Research Article

Cancer Prevention Research

Targeting Colon Luminal Lipid Peroxidation Limits Colon Carcinogenesis Associated with Red Meat Consumption

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Abstract

Red meat is probably carcinogenic to humans (WHO/IARC class 2A), in part through heme iron-induced lipoperoxidation. Here, we investigated whether red meat promotes carcinogenesis in rodents and modulates associated biomarkers in volunteers, speculating that an antioxidant marinade could suppress these effects via limitation of the heme induced lipid peroxidation. We gave marinated or non-marinated beef with various degrees of cooking to azoxymethane-initiated rats, *Min* mice, and human volunteers (crossover study). Mucin-depleted foci were scored in rats, adenoma in *Min* mice. Biomarkers of lipoperoxidation were measured in the feces and urine of rats, mice, and volunteers. The organoleptic properties of marinated meat were tested. Fresh beef increased colon carcinogenesis and lipoperoxidation in rats and mice and lipoperoxidation in humans. Without an adverse organoleptic effect on meat, marinade normalized peroxidation biomarkers in rat and mouse feces, reduced peroxidation in human feces and reduced the number of Mucin-depleted foci in rats and adenoma in female *Min* mice. This could lead to protective strategies to decrease the colorectal cancer burden associated with red meat consumption. *Cancer Prev Res; 11(9); 569–80. ©2018 AACR.*

Introduction

Colorectal cancer is the third most common type of cancer and fourth most common cause of death from cancer worldwide (1). In 2007 and confirmed in 2011 and 2017, the World Cancer Research Fund (WCRF) and American Institute for Cancer Research (AICR) panel stated that there is strong evidence that consuming red meat increases the risk of colorectal cancer (2, 3). In 2015, the International Agency for Research on Cancer (IARC), an agency of the World Health Organization (WHO), classified red meat consumption as "probably carcinogenic to humans" (4).

Epidemiological studies incited the WCRF to recommend limiting red meat consumption to less than 500 g/w (1, 2). These recommendations may reduce the colorectal cancer burden, but sociological studies have demonstrated that people with a lower socioeconomic status are less receptive to nutritional messages and are less likely to change risky behaviors than affluent people (5–9). Simply conveying information on risks and benefits has almost no effect on food choices among less-educated people and tends to enlarge the health inequalities to which colorectal cancer contributes (10, 11). Furthermore, adherence to the WCRF recommendation of limiting red meat intake is low in cancer survivors (8%; ref. 12). On the basis of these observations, we wanted to propose an alternative to the current recommendation of limiting consumption by adding protective additives directly in the commercial product.

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Different hypotheses have been explored to explain the link between red meat intake and colorectal cancer, including heterocyclic amine formation during high-temperature cooking (13), *N*-nitroso compound formation by endogenous nitrosation, and high heme iron content, principally through the catalysis of dietary lipid oxidation and formation of *N*-nitroso compounds involving gut microbiota (14, 15). We recently showed that, at nutritional doses, heme iron is the major actor in meat-induced promotion of colon cancer without an additive or synergistic effect of heterocyclic amines and endogenous *N*-nitroso compounds (16). The promoting effect of heme iron has been validated in rodent models using purified molecules (hemoglobin or hemin) and lyophilized red meat (16–20). Furthermore, this promoting effect of heme was confirmed at the epidemiological level in the French E3N prospective cohort of women (21). In humans and in rodent models, meat and heme intake have been associated with an increase in two lipoperoxidation biomarkers: thiobarbituric acid reactive substances (TBARS) and 1,4-dihydroxynonane mercapturic acid (DHN-MA; refs. 18–20, 22–24). Heme iron can catalyze dietary lipoperoxidation, a key downstream feature of ferroptosis, an iron-dependent form of non–apoptotic-regulated cell death (25–27) and a reaction leading to the production of genotoxic and/or cytotoxic aldehydes that could be involved in the promotion of carcinogenesis (16, 28–31). Moreover, we have shown that addition of antioxidants to diet is efficient to decrease meat-induced lipoperoxidation and carcinogenesis in animal models (19, 20, 32). The protective effect of the dietary antioxidant capacity was confirmed in humans in the French E3N prospective cohort of women (21).

We designed a project which aimed to limit the heme-induced colonic luminal lipoperoxidation to prevent the risk of colon cancer associated with red meat consumption. The project was divided in six sequential studies, including an industrial task of meat production and analysis, short-term and carcinogenesis animal studies, and clinical and organoleptic acceptability studies in human volunteers (Supplementary Fig. S1).

Materials and Methods

Ethics

All animal studies were performed in an accredited animal facility by approved staff and animal care conducted in accordance with the ARRIVE and European Council on Animals used in Experimental Studies guidelines. The human volunteer crossover study was allowed by a written decision of the ethical committee (French CPP Sud Est VI) and authorized by the French Ministry of Health (No. IDRCB 2013-A01692-43). The study was registered at Clinicaltrials.gov. under the number NCT02473302.

Study design

First, we selected the most effective marinade among the 12 tested (see Supplementary Materials for details) on the basis of a DPPH assay. The selected grape and olive marinade was then incorporated into cooked or uncooked meats to test the modulation of lipoperoxidation and iron content in meats. A 14-days nutritional experiment (see Supplementary Materials for details) was then performed in Fischer 344 male rats to test the effect of different marinated or non-marinated and cooked or uncooked meats on the modulation of lipoperoxidation and iron content in fecal water, two biomarkers associated with the promotion of colon carcinogenesis by red meat. These six meats were then tested in colorectal carcinogenesis studies in chemically induced rats, and four were selected for study in *Apc Min* mice. From these results, the biological effect of the most effective production process was tested in healthy human volunteers, as well as its organoleptic acceptability. A flowchart of the experiments is presented in Supplementary Fig. S1.

Carcinogenesis study in azoxymethane-induced rats

Male Fischer 344 rats ($N = 92$) purchased at 5 weeks of age from Charles River (France) were housed individually in stainless steel wire-bottom cages. The rats were kept at 22°C with a 12–12 hours light–dark cycle and allowed free access to standard AIN76 semipurified diet and tap water. After acclimatization, the rats received a single intraperotoneal injection of azoxymethane (Sigma, 20 mg/kg) in NaCl (9 g/L of water). Seven days later, the rats were randomly allocated to seven groups. Groups of twelve rats were given meats following the same 3×2 factorial protocol as for study 2: meats were raw, rare, or well-done and marinated or not-marinated for each level of cooking. As in the short-term experiment, pieces of meat were completed with low calcium modified AIN76. After evaluating the consumption of the pieces of meat and powder, we calculated that diets contained 47.3 g of meat (dry weight, per 100g total diet) and 52.7 g of low calcium modified AIN76 (See Supplementary Materials for details). One group ($N = 20$) received control diet without meat, composed only of low calcium–modified AIN76. Each diet was stored at −20°C under vacuum and dispensed daily at 5:00 p.m. Rats were fed the experimental diets daily for 98 days before CO_2 euthanasia. Body weight was monitored every week during the first 4 weeks, then every 2 weeks. Food and water intake was measured on days 25 and 60. Feces were collected on days 88 to 91 and kept at −20°C. Each rat was placed in a metabolic cage and urine collected on days 67 to 70 and kept at −20°C.

Carcinogenesis study in Apc Min mice

Five- to 8-week-old male and female *Apc Min* mice ($N = 97$; The Jackson Laboratory) were housed 2 to 5 mice per cage under standard laboratory conditions with free access to food and water. After 3 days of acclimatization, the mice

were randomly allocated to five experimental groups balanced for age and sex. Groups of 18 mice were given meats following a 2 × 2 factorial protocol: meats were raw or rare and marinated or not marinated for each level of cooking. As we could not have the same number of experimental groups as in rats, we decided to eliminate the well-done meat group because it is not as representative of human consumption (33). Unlike for rats, for the mice we had to grind and mix the meat with the modified AIN76 to ensure it was eaten entirely. The meat diets contained 60% (dry weight) meat and 40% low calcium–modified AIN76-base powder (see Supplementary Materials for more details on diets). One group ($N = 25$) received a control diet without meat, composed only of modified AIN76. Each diet was stored at −20°C under vacuum and dispensed daily at 5:00 p.m. Mice were fed the experimental diets for 45 days before euthanasia by cervical dislocation. Body weight was monitored every week during the first 3 weeks, then every 2 weeks. Feces were collected on days 35 to 39 and urine on day 39 and kept at −20°C.

The consumption of raw beef is too unusual to be representative of human consumption; therefore, for studies in humans, the meat was cooked to be consistent with the most common consumption methods (rare and well done).

Cross over study with human volunteers

The human study was performed in the Nutritional Investigation Unit of the Human Nutrition Center of Auvergne (Clermont-Ferrand, France). A single-blind randomized, controlled cross-over trial was performed in 24 human volunteers after obtaining written informed consent (inclusion criteria in Supplementary Materials). During the 1-week run-in period for adaptation, volunteers were asked to eat a diet without beef or pork and low in antioxidant products. Volunteers were then randomly submitted to three alternating 4-day intervention periods with 110 g/d of rare beef, marinated rare beef or marinated well-done beef in a random order. During the intervention periods, the volunteers must not eat meat, fish or eggs outside the provided beef. To facilitate the comparison between human and rodents studies of this project, meats for the human study were obtained from the same production batch as those given to rats and mice. Intervention periods were separated by a wash-out period of at least 3 days with the same diet as the run-in period. Urine and stool were collected during the last 3 days of each intervention period and at the end of each washout period. Each subject came to the Nutrition Investigation Unit four times in addition to the screening visit. Before the first intervention period (visit 1), volunteers were given cooking instructions. At each visit, meats were distributed for the following intervention period. During visits 2, 3, and 4, each volunteer brought back the meat packaging and frozen urine and stool samples. Compliance to diet was assessed after the collection of feces and urine samples at the end of the intervention periods.

Organoleptic acceptability of marinated meats by a consumer panel

Two consumer studies were organized in INRA-Dijon, France (see Supplementary Materials for details). The first study aimed at assessing a reference beef meat (beef) and the two marinated products (one marinade without grape–olive extracts and one marinade with these extracts) for overall liking by consumers during blind tasting. The second study aimed to replicate the first study (reference vs. grape–olive marinade) but also aimed to assess whether providing the consumers with information about the protective effect of the marinated product could improve their overall liking of the product. Consumers from both groups were regular eaters of beef. The consumers from the treatment group were first (at recruitment and at the beginning of the session) instructed about the risk of colon cancer promotion by excessive red meat consumption. Both consumer studies were designed according to the same rules. Data were collected by the Fizz system in a sensory room equipped with 16 booths and maintained according to ISO Standard (NF EN ISO 8589). Sessions occurred at lunch time. The order of serving the samples was balanced using Williams Latin squares, ensuring that each product was evaluated equally often in the different position orders and preceded each other product equally often. Each consumer had the products served either rare or well done according to his/her usual way of consuming beef. The liking scale was the 9-point hedonic scale with the first tick-box on the left labeled "I don't like it at all" and the ninth on the right labeled "I like it very much."

In the second study, the information about colon cancer risk and the protective effect of new products was provided to the consumer of the informed group (see Supplementary Table S9). This text was sent to them by mail before the experiment and included in a preliminary screen of the sensory session. For each product and subject, the difference of liking scores given in the informed condition (protective effect) versus no information was computed. The 3 differences of each subject were averaged providing individual scores of the effect of information on liking. A number of individual variables (demographics, usages, attitudes, . . .) were screened for differences in information scores among their levels.

Carcinogenesis endpoints

MDF scoring. At euthanasia, the rats' colons were removed, washed with cold Ringer, opened, coded, and fixed flat between two sheets of filter paper in 10% buffered formalin (Sigma). The number of MDF per colon and the number of crypts per MDF, as a measure of their size, were scored by two blinded investigators after high-iron diamine alcian blue staining according to Caderni and colleagues and Femia and colleagues (34, 35).

Tumor scoring. At euthanasia, the small intestine (from duodenum to ileum) and the colon of mice were removed. Sections of the duodenum, jejunum, ileum, and colon were opened along the longitudinal axis and washed in PBS. After fixation, the different sections were stained for 48 hours in a 300 ppm solution of methylene blue in formalin. Two blinded investigators scored tumors and determined their diameters using a binocular microscope at ×25 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 biomarkers

Fecal water preparation. To prepare fecal water of induced rats and mice, 1 mL of distilled water and 50 μL of butylated hydroxytoluene (BHT, Sigma) 0.45 mol/L were added to 0.4 g of fresh feces. The feces were ground using Fast-Prep (MP Biomedicals) for 30 seconds three times and then centrifuged at 5,500 g for 20 minutes. The fecal water was collected and kept at −20°C until use. For humans, 1 mL of sterilized water and 25 μL of BHT (0.90 mol/L) were added to 0.25 g of fresh feces and the same protocol followed.

Heme and TBARS assays. Heme was measured by fluorescence in fecal water according to Sesink and colleagues (36) as described previously (20). TBARS were measured in fecal water according to Ohkawa and colleagues as previously described (37).

HNE assay. Samples were prepared for free HNE determination as described previously by Lesgards and colleagues (38) and adapted for the analysis of rat fecal water samples (see Supplementary Materials for details).

Urinary biomarker

Urine preparation. Urine samples were diluted at 1:200 for animal studies and at 1:20 for human study in an EIA/BSA solution containing phosphate buffer (0.1 mol/L at pH 7.4) with NaCl (0.15 mol/L), 0.1% BSA and 0.01% sodium azide.

DHN-MA assay. DHN-MA assay was performed by competitive enzyme immunoassay as described previously using DHN-MA–linked acetylcholinesterase enzyme as tracer (24). Urine samples from non-induced rats study were pooled and analyzed in duplicate, whereas other urine samples were assayed individually.

Statistical analysis

Data were analyzed using Systat 12 software for Windows and reported as mean ± SEM or mean ± SD as noted in legends.

In study with induced rats, the effect of meat intake was analyzed by one-way ANOVA comparing the control animals to the meat-fed animals. Next, the control animals were removed and the effect of marinade and cooking analyzed in two-way ANOVA. The interaction between the two factors (marinade and cooking) was always checked. In study with *Min* mice, the effect of meat intake was analyzed by two-way ANOVA, including the factor sex. Next, the effect of the marinade, cooking, and gender was analyzed in three-way ANOVA. The interaction between the two or three factors was always checked. MDF and tumor scoring was performed in duplicate by two independent readers; therefore, these variables were tested first by 2-factor ANOVA (groups and readers). The group x reader interaction was never significant, and when total ANOVA was significant ($P < 0.05$), pairwise differences between groups were analyzed.

Human volunteer data were analyzed using the Wilcoxon's signed rank test with each volunteer being his own control. No Bonferronni correction was made for the multiple comparison analysis, as only three pairwise comparisons were made that had been decided beforehand (i.e., rare-cooked meat *vs.* no-meat control period, rare-cooked meat *vs.* rare-cooked meat marinated, and rare-cooked meat marinated *vs.* well-done meat marinated).

Liking data from the first panel and control group of the second panel were analyzed separately using two-way ANOVA model, including the product and the consumer effects. The information (classical/protective) and its interaction with the product effect were added to the model for the informed group of the second panel. Finally, the difference between liking scores with and without the protective information averaged over the two products was used to screen the items on the consumer questionnaires by one-way ANOVAs for the effect of this information.

Results

Associated to fecal and urinary lipoperoxidation biomarkers, consumption of red meat increases the size of colonic preneoplastic lesions in azoxymethane-initiated rats, but marinating meat protects against these modulations

Rats fed meats had significantly larger MDF ($P = 0.028$) and more fecal heme ($P < 0.0001$), TBARS ($P < 0.0001$), free and bound 4-hydroxynonenal (HNE; $P = 0.002$ and $P < 0.0001$, respectively), and urinary DHN-MA ($P < 0.0001$) than rats given the control diet with no meat (Table 1). Proteomic analysis showed that raw no-marinated meat fed rats had significantly more ferritin and annexin in the colon mucosa than control no-meat diet fed rats (Supplementary Table S6). All proteomics results as well as the metabolic pathway affected are presented in Supplementary Data (Supplementary Table S6 and Supplementary Fig. S3). Rats given marinated meats had significantly fewer MDF ($P = 0.018$) and less fecal heme ($P = 0.013$), TBARS ($P = 0.015$), free and bound HNE ($P = 0.003$ and $P = 0.031$, respectively), and urinary DHN-MA ($P = 0.001$)

Table 1. Effect of experimental meats on size and number of preneoplastic Mucin depleted foci (MDF) lesions and on fecal and urinary biomarkers associated with meat-induced promotion in rats previously injected with azoxymethane and fed for 98 days

		Experimental meats						Statistics[a]		
	Control diet	No marinade			Marinade					
	(no meat)	Raw	Rare	WD	Raw	Rare	WD	Meat	Marinade	Cooking
MDF size	2.3 ± 0.3	2.5 ± 0.6	2.7 ± 0.9	2.4 ± 0.6	2.6 ± 0.5	2.5 ± 0.5	2.7 ± 0.7	$P = 0.028$	$P = 0.855$	$P = 0.944$
MDF number	19 ± 9	21 ± 11	17 ± 10	15 ± 6	16 ± 9	14 ± 7	13 ± 7	$P = 0.067$	$P = 0.018$	$P = 0.042$
Heme (μmol/L in FW)	2 ± 2	193 ± 72	122 ± 43	90 ± 30	120 ± 58	101 ± 44	99 ± 26	$P < 0.0001$	$P = 0.013$	$P < 0.0001$
TBARS (μmol/L MDA eq. in FW)	13 ± 12	84 ± 21	75 ± 15	91 ± 17	67 ± 19	68 ± 14	81 ± 27	$P < 0.0001$	$P = 0.015$	$P = 0.034$
HNE free (nmol/g FW)	1.1 ± 0.4	1.7 ± 0.4	1.8 ± 0.5	1.7 ± 0.6	1.5 ± 0.3	1.2 ± 0.4	1.3 ± 0.6	$P = 0.002$	$P = 0.003$	$P = 0.824$
HNE bound (nmol/g proteins)	44 ± 17	72 ± 23	97 ± 28	66 ± 32	63 ± 21	62 ± 22	69 ± 28	$P < 0.0001$	$P = 0.031$	$P = 0.202$
DHN-MA (μg/24 h in urine)	178 ± 62	406 ± 173	564 ± 237	485 ± 229	335 ± 86	372 ± 170	310 ± 149	$P < 0.0001$	$P = 0.001$	$P = 0.172$

NOTE: Data are presented as mean ± SD.

Abbreviations: FW, fecal water; WD, well-done.

[a]The effect of meat intake was evaluated by a one-way ANOVA and the effect of marinated and cooked meats intake by a two-way ANOVA.

than rats given non-marinated meats (Table 1). Finally, rats given cooked meats had significantly fewer MDF ($P = 0.042$) and less fecal heme ($P < 0.0001$) and TBARS ($P = 0.034$) than rats given uncooked meats, without a significant difference in free and bound HNE ($P = 0.8$ and $P = 0.2$, respectively) and urinary DHN-MA ($P = 0.2$; Table 1). We identified significant interactions between marinade and cooking factors for fecal heme ($P = 0.014$) and fecal bound HNE ($P = 0.042$).

Red meat intake induces a simultaneous increase in the number of intestinal tumors, the tumor load and the fecal biomarker of peroxidation in *Apc Min* mice. Marinating red meat protects against these increases in female mice

The tumoral character of the enumerated lesions in the intestine after methylene blue staining (Fig. 1A) was evaluated in histological sections (Fig. 1B). Mice given meats had significantly more intestinal tumors and higher tumor load ($P = 0.048$ and $P = 0.001$, respectively; Fig. 1C and D) and more fecal heme ($P = 0.02$, Fig. 2A) and TBARS ($P < 0.0001$, Fig. 2B) than mice given the control diet with no meat. Taking into account the size of the tumors, the significant effect of meat in increasing the number of tumors was confirmed for medium and large tumors (Supplementary Table S7). Furthermore, female mice given meats had significantly more and larger intestinal tumors ($P = 0.047$, Fig. 1C and $P = 0.009$, respectively) than male mice given meats.

We found a significant interaction between marinade and sex factors for tumor number ($P = 0.015$). Female mice given marinated meats had significantly fewer intestinal tumors than female mice given non-marinated meats ($P = 0.019$, Fig. 1E), but no protecting effect of marinade was observed for male mice ($P = 0.47$, Fig. 1F).

Mice given marinated meats had significantly less fecal TBARS ($P = 0.043$) than rats given non-marinated meats without a significant effect on fecal heme ($P = 0.4$), and urinary DHN-MA ($P = 0.2$, Fig. 2). Proteomic analysis showed that rare marinated meat fed mice had significantly less vimentin in the colon mucosa than control no-meat diet fed mice (Supplementary Table S8). All proteomics results as well as the metabolic pathway affected are presented in Supplementary Data (Supplementary Table S8 and Supplementary Fig. S5). Mice given cooked meats had significantly more intestinal tumors and higher tumor load ($P = 0.001$ and $P = 0.001$, respectively; Fig. 1C and D) than mice given uncooked meats, without a significant difference for fecal heme ($P = 0.2$), TBARS ($P = 0.1$), and urinary DHN-MA ($P = 0.1$, Fig. 2). We found no significant interaction between marinade and cooking factors.

Rare cooked meat consumption increases luminal peroxidation in healthy volunteers and a grape–olive marinade reduces this effect

The nutritional intervention was scheduled with 24 healthy volunteers (see Supplementary Materials for details). Biomarker measurements revealed a significant increase in the heme content of fecal water for the two rare meat periods (Fig. 3A) and a tendency to increase with the well-done meat. We also observed a significant increase in the concentrations of TBARS in fecal water from human volunteers fed 110 g of non-marinated meat for 4 days compared with control periods ($P = 0.038$, Fig. 3B). Marinade significantly decreased fecal TBARS for rare cooking ($P = 0.046$) but not well-done cooking ($P = 1$). We found no significant difference between volunteers in regards to HNE content in fecal water, urinary DHN-MA, or fecal ATNC, and fecal and genotoxicity (Supplementary Fig. S6).

The olive–grape marinade does not alter the organoleptic acceptability of red meat but conversely increases the preference of the meat

To quantify the contribution of antioxidants in the effect of grape–olive marinade on organoleptic acceptability, an antioxidant-free marinade was compared with a marinade with antioxidants. A two-way (product and consumer) ANOVA model of liking scores from the first study demonstrated that the marinated meats were both significantly

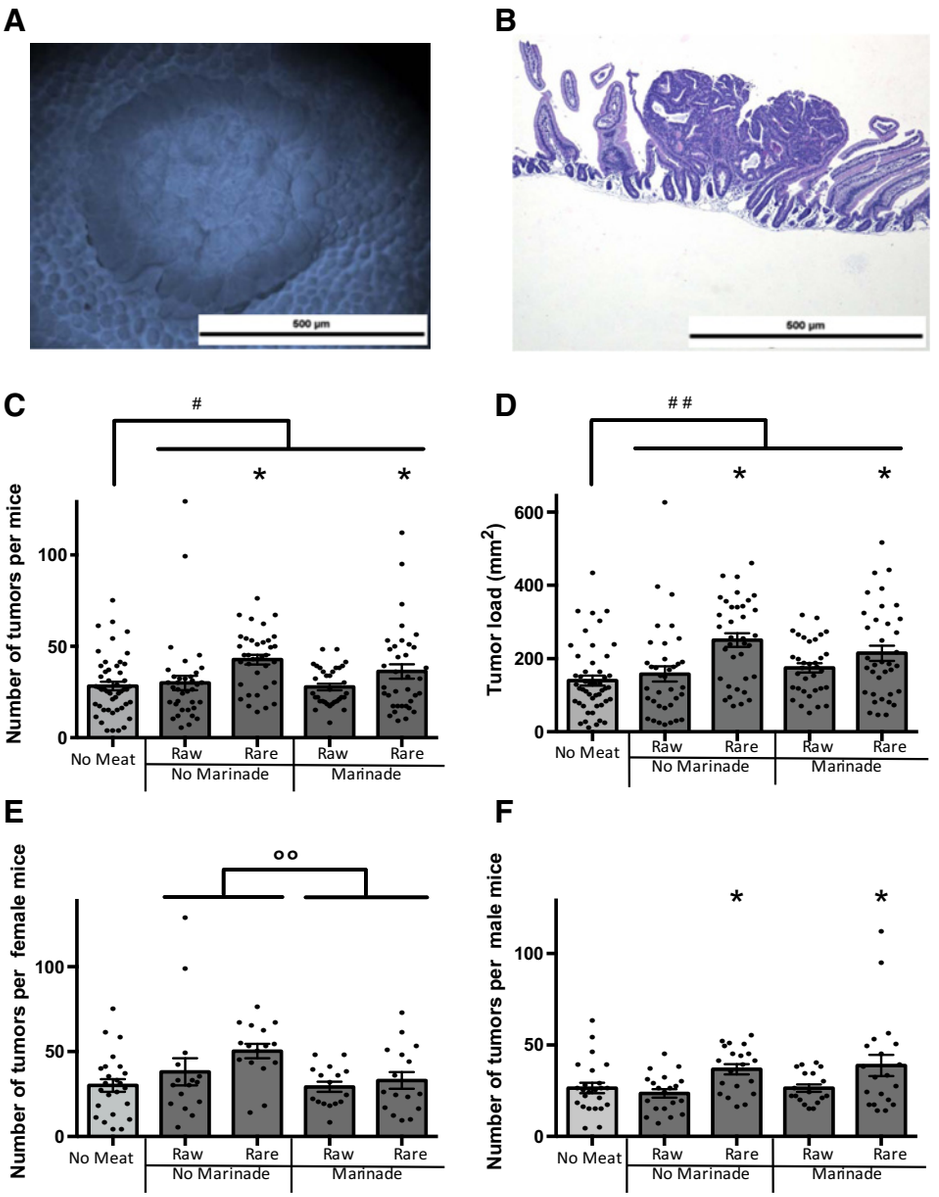

Figure 1.
Effect of experimental meats on small intestine tumors in *Apc Min* mice fed for 45 days. **A,** Adenoma after methylene blue staining. **B,** Histological section of adenoma (H&E staining). **C,** Number of tumors per mouse. **D,** Tumor load per mouse. **E,** Number of tumors per female mouse. **F,** Number of tumors per male mouse; scale bars, 500 µm. Data are presented in the graphs as mean ± SEM and the circle are the individuals. The effect of meat intake was evaluated by two-way ANOVA and the effect of marinated and cooked meat intake by three-way ANOVA. $^{\#}$, $P < 0.05$; $^{\#\#}$, $P < 0.01$ versus the no-meat-fed mice; *, $P < 0.05$ versus the raw condition with the same marinade status; $^{\circ\circ}$, $P < 0.01$ versus the no-marinated condition.

preferred to the reference meat ($P = 0.004$, Fig. 4A). A very slight similar trend was observed in the second study (Fig. 4B), although it did not reach statistical significance ($P = 0.283$). The marinated meat with antioxidants was slightly less appreciated than the marinated meat without antioxidants, though this difference was not significant (Fig. 4A).

The effect of information about colon cancer risk and the protective effect of new products was not significant and had almost ($P = 0.182$) no interaction with the product effect (Fig. 4C). However, looking precisely at the mean liking scores of the four products tested in the test group, a potential positive effect of the information on the liking of the marinated meat was observed ($P = 0.086$).

None of the variables on the usage, attitude, and knowledge questionnaire influenced the effect of the information on product-liking. However, the level of education and age of the consumers seemed to have impacted that effect. Indeed, the difference between liking scores with and without information is significantly ($P = 0.022$) higher in primary and secondary versus higher school levels (Fig. 4D). Also, Fig. 4E shows that the effect of information would be negative in consumers ages less than 35, about null in consumers between 36 et 55 and positive in consumer older than 56; these differences among the three age categories were almost significant ($P = 0.053$).

Discussion

The present study is the first to show that consumption of fresh red meat for few days increased fecal lipid oxidation

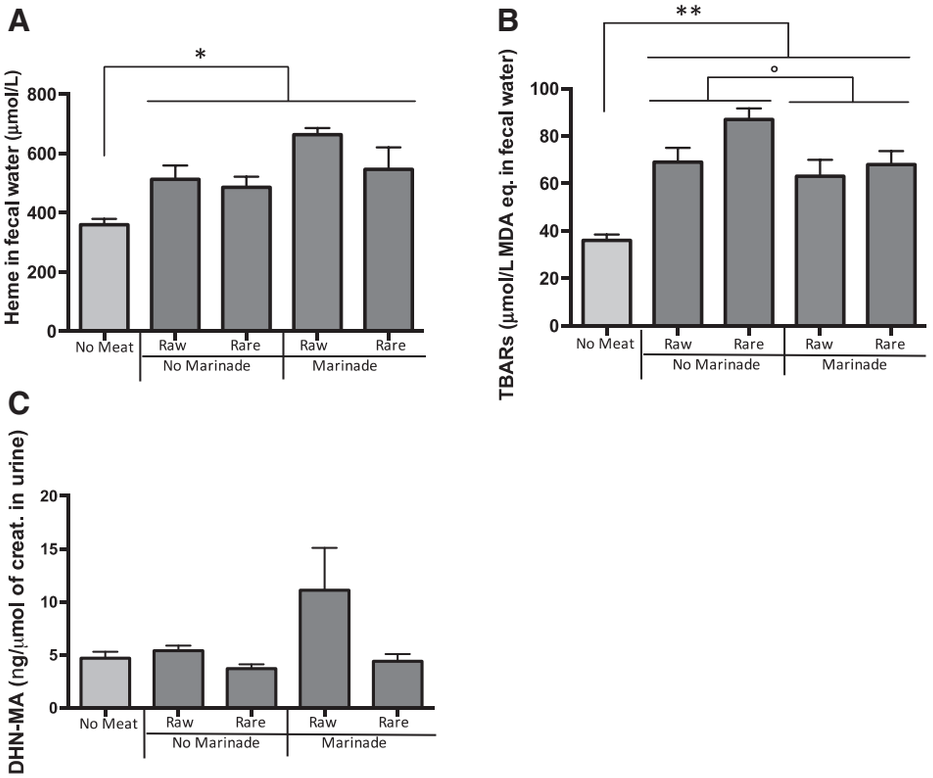

Figure 2.
Effect of experimental meats on fecal and urinary biomarkers associated with meat-induced promotion in *Apc Min* mice fed for 45 days. **A,** Heme in fecal water. **B,** TBARS in fecal water. **C,** DHN-MA in urine. Data are presented as mean ± SEM. The effect of meat intake was evaluated by two-way ANOVA and the effect of marinated and cooked meat intake evaluated by three-way ANOVA. *, $P < 0.05$; **, $P < 0{,}0001$ versus no meat-fed mice, °, $P < 0.05$ versus no-marinated condition.

biomarkers associated with heme-induced promotion of colon carcinogenesis in human volunteers and in two complementary animal models.

These findings agree with the epidemiological data and the conclusions of the WCRF and WHO (3, 4). This study also shows that this increase and the promotion of carcinogenesis in rats and female mice were decreased when meat was treated with an antioxidant marinade.

As people of a lower socioeconomic status are not receptive to nutritional messages and less likely to change risky behaviors than affluent people, we wanted to propose an alternative to the current recommendation of limiting consumption by working directly on the commercial product. To achieve this objective, we designed a project (Flow chart in Supplementary Fig. S1) allowing to highlight a beef antioxidant marinade limiting the heme-iron induced lipoperoxidation, a key step in the promotion of colon cancer associated with red meat consumption (11).

For this project, we associated two animal models of carcinogenesis: The AOM-induced rat and the *Apc Min* mice. Indeed, for 30 years, investigators have searched for dietary agents that could impact colon carcinogenesis tumors. For that, the azoxymethane (AOM) model was widely used and more recently associated to *Apc Mice* models. If carcinogenesis in the rat model results of a large bolus of AOM, it has many morphological as well as

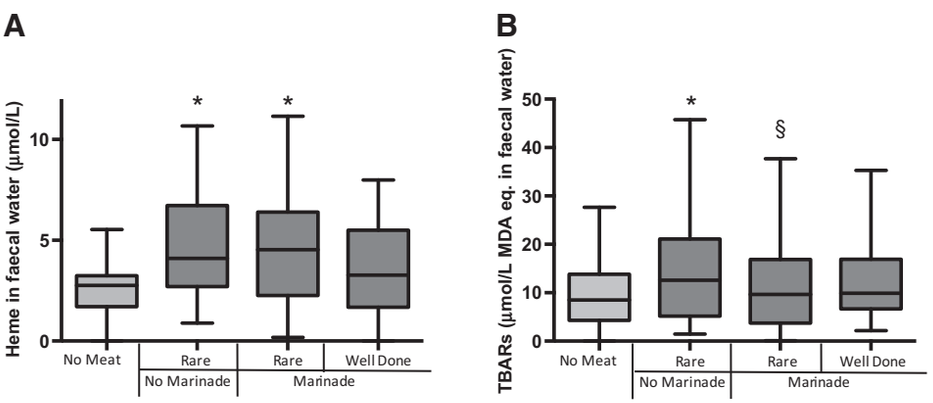

Figure 3.
Effect of experimental meats on fecal biomarkers associated with meat-induced promotion in human volunteers after 4 days of 110 g/d of meat. **A,** Heme. **B,** TBARS in fecal water. Data are presented as mean ± Min/Max, $N = 21$.*, $P < 0.05$ versus the no-meat control period (Wilcoxon's test). §, $P < 0.05$, versus the no-marinated meat period (Wilcoxon's test).

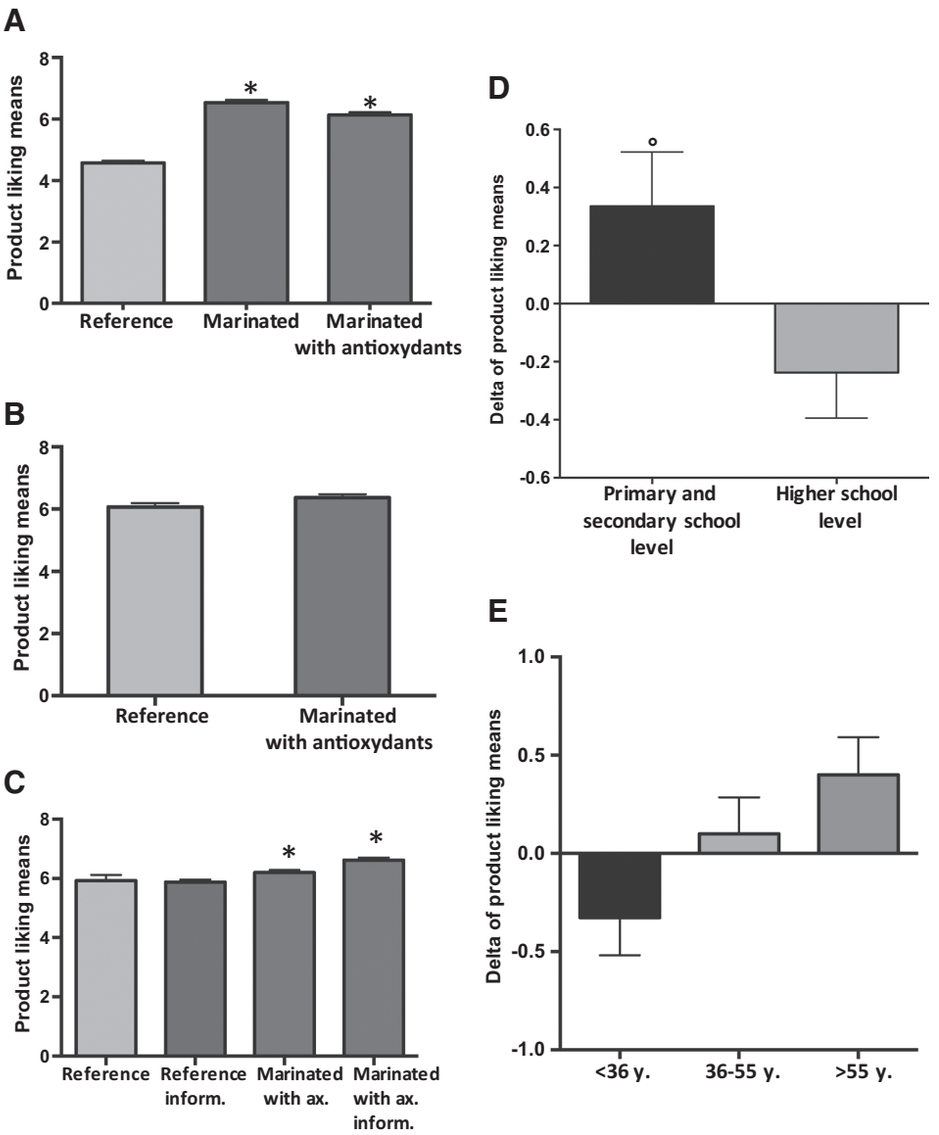

Figure 4.
Product liking means in the first sensory study (**A**), second sensory study (**B**), test groups (**C**). Effect of the information according to education level (**D**) and age (**E**). Data are presented as mean ± SEM. Statistical analysis was three-way ANOVA. *, $P < 0.05$, *vs.* the reference. °, $P < 0.05$, versus the reference Higher school level; ax.: antioxidants; inform.: after information.

molecular similarities to human sporadic colorectal cancer (39). Similarly, if the main location of tumors in the *Apc Min* mice is the small intestine, this mice is considered to be a model for human familial adenomatous polyposis with applications to better understand the molecular mechanisms of multistage carcinogenesis in the large bowel of humans (40). Furthermore, review of experimental data between diet and colon cancer demonstrated that there is a close agreement between many results obtained in the colons of AOM-initiated rats and in the small intestine of *Min* mice (41) and that there is a reasonable agreement between the results of these animal studies and the more limited clinical studies (42).

First, we defined an antioxidant marinade able to decrease lipoperoxidation in meats and rat fecal water after meat consumption. Several studies have shown that natural antioxidants can be used to reduce lipoperoxidation in meat, and that the antioxidant activity of a marinade can be determined by a DPPH assay (43–46). Twelve marinades were prepared to test the antioxidant activity of eight molecules, alone or in mixture, and grape–olive marinade had the highest (Supplementary Table S2). The addition of this marinade to beef sirloin led to a significant decrease in the concentrations of TBARS in meat (study 1, Supplementary Table S3). Although the grape–olive mixture has never been tested, these results are consistent with the literature regarding the properties of grapes and olives (47–49). In a short-term study in rats, we confirmed that the marinade can also decrease meat-induced colonic luminal lipoperoxidation (study 2, Supplementary Table S4), which is consistent with our previous studies (19, 20).

Thus, we wanted to check the effect of marinated and non-marinated meat on two stages of colorectal

carcinogenesis using two complementary animal models: AOM-initiated rats for the preneoplastic stage and *Apc Min* mice for the tumoral stage. Finally, we explored the impact of these meats on fecal and urinary biomarkers in human volunteers given 110 g of meat per day for 4 days.

Our results showed that fresh red meat intake significantly increased the size of pre-cancerous lesion MDF in initiated rats (Table 1) and the number and size of intestinal tumors and tumor load in *Apc Min* mice (Fig. 1). More precisely, fresh red meat intake significantly increased the number of medium and large tumors (Supplementary Table S7). In the two carcinogenesis animal models, meat-induced promotion was associated with a significant increase in fecal heme and lipoperoxidation biomarkers (TBARS, HNE in fecal water, and urinary DHN-MA in rats [Table 1]; TBARS in *Apc Min* mice [Fig. 2]). In mucosa, promotion in initiated rats was positively associated with the level of ferritin and annexin (Supplementary Table S6), two proteins for which expression was positively associated with the degree of dysplasia (50, 51). In our study, the level of ferritin protein in the colon mucosa correlated with the size of MDF ($r = 0.497$, $P = 0.002$), with the TBARS level in fecal water ($r = 0.511$, $P = 0.001$) and with the DHN-NA content in urine ($r = 0.409$, $P = 0.013$). The level of annexin protein in mucosa positively correlated with the size of MDF ($r = 0.452$, $P = 0.006$), with the heme content in fecal water ($r = 0.394$, $P = 0.017$) and with the TBARS level in fecal water ($r = 0.402$, $P = 0.015$). These results on fecal and urinary biomarker modulations are consistent with previous studies showing a promoting effect of hemoglobin or lyophilized red meat on pre-cancerous lesions or on tumors in association with increased fecal lipoperoxidation (16, 18–20). In human volunteers, the present crossover study showed that eating fresh red meat for 4 days was sufficient to increase lipoperoxidation biomarker concentrations in fecal water, compared with the control period without meat (Fig. 4). We did not observe a significant increase in urinary DHN-MA, which is consistent with our previous results with red meats (22). In humans, unlike in rodents, meat intake correlates with endogenous formation of fecal ATNC (52, 53). In this study, we did not observe a significant increase of fecal ATNC (Supplementary Fig. S6) and the level of ATNC was low overall, but this result is consistent with other human studies in which an increase in fecal ATNC was found only in subjects consuming at least 240 g of red meat per day and for a longer consumption period (52, 54, 55). Thus, the intake of fresh red meat can modulate lipoperoxidation biomarkers associated with the promotion of colon carcinogenesis in initiated rats and *Apc Min* mice, which gives experimental support to the epidemiology-based conclusion that red meat may be a cause of colorectal cancer (1, 2, 4).

Grape–olive marinade was efficient in initiated rats to decrease the number of pre-cancerous lesion MDF and was associated with a decrease in heme and lipoperoxidation biomarkers (Table 1). In *Apc Min* mice study, we observed a significant protective effect in female mice (Fig. 1E). To the best of our knowledge, this study is the first in which an antioxidant marinade was used to reduce the promotion of colorectal cancer by fresh red meat. Our results support epidemiological studies showing that adhesion to a Mediterranean diet, rich in olive oil and antioxidants, may reduce colorectal cancer risk, mainly in women (56, 57). The fact that we only observed a protective effect of the marinade in female may be consistent with epidemiologic evidences showing a gender-specific associations between antioxidants and rectal cancer risk, pointing a protective effect in women while no effect in men, probably due to the estrogen status (58). In our study, the protection due to the marinade in female *Apc Min* mice was associated with decreased expression of vimentin in the colon mucosa (Supplementary Table S8). Vimentin activates the Wnt signaling pathway, which is detectable as increased β-catenin accumulation in the nucleus with concomitant activation of β-catenin–dependent transcription of Wnt signaling downstream targets (59); thus, a decrease in the activation of this pathway largely involved in the colon carcinogenesis could participate in the observed protection. In human volunteers, as in the two carcinogenesis animal models, the marinade reduced significantly the increase of fecal TBARS when meat was cooked rare (Fig. 3). However, the protective effect was lost when the meat was well-done, likely reflecting an alteration in the antioxidant capacity during cooking at high temperature. In accordance with our initial aim to limit the risk in big meat eaters, in whom the risk of colon cancer increases the most, this absence of effect in well-done meat will not greatly limit the efficiency of the marinade because a majority of big eaters in France (60), in Europe (33), and in the United States (61) consume rare or medium red meat. Moreover, in parallel to the addition of antioxidant *via* a marinade, it may be interesting to test other solutions to provide the antioxidants with the meat: sell antioxidant extracts with the meat for example. These extracts could be used to dress the meat after cooking. However, if it is not certain that a sufficient amount of antioxidant is attained, this solution could be useful to limit the loss of antioxidant activity during "well-done" cooking.

The biological effectiveness of modifying a product is not useful if consumers do not like the product. For the marinated meat, it is important to note that the product had higher liking scores than the unmodified product (Fig. 4). Thus, modifying the product to limit the risk of cancer does not affect the acceptability of the product. Furthermore, we observed a potential positive effect of the information about colon cancer risk and the protective effect of new products on the liking score for the marinated

meat. This suggests that, if the consequences of the modification of the meat product are appreciated, then the consumers can positively value the information. Though the current communication of only limiting consumption does not seem to be well accepted (12), the strategy of associating the modification of the product with the communication on the potential benefit could be very effective in limiting the risk.

We have also evaluated the effect of cooking on meat-induced colorectal cancer. We studied rare and well-done meat in initiated rats and meat cooked rare in *Apc Min* mice because it is the cooking method most used by consumers (62). In a previous study, in which we demonstrated the promotive effect of heme iron (16), we did not observe a positive association between the number of MDF and PhiP and Me-IQx in an AIN-76-based diet. In the present study, cooking had a protective effect on the number of pre-cancerous lesions in initiated-rats. In contrast, in *Apc Min* mice, cooking had a promoting effect on the number and size of intestinal tumors. In our study, this promoting effect did not come from potentially carcinogenic heterocyclic amine formation because concentrations found in meats were very low. Among the 13 tested, 8 heterocyclic amines were not detected or under the quantification limit, only PhiP, IQx, MeIQx, DiMeIQx and DMIP were quantifiable in rare and well-cooked meats, but at low doses and well below the concentrations usually used to promote colorectal carcinogenesis in *Apc Min* mice (Supplementary Table S5; refs. 63, 64). In initiated rats, the protective effect of cooking was associated with a significant decrease in fecal heme, and in *Apc Min* mice the promoting effect of cooking was associated with a tendency of increase in fecal heme. Thus, as observed in our previous studies (16, 19), the promotion of carcinogenesis appears to be associated with the luminal heme content. Differences observed between rats and *Apc Min* mice suggest a difference in heme bioavailability in these two different rodent models. These opposing results make it difficult to come to a conclusion on the effect of cooking on heme bioavailability and the link with lipoperoxidation and the promotion of carcinogenesis. However, in human volunteers, if meat consumption increased the level of heme in fecal water, the cooking method did not significantly affect this level. Thus, the impact of marinated meat on lipoperoxidation in human volunteers could be assigned to the antioxidant capacity of the marinade and not the decrease of heme bioavailability, the catalyzer of lipid peroxidation.

In conclusion, consumption of fresh red meat for few days increased biomarkers associated with heme-induced promotion of colon carcinogenesis. Grape–olive marinade counteracted this promoting effect in carcinogen-injected rats and female *Apc Min* mice. This protection was associated with normalization of fecal biomarkers in the two animal models and a significant limitation in human volunteers. As in one of our previous studies with processed meat (32), these results suggest that it is possible to modify fresh red meat products by adding antioxidants to reduce the cancer-promoting properties of red meat, which could also improve, or at least not deteriorate, its sensory acceptability. The protective effect of meat antioxidant marinades need now to be validated by epidemiological studies and mechanisms need to be explored more precisely with for example the monitoring of the levels of DNA damage and DNA adducts to lipoperoxidation endproducts. Their effectiveness could lead to protective strategies to decrease the colorectal cancer burden in all populations, especially those who need it most.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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