

August 28, 2015

Dr. Veronique Bouvard, Responsible Officer  
Kurt Straif, Head of the IARC Monographs Programme  
IARC  
Lyon, France

**Re: Volume 114: Red Meat and Processed Meat – Call for Data – Systematic Review: Mechanisms of Dietary Heme Iron Intake in Colorectal Cancer**

Dear Drs. Bouvard and Straif:

Although the human observational data linking red meat and various cancers remains inconclusive, a number of potential mechanisms have been hypothesized related to a role for red and processed meat in various cancers, including compounds formed during the heating process (Demeyer et al., 2015). However, none of these substances are inherent to red meat and have numerous additional and more significant sources of exposure. An exception is the proposed mechanism related to the naturally occurring essential nutrient, iron, which is a component of heme iron as it occurs in red meat. The European Food Safety Authority (EFSA, 2015) recently reiterated earlier conclusions (EFSA, 2004) that iron is an essential nutrient, evidence is insufficient to establish a tolerable upper intake level (UL) for iron, and insufficient data exists to link total dietary iron to increased risk of colorectal cancer (CRC) (SACN, 2010). We are aware that IARC will be considering the "High Priority" evaluation of "Dietary iron and iron used as supplements or for medical purposes" sometime in the future, and we also see that haeme (heme), an iron-containing compound, will be a part of this evaluation (IARC, 2014). Consequently, **we urge IARC to first systematically evaluate heme, from all dietary sources, in a separate monograph process and avoid consideration of the role of heme derived from red and processed meat prior to a complete mechanistic evaluation of heme.** In as much as heme iron is a component of red and processed meat, we offer the following results of a systematic review of dietary heme iron and CRC.

**Systematic Review: Mechanisms of Dietary Heme Iron Intake in Colorectal Cancer**

**EXECUTIVE SUMMARY**

A search of the PubMed and Embase databases for peer-reviewed publications of mechanistic evidence relating dietary heme iron and CRC, revealed the following:

**Results**

- Of 210 search results since 2000, **only 13 publications met the full criteria for inclusion.** Eight studies were conducted in rodents (seven in rats and one in A/J Min/+ mice), one study was conducted in both humans and rats, and four studies in humans were case-cohort or case-control designs.
- The primary reasons for study exclusion at Tier 1 were:
  - Heme iron was not administered orally or as part of a diet (Criteria 1), and
  - No original data was provided, typically a review paper or abstract (Criteria 5)
- The primary reason for study exclusion at Tier 2 was:
  - Failure of the study design to accurately represent human colon physiology or metabolism (Criteria 8)

## Conclusions

- There are several critical research gaps in mechanistic evidence linking dietary heme iron, from red and processed meat, and CRC.
- The type of heme iron supplemented in animal diets, as surrogates for naturally occurring heme iron from hemoglobin and myoglobin in red and processed meat, independently affects outcomes.
- None of the various mechanisms tested by studies included in this review, including oxidative stress, inflammation, cytotoxicity and perturbations to the normal process of apoptosis, are supported by evidence sufficient to confirm a mechanistic link between red meat intake and CRC.
- **Evidence is weak and inadequate in both humans and animals concerning the mechanistic relationship between dietary heme iron, from red and processed meat, and the development of human CRC.**

## **STUDY PROCESS AND STUDY QUESTION**

The use of a systematic approach for retrieval and selection of literature to clarify scientific support for cancer mechanisms of action was described in a case study by Kushman et al. (2013). We used this approach and modified their general question “what are the mechanisms by which a chemical may cause carcinogenicity in the target tissue?” to the more specific question “**what is the mechanism by which naturally occurring heme iron intake from a food, or as part of an overall diet, may cause or promote carcinogenicity in colon and/or rectal tissue?**”

Investigations of heme-catalyzed nitrosation were not included in this review because nitrosation-related mechanisms, including endogenous nitrosation, are being evaluated separately in a future review (in preparation).

## **DEVELOPMENT OF EVALUATION CRITERIA FOR DIETARY HEME IRON INTAKE**

Kushman et al. (2013) reported the evaluation criteria used for studies of mechanisms of action for the agent of interest in their di(2-ethylhexyl)phthalate (DEHP) case study. We used these criteria, with adaptations, to accommodate intake from a whole food or as part of an overall diet. Our modified criteria allowed selection of only those studies with design and dietary detail sufficient for making mechanistic conclusions regarding human dietary heme iron intake and CRC. The following factors were considered:

### **A. Selection of appropriate research models**

*In vitro* - Data from mechanistic studies conducted exclusively *in vitro* have limited applicability to metabolism in the normal colon. Cells that line the colonic crypts in the intact animal are structurally polar, with strict orientation toward either the lumen or the circulatory system. As a result, in a normal system these cells receive exposure to carcinogenic and anti-carcinogenic agents from both luminal contents and the circulation. In addition, normal colonocytes have a short lifespan, fully-differentiating as they migrate to the top of the crypt structure before sloughing into the lumen. This complex and multilayer anatomy is poorly represented by monolayers of cells grown in culture; and, the rapid differentiation of normal colonic epithelial cells cannot be represented in transformed cell lines (Eisenbrand et al., 2002).

*In vivo* - The use of animal models has the advantage of representing dietary exposure via whole body metabolism and is particularly important when considering iron metabolism. Iron absorption in the proximal intestine is governed by multiple factors including the form of ingested iron and the presence of antioxidants or binding agents in the diet. Most important, iron absorption is influenced by the current iron status and iron binding capacity of the animal (Hunt, 2005; Sharp, 2010). In addition, the type, source and amount of residual iron in the colon are of interest for studies in cancer prevention.

Genetically modified animal strains may not accurately model normal iron metabolism. The extent of the genetic mutation must be clear in order to accurately interpret data. Iron absorption is regulated by complex intestinal system (Gulec et al., 2014; Shayeghi et al., 2005) with iron status being significant contributor to the activation and deactivation of various regulators. It is uncertain if the consistency with which genetically modified animals reflect normal iron transport and if iron deficiencies could exacerbate perturbations of iron regulation (Xue et al., 2012) that may already be present in various cancer models.

## **B. Selection of an appropriate testing agent**

The use of heme and other heme-containing chemicals as test agents may not accurately reflect heme iron intake from red and processed meat. Red meat contains heme iron from both myoglobin and hemoglobin. The content and proportion of these heme-containing proteins varies widely according to the species of origin, the age of the animal, and anatomical location of the source (Lawrie and Ledward, 2006). In many red meats, such as beef, myoglobin contributes up to 50% of the heme-iron, making myoglobin particularly important for studies intended to represent typical meat intake (McKenna et al., 2005; Joseph et al., 2012). Isolated forms of heme do not accurately represent the effect that cooking, and other common forms of denaturation used in food preparation, have on the heme-containing proteins from meat (Joseph et al., 2010; Suman et al., 2005; Suman and Joseph, 2014).

## **C. Reporting diet composition**

### ***Importance of the whole diet***

There are a large number of nutrients and compounds found within the diet that can prevent the occurrence of cancer (Yerba-Pimentel et al., 2015). Such dietary components are known to influence cell proliferation and cell death, as well as carcinogen activation and detoxification (Zanini et al., 2015). Intended or unintended modifications of these chemo-preventive compounds in study diets can invalidate diet-related results. The complete composition of the diet must be known and diet-related statistical analyses performed in order to determine the effect of a diet-related exposure on mechanistic pathways or events (Bidlack et al., 2009). In addition, recent information regarding diet–gene interactions may clarify biological mechanisms that link various dietary components with CRC in humans (Andersen et al., 2013).

### ***Relevance to a human diet***

Studies of colon carcinogenesis often utilize chemicals and methods of tumor initiation and promotion, which require severe restriction of the intake of certain nutrients, most notably calcium, fiber, and antioxidants. The diets used in such tumor protocols may have little relevance to human nutrient intakes.

While it is clear that iron has toxic effects when consumed at very high levels, it is also an essential nutrient required for life. Like many nutrients, iron is defined in terms of the serious health consequences, which result from intakes that are either too low (deficiency) or too high (toxicity). This so-called “U-shaped” curve is a well-recognized scientific principle in the fields of both nutrition and toxicology (Douron et al., 2010; IOM, 2001; Munro et al., 2006). Major scientific groups are in general agreement regarding the level of iron needed for prevention of deficiency. However, not all competent authorities agree that an upper limit (UL) for iron is justified. The European Food Safety Authority (EFSA, 2015) recently reiterated earlier conclusions (EFSA, 2004) that evidence is insufficient to establish a UL for iron and that insufficient data exists to link total dietary iron to increased risk of CRC (SACN, 2010).

## INCLUSION/EXCLUSION CRITERIA FOR MECHANISTIC STUDIES OF HEME IRON INTAKE

The resulting inclusion/exclusion criteria, based on the above considerations, are shown in **Appendix A, Table 1**. These criteria differ from those used in the Kushman case study with regard to: the inclusion of dietary heme iron as the agent of interest; designation of colon/rectum as tissues of interest; and a required description of any model which varied from normal colon physiology. The criteria also require studies to report diet composition in detail and to statistically analyze outcomes according to relevant dietary variables.

## SEARCH RESULTS

Results of PubMed and Embase searches following application of the dietary intake evaluation criteria are shown in the table below. **Appendix A, Table 2** provides the full list of citations and primary reasons for exclusion.

Database	Search terms	Number of citations
PubMed	(colorectal cancer) AND heme iron Filters: From 2000/01/01 to 2015/07/06	Initial search = 58 Tier 1 screen (abstract/full text) Include = 18 Exclude = 40 Tier 2 screen (full-text) Include = 9 Exclude = 9
Embase	(colorectal cancer) AND heme iron Filters: From 2000/01/01 to 2015/07/06	Initial search = 152 Tier 1 screen (abstract/full text) Include = 7 Exclude = 145 Tier 2 screen (full-text) Include = 4 Exclude = 3
Final Total		Total Included = 13

**Out of the original 210 results identified in this search, only 13 studies were considered acceptable, meeting full inclusion criteria with sufficient study design and diet detail, for evaluation of heme iron intake in relation to CRC.**

## EVIDENCE REGARDING DIETARY HEME IRON FROM INCLUDED STUDIES

Evidence from the thirteen included studies, as related to dietary heme, is presented below and summarized in **Table 3, Appendix A.**

- **Andersen et al. (2011)** studied the heme oxygenase-1 polymorphism, *HO-1 (HMOX1) A-413T (HMOX-1)* and the interaction with red and processed meat intake in relation to CRC risk. *HMOX-1* and meat intakes were determined in a nested case-cohort study of 383 CRC cases and 763 randomly selected participants from a prospective study of 57,053 individuals. Heme oxygenase-1, encoded by *HMOX-1*, is the rate-limiting enzyme in the degradation of heme to carbon monoxide, iron and bilirubin, and as a result is a key mechanism for reducing cellular oxidative stress and inhibiting inflammatory cytokine production. The CRC association of *HO-1 A-413T* polymorphism, as well as the interaction with meat intake, were not significant. Thus, the study provides no support for heme degradation as a mechanism of colon carcinogenesis and the authors conclude “The present null result suggests that oxidative stress induced by dietary heme is not a strong risk factor for CRC.” Further, the authors stated by way of a conclusion that “we found no association between the *HO-1* polymorphism and risk of CRC, no interactions between *HO-1* polymorphism and meat intake in this prospective Danish nested case-control study. Thus, this study does not support an important role of meat heme in relation to CRC.”
- **Andersen et al. (2015)** extended their study from 2011 to include a larger cohort of both cases (928) and controls (1,726). The purpose continued to be the assessment of *HMOX1* polymorphism modification of CRC risk and interactions with diet and lifestyle factors. In this larger case-cohort study, the authors found no association between *HMOX1 A-413T* and CRC risk and no interactions between diet and lifestyle and *HMOX1 A-413T*. Therefore, these results continue to provide no support for heme degradation as a mechanism of colon carcinogenesis and that oxidative stress induced by dietary heme or heme iron is not a risk factor for CRC in humans. Furthermore, in their systematic review of interactions between meat intake and genetic variation in relation to CRC, Anderson and Vogel (2015) conclude that “...no support for the involvement of heme and iron from meat or cooking mutagens was found”.
- **Bastide et al. (2015)** studied potential mechanisms linking dietary hemoglobin, heterocyclic amines, nitrites and nitrates with CRC using multiple animal and cell culture models in three separate studies reported in one publication. In study one, the inclusion of 1% hemoglobin in the diet of azoxymethane (AOM)-treated Fischer 344 rats increased the number of mucin-depleted foci (MDF) at 100 d as compared to rats fed diets without hemoglobin. There was an increased thiobarbituric acid reactive substances (TBARS) content in fecal water of the hemoglobin-fed rats. When tested *in vitro*, the fecal water from rats fed hemoglobin-containing diets was found to be more cytotoxic to non-mutated *Apc +/+* cells than to premalignant *Apc +/-* cells. Rats fed a hemoglobin-containing diet also had increased urinary excretion of DHN-MA. Further *in vitro* work indicated that aldehydes from the fecal water of hemoglobin-fed rats, not MDA and not heme, proved to be compounds which were more toxic to non-mutated than mutated cells. In their third study, dietary hemoglobin did not influence the development of colon tumors in *Apc +/-* mice, although this model is not readily susceptible to colon tumors.

Bastide et al. (2015) is the most recent in a series of studies employing a combination of laboratory methods which raise concern regarding applicability to normal colon physiology.

- First, the studies fail to report data from saline-treated (non-AOM) rats, i.e. no control group provided. While these control animals are not expected to develop tumors, parallel data from saline-treated animals for each diet group for all other outcomes is necessary for interpretation of the experimental results in a normal colon.
- A second concern is the interpretation of data using fecal water as an indicator of heme iron consumption. The quantity of heme and the level of TBARS in fecal water is not totally dependent upon heme from the diet. Other data from these same authors show that both the source (type) of heme, as well as the bacterial content and composition in the gut affect the content of fecal water (Martin et al., 2015; Pierre et al., 2003).
- A third concern is that the application of fecal water to cells in culture does not accurately reflect the impact that fecal content may have on colon mucosa *in vivo*; and, none of the studies in this series measures lipid peroxidation directly in colonic mucosa.
- In addition, cytotoxicity of fecal water in an *in vitro* culture is highly dependent on the characteristics of the cells in culture. In this study, the authors verify that APC-/+ cells are highly mutated and resistant to apoptosis, resulting in lower levels of cell death. The greater level of cell death measured in the more 'normal' cell line is to be expected and considered as a beneficial response in the context of carcinogenicity.

Therefore, linking results from carcinogen-treated rats without an appropriate control and highly mutated cell cultures without measuring direct effects on the colon leads to conclusions that may not relate to dietary effects in human CRC.

- **Gilsing et al. (2013)** investigated the association between dietary heme iron intake and risk of CRC with mutations in APC (adenomatous polyposis coli), KRAS (Kirsten ras) and P53 overexpression in the Netherlands Cohort Study. The authors reported that heme iron intake (estimated from meat intake assessed by food frequency questionnaire) was associated with an increased risk of colorectal tumors harboring G>A transitions in KRAS and APC, and with an overexpression of P53. The authors suggest alkylation of DNA as a novel mechanism requiring further research in humans and conclude that oxidative DNA damaging mechanisms do not relate dietary heme with CRC.
- **Gueraud et al. (2015)** measured multiple biomarkers of oxidative stress in Fischer 344 rats fed hemin or ferric citrate in diets containing either fish oil, safflower oil, or hydrogenated coconut oil. Urinary excretion of biomarkers of oxidative stress were modified by both the source of iron and the type of fat in the diet. Fecal water TBARS were greater in rats fed fish oil with either hemin or ferric citrate, and in those fed safflower oil with hemin. These diets also resulted in greater cytotoxicity in an *in vitro* application. It should also be noted, however, that these diets contained higher levels of TBARS indicating peroxidation of the diets prior to feeding, possibly due to the lack of adequate antioxidants in the dietary mixtures, thus making it impossible to make conclusions regarding oxidative damage due exclusively to heme iron.

- **Martin et al. (2015)** used a factorial design to study the role of intestinal microbiota in the development of colon carcinogenesis in Fisher 344 rats. Rats treated or not with an antibiotic cocktail were given a diet containing hemoglobin or ferric citrate. Fecal bacteria were quantitated and TBARS concentrations assayed in fecal water. There was decreased crypt height, reduced proliferation, and a fourfold increase in cecum size in the antibiotic-treated rats compared with controls. Higher fecal water TBARS were present from rats given the hemoglobin diet, which were suppressed by antibiotic treatment. A parallel experiment in rats fed dietary heme yielded similar results. The authors concluded that intestinal microbiota is involved in the generation of lipid peroxidation by heme iron. Thus, it is important to also account for other diet- and non-diet- related factors known to alter intestinal microflora, since red meat is just one such factor (Wlodarska et al., 2015).
- **Pierre et al. (2003)** tested the effects of an AIN-76-based low-calcium diet containing different levels of heme or hemoglobin in AOM-treated Fischer 344 rats. Separate diets tested the addition of calcium, olive oil, and other antioxidants. Aberrant crypt foci (ACF) were quantitated and fecal water was assayed for TBARS and cytotoxicity in erythrocytes. Heme increased the number and size of ACF in a dose-dependent manner, and also increased TBARS content and cytotoxicity of fecal water. Calcium, olive oil and the added antioxidants inhibited the heme-induced ACF promotion and normalized fecal TBARS and cytotoxicity. The hemoglobin-supplemented diets also increased the number of ACF, but not ACF size. In addition, there was a marked difference in the number and distribution of ACF vs major aberrant crypt foci (MACF) in heme vs hemoglobin supplemented rats. Interestingly, the hemoglobin-supplemented diets did not show an increase in fecal TBARS or fecal water cytotoxicity toward erythrocytes. It is important to note that heme and hemoglobin had different effects on the parameters measured in this study and that supplementation with heme inhibited weight gain and increased overall fecal excretion. In fact, overt toxic effects were observed in rats fed the high heme diet with body weights significantly (~10%) below control at 14 weeks. Overall, these results point to the protective action of the calcium, antioxidants, polyphenols and other potential dietary components which would normally be consumed by people in a complex diet, and the need for their consideration when extrapolating the findings of this study to humans.
- **Pierre et al. (2004)** quantitated ACF in colons of AOM-treated Fischer 344 rats fed low calcium diets containing varying concentrations of heme as supplemented from chicken (low heme), beef (medium heme), or blood sausage (high heme). The control diet was supplemented with ferric citrate and the heme control diet with hemoglobin. In addition, no data were included for an AOM control group (saline-treated), making relevance or comparison in a normal colon impossible. After 100 days, all meat diets were shown to promote ACF formation, but only heme-containing diets promoted the formation of MDF. ACF and MDF did not differ between rats fed the beef diet and those fed either the heme control diet (equal amount of heme) or the chicken diet (which purportedly contained no heme). However, the greatest number of aberrant foci as well as the highest level of fecal water heme and peroxidation products was found in animals fed blood sausage. It should be noted that blood sausage is not red meat, it has a nutrient profile that is different from muscle meats, and should not be confused as a surrogate for red meat. The level of heme in the diets of animals fed blood sausage was over 25 times greater than that in the other groups. Results in animals supplemented with blood sausage may represent a pharmacological, rather than dietary effect of heme iron. In addition, the total iron content of the individual diets was not provided in the paper but would be expected to be significantly higher in diets containing blood sausage. Equalization of total iron among the treatment groups is essential for the evaluation of heme iron on any outcome. Therefore, the design of the diets used in this study introduces confounders not considered in the analysis. The authors' conclusion that results "show the promotion of experimental carcinogenesis by dietary meat and the association with heme intake" is not supported by their evidence.

- **Pierre et al. (2006)** investigated, urinary excretion of 1, 4-dihydroxynonane mercapturic acid (DHN-MA) in Fisher 344 rats and humans. In the animal study, chicken, beef or blood sausage was included in the diet of AOM-treated Fischer 344 rats. The human study was a randomized crossover design in which two levels of hemoglobin were fed from red meat or from a diet supplemented with non-heme iron. DHN-MA excretion increased in rats fed blood sausage diets compared to all other diets, and the excretion paralleled the number of preneoplastic lesions in AOM-treated rats. Urinary 8-iso-PGF2A increased moderately in rats fed a high heme diet, but not in humans. Therefore, the evidence in humans indicates no link of dietary heme iron with systemic inflammation as measured by 8-iso-PGF2A excretion.

However, the heme-supplemented diet resulted in a 2-fold increase in DHN-MA in humans. In general, urinary excretion of DHN-MA is an indicator of a normal detoxification pathway, and without other comparators it is not possible to determine whether this level of excretion is associated with CRC. In fact, if this compound is present in the urine as the result of iron induced oxidation, the relationship would be with the whole body status of iron, not luminal content. It is well established that the type/source of iron in the diet, as well as other nutrients, significantly impacts iron bioavailability and status. Therefore, iron status as measured in blood is needed in both human and animal studies reporting urinary excretion of a compound in order to establish its relationship with dietary heme iron. This study did not (nor did any of the animal studies in this review) report the iron status of humans or animals.

Of most concern in the present study is the use of blood sausage as a source of heme iron and its use as a comparator with meat in the design of the experimental diets. As described in the summary above, the amount of heme iron from hemoglobin, as well as total iron, is dramatically higher in the blood sausage diet than in the other diets. The composition of blood is also drastically different with regard to a number of other nutrients, making it a poor comparator for meat. In this study the reported iron content of animal diets containing blood sausage is more than six-fold greater than control.

Due to these limitations in study design, linkage cannot be established for the use of urinary DHN-MA as a biomarker for determining CRC risk in relation to dietary heme, and therefore, no mechanistic link is established for dietary heme in human CRC.

- **Pierre et al. (2008)** utilized a Fisher 344 rat model that employed one injection of 1, 2-dimethylhydrazine (DMH) as an initiating carcinogen in this initiation-promotion protocol. Animals were fed diets based on a modified AIN-76 diet with red meat as a heme source at the level of 60% by weight in the finished ration. Diet variations included supplementation with calcium, olive oil, or other antioxidants. Aberrant crypt foci (ACF) and mucin-depleted foci (MDF) were quantitated; urinary 1, 4-dihydroxynonane mercapturic acid (DHN-MA) was measured; and fecal water TBARS were quantitated and cytotoxicity determined toward mouse tumor cells. Results showed increases in cytotoxicity, as well as increased fecal water TBARS and urinary DHN-MA with consumption of beef at the 60% of diet level (a level which exceeds typical human intake). Animals receiving the high beef diet had increased ACF and MDF compared to control, an effect which was inhibited by the addition of dietary calcium. Calcium supplementation also normalized fecal TBARS and cytotoxicity of fecal water, but it did not reduce DHN-MA levels in the urine. Unexpectedly, rats fed the high-calcium control diet had significantly more ACF and MDF in the colon as compared to those fed the low-calcium control diet. Supplementation with additional antioxidants or olive oil failed to normalize



ACF and MDF in the high meat diet group, and did not impact the other biochemical markers analyzed in this study. The disparate effects of calcium, in addition to the lack of effect from antioxidant/olive supplementation, bring into question any role of oxidative stress in this study. The authors used DMH to initiate CRC in all animals, therefore no comparisons were made to control and data cannot be interpreted for conditions in the normal human colon.

- **Ruder et al. (2014)** conducted two nested case-control studies within the Prostate, Lung, Colorectal and Ovarian Cancer Screening Trial; one was in subjects with advanced colorectal adenoma and one in those with colorectal cancer. The intake of heme iron was estimated from meat intake reported on a food frequency questionnaire and genotyping was performed for 21 genes known to be involved in iron homeostasis, as well as iron uptake, transport, absorption and storage. Dietary iron was positively associated with colorectal adenoma among several of the variations in SNPs and genotypes measured; and, dietary heme iron was positively associated with CRC in a certain genotype. Most importantly, however, none of the associations were statistically significant after adjustment for multiple comparisons. Future research to link dietary heme with CRC in these gene variants seems unwarranted.
- **Sesink et al. (2001)** investigated the relationship of dietary calcium and heme with colonic cytotoxicity *in vitro*, and with epithelial hyper-proliferation *in vivo*. Wistar rats were fed either zero or 1.3 mmol/g heme in diets containing a low or high level of calcium (from calcium phosphate). Fecal water from rats fed the low-calcium + heme diet was more toxic to cells *in vitro* than was fecal water from rats fed the high-calcium + heme diet. Colonic epithelial proliferation did not differ between the low- and high-calcium control groups. However, dietary heme increased proliferative activity in rats fed the low-calcium diet, but not in rats fed the high-calcium diet. The use of only one level of dietary heme prevents establishment of a dose-response. It is not clear whether the levels of heme or calcium used in this study relate to typical human intake, and the authors state: “Because our diets mimicked the composition of human western-style diets, our results may have implications for the human situation...Providing that the results from the present study can be extrapolated to humans, this suggests that a relationship between red meat consumption and colon cancer can only be found in populations with a relatively low calcium intake.”
- **Sodring et al. (2015)** conducted a study of the effects of hemin and nitrite on the formation of tumors in the A/J Min/+ mouse model. In this study, dietary hemin decreased the number of ACF and tumors of the colon. This decrease in tumor load brings into question any heme-related mechanism associating dietary heme with CRC in this model.

#### CRITICAL RESEARCH GAPS OBSERVED IN INCLUDED STUDIES

Although meeting our criteria for inclusion, these 13 studies include design features and subsequent results which limit their ability to link dietary intake of heme iron from red and processed meat with CRC in humans, particularly with regard to its action as an oxidative stressor in the colon. Consider the following:

## **A) The use of inappropriate test agents may introduce bias**

- **The source of heme iron tested influenced the outcomes.** While it seems possible that following digestion, any source of heme iron would ultimately be converted to the protoporphyrin IX molecule, and as such, be treated equally within the intestinal lumen. At least two studies included in this review demonstrate that this is not the case. Martin et al. (2015) and Pierre et al. (2003) provided a comparison of hemoglobin and hemin as test agents. Both studies reported differences in outcomes as the result of the type of heme iron used as the test agent.
  - Martin et al. (2015) stated that in previous studies hemin (0.2–0.5 mmol/g diet) increased the number of cells, and the number of Ki67 labeled cells, per crypt. In comparison, hemoglobin (1.5 mmol/g) did not raise these numbers in the current study. The authors conclude “[w]e can so suspect a difference of toxicity between hemin and hemoglobin. In this way, we have already shown that hemin and hemoglobin do not have the same effects on biomarkers associated with heme-induced promotion”.
  - The study by Pierre et al. (2003) provided a direct comparison of diets supplemented with hemin vs hemoglobin. Rats given a high-hemin diet gained less weight than controls. In contrast, high-hemoglobin diets did not depress growth, in spite of similar food and heme intake. There was also a higher concentration of heme in the feces of hemoglobin-fed rats than in hemin-fed rats. The authors stated “[the observed] differences in haem faecal concentrations were likely due to differences in faecal daily weight: high haemin diets increased the faecal excretion (+33%), a laxative effect noted previously by Sesink [1999], and haem was thus diluted in the faeces. In contrast, haemoglobin was not laxative, and the antioxidants and olive oil normalized the laxative effect of haemin”. The analysis of ACF and MACF data in the Pierre 2003 study showed that hemin promoted or induced the large MACF instead of classical ACF, while hemoglobin promoted the classical ACF and not the MACF. The cytolytic activity of fecal water increased 50-fold in rats fed a high-hemin diet, but according to the authors “surprisingly, absolutely no increase was seen in haemoglobin-fed rats”.
- **Few studies used test compounds that accurately reflect the sources of heme iron in red meat.** The combination of myoglobin and hemoglobin as they occur in red meat was evaluated only in those studies that estimated human meat intake (Andersen et al., 2011; Anderson et al., 2015; Gilsing et al., 2013; Ruder et al., 2014) or directly provided meat as a test agent (Pierre et al., 2004; Pierre et al. 2006; Pierre et al. 2008). The use of myoglobin per se may be impractical due to its availability and perhaps other factors, including cost and stability. Nonetheless, myoglobin is a ubiquitous and significant form of heme iron in red meat and the use of readily available surrogates, such as hemin or isolated hemoglobin, are not equivalent to heme iron from meat (Suman and Joseph, 2010).

- The use of blood sausage (blood pudding) as a source of heme iron and as a comparator for meat in these studies is problematic, introducing a number of confounders. Blood sausage is not a muscle meat, and according to the recipe reported by Pierre et al. (2004), contains no muscle meat products. Blood, by definition, contains a nutrient profile that is different from muscle meat and, as used in the animal diets in current studies, provides over 25 times the hemoglobin and over 6 times the iron content of the high-meat diets (Pierre et al., 2004; Pierre et al., 2006).

## **B) Results from CRC animal protocols are often misinterpreted as they relate to human consumption of red meat**

- **Hemoglobin itself, as a component of the diet, is not a carcinogen.** The majority of animal studies included in this review include data from AOM-treated rats. As the result of AOM injection, colon epithelial cells undergo pathogenesis from minor lesions (ACF), to adenoma and malignant adenocarcinoma over a period of approximately 14 weeks (Chen and Huang, 2009). The carcinogen, AOM, is complete and the process begins from the moment of injection. The ability to test the effect of outside factors on the ultimate tumor yield, make the model appropriate for diet studies of chemoprevention. However, sources of heme iron, as dietary components, are not themselves carcinogens. In their 2008 publication Pierre et al. stated “We chose to initiate all rats with the carcinogen, since the study was designed to show dietary promotion, and because a 2.5% Hb diet does not initiate ACF in rats (F Pierre and DE Corpet, unpublished results)” (Pierre et al., 2008). In addition, the use of parallel studies in a control (saline-only) injected rat are needed for comparison of normal colon metabolism vs. that in a cancer-initiated state.
- **The levels of heme-iron included in test diets more accurately reflect pharmacological rather than dietary effects.** The feeding of hemoglobin to rats at the level of 2.5% and 5.0% as included in the study design by Pierre et al. (2008) is in excess of normal human intake. For context, hemoglobin and myoglobin only constitute ~0.5% of the wet weight of muscle tissue, and red meat constitutes only a fraction of the total mass of the ingested diet (Aberle, 2001). Therefore, the doses of heme iron used in these studies does not reflect the normal human intake and should be considered a pharmacological, not dietary, effect.
- **The effects of heme iron and peroxidation products were rarely tested in normal colon; the limited results are conflicting and, thus, inconclusive.** Several of the included studies measured the amount of heme iron and TBARS in fecal water, followed by determining its cytotoxic potential in an *in vitro* cell culture. Implications are that as heme iron intake increases, the amount of heme iron in fecal water increases, which in turn, will cause oxidative damage to colon mucosa. However, **none of the studies measured lipid peroxidation in the colonic mucosa**; and, only two studies measured any growth parameter directly in colonic epithelium as the result of feeding various diets. Their findings were mixed:
  - Martin et al. (2015) conducted histo- and immune-histological characterization of colonic mucosa and reported no effect of dietary heme iron on crypt height or number of Ki67 positive cells.
  - Sesink et al. (2001) found no effect of heme iron or calcium on DNA or protein content in colon mucosal scrapings. However, they reported an increase in colonic epithelium proliferation in animals fed a diet containing heme iron in combination with low calcium.

- **Studies fail to assess iron status as a probable confounder.** Several studies report urinary excretion of degradation products of peroxidation. If the peroxidation is iron related, the relationship would exist with iron in circulation, not iron in the lumen. However, none of the studies report iron status of the animals. Since iron status is a major determinant in the rate of iron absorption, the short-term studies (10-20 days) may not be sufficient for the animals in the various diet groups to reach their steady-state of iron status (Sharp, 2010; Shils et al., 1999). This fluctuation in iron status could artificially create differences between groups.

The experimental and control diets used in these studies included an overall amount of iron which was in excess of that recommended for the species. In most of the test diets, heme iron was added to a base diet containing sufficient iron; and, the iron levels in control diets were equalized with the addition of supplemental iron from sources such as ferric citrate. No other dietary components, such as antioxidants, were correspondingly increased.

### **C) Modifications to animal test diets may not apply to the human diet**

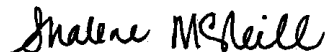
- **Calcium is restricted in the test diets.** It is well established that dietary calcium, and particularly residual calcium in the colon suppresses the efficiency of AOM-induced tumorigenesis. Therefore, most test diets in the included studies were based on a low-calcium modification of an AIN diet in an effort to exacerbate tumorigenesis. Such reductions are severe and poorly reflect typical human intake. Calcium content in test diets ranged from 16% (Geuraud et al., 2015) to 44% (Martin et al., 2015) of the level in the AIN76 diet. A similar rate of reduction in humans would result in deficient calcium intakes of approximately 128-352 mg/d.
- Discussion from Pierre et al. (2006) defines some of the reasons which explain why results from a diet study in animals are not always applicable to the human diet. According to Pierre et al., the reason that effects seen in humans were less than in rats in their study, could be explained by differences in heme doses, and in dietary protective agents. Effective doses of heme are much different in rats than in humans when considering body size, weight, and metabolic rates. In addition, diets given to human control groups rarely have an absolute exclusion of nutrients or food groups that can be achieved in experimental animals. Finally rat diets are composed of purified components and void of many antioxidant agents commonly found in the human diet from fruits and vegetables. Such antioxidant agents inhibit both peroxidation and carcinogenesis (Pierre et al., 2006).

## OVERALL CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE RESEARCH

Mechanisms by which heme iron may enhance biomarkers and other factors related to CRC remain open to serious experimental and theoretical gaps and weaknesses that require further research.

- There are several critical research gaps in mechanistic evidence linking dietary heme iron, from red and processed meat, and CRC.
- The type of heme iron supplemented in animal diets, as surrogates for naturally occurring heme iron from hemoglobin and myoglobin in red and processed meat, independently affect outcomes.
- None of the various mechanisms tested by studies included in this review, including oxidative stress, inflammation, cytotoxicity and perturbations to the normal process of apoptosis, are supported by evidence sufficient to confirm a mechanistic link between red meat intake and CRC.
- **Evidence is weak and inadequate in both humans and animals concerning the mechanistic relationship between dietary heme iron, from red and processed meat, and the development of human CRC.**

Sincerely,



Shalene McNeill, PhD, RD  
Executive Director, Human Nutrition Research,  
National Cattlemen's Beef Association, a contractor to the Beef Checkoff

In Cooperation with:

George E. Dunaif, PhD, DABT, Toxpro Solutions, LLC  
Connye Kuratko, PhD, RD, Kuratko Nutrition Research  
James R. Coughlin, PhD, CFS, Coughlin & Associates  
Mary E. Van Elswyk, PhD, RD, Van Elswyk Consulting, Inc.  
Charli A. Weatherford, MS, Weatherford Consulting Services

Attachments:

Zip file enclosure #1 – Mechanistic Evidence re: Heme-Iron and Colorectal Cancer; Appendix A  
Zip file enclosure #2 – Evidence Supporting Modified Evaluation Criteria

## REFERENCES

- Aberle, E. D., Forrest, J. C., Gerrard, D. E., and Mills, E. W. (2001). Principles of Meat Science. 4th edition, Kendall Hunt, Dubuque, IA, USA.
- Andersen, V., Christensen, J., Overvad, K., Tjønneland, A., and Vogel, U. (2011). Heme oxygenase-1 polymorphism is not associated with risk of colorectal cancer: A Danish prospective study. *European Journal of Gastroenterology and Hepatology*, 23(3), 282-285.
- Andersen, V., Holst, R., and Vogel, U. (2013). Systematic review: diet-gene interactions and the risk of colorectal cancer. *Alimentary Pharmacology and Therapeutics*, 37, 383-391.
- Andersen, V., Kopp, T.V., Tjønneland, A. and Vogel, U., (2015). No Association between HMOX1 and Risk of Colorectal Cancer and No Interaction with Diet and Lifestyle Factors in a Perspective Danish Case-Cohort Study. *International Journal of Molecular Sciences*, 16, 1375-1384.
- Andersen, V. and Vogel, U. (2015). Interactions between meat intake and genetic variation in relation to colorectal cancer. *Genes and Nutrition*, 10, 448-461.
- Bastide, N. M., Chenni, F., Audebert, M., Santarelli, R. L., Tache, S., Naud, N., Baradat, M., Jouanin, I., Surya, R., Hobbs, D. A., Kuhnle, G. G., Raymond-Letron, I., Gueraud, F., Corpet, D. E., and Pierre, F. H. F. (2015). A central role for heme iron in colon carcinogenesis associated with red meat intake. *Cancer Research*, 75(5), 870-879.
- Bidlack, W. R., Birt, D., Borzelleca, J., Clemens, R., Coutrelis, N., Coughlin, J. R., and Trautman, T. (2009). Expert report: making decisions about the risks of chemicals in foods with limited scientific information. *Comprehensive Reviews in Food Science and Food Safety*, 8(3), 269-303.
- Chen, J. and Huang, X. F. (2009). The signal pathways in azoxymethane-induced colon cancer and preventive implications. *Cancer Biology and Therapy*, 8(14), 1313-1317.
- Demeyer, D., Mertens, B., De Smet, S., and Ulens, M. (2015). Mechanisms linking colorectal cancer to the consumption of (processed) red meat: a review. *Critical Reviews in Food Science and Nutrition*, 15:0. Epub ahead of print.
- Douron, M. (2010). U-shaped dose response curves: implications for risk characterization of essential elements and other chemicals. *Journal of Toxicology and Environmental Health, Part A* 73, 181-186.
- EFSA Opinion. (2004). Opinion of the Scientific Panel on Dietetic Products, Nutrition and Allergies on a request from the Commission related to Tolerable Upper Intake Level of Iron. *The EFSA Journal*, 125, 1-34.
- EFSA Opinion. (2015). Scientific Opinion on Dietary Reference Values for iron. *The EFSA Journal*, In press.
- Eisenbrand, G., Pool-Zobel, B., Baker, V., Balls, M., Blaauboer, B. J., Boobis, A., Carere, A., Kevekordes, S., Lhuguenot, J. C., Pieters, R., and Kleiner, J. (2002). Methods of in vitro toxicology. *Food and Chemical Toxicology*, 40, 193-236.

- Gilting, A. M. J., Fransen, F., de Kok, T. M., Goldbohm, A. R., Schouten, L. J., de Bruine, A. P., van Engeland, M., van den Brandt, P. A., de Goeij, A. F. P. M., and Weijnen, M. P. (2013). Dietary heme iron and the risk of colorectal cancer with specific mutations in KRAS and APC. *Carcinogenesis*, 34(12), 2757-2766.
- Gueraud, F., Tache, S., Steghens, J. P., Milkovic, L., Borovic-Sunjic, S., Zarkovic, N., Gaultier, E., Naud, N., Helies-Toussaint, C., Pierre, F., and Priymenko, N. (2015). Dietary polyunsaturated fatty acids and heme iron induce oxidative stress biomarkers and a cancer promoting environment in the colon of rats. *Free Radical Biology and Medicine*, 83, 192-200.
- Gulec, S., Anderson, G. J., and Collins, J. F. (2014). Mechanistic and regulatory aspects of intestinal iron absorption. *American Journal of Physiology Gastrointestinal and Liver Physiology*, 307, G397-G409.
- IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. (2014). Report of the Advisory Group to Recommend Priorities for IARC Monographs during 2015–2019.
- Institute of Medicine (IOM). Food and Nutrition Board. (2001). Dietary reference intakes for vitamin A, vitamin K, arsenic, boron, chromium, copper, iodine, iron, manganese, molybdenum, nickel, silicon, vanadium, and zinc: a report of the panel on micronutrients. Washington, DC: National Academy Press.
- Hunt, J. R. (2005). Dietary and physiological factors that affect the absorption and bioavailability of iron. *International Journal for Vitamin and Nutrition Research*, 75(6), 375-384.
- Joseph, P., Suman, S. P., Li, S., Beach, C. M., and Claus, J. R. (2010). Mass spectrometric characterization and thermostability of turkey myoglobin. *LWT-Food Science and Technology*, 43, 273–278.
- Joseph, P., Suman, S. P., Rentfrow, G., Li, S., and Beach, C. M. (2012). Proteomics of muscle-specific beef color stability. *Journal of Agricultural and Food Chemistry*, 60, 3196–3203.
- Kushman, M. E., Kraft, A. D., Guyton, K. Z., Chiu, W. A., Makris, S. L., and Rusyn, I. (2013). A systematic approach for identifying and presenting mechanistic evidence in human health assessments. *Regulatory Toxicology and Pharmacology*, 67, 266-277.
- Lawrie, R. A., and D. A. Ledward. (2006). *Lawrie's Meat Science*. 7th edition, Woodhead Publishing Limited, Abington Hall, Abington, Cambridge CB1 6AH, England.
- Martin, O. C. B., Naud, N., Tache, S., Raymond-Letron, I., Corpet, D. E., and Pierre, F. H. (2015). Antibiotic suppression of intestinal microbiota reduces heme-induced lipoperoxidation associated with colon carcinogenesis in rats. *Nutrition and Cancer*, 67(1), 119-125.
- McKenna, D. R., Mies, P. D., Baird, B. E., Pfeiffer, K. D., Ellebracht, J. W., Savell, J. W. (2005). Biochemical and physical factors affecting discoloration characteristics of 19 bovine muscles. *Meat Science*, 70, 665-682.
- Munro, I. C. (2006). Setting tolerable upper intake levels for nutrients. *Journal of Nutrition*, 136, 490S-492S.
- Pierre, F., Tache, S., Petit, C. R., Van der Meer, R., and Corpet, D. E. (2003). Meat and cancer: haemoglobin and haemin in a low-calcium diet promote colorectal carcinogenesis at the aberrant crypt stage in rats. *Carcinogenesis*, 24(10), 1683-1690.

- Pierre, F., Freeman, A., Tache, S., Van der Meer, R., and Corpet, D. E. (2004). Beef meat and blood sausage promote the formation of azoxymethane-induced mucin-depleted foci and aberrant crypt foci in rat colons. *Journal of Nutrition*, 134(10), 2711-2716.
- Pierre, F., Peiro, G., Tache, S., Cross, A. J., Bingham, S. A., Gasc, N., Gottardi, G., Corpet, D. E., and Gueraud, F. (2006). New marker of colon cancer risk associated with heme intake: 1, 4-dihydroxynonane mercapturic acid. *Cancer Epidemiology, Biomarkers and Prevention*, 15(11), 2274-2279.
- Pierre, F., Santarelli, R., Tache, S., Gueraud, F., and Corpet, D. E. (2008). Beef meat promotion of dimethylhydrazine-induced colorectal carcinogenesis biomarkers is suppressed by dietary calcium. *British Journal of Nutrition*, 99(5), 1000-1006.
- Ruder, E. H., Berndt, S. I., Gilsing, A. M. J., Graubard, B. I., Burdett, L., Hayes, R. B., Weissfeld, J. L., Ferrucci, L. M., Sinha, R., and Cross, A. J. (2014). Dietary iron, iron homeostatic gene polymorphisms and the risk of advanced colorectal adenoma and cancer. *Carcinogenesis*, 35(6), 1276-1283.
- Scientific Committee on Nutrition (SCAN). (2010). Iron and Health Report, 102-105.
- Sesink, A. L. A., Termont, D. S. M. L., Kleibeuker, J. H., and Van der Meer, R. (1999). Red meat and colon cancer: the cytotoxic and hyperproliferative effects of dietary heme. *Cancer Research*, 59, 5704-5709.
- Sesink, A. L. A., Termont, D. S. M. L., Kleibeuker, J. H., and Van der Meer, R. (2001). Red meat and colon cancer: Dietary haem-induced colonic cytotoxicity and epithelial hyperproliferation are inhibited by calcium. *Carcinogenesis*, 22(10), 1653-1659.
- Sharp, P. A. (2010). Intestinal iron absorption: regulation by dietary and systemic factors. *International Journal of Vitamin and Nutrition Research*, 80(4-5), 231-242.
- Shayeghi, M., Latunde-Dada, G. O., Oakhill, J. S., Laftah, A. H., Takeuchi, K., Halliday, N., Khan, Y., Warley, A., McCann, F. E., Hider, R. C., Frazer, D. M., Anderson, G. J., Vulpe, C. D., Simpson, R. J., and McKie, A. T. (2005). Identification of an intestinal heme transporter. *Cell*, 122, 789-801.
- Shils, M., Olson, J., and Shike, M. (1999). "Iron in medicine and nutrition." *Modern Nutrition in Health and Disease*. 9th edition, Lippincott Williams and Wilkins.
- Sodring, M., Oostindjer, M., Egelandsdal, B., and Paulsen, J. E. (2015). Effects of hemin and nitrite on intestinal tumorigenesis in the A/J Min/+ mouse model. *PLoS One*, 10(4), 1-15.
- Suman, S. P., Faustman, C., Lee, S., Tang, J., Sepe, H. A., Vasudevan, P., Annamalai, T., Manojkuman, M., Marek, P., Venkitanarayanan, K. S. (2005). Effect of erythorbate, storage and high-oxygen packaging on premature browning in ground beef. *Meat Science*, 69, 363-369.



- Suman, S. P., Rentfrow, G., Nair, M. N. and Joseph, P. (2014). Proteomics of muscle- and species-specificity in meat color stability. *Journal of Animal Science*, 92, 875-882.
- Wlodarska, M., Kostic, A. D. and Xavier, R. (2015). An Integrative View of Microbiome-Host Interactions in Inflammatory Bowel Diseases. *Cell Host and Microbe*, 17, 577-591.
- Xue, X., Taylor, M., Anderson, E., Hao, C., Qu, A., Greenson, J. K., Zimmermann, E. M., Gonzalez, F. J., and Shah, Y. M. (2012). Hypoxia-inducible factor-2a activation promotes colorectal cancer progression by dysregulating iron homeostasis. *Cancer Research*, 72(9), 2285-2293.
- Yebra-Pimentel, I., Fernández-González, R., Martínez-Carballo, E., and Simal-Gándara, J. (2015). A critical review about the health risk assessment of PAHs and their metabolites in foods. *Critical Reviews in Food Science and Nutrition*, 55, 1383-1405.
- Zanini, S., Marzotto, M., Giovinazzo, F., Bassi, C., and Bellavite, P. (2015). Effects of dietary components on cancer of the digestive system. *Critical Reviews in Food Science and Nutrition*, 55, 1870-1885.