents including ascorbic acid and α-tocopherol (Mirvish, 1986). The inhibitory effects of nitrite, L-ascorbic acid, and α-tocopherol on the formation of NOCs in processed meat products were evaluated by comparing samples of sausage with different concentrations of these reductants (Pourazrang et al., 2002). Revertants in the *S. typhimurium* (TA100) microsome assay were significantly reduced ($P < 0.05$) by 60% when the reductants were added to the samples. In the study of Hughes et al. (2002) described in Section 4.2 evaluated the effect of soy and other dietary components on faecal NOC excretion with consumption of a high–red meat (420 g/day) diet in 11 male volunteers randomized to 15-day dietary periods. Soy significantly suppressed faecal ATNC concentrations ($P = 0.02$), but vegetables and tea extract did not affect mean faecal ATNC concentrations or faecal water genotoxicity. However, faecal weight increased and was associated with reduced transit time, decreasing contact between ATNC concentrations, nitrite, and ammonia and the large bowel mucosa.

(b) Mechanisms of carcinogenesis

For decades, experimental animal data have afforded insight into the increased risk of cancer in humans that is attributable to the consumption of different categories of meat, and specifically the possible role of NOCs (Olsen et al., 1984); see also Santarelli et al. (2010), as discussed in Section 4.3.

G→A transitions in *K-RAS* occur in cancer of the colorectum and are characteristic of the effects of alkylating agents such as NOCs (Bingham et al., 1996). The methylating agent *N*-methyl-*N*-nitrosourea produced predominantly (> 80%) transitions (GC→AT), whereas potassium diazoacetate, a stable form of nitrosated glycine, produced transitions (GC→AT) and transversions (GC→TA and AT→TA) in equivalent amounts (Gottschalg et al., 2007). The similarity in the patterns of mutations induced by potassium diazoacetate with those observed in mutated *P53* in human gastrointestinal tract tumours suggests that nitrosation of glycine (or glycine derivatives) may contribute to characteristic human *P53* mutation profiles.

(c) Epidemiological studies

Studies addressing the association between risk of cancer and dietary intake of nitrate, nitrite, or nitrosamines refer to meat as well as other relevant foods (Loh et al., 2011). [The Working Group noted that dietary intake of nitrate and nitrite does not necessarily reflect NOC intake.]

In the EPIC-Norfolk study (Loh et al., 2011), dietary NDMA intake was significantly associated with an increased risk of cancer of the rectum in women. There was no significant association between cancer risk across quartiles and dietary nitrite and endogenous NOCs. In a case–control study in Canada, NDMA intake was associated with a higher risk of cancer of the colorectum, specifically rectal carcinoma. Risk of cancer of the colorectum also increased with the consumption of NDMA-containing meats (Zhu et al., 2014). Individuals with high-NDMA and low–vitamin E intake had a significantly higher risk than those with both low-NDMA and low–vitamin E intake.

Intake of dietary nitrites and nitrosamines was positively associated with risk of cancer of the lower urinary tract in American men of Japanese ancestry (Wilkens et al., 1996). Consumption of processed meats, in particular bacon, sausage, and ham, was also significantly associated with an increased risk in American men of Japanese ancestry. Three food items accounted for all of the NOC intake: sausage (46%), bacon (33%), and luncheon meats (21%).

The association between nitrate, nitrite, and nitrosamine intake and glioma was examined by Michaud et al. (2009). Risk of glioma was not elevated among individuals in the highest intake category of nitrate, nitrite, or NDMA compared with those in the lowest intake category. In a population-based case–control study of glioma in adults, increased odds ratios were observed in males who consumed high levels of bacon, corned meats, apples, melons, and oil (Giles et al., 1994). Elevated odds ratio in men, but not women, were associated with the intake of NDMA.

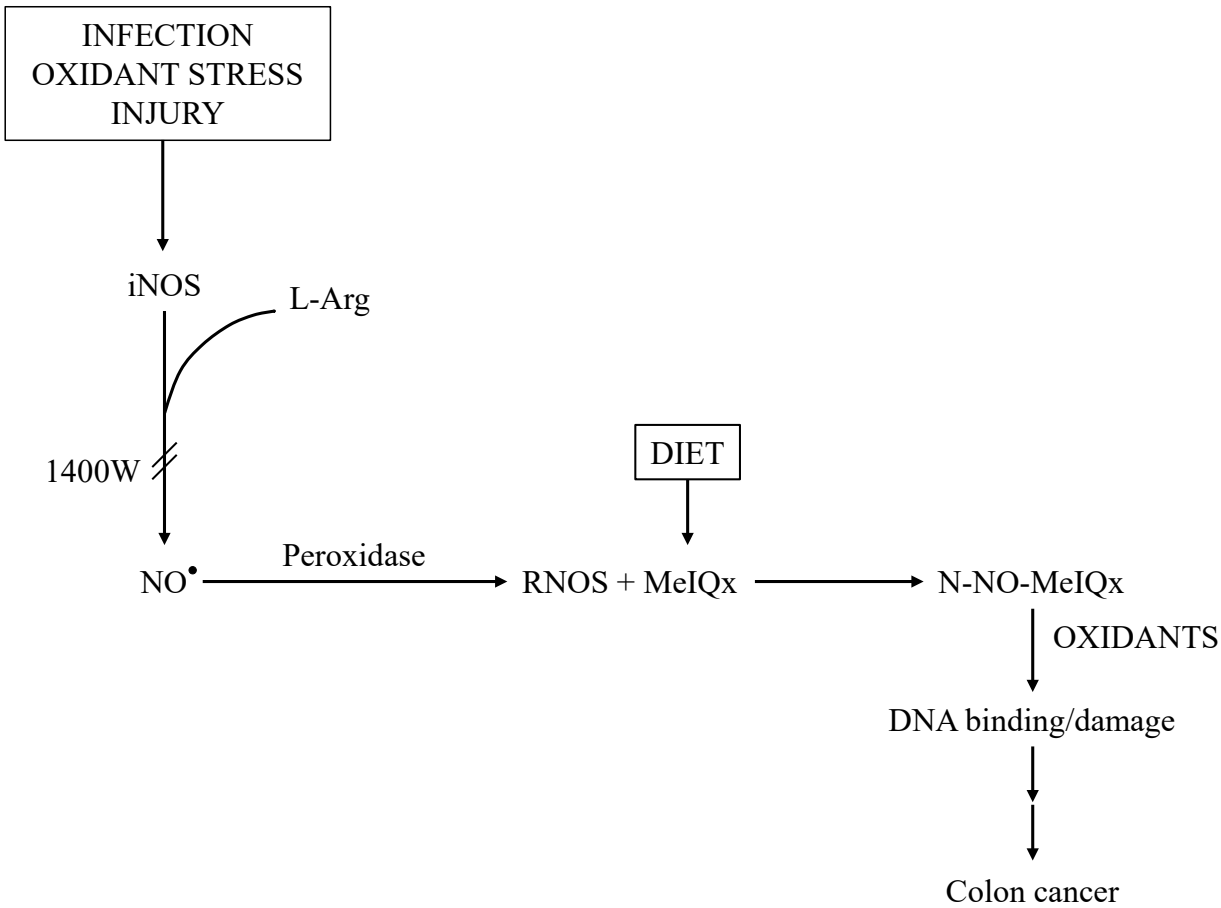
4.5.6 Interactions between NOCs, haem iron, and HAAs

Haem in red meat stimulates the endogenous production of NOCs. The effect of red meat and processed meat on endogenous nitrosation, as well as DNA damage (see Section 4.2), was investigated by Joosen et al. (2009). Faecal NOC concentrations in 5 males and 11 females on vegetarian diets were low (2.6 and 3.5 mmol/g, respectively), but significantly increased in those fed meat diets (preserved red meat, 175 ± 19 nmol/g; red meat, 185 ± 22 nmol/g; $P = 0.75$). The meat diets contained 420 g/day (males) or 366 g/day (females) of meat. The nitrite-cured meat diet had the same effect as the fresh red meat diet on endogenous nitrosation, but increased faecal water–induced oxidative DNA damage.

A high–red meat diet (420 g/day) significantly increased nitrosyl iron and nitrosothiols in ileal and faecal samples compared with a vegetarian diet (Kuhnle et al., 2007). Faecal nitrosyl iron and haem were strongly correlated ($r = 0.776$; $P < 0.0001$), suggesting that nitrosyl haem is the main source of nitrosyl iron. Nitrosation of HAAs is depicted in Fig. 4.3 (Lakshmi et al., 2005b). Lakshmi et al. (2005a) demonstrated hemin potentiation of NO-mediated nitrosation using the HAA IQ as a target and by monitoring the formation of ^{14}C-2-nitrosoamino-3-methylimidazo[4,5-*f*]quinoline (N-NO-IQ) by high-performance liquid chromatography. Faecal NOCs (Mirvish et al., 2003) and urinary nitrite and nitrate were increased in mice with dextran sulfate sodium–induced colitis, which was consistent with increased expression of inducible NO synthase and NO synthesis.

IQ and MeIQx can be converted to their corresponding *N*-nitrosamines, N-NO-IQ and 2-nitrosoamino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (N-NO-MeIQx) (Zenser et al., 2009). N-NO-IQ and N-NO-MeIQx have been shown to form several putative adducts in common with those formed by 2-hydroxyamino-3-methylimidazo[4,5-*f*]quinoline (N-OH-IQ) and 2-hydroxyamino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (N-OH-MeIQx). These *N*-nitrosamines might be alternatives to their hydroxylamine analogues, as

Fig. 4.3 Nitrosation of heterocyclic aromatic amines



The relationship between chronic inflammation/infection and injury, well-done red meat in the diet, and colon cancer is depicted. The inflammatory process provides NO, MPO, H_2O_2, and HOCl. Well-done red meat provides haem and HAAs (MeIQx). Together, these pathways yield *N*-nitroso compounds (N-NO-MeIQx).
Reprinted with permission from Lakshmi et al. (2005a). Hemin potentiates nitric oxide-mediated nitrosation of 2-amino-3-methylimidazo[4,5-*f*] quinoline (IQ) to 2-nitrosoamino-3-methylimidazo[4,5-*f*]quinoline. *Chem Res Toxicol*, 18(3):528–35. doi:10.1021/tx049792r PMID:15777093. Copyright (2005) American Chemical Society

activated intermediates leading to the initiation of cancer of the colon in individuals with colitis.

4.5.7 Other components

The following subsections address components or contaminants of red meat or processed meat not considered elsewhere in Section 4.5.

(a) Advanced glycation end products

AGEPs form by Maillard reaction after the initial binding of aldehydes with amines or amides in, among other places, heated foods. Within proteins, high molecular-mass AGEPs are formed whereas reactions among small molecules yield low-molecular-mass AGEPs. Some of these compounds interact with specific pro- or anti-inflammatory receptors. In observational studies, dietary AGEPs were strongly associated with late complications in diabetes (e.g., Poulsen et al., 2013).

Levels of representative AGEPs are similar in certain cheeses, fried eggs, cereal products, and broiled steak (Uribarri et al., 2010). Monitoring of representative AGEPs in 19 healthy, overweight

individuals who were fed meals of identical ingredients, prepared by either roasting or steaming, indicated that AGEPs may affect postprandial ghrelin, oxidative stress, and glucose responses (Poulsen et al., 2014). In a prospective case–control study of cancer of the colorectum, higher prediagnostic levels of the serum-soluble receptor for AGEPs were associated with a lower risk of cancer of the colorectum in male smokers; no specific relationship with any dietary constituent was reported (Jiao et al., 2011).

(b) N-Glycolylneuraminic acid

Neu5Gc is a predominant sialic acid on most mammalian cells. Humans are genetically deficient in Neu5Gc production, and the compound is metabolically incorporated into human tissue from dietary sources, particularly red meat. Neu5Gc is thus detectable on the surface of human epithelia and endothelia, and in higher amounts in malignant tissues. This xeno-autoantigen can react with circulating anti-Neu5Gc antibodies in humans. The compound has been proposed as a cancer biomarker (Samraj et al., 2014). Among the evidence for its role in tumour progression, Hedlund et al. (2008) reported that murine tumours expressing human-like levels of Neu5Gc showed accelerated growth in syngeneic mice with a human-like Neu5Gc deficiency, which coincided with the induction of anti-Neu5Gc antibodies and increased infiltration of inflammatory cells.

Samraj et al. (2015) employed what was described as an improved method to survey foods for Neu5Gc. They showed that Neu5Gc was highly and selectively enriched in red meat. In the study, Neu5Gc-deficient mice, immunized against Neu5Gc and fed bioavailable Neu5Gc from porcine saliva, developed a much higher incidence of hepatocellular carcinoma than three groups of variously identified control mice.

(c) Proposed oncogenic bovine virus

Noting the cancer incidence in Asian communities known to consume undercooked beef, zur Hausen (2012) hypothesized that the presence of one or more thermoresistant, potentially oncogenic bovine viruses contaminates beef preparations and contributes to development of cancer of the colorectum. The same, or comparable, factors were proposed to be transmitted by the consumption of milk products (zur Hausen & de Villiers, 2015). [The Working Group took note of the lack of supporting evidence for this hypothesis.]

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