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# **A central role for heme iron in colon carcinogenesis associated with red meat intake**

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## ABSTRACT

Epidemiology shows that red and processed meat intake is associated with an increased risk of colorectal cancer. Heme iron, heterocyclic amines and endogenous N-nitroso compounds (NOC) are proposed to explain this effect, but their relative contribution is unknown. Our study aimed at determining, at nutritional doses, which is the main factor involved and proposing a mechanism of cancer promotion by red meat. The relative part of heme iron (1% in diet), heterocyclic amines (PhIP+MeIQx, 50+25  $\mu\text{g}/\text{kg}$  in diet) and NOC (induced by  $\text{NaNO}_2+\text{NaNO}_3$  0.17+0.23 g/l of drinking water) was determined by a factorial design and preneoplastic endpoints in chemically-induced rats and validated on tumors in *Min* mice. The molecular mechanisms (genotoxicity, cytotoxicity) were analyzed *in vitro* in normal and *Apc*-deficient cell lines and confirmed on colon mucosa. Heme iron increased the number of preneoplastic lesions but dietary heterocyclic amines and NOC had no effect on carcinogenesis in rats. Dietary hemoglobin increased tumor load in *Min* mice (control diet:  $67\pm 39$   $\text{mm}^2$ ; 2,5% hemoglobin diet:  $114\pm 47$   $\text{mm}^2$ ,  $p=0.004$ ). *In vitro*, fecal water from rats given hemoglobin was rich in aldehydes and was cytotoxic to normal cells, but not to premalignant cells. The aldehydes 4-hydroxynonenal and 4-hydroxyhexenal were more toxic to normal versus mutated cells and were only genotoxic to normal cells. Genotoxicity was also observed in colon mucosa of mice given hemoglobin. These results highlight the role of heme iron in the promotion of colon cancer by red meat and suggest that heme iron could initiate carcinogenesis through lipid peroxidation.

**Precis:** Elevated risk of colon cancer associated with red meat consumption is linked to heme iron, which may initiate carcinogenesis by enabling lipid peroxidation, providing a possible etiological basis to understand this connection.

## INTRODUCTION

Colorectal cancer (CRC) is the third most common type of cancer worldwide after lung and prostate cancer in men and after lung and breast cancer in women (1). Environmental factors, particularly diet, play roles in the development of CRC (2,3). Based on epidemiological studies, the World Cancer Research Fund panel considers the colorectal cancer risk associated with red and processed meat intake to be convincing and recommends limiting the consumption of red meat and avoiding the consumption of processed meat (2,3). Our previous works showed that red and processed meats promote precancerous lesions (aberrant crypt foci and mucin-depleted foci, MDF) in the colons of rats fed a low-calcium diet (4-6). These data strongly support the results from epidemiological studies.

Three major mechanisms may explain the association between meat and CRC (7). First, potentially carcinogenic *N*-nitroso-compounds can form in the gastrointestinal tract by *N*-nitrosation of peptide-derived amine or by nitrosylation yielding *S*-nitrosothiols and nitrosyl iron (FeNO). Collectively, these are measured as the apparent total *N*-nitroso-compounds (ATNCs) (8). Second, meat cooked at high temperatures contains mutagenic heterocyclic amines (HCA) like 2-amino-1-methyl-6-phenylimidazo(4,5-*b*)pyridine (PhIP) and 2-amino-3,8-dimethylimidazo [4,5-*f*]quinoxaline (MeIQx) (9). Third, epidemiological and experimental data support the hypothesis that heme iron present in red and processed meats promotes CRC (5-7,10). This effect can be explained by the direct cytotoxic, genotoxic effects of heme on epithelial cells, and by the catalytic effect of heme iron on the formation of ATNC, and lipid peroxidation end-products like 4-hydroxynonenal (4-HNE) (4,5,7,11-14). Cross *et al.* investigated these three hypotheses in a cohort study and found a significant association between CRC and the intake of heme iron, nitrate from processed meat, and HCA (15). Nevertheless, numerous biases are possible in the determination of risk factors using

epidemiological approach, and contribution of each of these factors has never been evaluated experimentally in the same study.

The present study aimed at investigating the roles of these three potential mechanisms, namely heme iron, NOC and HCA, in CRC *in vivo* at a precancerous lesion stage (MDF) in carcinogen-induced rats (see a flow chart in **Supplementary Fig 1**). Doses were chosen to mimic red meat consumption. Subsequently, the results were confirmed at the tumor stage using C57BL/6J *Apc<sup>Min/+</sup>* mice, a genetic model of CRC. The tumor incidence was associated with genotoxicity endpoints in mucosa as  $\gamma$ H2AX and anaphase bridges. Like tumors in the majority of human CRC cases and in *Apc<sup>Min/+</sup>* mice, the preneoplastic lesion MDF in rats show activation of the Wnt signaling pathway driven by mutations in *Apc* and/or in the beta-catenin gene. We also used an intestinal cellular model with normal and premalignant cells (*Apc<sup>+/+</sup>* and *Apc<sup>-/+</sup>* cells) to complement the *in vivo* studies. In combination with animal models, such cellular models allowed us (i) to understand the effect of dietary compounds on cancer promotion at early stages of carcinogenesis and (ii) to explain and further investigate the effects observed *in vivo*.

## Materials and Methods

**Animals and diets.** Male 4-week-old F344 rats (n=80; Charles Rivers), male and female 4-week-old C57BL/6J *Apc*<sup>Min/+</sup> mice (Jackson, Laboratory, n=35) and *Apc*<sup>+/+</sup> mice (Charles River, n=33), and male and female C57BL/6J mice (n=10) were housed (two rats per cage and two to three mice per cage) under standard laboratory conditions with free access to food and water. After acclimatization with AIN76 diet, rats were fed experimental diets for 100 days, *Apc*<sup>Min/+</sup> and *Apc*<sup>+/+</sup> mice were fed experimental diets for 49 days, and C57BL/6J mice were fed experimental diets for 14 days. Rats were killed by CO<sub>2</sub> asphyxiation, and mice were killed by cervical dislocation. Animal care was in accordance with the European Council and ARRIVE guidelines.

To assess the relative contributions of the three potential mechanisms (heme iron, NOC, and HCA), we conducted a 2×2×2 protocol on azoxymethane-induced F344 rats fed a diet containing 1% hemoglobin, HCA (PhIP, 50 µg/kg; MeIQx, 25 µg/kg), or both. To induce a strong endogenous NOC formation, drinking water was supplemented with sodium nitrate and nitrite (0.17 g/l of NaNO<sub>2</sub> and 0.23 g/l of NaNO<sub>3</sub>) and compared with a nitrate-free water, according to the experimental groups described in **Table 1A**. (16). Mice were fed a control diet or a 2.5 % hemoglobin diet (**Table 1B**).

**Azoxymethane-induced Colon Carcinogenesis in Rat.** After one week on the experimental diet, rats received intraperitoneal injection of azoxymethane (Sigma; 20 mg/kg body weight).

**Neoplastic Lesions.** The large intestines of rats and large and small intestines of mice were removed and fixed in 0.05% buffered formalin (Sigma).



### ***MDF Scoring in Colon of Rats***

MDF were scored in duplicate by two readers who were blinded to the origin of the colon following the high-iron diamine Alcian blue procedure (17) described by Santarelli *et al* (4).

### ***Tumor Scoring in Small Intestine and Colon of Mice***

At sacrifice, the intestinal tract from duodenum to colon was removed. Sections of duodenum, jejunum, and ileum were harvested, opened along the longitudinal axis, and washed in phosphate-buffered saline. After fixation in 10% formalin, mouse colons were stained for 6 minutes in a 0.05% filtered methylene blue solution, and small intestines were stained for 48 hours in a 300 ppm solution of methylene blue in formalin. One reader who was blinded to the origin of the sample scored tumors and determined their diameters using a binocular microscope at 25x magnification. All tumors in each section of the intestines were counted, the smallest tumors that could be counted were approximately 0.5 mm in diameter.

**Fecal Assays in Rat and Mice.** Feces were collected during the last 10 days and frozen at -20°C. Urine was collected on days 67–70 for rats and on days 44–45 for mice and frozen at -20°C before DHN-MA assay (**Supplementary Materials and Methods**).

### ***Fecal Water Preparation***

Feces of 24h were collected. To prepare fecal water, distilled water (1 ml for rats or 0.85 ml for mice) was added to 0.3 g of dried feces. Fecal water was prepared as described by Pierre *et al* (5).

### ***Heme, TBARS in Fecal Water of Rats and Mice***

The heme concentration in the fecal water was measured by fluorescence according to Van den Berg *et al* (18) and as described by Pierre *et al* (5). To determine the lipid peroxides in the lumen, TBARS were quantified in fecal water according to the technique of Ohkawa *et al* (19) as described previously (20). The results are expressed as the MDA equivalent.

### ***Apparent Total N-nitroso Compound in Fecal Water of Rats***

ATNCs include N-nitroso compounds, S-nitrosothiols, and FeNO nitrosyl heme. They were analyzed as described previously (11) with an Ecomedics CLD Exhalyzer (Ecomedics, Duernten, Switzerland). The values measured in 100  $\mu$ L of the sample are expressed as concentration (in  $\mu$ M).

**Cell Lines.** *Apc*<sup>+/+</sup> (derived from C57BL/6J mice) and *Apc*<sup>-/+</sup> (derived from C57BL/6J *Apc*<sup>Min/+</sup> mice) colon epithelial cells (21) express the heat-labile SV40 large T antigen (AgTtsa58) under the control of an interferon  $\gamma$ -inducible promoter. Both cell lines expressed cytokeratin 18, a marker of their epithelial phenotype (Forest 2003). Consequences of the *Apc* mutation were also detected in the *Apc*<sup>-/+</sup> cell line. As expected, actin network was disorganized in *Apc*<sup>-/+</sup> cells ((21,22); **Supplementary Fig 2A**), accumulation of multinucleated cells was observed in *Apc*<sup>-/+</sup> cells (**Supplementary Fig 2A**). As expected, the culture conditions affected cell proliferation due to the thermolabile tsA58 T antigen, which confers conditional immortalization: at 33°C with interferon  $\gamma$ , the large T antigen is active and drives cellular proliferation, and at 37°C, the temperature-sensitive mutation yields an inactive protein, and cells act like non-proliferating epithelial cells (**Supplementary Fig 2B**).

**Aldehydes for Cytotoxicity and Genotoxicity Assays.** 4-HNE derived from the oxidation of n-6 poly-unsaturated fatty acids and 4-hydroxy-2-hexenal (4-HHE) derived n-3 poly-unsaturated fatty acids were synthesized as described by Chandra and Srivastava (23). Malondialdehyde (MDA) derived from poly-unsaturated fatty acids with three or more double bonds was prepared as described by Fenaille *et al* (24).

### **Aldehyde Trapping of Fecal Water from Hemoglobin fed Rats for Cytotoxicity Assay.**

Polymer resin (4-Fmoc-hydrazinobenzoyl AM NovaGel™, Nobabiochem ® Merck Chemicals, Nottingham, UK) contains hydrazine functional groups protected by Fmoc groups. In order to unprotect them, the resin was washed with 0.8 ml DMSO + 0.2 ml piperidine, vortexed for 1 minute, and allowed to settle for 15 minutes. The settled resin was washed twice with DMSO, 4 times with ethanol, and with distilled water. The amount of polymer resin used for each sample was based on using 100x the amount of MDA equivalents present in the fecal water sample. Polymer resin was added to fecal water, and the samples were agitated for 2 hours at 4°C. After letting the resin settle for 15 minutes, supernatant was transferred to a new tube with polymer resin and agitated for 2 hours at 4°C. After settling, the supernatant was diluted into culture medium without fetal calf serum and used for the MTT assay.

### **Cytotoxicity and Genotoxicity Assays on Cell Lines Treated with Fecal Water, Heme or Aldehydes.**

To determine cytotoxic activity of fecal water, of HNE (20µM) and of hemin (100 µM), the MTT assay on *Apc*<sup>+/+</sup> and *Apc*<sup>-/+</sup> cells was used, as described previously (5). H2AX phosphorylation ( $\gamma$ H2AX) is a rapid and sensitive cellular response to genotoxicity (25,26). Genotoxicity and cytotoxicity of aldehydes were measured after 24 hours of treatment of *Apc*<sup>+/+</sup> and *Apc*<sup>-/+</sup> cells using a  $\gamma$ H2AX in-cell Western assay according to Audebert *et al* (27,28). Graillet *et al* (28) demonstrated that this assay can be used to measure cell viability via DNA quantification. Cells were seeded into 96-well plates at  $5 \times 10^3$  cells per well in DMEM supplemented with 10% (v/v) fetal calf serum, 1% (v/v) penicillin/streptomycin, and 10 U/mL interferon  $\gamma$  at the permissive temperature of 33°C. After 72 h, cells were transferred to 37°C without interferon  $\gamma$  for 24 h then treated with

aldehyde (5, 10 and 20  $\mu$ M) in duplicate. Culture medium without interferon  $\gamma$  and fetal calf serum was used for untreated control wells. This assay was repeated 4 times.

**Apoptosis Assay on Cell Lines Treated Aldehydes.** Apoptosis was measured in *Apc*<sup>+/+</sup> and *Apc*<sup>-/+</sup> cells using a luminescent assay (Caspase-Glo3/7; Promega). Cells were treated with aldehydes for 6 h. After cell lysis, plates were incubated at RT for 2 hours, and the luminescence intensity of each well was determined using an INFINITEM200 plate reader (TECAN). This measure was performed in triplicate with aldehydes at 2.5, 5, 10, 20, 40, and 80  $\mu$ M.

## **Histological analyses of the Small Intestine of Mice**

### ***Immunohistochemistry H2AX***

Four-micrometer paraffin-embedded sections from formalin-fixed mouse small intestine (Swiss rolls) specimens were de-waxed in toluene and rehydrated. Sections were incubated in Dako peroxidase blocking solution (Dako S2023) and in goat serum (1:10, Dako X0907) for 20 min at room temperature (RT). Sections were incubated with the rabbit polyclonal anti- $\gamma$ H2AX antibody (1:400, Cell Signaling Technology #9718) 50 min at RT. The secondary antibody (biotinylated goat anti-rabbit, Thermo Scientific TR-060-BN) was applied for 30 min at RT followed by HRP-streptavidin solution (DAB, Dako K0675) for 25 min. Peroxidase activity was revealed by DAB substrate (DAKO, K3468). Sections were counterstained with Harris hematoxylin, dehydrated, and coverslipped.

Enterocytes with nuclear  $\gamma$ H2AX-positive foci or complete nuclear labeling were considered positive cells. Cells were assessed by counting the positive nuclei in segments of the small intestine specimen that were at least 200 glands long. The positive counts were expressed as counts per one villi-gland unit.

### ***Anaphase bridges (AB)***

Chromosomal or mitotic alterations can arise from numerous events, including errors during cell division or repair of damaged DNA. As a consequence, the separating sister chromatids are often connected by DNA bridges in anaphase. AB were evaluated on four-micrometer paraffin-embedded sections from formalin-fixed mouse small intestine (Swiss rolls). Sections were stained with hematoxylin and eosin and AB were evaluated under light microscope using 400x magnification. Four segments from the duodenum, jejunum, and ileum that were at least 100 consecutive glands long were selected for counting. Criteria for anaphase bridges included having a well-separated parallel anaphase plate displaying a perpendicularly aligned amphophilic (stretched) connecting filament (29). The scores were expressed as number of AB per villi-gland unit.

**Statistical analysis.** Results were analyzed using Systat 10 software for Windows, and all data are reported as mean  $\pm$  SEM. For the *in vivo* experiments on chemically-induced rats, the importance of each factor was tested independently of the experimental groups (ANOVA per factor). If a significant difference was found between groups ( $p < 0.05$ ), each experimental group was compared with the control using Dunnett's test, the difference between control and hemoglobin diets effect on Min mice tumors was analyzed using the Student's t-test. For the *in vitro* study, the dose-effect of aldehydes was analyzed using one-way analysis of variance (ANOVA). If a significant difference was found between groups ( $p < 0.05$ ), each experimental group was compared with the control treatment using Dunnett's test. Secondly, the effect of the mutation effect at each concentration of aldehyde was analyzed with the comparison between the *Apc*<sup>+/+</sup> and *Apc*<sup>-/+</sup> cell lines using the Student's t-test.

## RESULTS

### Heme Iron Plays a Major Role in Mucin-depleted Foci Formation

Only diets containing hemoglobin significantly increased the number of MDF per colon ( $p < 0.001$ ) independent of the two other factors (**Fig 1A**). Indeed, although nitrates/nitrites in drinking water induced a considerable increase in fecal ATNCs in all groups, they failed to increase the number of MDF per colon (**Fig 1A, D**). Nevertheless, we noticed that the ATNC composition was different between groups, containing 30–80% FeNO and no S-nitrosothiols in the hemoglobin-fed groups compared to no FeNO and about 30% of S-nitrosothiols in other groups.

Diets containing hemoglobin significantly increase the amount of thiobarbituric acid reactive substances (TBARS) in fecal water (**Fig 1B**) and the amount of urinary 1,4-dihydroxynonemercapturic acid (DHN-MA), a metabolite of the lipid oxidation product 4-HNE (**Supplementary Fig 3B**). These oxidation biomarkers depended only on dietary and fecal heme (**Supplementary Fig 3A**) and remained unchanged when the diet contained nitrates/nitrites or HCA without hemoglobin (**Fig 1B, Supplementary Fig 3B**).

### Premalignant Epithelial Cells Resist Cytotoxicity Induced by Fecal Water from Heme-fed Rats: The Central Role of Aldehydes

Fecal water from rats fed hemoglobin-containing diets was more cytotoxic to the non-mutated *Apc*<sup>+/+</sup> cells than to premalignant *Apc*<sup>-/+</sup> cells (**Fig 1C**). This data is consistent with previous results (20). Fecal water from rats fed HCA or nitrites/nitrates without hemoglobin was not cytotoxic to these cells (**Fig 1C**).

With the trapping of aldehydes from fecal water of rats fed heme with a polymer resin with hydrazine functional groups, we found that in *Apc*<sup>+/+</sup> and *Apc*<sup>-/+</sup> cells, a 95% reduction in

fecal water peroxidation was associated with a 75% reduction in cytotoxicity (**Fig 2A**). Furthermore, we observed that only 4-HNE, but not heme, had differential cytotoxic effects in *Apc*<sup>+/+</sup> and *Apc*<sup>-/+</sup> cells that was similar to that observed with fecal water of rats fed heme (**Fig 2B**).

We then measured the cytotoxic and genotoxic effects of three main lipid peroxidation end products (4-HNE, 4-HHE and MDA) on *Apc*<sup>+/+</sup> and *Apc*<sup>-/+</sup> cells using a  $\gamma$ H2AX in-cell Western assay. HNE and HHE were more cytotoxic and more genotoxic to normal *Apc*<sup>+/+</sup> cells than to premalignant *Apc*<sup>-/+</sup> ones (**Fig 2C**,  $p < 0.05$ ). HNE at 10 and 20  $\mu$ M and HHE at 20  $\mu$ M were significantly more genotoxic to *Apc*<sup>+/+</sup> cells than to *Apc*<sup>-/+</sup> ones (**Fig 2C**,  $p < 0.05$ ). MDA was neither cytotoxic nor genotoxic in the tested cell lines (**Fig 2C**). We confirmed these viability results with the MTT assay and with the CellTiter-Glo® assay (**Supplementary Fig 4**), and we used an expanded range of treatment concentrations (from 0 to 80  $\mu$ M) in these assays. We confirmed that HNE and HHE were more cytotoxic (from 10 to 80  $\mu$ M and from 40 to 80  $\mu$ M, respectively) to normal cells than to premalignant ones, while MDA had no effect (**Supplementary Fig 4**).

We determined the caspase 3/7 activity and again found a significant difference between *Apc*<sup>+/+</sup> and *Apc*<sup>-/+</sup> cells after HNE and HHE treatment at 80 and 40  $\mu$ M, respectively (**Fig 3D**,  $p < 0.05$ ). Specifically, caspase 3/7 activity was higher in *Apc*<sup>+/+</sup> cells than in *Apc*<sup>-/+</sup> cells, and MDA treatment had no effect (**Fig 2D**).

### **Heme Iron and Tumoral promotion: Hemoglobin Increases Intestinal Tumorigenesis in *Apc*<sup>Min/+</sup> Mice**

A diet containing 2.5% hemoglobin given to *Apc*<sup>Min/+</sup> mice significantly increased the intestinal tumor load (control diet:  $67 \pm 39$  mm<sup>2</sup>; hemoglobin diet:  $114 \pm 47$  mm<sup>2</sup>,  $p = 0.004$ ; **Fig 3A**). These mice develop polyps mainly in the small intestine, and in our study we did not

observe any effects of hemoglobin diet in the colon. The hemoglobin diet also significantly increased the number of all tumors in the jejunum (**Supplementary Fig 5A**). In the entire small intestine, the hemoglobin diet significantly increased the number of tumors with a diameter greater than 1 mm ( $1\text{ mm} < \text{tumor size} \leq 2\text{ mm}$ ,  $p=0.04$ ;  $2\text{ mm} < \text{tumor size}$ ,  $p=0.006$ ) (**Supplementary Fig 5B**). Giving the same hemoglobin diet to normal C57BL/6J *Apc*<sup>+/+</sup> mice induced no neoplasia. As observed in rats, the effect of dietary heme on mice tumors was associated with a significant increase in fecal heme (from  $20 \pm 22$  to  $198 \pm 29\ \mu\text{M}$ ) and with increases in lipoperoxidation biomarkers: fecal TBARS (Fig 3B) and urinary DHN-MA (**Supplementary Fig 5C**). We also assessed TBARS and the cytotoxicity of fecal water in the small intestine to measure biomarkers at the same location as tumors. As *Min* mice have a mutation in the *Apc* gene, we decided to use the mouse *Apc*<sup>-/+</sup> model to investigate the cytotoxic activity of fecal water *in vivo*. Heme diet was associated with a significant increase in fecal TBARS and cytotoxicity in the small intestine (**Fig 3C**).

#### **A Heme Diet is Genotoxic *in vivo* in the Epithelium of C57BL/6J *Apc*<sup>+/+</sup> Mice**

The induction of luminal lipid peroxidation by the hemoglobin diet (**Fig 3B, C**) was associated with increased genotoxicity only in non-mutated C57BL/6J *Apc*<sup>+/+</sup> mice with a higher anaphase bridge index in the epithelium (**Fig 4A and 4B**). As expected the anaphase bridge index was higher in C57BL/6J *Apc*<sup>Min/+</sup> mice than in C57BL/6J *Apc*<sup>+/+</sup> mice fed a control diet (**Fig 4A**) because of the *Apc* mutation. In *Apc*<sup>Min/+</sup> mice, the hemoglobin diet had no additional genotoxic effect (**Fig 4A**). The genotoxic effect of the hemoglobin diet in C57BL/6J *Apc*<sup>+/+</sup> was confirmed by  $\gamma\text{H2AX}$  induction (**Fig 4C, D**).



## DISCUSSION

This study examines *in vivo* the relative contributions of the three main factors that may explain how consumption of red and processed meat promotes CRC. Cross *et al.* showed that these factors, i.e. heme, HCA, and NOC, were associated with CRC in a prospective cohort study in humans (15). However, the identification of risk factors using an epidemiological approach has to be correlated with the experimental approach in order to establish the causative effect of such factors. Here, heme iron was the only experimental factor associated with a significant increase in precancerous lesions (MDF) in rats. Heme iron showed no additive or synergic effects with nitrates/nitrites or with HCA. Using a complementary approach that included two animal models and a cellular model, we found that heme is the determining factor in the promotion of colorectal carcinogenesis, and that the selective toxicity of heme-induced alkenals to non-mutated cells seemed to play an important role in this mechanism.

HCA are complete carcinogens that induce colon, mammary, and prostate tumors in rodents and monkeys (30). The absence of effects of HCA in this study could be explained by the dose we chose, which was based on the estimated dietary exposure to HCA in a diet that is high in red meat and was relevant of the human food exposure. Indeed, carcinogenic doses of HCAs in rodents are 1000 to 100,000 times higher than levels found in human foods (31). Nitrite undergoes an enterosalivary cycle in humans but not in rats. We hypothesized that the addition of sodium nitrates/nitrites to the rodents' drinking water, which mimics human saliva, would increase the effects of heme iron in rats by boosting nitrosation in the gut. In humans, red meat consumption increases fecal ATNC concentrations (11), as in our study with rats. Nevertheless, we could not detect any association between the ATNC level (**Fig 1D**) and carcinogenesis (**Fig 1A**). The highest level of ATNC was seen in the control group

given nitrates/nitrites-supplemented water; this group had the fewest MDF. The lack of a relationship between ATNCs and the number of MDF does not support a strong role for ATNCs in the promotion of colon carcinogenesis by red meat.

The present results strongly suggest that at concentrations that are in line with human red meat consumption, heme iron is associated with the promotion of colon carcinogenesis at a preneoplastic stage. Most human colon cancers have an *Apc* mutation, as do MDF in humans and rats (32). In order to unravel the mechanisms, we used a cellular model that represented the CRC stages that we investigated *in vivo*. We chose a cellular model that mimicked the early steps of carcinogenesis. This conditionally immortalized intestinal cellular model uses premalignant *Apc*<sup>-/+</sup> cells derived from C57BL/6J *Apc*<sup>Min/+</sup> mice and “normal” *Apc*<sup>+/+</sup> cells from C57BL/6J mice (33). Characterization of both cell lines showed the expected consequences of *Apc* mutation, such as actin network disassembly, aneuploidy, and multi-nucleated cells (**Supplementary Fig 2**). These cell lines can therefore be used to study the mechanisms involved in the early steps of CRC and thus, comprise a cellular model that is a relevant complement to our *in vivo* model.

In rats, promotion of colon carcinogenesis by dietary hemoglobin was associated with changes in non-invasive biomarkers: fecal water heme iron, TBARs, and cytotoxic activity. Only the hemoglobin diet increased TBARs levels in fecal water (**Fig 1B**). We speculated that the cytotoxic effects of fecal water on normal and premalignant colonic cells *in vitro* mimics the *in vivo* situation with normal epithelium (*Apc*<sup>+/+</sup>) and with *Apc*-mutated MDF. In this study, only fecal water from hemoglobin-fed rats was more cytotoxic to *Apc*<sup>+/+</sup> cells than to mutated cells (**Fig 1C**). We propose that premalignant cell selection explains the heme-induced promotion of MDF. Aldehydes or heme iron itself, both present at high concentration in feces from hemoglobin-fed rats, might be responsible for this differential cytotoxicity. Using a resin to specifically trap fecal aldehydes, we showed that aldehydes alone are

responsible for fecal water cytotoxicity. In addition, we observed that 4-HNE, but not heme iron, induced differential cytotoxicity in *Apc*<sup>+/+</sup> and *Apc*<sup>-/+</sup> cells similar to that observed with fecal water (**Fig 2B**). Therefore, we propose that heme-induced lipid peroxidation in the gut explains the observed differential cytotoxicity and the CRC-promoting effects of heme that are observed *in vivo*.

To explore the link between aldehydes and the promotion of colon carcinogenesis, we tested the effects of three relevant aldehydes, 4-HNE, HHE, and MDA, in *Apc*<sup>+/+</sup> and *Apc*<sup>-/+</sup> cells. These  $\alpha,\beta$ -unsaturated hydroxyalkenals are highly reactive compounds with proteins and nucleic acids (34), and they are potentially cytotoxic and genotoxic. In our cellular model, 4-HNE and HHE were more cytotoxic to normal cells than to premalignant cells, and induced higher levels of apoptosis in normal cells than in premalignant cells. HNE was more cytotoxic than HHE, as reported previously (35). Furthermore, HNE, like HHE, was more genotoxic to normal cells than to premalignant ones (**Fig 2C**), with a higher index of DNA double-strand breaks as revealed by the phosphorylation of histone H2AX. DNA double-strand breaks pose a critical hazard to the genome, and erroneous rejoining of DNA double-strand breaks can lead to mutation. These results thus suggest that at concentrations higher than 20  $\mu$ M, HNE and HHE will kill normal cells, while at lower concentration; they could create mutations in *Apc*<sup>+/+</sup> cells and might thus initiate carcinogenesis. Therefore, *Apc*-mutated cells are resistant to apoptosis and can survive to contact with cytotoxic and genotoxic aldehydes, which allows them to undergo further mutation and to become more malignant. Surprisingly, MDA was not toxic to the cells tested in this study, but others found also that MDA had little or no toxicity in cells (35,36). The results obtained by aldehyde trapping and *in vitro* with HNE and HHE, confirmed our hypothesis that aldehydes are responsible for the differential cytotoxic effects of fecal water from heme-fed rats. Heme iron catalyzes the formation of aldehydes in the

gastrointestinal tract, which would “select” premalignant cells and also increase the mutation frequency in normal cells (22).

This study shows that a hemoglobin-rich diet significantly increased the tumor load in the small intestine of *Apc*<sup>Min/+</sup> mice. In contrast, tumor load was not changed by heme diet in the colon of mice, despite the expected modulation of biochemical markers. The number of tumors in the colon of Min mice is low (less than 0.5 tumors per mouse), which reduces statistical power (37). These mice have a truncated *Apc* gene as in human familial adenomatous polyposis (FAP) (38). Moreover, sporadic CRCs tumors have the same early *Apc* mutation in 50–80 % of cases (37). This mutation is also present in MDF (39). Our nutritional experiments in rats and mice were thus conducted in the defined genetic context of the *Apc* mutation. Promotion of carcinogenesis in rats and in *Apc*<sup>Min/+</sup> mice (**Fig 3A**) was associated with two non-invasive biomarkers, fecal water TBARs and cytotoxic activity, in colon and in small intestine (**Fig 3B, C**).

Furthermore, as we found that HNE is more genotoxic *in vitro* to wild type cells than to *Apc*<sup>-/+</sup> cells (**Fig 2C**), we decided to investigate the genotoxicity of dietary heme *in vivo* by measuring (i) the anaphase bridge index in the epithelium of C57BL/6J *Apc*<sup>+/+</sup> mice and C57BL/6J *Apc*<sup>Min/+</sup> mice and (ii) by assessing  $\gamma$ H2AX induction in the epithelium of C57BL/6J *Apc*<sup>+/+</sup> mice (**Fig 4**). In these two studies, the induction of lipid peroxidation in the gut by heme (**Fig 3B, C**) was associated with increased epithelial genotoxicity in *Apc*<sup>+/+</sup> mice but not in *Apc*<sup>Min/+</sup> mice. Together with *in vitro* data, these data show that dietary hemoglobin can induce DNA damage. We also observed anaphase bridges, which are biomarkers of chromosomal instability and a major consequence of *Apc* mutation. As expected, in mice fed the control diet, more anaphase bridges were seen in *Apc*<sup>Min/+</sup> mice than in *Apc*<sup>+/+</sup> mice (**Fig 4A**). Moreover, the hemoglobin diet increased the anaphase bridge index in *Apc*<sup>+/+</sup> mice (**Fig 4A**). The hemoglobin diet induced the same number of anaphase bridges as the *Apc* mutation,

suggesting that dietary hemoglobin generates strong initiators. Taken together, these data suggest that heme-induced aldehydes can induce mutations *in vitro* and *in vivo* and may initiate carcinogenesis.

In conclusion, we identified heme iron as the main factor responsible for the promotion of CRC by red meat and showed that aldehydes such as 4-HNE or HHE play roles in the underlying mechanism of action. Furthermore, we suggest that dietary heme could result in initiating agents in the gut. Improved dietary recommendations should focus (i) on the amount of heme iron in meat-based diets rather than on the modes of cooking or preparation and (ii) on dietary changes that could reduce the heme effect in the gut (i.e. on changes that limit the bioavailability of heme and of heme-induced peroxidation) (40).

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**Table 1: Experimental Diets (g /100 g). (A) Study in F344 rats. (B) Study in *Min* mice.**

<i>A</i>	Study in F344 rats				<i>B</i>	Study in <i>Min</i> mice	
	Control	Heme	HCA	Heme + HCA		Control	Hemoglobin
<b>AIN 76 base</b>	89	89	89	89	65.2	65.3	
<b>Sucrose</b>	5.7	4.7	5.7	4.7	29.7	27.2	
<b>Casein</b>	0.33	0.33	-	-			
<b>Hemoglobin</b>	-	1	-	1	-	2.5	
<b>Ferric citrate</b>	0.015	-	0.015	-	0.035	-	
<b>PhIP + MeIQx</b>	-	-	$7.5 \times 10^{-6}$	$7.5 \times 10^{-6}$			
<b>Safflower oil</b>	5	5	5	5	5	5	

**Note:** Heterocyclic amines (HCA): 2-amino-1-methyl-6-phenylimidazo(4,5-b)pyridine (PhIP) and 2-amino-3,8-dimethylimidazo [4,5-f]quinoxaline (MeIQx) were obtained from TRC (Toronto Research Chemicals). All other chemicals were analytical grade and were obtained from either Merck or Sigma. For their drinking water, each dietary group of rats was split in two sub-groups : half of them received tap water in their drinking bottle. The other half received NaNO<sub>2</sub> and NaNO<sub>3</sub> solutions in tap water. The NaNO<sub>2</sub> and NaNO<sub>3</sub> solutions were prepared with 170 mg NaNO<sub>2</sub>/L water and 230 mg NaNO<sub>3</sub>/L water. Water was replaced twice a week.

**Figure 1. Heme iron plays a major role in mucin-depleted foci (MDF) formation in rats and is associated with fecal water lipid peroxidation biomarkers independent of the fecal concentration of apparent total N-nitroso-compounds (ATNC).** (A) The number of MDF per rat colon. A three-factor Anova was used and showed only an effect of heme. (B) Lipid peroxidation in fecal water [measured as TBARs (MDA equivalents)]. (C) Fecal water cytotoxicity. The cytotoxic effects of fecal water from the eight groups of rats on *Apc*<sup>+/+</sup> cells (gray bars) and on *Apc*<sup>-/+</sup> cells (black bars). Viability was measured by MTT assay after 24 h of incubation with a 1/50 dilution of fecal water. (D) ATNCs in fecal water shown as nitrosyl iron (FeNO), S-nitrosothiols (RSNO), and other ATNCs. TEM: control diet; HCA: diet with PhIP (50 µg/kg) and MeIQx (25 µg/kg); Heme: diet containing 1% hemoglobin; Heme + HCA: diet containing 1% hemoglobin, PhIP, and MeIQx: 50+25 µg/kg; 0: drinking water control; N: drinking water with nitrites and nitrates at 0.4 g/l. Values are means ± SEM, n=10; \*Significantly different from TEM and HCA, p<0.001. °Significantly different from *Apc*<sup>-/+</sup> cells, p<0.01.

**Figure 2. The central role of aldehydes in the cytotoxic effect of fecal water of hemoglobin fed rats and the cytotoxic, genotoxic, and pro-apoptotic dose-dependent effects of HNE, HHE, and MDA in *Apc*<sup>+/+</sup> and *Apc*<sup>-/+</sup> cells.** (A) TBARs and cytotoxicity on *Apc*<sup>+/+</sup> and *Apc*<sup>-/+</sup> cells of fecal water of hemoglobin fed rats treated or not with trapping aldehydes resin. Solid bars: fecal water of hemoglobin group, hatched bars: fecal water of hemoglobin group treated with resin. Values are means ± SEM, n=3. (B) Cytotoxicity on *Apc*<sup>+/+</sup> (gray bars) and *Apc*<sup>-/+</sup> (black bars) cells of HNE (20 µM) and hemin (100 µM). Values are means ± SEM, n=3. (C) Genotoxic and cytotoxic effects of aldehydes on *Apc*<sup>+/+</sup> and *Apc*<sup>-/+</sup> cells. Values are means ± SEM, n=4; (D) Pro-apoptotic dose-dependent effects of aldehydes on *Apc*<sup>+/+</sup> and *Apc*<sup>-/+</sup> cells. Values are means ± SEM, n=3;

\*Significant difference between fecal water treated or not with resin, using Student's test (\* $p < 0.05$ ). °Significant difference between *Apc*<sup>+/+</sup> and *Apc*<sup>-/+</sup> using Student's test (° $p < 0.05$ ; °° $p < 0.01$ ; °°° $p < 0.001$ ).

**Figure 3. The effect of hemoglobin on intestinal tumorigenesis in *Min* mice and changes in fecal and luminal biomarkers associated with this effect.** (A) Intestinal tumor load (mm<sup>2</sup> per intestine). Values are means  $\pm$  SEM, n= 14 for the control diet and n=21 for the haemoglobin diet. (B) Lipid peroxidation in fecal water from feces [measured as TBARs (MDA equivalents)]. n= 5 for the control diet and n=7 for the haemoglobin diet. (C) Cytotoxic activity and peroxidation in small intestine contents from mouse. Cytotoxicity was measured in the *Apc*<sup>-/+</sup> cell line (fecal water diluted 1:50, MTT assay). n= 5 for the control diet and n=7 for the haemoglobin diet. \*\* and \*\*\*Significantly different from control diet (\*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ).

**Figure 4. The effect of a hemoglobin-containing diet on anaphase bridges and on the induction of  $\gamma$ H2AX in the small intestinal epithelium in mice.** (A) The number of anaphase bridges per gland in *Apc*<sup>Min/+</sup> and *Apc*<sup>+/+</sup> mice after 50 days of the indicated experimental diet. \*Significantly different from the control diet in the same genetic context, °Significantly different from *Apc*<sup>+/+</sup> mice on the same diet. (B) An anaphase bridge (arrow) in an *Apc*<sup>+/+</sup> mouse fed a hemoglobin diet, scale bar: 2  $\mu$ m. (C) The number of  $\gamma$ H2AX positive cells per gland in C57BL/6J mice after 14 days on the experimental diet. (D)  $\gamma$ H2AX-positive cells (arrows) in *Apc*<sup>+/+</sup> mice fed the heme diet, scale bar: 4  $\mu$ m.









