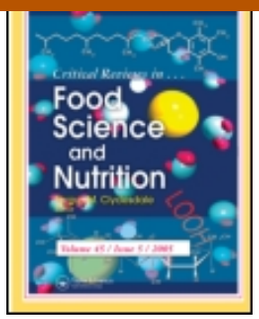

Critical Reviews in Food Science and Nutrition

ISSN: 1040-8398 (Print) 1549-7852 (Online) Journal homepage: http://www.tandfonline.com/loi/bfsn20

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To cite this article: Daniel Demeyer, Birgit Mertens, Stefaan De Smet & Michèle Ulens (2016) Mechanisms Linking Colorectal Cancer to the Consumption of (Processed) Red Meat: A Review, Critical Reviews in Food Science and Nutrition, 56:16, 2747-2766, DOI: 10.1080/10408398.2013.873886

To link to this article: http://dx.doi.org/10.1080/10408398.2013.873886

Accepted author version posted online: 15 May 2015.
Published online: 15 May 2015.

Critical Reviews in Food Science and Nutrition, 56:2747–2766, (2016)
Published with license by Taylor and Francis Group
ISSN: 1040-8398 / 1549-7852 online
DOI: 10.1080/10408398.2013.873886

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Mechanisms Linking Colorectal Cancer to the Consumption of (Processed) Red Meat: A Review

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Colorectal cancer (CRC) is the third most commonly diagnosed cancer in the world. The vast majority of CRC cases have been linked to environmental causes rather than to heritable genetic changes. Over the last decades, epidemiological evidence linking the consumption of red and, more convincingly, of processed red meat to CRC has accumulated. In parallel, hypotheses on carcinogenic mechanisms underlying an association between CRC and the intake of red and processed red meat have been proposed and investigated in biological studies. The hypotheses that have received most attention until now include (1) the presence of polycyclic aromatic hydrocarbons and heterocyclic aromatic amines, two groups of compounds recognized as carcinogenic, (2) the enhancing effect of (nitrosyl)heme on the formation of carcinogenic N-nitroso compounds and lipid peroxidation. However, none of these hypotheses completely explains the link between red and processed red meat intake and the CRC risk. Consequently, scientists have proposed additional mechanisms or refined their hypotheses. This review first briefly summarizes the development of CRC followed by an in-depth overview and critical discussion of the different potential carcinogenic mechanisms underlying the increased CRC risk associated with the consumption of red and processed red meat.

Keywords Polycyclic aromatic hydrocarbons, heterocyclic aromatic amines, N-nitroso compounds, lipid oxidation, heme, N-glycolylneuraminic acid

INTRODUCTION

Colorectal cancer (CRC) is the third most commonly diagnosed cancer in the world. Approximately 5% of all CRC cases are due to inherited genetic mutations, including many syndromes such as Familial Adenomatous Polyposis and Lynch syndrome (Power et al., 2010; Al-Sohaily et al., 2012); however, the vast majority of CRC cases have been linked to environmental causes rather than to heritable genetic changes (Lund et al., 2011; Watson and Collins, 2011). For many years, diet is recognized as an important factor in disease etiology and risk of CRC (Modan, 1977), including the consumption of red and processed red meat.[1] Convincing epidemiological evidence linking red and processed red meat

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[1]Although the literature is not always clear, "red meat" in this report refers to home prepared and cooked fresh red meat products (beef, pork, sheep and goat) that have not been subjected to treatments other than cooling, freezing and/or comminution for mixing with other red meats and salt (e.g. ground meat). Processed meat products are defined by the "World Cancer Research Fund/American Institute for Cancer Research" (WCRF/AICR, 2012) as "meat preserved by smoking, curing or salting or by addition of chemical preservatives" and are here more simply specified as "red meats at least preserved by nitrite (and/or nitrate) curing and possibly other treatments." In the literature, processed meats do clearly (although tacitly) not cover poultry. To prevent possible confusion with definitions of meat covering poultry the term "processed meats" is replaced by "processed red meats" in this review.

intake to CRC risk has accumulated over the last decades (Cross et al., 2010; Fung et al., 2010; Reedy et al., 2010; Chan et al., 2011; Shin et al., 2011; Ferrucci et al., 2012; Magalhaes et al., 2012; Johnson et al., 2013), and suggests that the CRC inducing effect is more pronounced for processed red meat compared to red meat. In parallel with these epidemiological observations, hypotheses on carcinogenic mechanisms underlying an association between CRC and the intake of red and processed red meat have been proposed and investigated in biological studies. Despite all efforts made, the underlying carcinogenic mechanisms remain unclear. Like other types of cancer, the development of CRC is a multistep process and compounds present in red an processed red meat may interfere with this process at different levels. In order to better understand these interactions, the current state of knowledge on the molecular pathogenesis of CRC is first briefly summarized.

COLORECTAL CANCER

GENOMIC INSTABILITY. CRC arises in most cases as a benign adenomatous polyp, which develops into an advanced adenoma with high-grade dysplasia and finally progresses to an invasive cancer (Sanford and McPherson, 2009). Genomic instability appears to be an integral part in the transformation process (Al-Sohaily et al., 2012). In an initial attempt to characterize multistep carcinogenesis, Fearon and Vogelstein proposed a model in which specific genetic alterations (i.e. mutations in the *APC, K-ras* and *p53* genes) are associated with the sequential evolution of the neoplastic phenotype in the colon (Fearon and Vogelstein, 1990; Boland, 2010). Although this model of Fearon and Vogelstein has been useful, combination of mutations of the different genes in the same cancer is uncommon and additional CRC pathways have been recognized (Al-Sohaily et al., 2012). Today, three distinct molecular pathways of genomic instability are considered to occur in colon carcinogenesis: the chromosomal instability pathway, the microsatellite instability pathway and the CpG island methylator phenotype pathway (Itzkowitz and Yio, 2004). These three pathways have been extensively reviewed elsewhere (Markowitz and Bertagnolli, 2009; Boland and Goel, 2010; Migliore et al., 2011; Al-Sohaily et al., 2012).

Environmental and food-borne mutagens including compounds present in red and processed red meat products can contribute to genomic instability and finally CRC by inducing DNA damage. In addition to an effect on the genetic material, interference of these compounds with other processes such as intestinal inflammation and the intestinal microflora may also be involved in the development of CRC as both processes play an important role in CRC development.

INFLAMMATION. Like for other types of cancer (Coussens and Werb, 2002; Marnett, 2012), inflammation has been suggested to play a role in the development of CRC. Epidemiologic observations have clearly identified inflammation as an important risk factor for developing CRC in patients suffering from inflammatory bowel disease (IBD, including both ulcerative colitis and Crohn's colitis) (Itzkowitz and Yio, 2004). More recent evidence, however, indicates that also in other forms of sporadic as well as heritable CRC, inflammation is likely to be involved (Terzic et al., 2010). Considering the large number of pathways disturbed (Westbrook et al., 2010), chronic inflammation has been proposed to affect the three stages of cancer: tumor initiation, tumor promotion and tumor progression.

Tumor initiation, the process by which a normal cell starts to become malignant, is associated with accumulation of genetic alterations (Grivennikov et al., 2010; Westbrook et al., 2010). Research data indicate that inflammation can induce genetic alterations through several mechanisms. Activated inflammatory cells for example trigger oxidant-generating enzymes such as NADPH oxidase and myeloperoxidase to produce high concentrations of oxygen species (ROS) and reactive nitrogen species (RNS) (Coussens and Werb, 2002), compounds known to induce DNA single- and double-strand breaks (Murata et al., 2012). Nonetheless, it is rather unlikely that ROS produced by immune cells diffuse and induce mutations in adjacent epithelial cells. A more plausible hypothesis is that the cytokines and other factors released by activated immune cells stimulate ROS production within epithelial cells (Hussain et al., 2003). In colitis-associated cancer (CAC), which is the CRC subtype associated with IBD, chronic inflammation precedes colitis-associated tumor development and therefore oxidative damage to DNA due to chronic inflammation may be responsible for tumor initiation (Kraus and Arber, 2009). However, in sporadic CRC, most intratumoral immune cells are only recruited after the tumor is formed indicating that chronic inflammation does not precede but follows tumor development. Inflammation is thus probably not involved in tumor initiation in sporadic CRC although after tumor formation, the localized inflammatory microenvironment can promote accumulation of additional mutations and epigenetic changes (Meira et al., 2008; Terzic et al., 2010; Westbrook et al., 2010). Furthermore, cytokines released by activated immune cells can play a role during tumor promotion and progression by stimulating angiogenesis and suppressing immune-mediated tumor elimination (Karin, 2006; Yu et al, 2009; Grivennikov et al, 2010).

INTESTINAL MICROBIOTA AND TOLL-LIKE RECEPTORS. Both studies with human CRC tissue (Marchesi et al., 2011; Castellarin et al., 2012; Kostic et al., 2012) and animal studies (Kado et al., 2001; Engle et al., 2002; Vannucci et al., 2008) indicate that the intestinal microflora influences the development of sporadic CRC and CAC. In normal healthy conditions, the intestinal microflora has important homeostatic immune and metabolic functions, affects the proliferation and survival of epithelial cells and provides protection against pathogens (Boleij and Tjalsma, 2012). Disruption of the homeostasis (both qualitatively and quantitatively) of the intestinal microflora could promote cancer through different

ways, for example by altering the number, diversity, and stability of commensal bacteria or by the action of pathogens (i.e. *Bacteroides fragilis*) or conditional commensals (Terzic et al., 2010; Zhu et al., 2013). Over the last years, evidence for a role of microbiota and toll-like receptors, a type of pattern recognition receptors, in CRC is emerging and literature on the different potential mechanisms involved has been reviewed recently (Moossavi and Rezaei, 2013).

RED AND PROCESSED RED MEAT INTAKE AND COLORECTAL CANCER RISK

Several mechanisms through which compounds present in red and processed red meat can interfere with the different processes occurring during the CRC development have been proposed and the most important ones will be discussed.

Unlikely Hypotheses: Fat, Protein and Virus

Excess of Protein and Fat Intake

A diet high in red meat and/or in processed red meat products potentially contains high levels of fat. Evidence from both epidemiologic studies (Boyle et al., 1985) and laboratory animal studies (Reddy, 1992) has suggested a promoting role of dietary fat for CRC development. Several mechanisms have been postulated for the possible relationship between high-fat intake and CRC risk among which the association between fat intake and the production of bile acids has received most attention. High fat intake indeed stimulates the secretion of secondary bile acids in the gut. These bile acids can promote tumor formation by acting as aggressive surfactants for the mucosa thus increasing cell loss and proliferation (Bruce, 1987; Owen, 1997). Other hypotheses for the promoting role of dietary fat include (i) an increase in the amount of free fatty acids in the colonic lumen which may damage the colonic epithelium and induce proliferation (discussed more in detail later in this article) and (ii) an augmented risk for obesity, a status associated with CRC and other diseases (Calle and Kaaks, 2004). Although a promoting effect of high-fat diet has been repeatedly shown in animal models of carcinogenesis (Zhao et al., 1991), most studies reporting a correlation between dietary fat intake and CRC risk are quite old and more recent assessments failed to detect an effect once confounding factors had been taken into account, in particular body weight (Santarelli et al., 2008; Liu et al., 2011).

Meat is also a rich source of dietary protein. Fermentation of the excess of proteins in the gut yields metabolites such as NH_3 and H_2S, compounds known to be toxic to the mucosa (Corpet et al., 1995). However, human epidemiologic studies do not support an association between protein intake and CRC (Windey et al., 2012).

Both an excess of neither fat nor protein can explain the link between consumption of red and processed red meat and CRC risk since these macro-nutrients are also present in dairy products, fish and poultry, foods not found to be associated with CRC incidence in epidemiology. A meta-analysis, though subject to some criticism, also showed that neither animal fat nor animal protein are CRC risk factors (Alexander et al., 2009).

Thermoresistant Oncogenic Bovine Viruses

An elegant hypothesis links red and processed red meat to CRC through "thermoresistant potentially oncogenic bovine viruses" (zur Hausen, 2012). The hypothesis however is not based on experimental work and does not explain a more outspoken cancer inducing effect of processed (cured) red meats. In contrast to red meat, these processed red meat products are indeed mainly obtained from pig meat, rendering such hypothesis improbable though elegant. Nevertheless, a synergistic action of a thermoresistant infectious agent with other carcinogenic mechanisms (e.g. carcinogenic chemicals) cannot be excluded.

Alternative Unlikely Hypotheses

Colorectal cancer has been linked to several other agents that may be present in (processed) red meat. Such compounds include, amongst others, arachidonic acid (Phinney, 1996), methionine (Duranton et al., 1999), trans-fatty acids (Smith et al., 2009), endogenous hormones (e.g. IGF1) (Toden et al., 2010), exogenous hormonal growth-promoters (Galbraith, 2002), man-made contaminants e.g. pesticides (Vogt et al., 2012), and formaldehyde (Zhu et al., 2012). As evidence for these hypotheses is limited and most of the compounds are not specific for (processed) red meat, they are not further discussed.

Polycyclic Aromatic Hydrocarbons and Heterocyclic Amines

Polycyclic aromatic hydrocarbons (PAHs) and heterocyclic amines (HCAs) can be present in food, including red and processed red meat products. In animal studies, both types of compounds have been shown to be carcinogenic through the induction of mutations (de Kok and van Maanen, 2000; Goldman and Shields, 2003; Cross and Sinha, 2004).

Polycyclic Aromatic Hydrocarbons

PAHs are ubiquitous environmental toxicants produced by incomplete combustion of organic compounds. They can be emitted during processing of coal, crude oil, petroleum, and natural gas, as by-products of industrial production, from heating in power plants and homes (oil, gas, charcoal-fired stoves, wood stoves), burning of refuse, wood fires, and from motor

vehicle exhausts. Humans are exposed to PAHs through different routes since they enter the body by consumption of contaminated foods or drinking water, inhalation of cigarette smoke, automobile exhausts, and contaminated air from occupational settings (Alexander et al., 2008). For non-smoking humans, the major routes of exposure to PAHs are food and to some extent inhaled air. In cigarette smokers, the contributions from smoking and food may be of a similar magnitude (Menzie et al., 1992). Food can be contaminated both with PAHs from environmental sources and PAHs formed during food cooking. In case meat is grilled over a direct flame, fat/meat juices often drip onto the hot fire, yielding flames containing a number of PAHs which can adhere to the surface of the food. However, PAHs can also be produced during the curing and the processing of the food, for example, when smoking is used as a preservation method (Phillips, 1999; Cross and Sinha, 2004). The best studied PAH is benzo[*a*]pyrene (BaP), recognized since 1987 as a probable human carcinogen, and recently upgraded to Group 1 based on mechanistic and other relevant data by the International Agency for Research on Cancer (IARC, 2012). In addition, three PAHs have been categorized as probably and twelve PAHs as possibly carcinogenic to humans (IARC, 2013). PAHs are not genotoxic as such, but are activated through metabolization. During the metabolization process of different PAHs, reactive metabolites are formed which can covalently bind to DNA (mainly to the guanine bases), inducing DNA damage (Phillips, 1983; Phillips and Grover, 1994). If the DNA damage is not or incorrectly repaired, mutations can be induced which may contribute to the development of CRC. Risk evaluation of long-term adverse health effects following dietary intake of PAHs often considers the total of up to 8 different PAH compounds. The 4 major compounds often considered together are benz[a]anthracene, benzo[b]fluoranthene, BaP and chrysene (Alexander et al., 2008).

Although PAHs are present in meat and meat products, they are usually not considered as a specific causal agent for the carcinogenic activity of red and processed red meat products as they are present in all foods (Cirillo et al., 2010). Indeed, comparable levels of PAHs are found in cereal products and in grilled poultry and fish, foods not found to be associated with an increased risk for CRC. Epidemiologic studies do not clearly support the hypothesis of PAH intake as cause of the increased risk for CRC associated with the consumption of red and processed red meat either. In a limited clinical based case/control study (374 subjects) and after correction for possible confounders, Sinha and others (2005a) found that BaP intake was associated with colorectal adenoma incidence. However, the relation was stronger for BaP intake from all foods than for BaP from meat with relative risk rates (highest versus lowest intake) of 5.60 and 2.82 respectively. Within this study, dietary BaP intakes were derived from previously established databases and from information on meat cooking methods and degrees of doneness/browning obtained in preparation as collected from the literature and illustrated by photography of the prepared meat. Meat and meat products represented 19% of total dietary BaP intake. In line with earlier work (Kazerouni et al., 2001), only grilling/barbecuing (gas barbecue unit with ceramic briquettes) but not cooking increased PAHs in meat and the major source of meat associated BaP intake was grilled steak and hamburger (69%) whereas grilled chicken represented 23%. In related work, Gunter and others (2005) confirmed these results and stated that "an incremental increase of 10 g of barbecued red meat per day was associated with a 29% increased risk of large adenoma." As no such association was found for oven-broiled red meat, the authors concluded that the way the meat is cooked, i.e. exposure "to a naked flame", rather than meat per se, contributes to the increased risk for CRC. In similar Australian work involving 1280 subjects, however, a risk of CRC could not be associated with BaP intake (Tabatabaei et al., 2010). The authors related this contradiction to the possible differences in fat content of the meat consumed.

Based on this information, it would seem that concern for PAHs as a clear causal agent of an increased risk for CRC through meat consumption should be limited to the cooking process. Consequently, a considerable reduction in PAH concentrations in foods (including meat products) can be obtained by avoiding the pyrolysis of fat that drops into the flames applied (Alexander et al., 2008).

Heterocyclic Amines

The final concentration of HCAs formed in heated meat and fish via the Maillard reaction with creati(ni)ne, amino acids, and sugars depends on many factors including cooking method, cooking time and temperature, the concentration of HCA precursors, and presence of water and fat in the raw product (Alaejos and Afonso, 2011). More than 25 HCAs have been isolated from different cooked muscle foods and the IARC categorized eight of them [including 2-Amino-3,4-dimethylimidazo[4,5-f]quinoline (MeIQ), 2-Amino-3,8-dimethyl imidazo[4,5-f]quinoxaline (MeIQx) and 2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP)] as possibly carcinogenic to humans and 2-Amino-3-methylimidazo[4,5-f]quinoline (IQ) as a probable human carcinogen (IARC, 2013). The nitrenium ion formed during metabolization is considered the likely ultimate genotoxic compound binding to the DNA bases producing DNA adducts through the formation of N-C bonds at guanine bases (Goldman and Shields, 2003; Jägerstad and Skog, 2005). The most abundant HCAs in cooked meat are PhIP and MeIQx. They are also the two major absorbed HCAs after intake of a cooked meat meal (Lynch et al., 1992). When comparing the effects of type of cooking on HCA, levels of MeIQx and PhIP appear to be similar if not lower in beef and pork compared to those found in cooked poultry and fish (Puangsombat et al., 2012). For both poultry and fish, epidemiology did not show an association with CRC risk. By the same token, Viegas and others (2012) demonstrated that whereas quantitative HCA and PAH profiles were different for

barbecued beef and salmon using the same type of charcoal, higher levels of HCAs and PAHs were found in salmon samples. Nevertheless, several studies provide some evidence for a positive association of well-done meat intake and HCA exposure with the risk of CRC. In a German European Prospective Investigation into Cancer and Nutrition (EPIC) observational study involving 25540 participants followed between 1998 and 2007, dietary HCAs (PhIP but not MeIQx) were found to be significantly associated with the CRC incidence (relative risk: 1.47, highest vs lowest intake), even after adjustment for total red and processed red meat consumption. This suggests a specific effect of PhIP that cannot be completely explained by other components in red or processed red meat (Rohrmann et al., 2009). In a smaller US scale case-control study involving 6307 subjects between 2003 and 2010 (Fu et al., 2011), a significant positive association was found between exposures to all meat-derived HCA studied and risk of colorectal polyps, again after adjusting for potential confounders (relative risk: 1.3–1.4, highest versus lowest intake). As described above for PAH, HCA intake levels were derived in both studies from information on cooking methods and the degree of doneness/browning obtained in preparation as collected from the literature and illustrated by photography of the prepared meat (Sinha et al., 2005b). For EPIC, similar data are available in relation to cooking method at the EPIC website (EPIC, 2012a) and photographs illustrating "meat doneness" were developed for European environment by e.g. Augustsson and others (1997). However, data from studies using photographs to indirectly estimate the intake of HCAs should be interpreted with caution as the correlation between these parameters is not clear (Solyakov and Skog, 2002). This is also illustrated by the range of HCA intake in the US study performed by Fu and others and the German observations done by Rohrmann and others (Table 1). Furthermore, Deziel and others (2012) demonstrated that estimates for dietary HCA exposure obtained from food frequency questionnaire (FFQ) collections, diary collections, and measurements of urinary PhIP were not related. More consistent cancer risk estimates of dietary HCA exposure may therefore require improved HCA assessment tools.

Results from animal experiments do not fully explain the role of HCAs in the increased risk of CRC related to consumption of red and processed red meat either as the well-known carcinogenic effects of HCA have been demonstrated in rodents at levels more than 1000 times higher than HCA levels found in foods (Stavric, 1994; Schwab et al., 2000). For these reasons, HCAs are generally not considered very important in relation to the carcinogenic properties of meat (Corpet, 2011). Furthermore, as HCAs require metabolic activation to function as mutagens/carcinogens, the carcinogenic potential of HCA may depend on the extent of metabolization (Cross and Sinha, 2004). Cancer risk posed by dietary HCAs was indeed shown to vary with genetic differences in the drug metabolizing enzymes involved e.g. in acetylation and sulfonation (Le Marchand et al., 2002). Besides genetic factors, evidence is available that individual differences in the intestinal microflora may affect HCA carcinogenic activity as well (Kassie et al., 2004; Vanhaecke et al., 2008). In a comprehensive review Alaejos and others (2007) therefore conclude that there is no sufficient scientific evidence to definitely accept that dietary HCA intake specifically causes cancer but that "Epidemiological evidence points to genetic predisposition as the main factor in meat associated HCA related cancer development."

Table 1 Intake (ng/day) of two heterocyclic amines in German and US observational studies

	German observational study (Rohrmann et al., 2009)	US observational study (Fu et al., 2011)
PhIP	≤6.5 – ≥41.4	≤73.3 – ≥339.4
MeIQx	≤3.8 – ≥19.9	≤12.2 – ≥70.1

[1]Levels of lowest–highest quartiles.

Conclusion

The evidence listed above indicates that both HCA and PAH intake is determined by the meat-cooking technique and doneness level. Nevertheless, the formulation of guidelines for meat cooking may still be considered appropriate. For industrially cooked meat products, a decrease of HCA content could be aimed at using processing variables that minimize HCA production as determined e.g. by Dundar and others (2012). In this respect, the finding that the ripening time of meat affects HCA formation during heating (Szterk et al., 2012) may also be relevant. People involved in meat preparation in restaurants and catering should also consider such information as it has been shown that cooks are potentially exposed to relatively high levels of airborne HCA (Thiébaud et al., 1995). For household cooking, an efficient method to reduce exposure to PAH and HCA consists of the mechanical removal of charred and blackened material from the surface of broiled meat and fish on the dish (Sugimura, 1997). Furthermore, discouragement of "well done meat" should be considered and longer time/lower temperature treatments should be encouraged.

Nitrosamines and Other N-nitroso Compounds

The general term 'N-nitroso compounds' (NOCs) covers all substances with N-nitroso groups, including N-nitrosamines and N-nitrosamides. Twenty years ago, Tricker and Preussmann (1991) referred to established carcinogenic activities of over 300 NOCs in one or more animal species including higher primates. Since NOCs are alkylating agents that can react with the DNA of the target tissue, they can induce mutations and therefore, like PAHs and HCAs, could potentially initiate carcinogenesis (Saffhill et al., 1985). Alkylation of the O^6-position of guanine by NOCs may lead to G → A transitions

(Singer and Essigmann, 1991; Romach et al., 1994), a common mutation in CRC found in codons 12 or 13 of *K-Ras* (Bos, 1989), if not repaired by O^6-methylguanine transferase (Lindahl, 1982; Rydberg et al., 1990). N-nitrosamides are alkylating agents, and thus are more likely to cause damage at the site of exposure. In contrast, N-nitrosamines require metabolic activation to be mutagenic and have thus the potential to cause damage at any activation site. During food consumption, humans can be exposed to NOCs both of exogenous and endogenous origin (Cross and Sinha, 2004; Jägerstad and Skog, 2005).

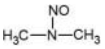
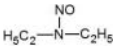
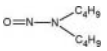
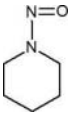
Exposure by Exogenous Routes

Meat products, and especially processed or heat-treated foods, often contain NOCs. NOCs have for example been detected in foods processed by smoking or direct fire-drying. When these production processes are performed at high temperature, molecular nitrogen can be oxidized forming nitrogen oxides, compounds able to nitrosate secondary amines and amides present in foods such as meat (Mirvish, 1995; Cross and Sinha, 2004). As will be discussed later in more detail, nitrite, present in processed foods also contributes to the formation of NOCs.

An important group of NOCs in food are the volatile N-nitrosoamines including N-nitrosodimethylamine (NDMA) and N-nitrosodiethylamine (NDEA), two compounds classified by IARC as probably carcinogenic to humans, and N-nitrosopyrrolidine (NPYR), N- nitrosopiperidine (NPIP), and N-nitrosodibutylamine (NDBA) which are considered as possible human carcinogens (Jägerstad and Skog, 2005; IARC, 2013). Tricker (1997) estimated that dietary sources accounted for about 70% of exogenous human exposure to N-nitrosamines, the rest being due to occupational exposure (20%), smoking (2%) and various sources (8%). The data assembled in Table 2 illustrate that processed and heated meats (e.g. heated bacon) are indeed major dietary sources of these volatile NOCs but dairy products, fish and products such as spices may also be important contributors (Tricker and Preussmann, 1991; Jakszyn et al., 2004; Stuff et al., 2009). Despite the fact that most information is available on volatile nitrosamines, the main forms of NOCs occurring in food are non-volatile NOCs including for example *N*-nitrosoproline. Until now, non-volatile NOCs have not yet been reported as mutagenic or carcinogenic, but they might act as precursors to volatile carcinogenic nitrosamines (Jägerstad and Skog, 2005).

A striking finding is the possible presence of NDMA in fresh and minced meat (EPIC, 2012a). As almost no amines are present in fresh meat and its nitrite concentration is rather low, formation of nitrosamines in fresh meat has indeed been considered to be rather unlikely (Honikel, 2008). Nevertheless, values of NDMA up to 14 μg/kg have been reported for bulls but were shown to originate from contamination in meat production systems (Rywotycki, 2003). Heating of such meat further increases the levels of nitrosamines (Yurchenko and Mölder, 2007). In line with established carcinogenic activities, a significant positive association was observed between intake of NDMA and subsequent occurrence of CRC in a large scale Finnish study (Knekt et al., 1999). However, within the various sources of NOCs, intake of smoked and salted fish was significantly and intake of cured meat was non-significantly associated with risk of CRC.

Table 2 Levels of nitrosamines (μg/kg) in foods

		Reference		
Nitrosamine	Food item	(1)	(2)	(3)
N-Nitrosodimethy lamine (NDMA) 	Meat			0 – 4.0
	Cured meats	1.0 – 5.0	4.5	0 – 84
	Fried Bacon	4.0	1.9	0.5 – 5.0
	Frankfurt	3.0 – 38.0		0 – 4.5
	Dried Fish (Japan)	1.0 – 6.0		
	Dairy products			
	Cheese			
	Non fat milk			
N-Nitrosodiethylamine (NDEA) 	Cured meats	<2.4		
	Ham		1.5	
N-Nitrosodibutylamine (NDBA) 	Cured meats	1.0−56.0		
	Smoked chicken	<5.3	13.6	
	Bacon			
N-Nitrosopyrrolydine (NPYR) 	Cured meats	1.0−5.0		
	Fried bacon	<130.0		
	Mixed spices	<10.0		
N-Nitrosopiperidine (NPIP) 	Cured meats	<20.0		
	Fried bacon	<9.2		

References: (1) Tricker & Preussmann (1991), (2) Stuff et al. (2009), (3) Jakszyn et al. (2004) and EPIC (2012a).

Endogenous Production of NOCs

About 45–75% of the total NOC exposure is estimated to be the result of endogenous production from nitrosatable precursors (Tricker, 1997). Besides secondary amines, other

nitrosatable precursors present in food are essential nutrients such as proteins and trace compounds including urea derivatives (de Kok and van Maanen, 2000). Endogenous formation of NOCs may occur through several mechanisms including acid catalyzed, bacterial or colon cell-mediated pathways (Cross and Sinha, 2004). Acid catalyzed endogenous NOC formation is considered to occur mainly in the stomach. About 5% of the exogenous nitrate is absorbed in the small intestine, recirculated into the saliva and reduced to nitrite by oral bacteria. In the acidic environment of the stomach, the salivary nitrite is transformed into the nitrosating agent, nitrous acid (Spiegelhalder et al., 1976; de Kok and van Maanen, 2000). However, levels of NOCs in gastric juices are ten times lower than fecal levels and gastric NOC formation is consequently considered to be minimal (Pignatelli et al., 1993). In conditions where the pH is too high for nitrous acid mediated nitrosation, NOC formation may result from biological catalysis by bacterial growth (Leach et al., 1987). Considering the neutral pH of the bowel, the bacteria mediated NOC formation is most likely. The involvement of the colonic flora in the NOC formation was supported by the observation that in germ free rats, nitrate did not stimulate endogenous NOC formation (Massey et al, 1988). However, little is known regarding the bacterial species involved in endogenous nitrosation and different bacterial enzymes responsible for the nitrosation reaction have been described. A recent study in a pig cecum model indicated that intestinal formation of NOCs depends on the intake of nitrate and the capacity of the microbiota to reduce nitrate into nitrite (Engemann et al., 2013). In the colon cell-mediated catalysis, nitric oxide formed via the inducible form of NO synthase appears to act as the nitrosating species. Nitrosation of amines by activated macrophages has been reported (Ohshima and Bartsch, 1994) and may thus occur at sites of (low-grade) inflammation (Cross and Sinha, 2004).

As discussed later in this review, processed red meat does contain residual nitrite which can contribute to the formation of NOCs. In addition, heme present in red and processed red meat has also been suggested to stimulate endogenous production of NOCs.

Heme

Evidence from both epidemiological and experimental studies suggests that heme plays a crucial role in the link between the risk of CRC and red and processed red meat intake (Santarelli et al., 2008; Bastide et al., 2011; Corpet, 2011). Heme consists of an iron atom contained in the center of a large heterocyclic organic ring called a porphyrin (Santarelli et al., 2008). In red meat, heme is present in high concentrations in the form of myoglobin, resulting in the red color. Due to the nitrate and nitrite present in the curing salt, heme iron occurs in its nitrosylated form in processed red meat (Bastide et al., 2011). When heme compounds are included in the diet for experimental studies, hemin is often used (Sesink et al., 2001). Hemin is a chemical derivative of hemoglobin formed by removal of the protein part of the molecule, oxidation of the iron atom, and combination with an acid to form a salt.

Four large prospective studies found that a high intake of heme iron was associated with a higher risk of CRC (Lee et al., 2004; Larsson et al., 2005; Cross et al., 2010; Ferrucci et al., 2012) but no evidence for such an association was provided in two other studies (Kabat et al., 2007; Zhang et al., 2011). Estimation of heme iron from food intake is however difficult and subject to error as the proportion of heme iron in total Fe decreases with aging in beef (Ramos et al., 2012) and with heat treatment (Lombardi-Boccia et al., 2002; Purchas et al., 2004; D'evoli et al., 2009). In a recent study involving 185 archival CRC samples collected from participants of the EPIC-Norfolk study, Gay and others (2012) found that CRC cases associated with *APC* gene aberrations consumed higher levels of processed red meat and iron from red meat. In contrast, an association between heme iron intakes and CRC risk was not observed in Japanese men or women as reported from a large population based prospective study (Hara et al., 2012). However, lower intakes and different major food sources of heme iron may explain the lack of association. Indeed, fish was the main food source of heme iron in the study, whereas maximal daily intakes of fish and red meat were 125 and 85 g respectively.

As discussed by Corpet (2011), animal studies published before 2004 did not show red meat promotion of CRC because of the high calcium content of experimental diets, known to suppress heme induced colon carcinogenesis (Sesink et al., 2001). In a series of later experiments on low calcium diets however the research group of Fabrice Pierre (Toulouse, FR) linked red meat consumption to the development of aberrant crypt foci (ACF) and mucin-depleted foci (MDF), precancerous lesions, in the colon of azoxymethane sensitized rats. The development of precancerous lesions was shown to be related to red meat intake in a dose-response manner and the effect was mimicked by heme (Pierre et al., 2004). It should be noted that very large amounts of "freeze dried beef", chicken or "black pudding" were included into the experimental diets (600 g/kg). Later, the same research group demonstrated that also processed red meat products promote colon carcinogenesis in a rodent animal model (Pierre et al., 2010; Santarelli et al., 2010). The lack of an enterosalivary recycling of nitrate in rats has however cast doubt on the relevance of the results of nutrition and cancer studies in this animal species. Recently, the effect of the addition of nitrite to the drinking water of rats mimicking the enterosalivary cycle on biochemical markers linked to colon carcinogenesis was investigated (Chenni et al., 2013). As no changes were observed, the authors concluded that despite the lack of nitrite in the saliva, the rat can be relevant to study the effects of red and processed red meat on CRC.

Three mechanistic hypotheses have been put forward underlying the promotion of CRC by heme: (1) the catalytic

effect of heme iron on the endogenous formation of NOCs, (2) the catalytic effect of heme iron on the formation of lipid oxidation endpoints (Bastide et al., 2011) and (3) the metabolization of heme in the gut into a cytotoxic and promoting factor (Sesink et al., 1999).

Effect of Heme on the Endogenous Production of NOC

In a series of human intervention studies, the research group of the late Sheila Bingham (Cambridge, UK) related the extensive fecal excretion of endogenously produced NOCs to red meat and processed red meat consumption. They demonstrated a dose-response increase in fecal excretion of NOCs with red meat intake, not observed with vegetable proteins, white meat or an Fe^{++} supplement, but clearly mimicked by a heme supplement. Also in animal studies, inclusion of red or processed red meat into the diet increased the fecal concentration of NOCs (Parnaud et al., 2000; Mirvish et al., 2003). However, the analytical method used to analyze NOCs did not differentiate between N-nitrosamines and other compounds such as S-nitrosothiols, O-nitroso compounds and nitrosyl iron. As discussed previously, certain N-nitroso species such as most of the N-nitrosamines, are known to be profoundly tumorogenic through the formation of DNA adducts, whereas such activity has not been reported for iron nitrosyls and S-nitrosothiols, compounds that are continuously formed in biological systems through the action of nitric oxide synthase (Hogg, 2007). Given the lack of specificity, the term ATNC (apparent total N-nitroso compounds) was used to describe the substances measured by this technique.

Several mechanisms have been proposed to explain the effect of heme and heme-containing meat products on the fecal ATNC content. First, the increased fecal ATNC content may be related to heme-induced changes in microbiota. In a recent study, dietary heme changed the microbiota with a major increase in the ratio of Gram-negative to Gram-positive bacteria (IJssennagger et al., 2012b). However, the selective shift to gram-negative bacteria was not accompanied by heme-dependent inflammation or functional change in epithelial microbe sensing. Previously, Lunn and others (2006) had already observed comparable fecal ATNC content in healthy and ileostomized volunteers. As microbiota are mainly active in the colon, they concluded that endogenous formation of NOCs after red meat intake is more likely to occur in the small intestine through another mechanism. By using an improved analysis technique for the detection of NOCs, the same research group was also able to show that nitrosyl heme and nitrosothiols are major constituents of both fecal and ileal ATNC (Kuhnle et al., 2007). Based on these observations, an alternative hypothesis was formulated according to which acid-catalyzed thionitrosation in the stomach is considered to be the initial step in the endogenous formation of NOCs. Once exposed to the alkaline and reductive conditions of the small and large bowel, NO can be released from S-nitrosothiols to be taken up by heme derived from the red and processed red meat intake. Both nitrosyl heme and S-nitrosothiols can act as nitrosating agents, promoting the formation of potentially carcinogenic NOCs in the gut epithelium (Kuhnle and Bingham, 2007). However, S-nitrosothiols and iron nitrosyls may also act as a protective mechanism by capturing NO thereby limiting the formation of DNA alkylating agents and facilitating excretion (Hogg, 2007). Further investigation towards the relative role of the different ATNC involved and the origin and regulation of the NO supply is thus required.

Despite the data from human intervention and animal studies showing an increase in ATNC after the consumption of red or processed red meat, the carcinogenicity of the NOCs formed in the gut remains unclear. Mutations induced by diazoacetate, nitrosated glycine, in a yeast functional assay, are very similar to those observed in colorectal tumors, suggesting that NOCs may be involved in CRC (Gottschalg et al., 2006). Furthermore, fecal colon cells containing an O^6-methylguanine DNA adduct, a characteristic promutagenic and toxic adduct formed by many NOCs, have been numbered and found to be significantly related to fecal excretion of ATNC in the stool of volunteers consuming meat in various diets (Lewin et al., 2006). In 2011, a large prospective study investigating the relation between dietary NOCs and the risk for cancer was performed (Loh et al., 2011). Exposure to endogenous NOCs was derived based on the estimated iron content from meat intake considering the relationship between the fecal ATNC and iron intake observed in several human controlled-diet studies (discussed above). Dietary NDMA intake (ng/d) was calculated using a food database of potential carcinogens (EPIC, 2012a). Whereas dietary NDMA intake was associated with a higher incidence of rectal cancer, this association could not be found neither for endogenous NOC exposure nor for dietary nitrite. Large errors associated with the various estimates calculated can explain the lack of a relationship between endogenous NOC exposure and rectal cancer. Furthermore, it cannot be excluded that although the study confirms the previously reported carcinogenic activity of exogenous exposure to NDMA, other food sources than red and processed red meats may be involved. However, increased endogenous formation as the underlying cause for the association between red and processed red meat intake and CRC risk was also not supported by the observation that in the dietary intervention studies of Bingham's group (Cross et al., 2006; Joosen et al., 2009; Joosen et al., 2010), no difference in fecal water genotoxicity in relation to meat intake was detected despite the increased fecal ATNC concentrations. Both masking of the genotoxic effect by genoprotective compounds present in the complex mixture of the fecal water and the inability of the used Comet assay in human-derived colonocyte cell lines to detect genotoxic chemicals in fecal water have been suggested in order to explain this unexpected result (Gratz et al., 2011). Furthermore, NOCs measured as ATNC may promote cancer through a non-genotoxic mode of action. In contrast, a red-meat intake-induced increase in fecal water genotoxicity which was not related to fecal ATNC content nor influenced

by inflammation of the colon was observed in patients suffering from intestinal inflammation as compared to inflammatory bowel disease (Hebels et al., 2011). Based on these results, the authors suggest that the increased genotoxicity is rather linked to heme-catalyzed oxidative stress than heme-stimulated NOC formation.

Effect of Heme on the Oxidation of Polyunsaturated Fats

In rats, carcinogenesis promotion by dietary heme was found to be associated with the urinary excretion of 1,4-dihydroxynonane mercapturic acid (DHN-MA), a fat peroxidation biomarker (Pierre et al., 2004). An increase in this biomarker was also observed in the urine of volunteers given black pudding, a heme loaded blood sausage (Pierre et al., 2006), suggesting that the consumption of red meat stimulates the fat peroxidation pathway. During lipid peroxidation, polyunsaturated fatty acids derived from the phospholipids within the intestinal contents and/or colonocyte cells are oxidized by ROS and RNS. Due to the abstraction of the hydrogen atom from the fatty acid carbon chain, a carbon radical is formed that tends to be stabilized by production of a conjugated diene. The latter rapidly reacts with O_2 to give a hydroperoxy radical that oxidizes other lipid molecules and continues the chain reaction of lipid peroxidation. Lipid peroxides are further degraded generating reactive aldehydes of three to nine carbons in length unsaturated in alpha, beta- positions which are relatively stable and can diffuse throughout the cell (Halliwell and Gutteridge, 1984a; Halliwell and Gutteridge, 1984b; Bartsch and Nair, 2004). Reactive aldehydes, among which malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) are the two most important ones, can either interact directly with DNA and proteins, or undergo further oxidation to more reactive epoxy derivatives (Marnett, 2000). MDA has been shown to be mutagenic in bacterial and mammalian systems (Basu and Marnett, 1983) through the formation of DNA adducts, such as the malondialdehyde-deoxyguanosine adduct (Marnett, 1994). Increased levels of this adduct have been found in cells of colorectal biopsies from adenoma patients compared with adenoma-free subjects (Leuratti et al., 2002). 4-HNE has weak mutagenic activity but induces apoptosis killing of normal but not precancerous cells, possibly explaining tumor promotion by a selection process (Baradat et al., 2011).

Heme has been demonstrated to catalyze lipid (per)oxidation in several conditions, including digestion as well as processing of food. In a simulated gastric compartment (Lorrain et al., 2009) and in the rat stomach (Kanner et al., 2012), heme compounds such as myoglobin or metmyoglobin were found to accelerate further degradation of lipid peroxides, either ingested with the diet or formed in the stomach (Kanner, 2007). The lipid hydroperoxide decomposition products including hydroxyl fatty acids and potentially toxic electrophiles such as the aldehydes and epoxides formed in the stomach can afterwards be incorporated in gastric and intestinal tissues (Kanazawa and Ashida, 1998). An analogous myoglobin-induced oxidation/peroxidation of polyunsaturated fatty acids has been widely recognized to occur during the storage of meat but the mechanisms involved are very complex and still a matter of dispute (Baron and Andersen, 2002). Nitric oxide modifies and in most cases moderates the prooxidative activity of the heme pigments (Carlsen et al., 2005), as will be discussed more in detail later in this article.

The link of lipid peroxidation with heme in relation to CRC has been further supported by the observation that hemoglobin treatment promoted DNA damage induced by linoleic acid hydroperoxide derived compounds in colon adenocarcinoma cells (Angeli et al., 2011). In a more general context, lipid hydroperoxides of dietary origin have been identified as an important driving force for liver carcinogenesis (Rohr-Udilova et al., 2008) whereas unsaturated fatty acid oxidation contributes to the inflammatory response (Marnett, 2012), a condition associated with CRC.

Metabolization of Heme in the Gut into a Cytotoxic and Promoting Factor

A third possible explanation for the role of heme in the increased risk for CRC associated with consumption of red and processed red meat involves a direct effect of heme (or one of its metabolites) on colonic cells and was formulated by the research group of Van der Meer. They showed that addition of hemin to a low-calcium diet of rats increased epithelial proliferation in the colonic mucosa and induced cytotoxicity of fecal water. Hyperproliferation was considered as a compensation for cytotoxicity (Sesink et al., 1999). Furthermore, hemin-fed rats excreted much less host DNA in feces compared to controls, suggesting that hemin decreased cell differentiation and exfoliation of colonocytes in the gut lumen (Van Lieshout et al., 2004). Recently, the research group investigated the role of the PPARα, a nuclear hormone receptor known to protect against oxidative stress and lipid peroxidation, in heme-induced hyperproliferation and hyperplasia. Since the five investigated PPARα target genes did not respond to heme, they concluded that probably not ROS-induced stress but cytotoxicity-induced stress initiates colonic hyperproliferation (IJssennagger et al., 2012a). This hypothesis was further supported by the later observation that not ROS production but luminal cytotoxicity coincided the changes in crypt signaling and hyperproliferation in heme-fed mice (IJssennagger et al., 2013). However, until now, the speculated heme-based cytotoxic factor has not yet been identified (Santarelli et al., 2008). Within this respect, it is important to note that in most of the experiments of this research group hemin was used instead of food heme. In previous work, Glei and others (2006) demonstrated that hemoglobin and hemin were genotoxic in an in vitro model of human colon cells. However, the genotoxic effect of hemoglobin was observed at non-cytotoxic concentrations whereas the DNA damage induced by hemin always coincided with cytotoxicity. These data suggest that heme compounds derived from red and

processed red meat intake may have other modes of activities in the gut lumen.

Effect of Processing on Red Meat Toxicity

Epidemiological studies indicate that the risk of CRC incidence is higher per g increase in intake of processed (cured) meat than per g of unprocessed red meat (van den Brandt and Goldbohm, 2006; Boyle et al., 2008; Santarelli et al., 2008; Moore, 2010). In a recent report summarizing evidence of the EPIC study involving 448,568 men and women, Rohrmann and others (2013) concluded that after correction for measurement error, higher all-cause mortality was significantly related only to processed red meat and not to red meat intake. The authors even estimated that 3.3% (95% CI 1.5% to 5.0%) of deaths could be prevented if all participants had a processed red meat consumption of less than 20 g/day. Furthermore, based on the results of animal studies, it has been suggested that nitrosyl heme present in processed red meat and hemin used in experimental diets are more toxic than the native heme from fresh meat myoglobin (Santarelli et al., 2008; Pierre et al., 2010). The important impact of these findings justifies a closer look at meat processing and the reactions involved. Pig meat is the world's most widely eaten meat. In Western countries, at least about 50% of pig meat is processed and pig meat is estimated to provide about 80% of processed meats (FOD economie, 2010; US Pork Center of Excellence, 2013). As the latter contains less heme iron than beef, the presence of heme cannot be the sole factor responsible for the increased risk of CRC associated with processed red meat as compared to fresh meat. Possible explanations most likely relate to the changes provoked by the addition of processing additives and by processing conditions.

The Curing Process

Apart from smoking, meat processing basically involves "curing", a process dating back to ancient times and mainly characterized by the addition of salt (NaCl) containing sodium nitrite (and/or potassium nitrate as a source of nitrite) (Honikel, 2008). Whole meat cuts (hams) are cured by covering with dry salts or by immersion in brines whereas minced (ground) meat is cured by mixing ("cuttering") meat and fat with dry salts. Sodium nitrite should be added as a mixture with NaCl (nitrite or curing salt e.g. NaCl containing 0.6% $NaNO_2$) in order to prevent instantaneous production of toxic nitrous vapors (NO_2). Permitted levels of sodium nitrite in food in Europe and the US are respectively 150 ppm (EC, 2006) and 200 ppm (CFR, 2012). Other additives often used are ascorbate or erythorbate and sugars. The presence of salt dissolves proteins and leads to osmotic drying followed by coagulation of proteins and weight loss during further air drying for periods ranging from some weeks to several months (Heinz and Hautzinger, 2007).

Nitrite is mainly used to produce the characteristic bright red (pink when cooked) color originating from its reaction with myoglobin and/or for its anti-bacterial effect, specifically against *Clostridium botulinum*. Furthermore, nitrite effectively controls rancidity by inhibiting lipid peroxidation (Pearson and Gillet, 1996). Central for all these functions of nitrite are the complex reactions between the heme cavity in the meat pigment myoglobin and reactive oxygen and nitrogen species (Skibsted, 2011). Considering the eight possible states of oxidation of N ranging between -III (NH_3) to +V (HNO_3) together with the existence of intermediate perferryl ions (Fe:+IV and Fe:+V) (Qian and Buettner, 1999) apart from the ferrous (Fe: +II) and ferri (Fe: +III) ions, it is not surprising that a very complex set of possible reactions involving interactions with numerous food components in different environmental conditions has been reported (Honikel, 2008; Skibsted, 2011).

After addition to meat, nitrite primarily acts as an oxidant ($NO_2^- + H_2O + e^- \rightarrow NO + 2OH^-$). During this reaction, endogenous (e.g. NADH) or intentionally added (e.g. ascorbate) reductants become oxidized. In acidic conditions such as those often found during food processing operations, nitrous acid (N_2O_3) is formed from nitrite. This nitrous acid can on its turn be transformed to its anhydride which is in equilibrium with the oxides NO and NO_2. Myoglobin (Mb) can also act as a reductant in this reaction due to oxidation of the Fe^{2+} to Fe^{3+} yielding metmyoglobin (MetMb), inducing the initial colour change from fresh red to the brown colour. In presence of reductants such as NADH and ascorbate, MetMb can be transformed back to Mb. The NO formed in one of the previous reactions can then bind strongly to Mb with formation of a heat stable NO-myoglobin inducing a second colour transformation yielding the characteristic red color of cured meat (Honikel, 2008; Skibsted, 2011). This compound loses the globin bond to iron upon heating (Sun et al., 2009) resulting in nitrosylmyochromogen (or nitrosylprotoheme), the pigment changing the product color from bright red to the characteristic pink colour of cooked cured meat. Some researchers speculate that free nitrosylheme is more toxic than fresh meat myoglobin (Santarelli et al., 2008) as weak mutagenic activity for this compound has been reported in the Ames test (Stevanovic et al., 2000). However, in vivo genotoxicity data on nitrosylheme are still lacking.

Nitrite is also involved in the complex reactions which occur during lipid oxidation. Within this context, Nicolescu and others (2004) concluded that the reaction of NO derived from nitrite, with the free radical intermediates in lipid oxidation acts as a lipid radical chain termination agent. The "lipid nitrites" are labile and will function both as NO donors and antioxidants. Both these aspects are obviously more important for minced cured meat products, containing large amounts of adipose tissue susceptible to oxidation and possibly acting as NO donors during processing and conservation but also during digestion. Production of lipid nitrites contributes to the well recognized antioxidant effect of nitrite that effectively controls

rancidity in processed red meats by inhibiting lipid peroxidation (Freybler et al., 1993). It is indeed remarkable that in spite of the oxidative properties of nitrite, and their possible strengthening by the presence of iron and chloride, little lipid oxidation is observed in processed red meats. It should however be clear that the oxidation status of lipids is also determined by other reactions involving nitrosomyoglobin with depletion of oxygen or peroxides and production of NO as described in detail by Skibsted (2011). Peroxynitrite, formed by the reaction of NO with the superoxide radical anion ($NO + O_2^{\cdot -} \rightarrow ONOO^- \rightarrow NO_3^-$) may initiate lipid oxidation. Antioxidant activity on the other hand also involves NO binding to the heme ferrous ion forming nitrosomyoglobin and to other non-heme iron proteins preventing pro-oxidant iron release (e.g. from the porphyrin) (Morrissey and Tichivangana, 1985) during an attack of H_2O_2 or hydroperoxides (Kanner et al., 1994). The importance of the latter mechanism is suggested by the antioxidant effect of the isolated cured meat pigment in the absence of nitrite (Shahidi and Pegg, 1992). Many other possible reactions may be involved however as e.g. a contribution to an antioxidant medium by initial oxidation of NO_2 in the presence of oxygen (Honikel, 2008). Also, the pro-oxidant activities of heme pigments present are strongly affected by low pH, the initial prevalence of deoxygenated heme pigment and the oxidative modification of the heme pigments as well as the degree of proteolysis (Carlsen et al., 2005). Last but not least, it is clear that during conservation of processed red meats, the presence of O_2 and temperature conditions affect further oxidation reflected in browning of the products. Exposure to light intensifies oxidation but only in the presence of O_2 as discussed in detail by Skibsted (2011).

Effect of Nitrite on the Formation of NOCs and Lipid Peroxidation

Apart from the well-studied reactions described above, the use of ^{15}N-labelled sodium nitrite has shown that 73–87% of added nitrite is retained in the muscle proteins of bacon (Woolford and Cassens, 1977). This underlines the importance of residual nitrite in cured meat proteins as a "hidden NO generating pool providing nitric oxide for the numerous reactions occurring during storage and cooking of cured meats" (Skibsted, 2011). One important reaction is the formation of NOCs. Nitrite used for meat curing can stimulate both exogenous and endogenous formation of NOCs. NO and NO_2 generated from N_2O_3 in acidic conditions during processing or in the stomach can react with secondary amines or amides forming potentially carcinogenic NOCs (d'Ischia et al., 2011). Residual nitrite in processed red meat is not limited to the protein fraction as added ^{15}N-labelled sodium nitrite was recovered in cured whole adipose tissue (Goutefongea et al., 1977) and for up to 20 -25% in the adipose part of cured bacon (Woolford and Cassens, 1977). Nitrite present in the fat tissue can contribute to lipid peroxidation. Later work in model systems has demonstrated the formation of nitrosated lipids by addition of nitro groups to the double bonds of unsaturated lipids (Mouloud et al., 1992), and recognized these compounds as agents responsible for nitrosamine formation during frying of cured meat (Ross et al., 1987).

Most of the available evidence from epidemiology and animal experiments supports an enhancing effect of nitrite on the formation of NOCs both in food and endogenously. Haorah and others (2001) showed that hotdogs contained 10 times more NOCs than fresh red meat. Furthermore, ATNC derived by nitrosation of NOC precursors present in hotdogs induced colonic aberrant crypts in the mouse colon (Davis et al., 2012). However, Drabik-Markiewicz et al. (2009) observed that although levels of volatile nitrosamines in a cured meat model increased with temperature and amounts of nitrite added during processing, they stayed < 10 μg/kg as long as the nitrite level of 120 mg/kg was not surpassed. This observation justifies strict regulations on the use of nitrite in meat processing as well as the common addition of ascorbate as originally suggested by Mirvish and colleagues (1972). Indeed, according to Skibsted (2011), the addition of 500 mg/kg of sodium ascorbate or erythorbate (isoascorbate) may prevent formation of potentially carcinogenic NOCs as ascorbate reacts faster than secondary amines with the nitrosating agent N_2O_3. Interestingly, ascorbate in batters contributes together with nitrite and salt also to the reduced toxin production by proteolytic *Clostridium botulinum* types A and B (Robinson et al., 1982).

Several studies indicate that the presence of nitrite in processed food can also increase endogenous formation of NOCs. As for red meat, diets based on processed red meat have been reported to increase the fecal ATNC content but the effect of processed red meat appeared to be more pronounced. In mice, diets containing 18% hot dogs increased fecal ATNC excretion 3.7–5.0 fold compared to controls whereas equal amounts of beef resulted in a 2.6–2.9 fold increase (Mirvish et al., 2003). As fecal ATNC contain both unabsorbed dietary NOCs and endogenously formed NOCs, it was not clear if the observed increase was related to the increased dietary NOCs content of processed red meat. However, the observed effect on fecal ATNC levels does not appear to be specific for nitrite from processed red meat. In a later experiment, the same research group showed that fecal ATNC excretion in mice increased with nitrite in the drinking water, an effect even more increased by the presence of heme (Mirvish et al., 2008). Until now there is no or inconclusive evidence for nitrite being a colon carcinogen. In vitro work showed that high concentrations of nitrite promote cancer cell progression in cells representing stage 4 colon carcinomas whereas low concentrations of nitrite inhibit cancer cell progression at early stage (Jiang et al., 2012). The lack of a clear carcinogenic action of nitrite is not surprising as nitrite is ubiquitously present in food including vegetables and polluted drinking waters. Furthermore, humans are exposed to 4.5–13.5 mg nitrite/d from the

saliva derived from enterosalivary recirculation of dietary nitrate reduction in the oral cavity (d'Ischia et al., 2011). Swallowing saliva in combination with virtually any food would thus increase endogenous NOC formation (Sindelar and Milkowski, 2012). The general safety and even beneficial role of nitrate/nitrite in human health has indeed been extensively confirmed (Parthasarathy and Bryan, 2012; Sindelar and Milkowski, 2012). Endogenous production of nitrite and the important intake of nitrate through other foods considerably reduces the probability that these compounds are solely responsible for the increased CRC risk associated with the consumption of processed red meat. A synergistic effect of multiple compounds present in processed red meat (including nitrosatable precursors, heme and nitrite) in an acidic environment may be more likely. One hypothesis involves that in such conditions a specific carcinogenic component within the ATNC is produced which is different from those generated when only nitrite is present. Obviously more advanced analytical techniques are required for identification of such compounds in colon/fecal contents. Research within this domain is progressing and recently over 30 putative NOCs have been identified in fecal material (Clarke et al., 2011). A Danish study involving 185 archival CRC samples collected from participants of the EPIC-Norfolk study supports the hypothesis that the increased risk for CRC associated with the consumption of processed red meat products is related to an increase in the levels of NOCs. Indeed, CRC cases harboring GC-to-AT transition mutations were associated with processed red meat consumption, suggesting a dietary link with alkylating agents such as NOCs (Gay et al., 2012). The hypothesis was further supported by the observation that the promoting effect of nitrite treatment on preneoplastic lesion in the rat colon was linked with an increased fecal ATNC content. As the amount of lipoperoxides present in the fecal water and its cytotoxicity did not correlate with the increased occurrence of preneoplastic lesions, the authors concluded that not increased lipid peroxidation but an effect on NOC formation is responsible for the procarcinogenic effect of processed red meat (Santarelli et al., 2010). These results were recently confirmed by another study from the same research group (Santarelli et al., 2013) investigating the effect of nine different cured meat products on fecal and urinary biomarkers associated with heme-induced carcinogenesis promotion in rats. Two diets, i.e. the ones that included hot dogs and dry fermented sausage, induced a significant increase in cytotoxicity of the fecal water and both diets also increased the number of preneoplastic lesions. Although dry fermented sausage contained eight times more thiobarbituric acid reactive substances (TBARS, byproducts of lipid peroxidation) than hot dogs, again no association was found between the occurrence of preneoplastic lesions and the biomarkers for lipid oxidation. Hence, the authors suggest that nitroso-compounds are major pro-cancer factors in the gut of processed-meat eaters.

An Effect of NaCl?

A major effect of meat processing (nitrite curing) is of course the increased salt (NaCl) content reaching values ranging from 1 to 10%. The presence of Cl^- leads to the production of nitrosyl chloride following $HNO_2 + H^+ + Cl^- \rightarrow NOCl + H_2O$. NOCl is more reactive than N_2O_3 and chlorine may contribute with nitrite and ascorbate to the Fenton reaction producing hydroxyl radicals from ROS formed in biological tissues (Skibsted, 2011). Salty diets and salted foods have consistently been related to stomach cancer, mainly in Japan. Furthermore, NaCl enhances experimental gastrocarcinogenesis by N-methyl-N'-nitro-N-nitrosoguanidine in rats (Takahashi et al., 1994). Until now, no link has been published between salt intake and CRC (Santarelli et al., 2008). However, it has been suggested (Demeyer and De Smet, 2011) that free radical damage in the gut by myeloperoxidase (MP) catalyzed generation of reactive chlorinated intermediates may be linked to plasma chloride levels increased by salt in consumed processed red meats. This hypothesis deserves further consideration and research.

The White Meat Controversy

The evidence listed above identifies heme as the major responsible carcinogenic compound in red meat (mainly beef

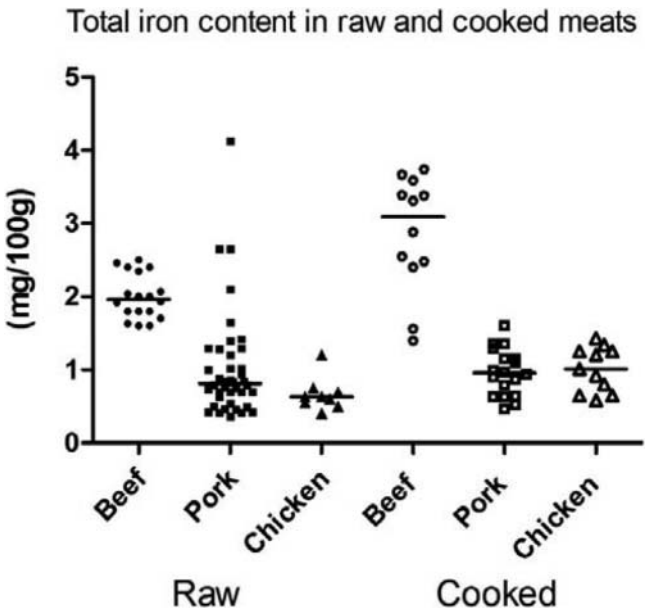


Figure 1 Total iron (mg/100 g) in raw and cooked beef, pork and chicken. Each dot represents a mean value and horizontal lines are median values. References: (Clark et al., 1997; Lombardi-Boccia et al., 2002; Purchas et al., 2003; Williamson et al., 2005; Ventanas et al., 2006; Dannenberger et al., 2007; López-Alonso et al., 2007; Gerber et al., 2009; Greenfield et al., 2009; Rooke et al., 2010; Schönfeldt and Hall, 2011; Tomovic et al., 2011; Lopez-Alonso et al., 2012 and Pretorius et al., 2013).

and pork) for the increase in CRC risk. Addition of nitrite during red meat processing may reinforce the effects of heme or even increase the toxic effect of red meat through a separate mechanism. However, one important discrepancy still exists, i.e. the so-called "white meat" controversy. Before entering into this discussion, it is important to review what is understood by 'red meat'. Red meat is often used as a culinary term referring to meat which is red when raw, and not white when cooked. It includes the meat of most adult mammals and some fowl (e.g. ducks). In a more precise manner, the terminology "red" and "white" reflects the relative proportion of red and white muscle fibers known to be more in favor of white fibers in pigs than in beef. Concomitantly, the heme content is higher in red than in white muscle fibers. It was based on this definition that the U.S. National Pork Board started an advertising campaign positioning pork as "the other white meat", a slogan retired in 2011 whereas pork is clearly considered as red meat by USDA (USDA, 2013). Meat from pigs together with that derived from calves, cattle and sheep (including lamb) was also defined as red meat within the EPIC study (EPIC, 2012b). Furthermore, the WCRF/AICR considers meat from cattle, goats, sheep and pigs as red meat (WCRF/AIRC, 2012).

The major concern regarding the hypothesis of heme as the carcinogenic compound in red meat relates to the observation that heme contents of chicken and pork are of similar magnitude and both lower than beef (Lombardi-Boccia et al., 2002). A wider literature survey suggests that iron contents of raw and cooked chicken and pig muscle are of similar magnitude and are considerably lower than total iron contents of beef, although widely varying with the nature of muscle and the type of production (Fig. 1). Median values for total iron in raw beef, pork and chicken from this survey were 2.0, 0.8 and 0.6 mg/100 g respectively (3.1, 1.0 and 1.0 mg/100 g for cooked beef, pork and chicken respectively). The proportion of heme iron (% of total iron) decreases from beef over pork to chicken, and was on average 87 (77), 61 (62) and 38 (28)% for raw (cooked) beef, pork and chicken respectively in the study of Lombardi-Boccia and colleagues (2002). These difference in heme iron content do not seem large enough however to account for the consistent absence of a carcinogenic effect of chicken consumption in contrast to red meat (mainly beef and pork) consumption. Within this context, the increased risk of CRC associated with the consumption of red meat may be related to a specific component of red meat (mainly beef and pork) other than heme: the mammalian cell surface sialic

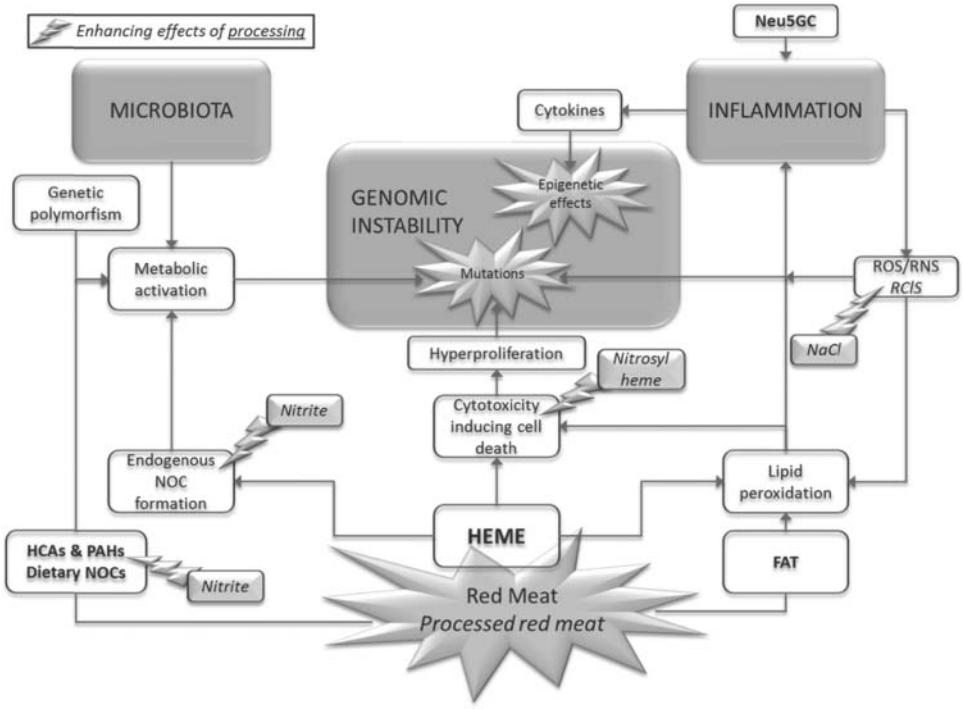


Figure 2 Combined effects of multiple compounds present in red and/or processed red meat on the development of colorectal cancer (CRC). Mutagens present in meat or formed during processing or preparation such as polycyclic aromatic hydrocarbons (PAHs), heterocyclic amines (HCAs) and dietary N-nitroso compounds (NOCs) can initiate mutations in epithelial cells after metabolic activation. In addition, heme catalyzes endogenous formation of NOCs. Hyperproliferation of epithelial cells resulting from the cytotoxic effects of secondary bile acids generated from fat, end products of fat peroxidation and heme may lead to further accumulation of mutations. Next, although not the initiating event, inflammation may further enhance the development of CRC by the production of reactive oxygen species (ROS)/reactive nitrogen species (RNS) and cytokines. Compounds present in red and processed red meat that can trigger inflammation include heme-catalyzed lipid peroxidation products and Neu5GC. The more pronounced effects of processed red meat on CRC risk may be related to the enhancing effect of nitrite on exogenous and endogenous NOC formation, by an increased cytotoxic effect of nitrosyl heme and/or the increased formation of reactive chlorine species (RClS) enhancing inflammation.

acid N-glycolylneuraminic acid (Neu5Gc). This compound is not produced by bacteria or plants, is low or absent in poultry and fish, but abundant in red meats (lamb, pork and beef) and in bovine milk (Byres et al., 2008). Humans however are genetically deficient in Neu5Gc production and instead metabolically accumulate it from dietary sources, particularly red meat and milk products. This metabolically-accumulated dietary Neu5Gc results in the production of circulating anti-Neu5Gc antibodies leading to local chronic inflammation (Hedlund et al., 2008). Intake of Neu5Gc alone may be insufficient to induce CRC as milk products have not or even inversely been associated with an increased CRC risk. In (processed) red meat products however, the combined presence of Neu5Gc with heme and genotoxic compounds (e.g. PAHs, HCAs and NOCs) may increase the propensity to develop diet-related carcinomas. Interestingly, high calcium contents of experimental diets have been shown to reduce CRC risk associated with (processed) red meat intake both in animals (Sesink et al., 2001) and in humans (Pierre et al., 2013), probably through interference with the effects of heme. In spite of the presence of inflammatory Neu5Gc, the significantly higher calcium levels in processed dairy products (~500 mg/100g) compared to processed red meat (~10 mg/100 g) (NUBEL, 2013) may thus account for the protective effects of dairy foods as reported earlier (Park et al., 2009; Pala et al., 2011). Anyway, the near absence of Neu5Gc in poultry and fish could provide an explanation for the "white meat controversy" and its importance justifies further research efforts.

CONCLUSION

Over the last decades, epidemiological evidence linking the consumption of red and, more convincingly, of processed red meat to CRC has accumulated which resulted in dietary recommendations, presenting a challenge to the meat processing industry (Demeyer et al., 2008). Multiple hypotheses have been put forward to explain the increased CRC risk associated with red and processed red meat intake. Yet, none of these appears to be sufficient as discrepancies (e.g. the "white meat" controversy) still exist. Considering the different hazardous compounds that may be present in red and processed red meat, the increased risk for CRC may not be associated with one single causative agent, but with the presence of a mixture of different carcinogenic compounds, acting on multiple stages of CRC development (Fig. 2). As discussed in the introduction, CRC is a multistep process; tumor initiation by the induction of mutations is not sufficient to develop cancer and additional processes such as tumor promotion and progression are required. High intakes of red and in particular processed red meat in unbalanced diets may create an environment which favors the different steps of CRC development. Environmental mutagens present in red meat such as PAHs, HCAs and dietary NOCs can initiate mutations in epithelial cells. Within this process, genetic polymorphism and microbiota also play an important role as the three classes of compounds are only mutagenic after metabolization. Besides acting as an exogenous source of NOCs, red meat further contributes to the endogenous formation of these compounds through a catalytic action of heme. In processed red meat, NOC formation is even further enhanced due to the presence of nitrite. Consequently, it is clear that both red and processed red meat contain compounds capable of inducing mutations. Hyperproliferation of epithelial cells due to cytotoxic effects can lead to accumulation of mutations. Secondary bile acids generated from fat, the end products of fat peroxidation and heme have all been reported to exert a cytotoxic effect on the intestinal mucosa. This mechanism may be in particular important for nitrosyl heme, present in processed red meat, and may explain the increased risk associated with processed red meat compared to red meat. Finally, inflammation may also be involved. Although probably not the initiating event in CRC induced by red and processed red meat intake, inflammation can contribute to the further development of CRC. Indeed, ROS and RNS released by activated inflammatory cells can cause further DNA damage whereas cytokines may result in epigenetic changes that silence tumor suppressors or promote tumor initiation. Compounds in red and processed red meat that can trigger inflammation include heme-catalyzed lipid peroxidation products and Neu5GC. Of particular interest is the fact that the latter could provide an answer for the "white meat" controversy. Additional mechanistic studies are needed to investigate the combined CRC promoting effects of compounds present in red and processed red meat. A possible role of NaCl (e.g. by enhancing the production of reactive chlorine species) should not be ignored and, interestingly, a combined effect has been recently described for a PAH (BaP) and a HCA (PhIP) (Jamin et al., 2013), but much more research in this area is necessary.

ACKNOWLEDGMENT

This literature review has been performed within the context of the formulation of a scientific advice on 'Red meat, processed red meats and the prevention of colorectal cancer' by the Belgian Superior Health Council. The authors were member of the ad hoc workgroup, and sincerely thank the other members of this workgroup (G. De Backer, S. De Henauw, N. Delzenne, L. Herman, G. Maghuin-Rogister, N. Paquot, M. Peeters and J. Van Camp).

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