

*Red wine and pomegranate extracts
suppress cured meat promotion of colonic
mucin-depleted foci in carcinogen-induced
rats*

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Red wine and pomegranate extracts suppress cured meat promotion of colonic mucin-depleted foci in carcinogen-induced rats

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2 1 **Red wine and pomegranate extracts suppress cured meat promotion of**
3 2 **colonic mucin-depleted foci in carcinogen-induced rats**
4 3

5 4 Nadia M. Bastide*¹, Nathalie Naud*¹, Gilles Nassy², Jean-Luc Vendeuvre^{2,†}, Sylviane
6 5 Taché¹, Françoise Guéraud¹, Ditte A. Hobbs³, Gunter G. Kuhnle³, Denis E. Corpet^{1,4}, and
7 6 Fabrice H.F. Pierre¹

8 7 ¹INRA UMR1331; TOXALIM (Research Center in Food Toxicology); Université de
9 8 Toulouse; ENVT; INP; 180 chemin de Tournefeuille, F-31027 Toulouse, France

10 9 ²IFIP-Institut du Porc, Paris, France

11 10 ³Department of Food and Nutritional Sciences, University of Reading, Whiteknights, PO
12 11 Box 226, Reading, RG6 6AP, UK

13 12 ⁴Corresponding author: D. Corpet; E-mail: prof.corpet@gmail.com

14 13 Phone: +33 6953 62223; Fax: +33 561 285 244;

15 14 *NMB and NN contributed equally to this work
16 15

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Abstract

Processed meat intake is carcinogenic to humans. We have shown that intake of a workshop-made cured meat with erythorbate promotes colon carcinogenesis in rats. We speculated that polyphenols could inhibit this effect by limitation of endogenous lipid peroxidation and nitrosation.

Polyphenol-rich plant extracts were added to the workshop-made cured meat and given for 14-days to rats and 100-days to azoxymethane-induced rats to evaluate the inhibition of preneoplastic lesions. Colons of 100d study were scored for precancerous lesions (mucin-depleted foci, MDF) and biochemical endpoints of peroxidation and nitrosation were measured in urinary and faecal samples.

In comparison with cured meat-fed rats, dried red wine, pomegranate extract, α -tocopherol added at one dose to cured meat and withdrawal of erythorbate significantly decreased the number of MDF per colon (but white grape and rosemary extracts did not). This protection was associated with the full suppression of faecal excretion of nitrosyl iron, suggesting this nitroso-compound might be a promoter of carcinogenesis.

At optimised concentrations, the incorporation of these plant extracts in cured meat might reduce the risk of colorectal cancer associated with processed meat consumption.

Keywords: Colorectal Cancer, Cancer Prevention, Polyphenols, Processed Meat, Mucin Depleted Foci

41 Introduction

42
43 Colorectal cancer (CRC) is the third most common type of cancer worldwide, and the
44 second cause of cancer death in affluent countries (1). Epidemiological studies show that
45 processed meat intake is linked to the risk of CRC (2). The World Cancer Research Fund
46 panel considers this risk as convincing and recommends avoiding processed meat
47 consumption (3, 4). The World Health Organization classified consumption of processed
48 meat as “carcinogenic to humans” (IARC Group 1) based on sufficient evidence for
49 colorectal cancer (5). Making safer meat products could be an alternative to banning cured
50 meat (6, 7). In carcinogen-initiated rats given a low-calcium diet freeze-dried cooked ham
51 and moist hot-dog increase significantly the number of mucin depleted foci (MDF) (8, 9).
52 The intake of an experimental cured pork meat, similar to an air-exposed cooked shoulder-
53 ham (DCNO for dark cooked meat with nitrite, oxidized, described below), also promotes
54 carcinogenesis in rats (10). In human volunteers, cured meat intake increases
55 endogenous nitrosation and fat peroxidation and faecal water-induced oxidative DNA
56 damage (7, 11).

57
58 We have speculated that haem iron could explain in part the promoting effect of processed
59 meat (12, 13), and added experimental support to this hypothesis: Dietary hemin (free
60 haem stabilized by a chloride ion) promotes azoxymethane-induced aberrant crypt foci
61 (ACF) in the colon of rats (14). Hemin, but not haemoglobin, mimics the effect of ham on
62 biomarkers associated with carcinogenesis (8).

63
64 Haem iron catalyses the formation of apparent total nitroso compounds (ATNC) (15) and
65 of lipid peroxidation endproducts, e.g., 4-hydroxynonenal and other alkenals (16). 4-
66 hydroxynonenal is cytotoxic and genotoxic to the intestinal epithelial cells (17, 18).
67 Potentially carcinogenic ATNC are formed in the gastrointestinal tract by N-nitrosation of
68 peptides derived amines or amides. Nitrosylated haem iron present in processed red meat
69 also represents a significant part of measured ATNC (13, 19, 20). ATNC and alkenals
70 could explain tumour promotion by dietary haem and by cured meat (6, 21, 22).

71
72 Our starting hypothesis here was that lipid peroxidation endproducts would promote
73 carcinogenesis (23), and that polyphenols would decrease haem-induced luminal
74 peroxidation (24) and hence carcinogenesis. There is ample evidence that polyphenols
75 and plant extract can block haem-induced fat peroxidation: For instance, quercetin, red

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2 76 wine and α -tocopherol suppress myoglobin-induced peroxidation in a fat/water emulsion
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4 77 that mimics the gastric environment and block the accumulation of conjugated dienes (25,
5
6 78 26). In human volunteers, red wine polyphenols strongly decrease postprandial plasma
7
8 79 malondialdehyde after a red meat meal, probably by suppressing haem-induced
9
10 80 peroxidation in the stomach (27-29). In rats, a mix of rutin and butylated hydroxyanisole
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12 81 inhibits hemin-induced lipid peroxidation in the gut, and suppress carcinogenesis
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14 82 promotion (14). In addition, polyphenols such as punicalagin and ellagic acid from
15
16 83 pomegranate can chelate iron through catechol groups (30, 31), while propyl gallate,
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18 84 tannic acid, thymol, vanillin, and ascorbate and α -tocopherol can inhibit nitrosation and
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20 85 ATNC formation (32, 33).
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24 87 The present study was designed to test the hypothesis that polyphenols can prevent the
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26 88 promotion of colon tumorigenesis by processed meat, by suppressing lipid peroxidation in
27
28 89 the gut. In a short-term screening study, several agents were added to DCNO cured meat
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30 90 during the manufacturing process. Such diets were given to rats for 14 days. Early lipid
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32 91 peroxidation endpoints were measured in faeces and urine. Most promising agents
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34 92 selected during these screening studies were added to DCNO and tested for
35
36 93 chemoprevention in a 100-day carcinogenesis study in rats. Tumorigenesis end points
37
38 94 were azoxymethane-induced preneoplastic lesions (ACF and MDF) in rats. The results
39
40 95 showed that dried red wine, pomegranate extract and α -tocopherol prevented meat-
41
42 96 associated formation of fecal nitrosyl iron and promotion of preneoplastic lesions.
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98 **Materials and methods**

99 *100 Animal study design*

101 Two sequential studies were performed on male Fisher 344 rats purchased at 4-5 weeks
102 of age from Charles River (St Germain l'Arbresle, France): A 14-day study investigated the
103 effect of plant extracts added to an experimental cured meat on early faecal and urinary
104 biomarkers in rats. A 100-day study measured the anti-promoting effect of four plant
105 extracts added to the same cured meat, on preneoplastic lesions in carcinogen-initiated
106 rats. Animal care was in accordance with the guidelines of the European Council on
107 animals used in experimental studies. Study was done in an accredited animal colony
108 (French A 31504) by approved staff (e.g., P.I. Corpet: Certificat d'autorisation
109 d'expérimenter sur animaux vertébrés vivants #31-121).

110 *Short-term study design (14 days-long)*

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2 111 Forty three rats were housed individually in metabolic cages. They were kept at 22°C and
3 112 12h-12h light-dark cycle. After 3 days of acclimatization to the animal colony and to a
4 113 standard AIN76 diet, rats were randomly allocated to eight groups. There were five rats in
5 114 each experimental group given DCNO cured meat with plant extracts (described below),
6 115 and eight rats in the control group fed DCNO. Rats were fed the experimental diets
7 116 described below during 14 days, and allowed free access to tap water. Body weight was
8 117 monitored every week. Food and water intakes were measured at days 13. Faeces and
9 118 urine were collected at days 11 and 12 and frozen at -20°C. Animals were terminated by
10 119 CO₂ asphyxiation on day 14. Faecal water samples (preparation described below) were
11 120 analyzed for haem, cytotoxicity and thiobarbituric acid reactive substances (TBARS). Urine
12 121 samples were analyzed for 1,4-dihydroxynonane mercapturic acid (DHN-MA).

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21 122 *Carcinogenesis study (100 day-long): Animals and design.*

22 123 Eighty six rats were housed individually in stainless steel, wire-bottomed cage (same
23 124 animal colony as above). After 7 days of acclimatization each rat received a single i.p.
24 125 injection of azoxymethane (20 mg/kg i.p.; Sigma Chemical) in NaCl (9 g/L). Seven days
25 126 later, they were randomly allocated to seven groups (N=10 rats per group, except control
26 127 group, N=26) and fed the experimental DCNO-based diets described below. Body weights
27 128 were monitored every week for four weeks, then every two-week. Food and water intakes
28 129 were measured at days 20 and 80. Faeces were collected daily between days 18 and 21,
29 130 and 80 and 91 and frozen at -20°C. Between days 74 and 76 each rat was put in a
30 131 metabolic cage and urine was collected and frozen at -20°C. Rats were killed by CO₂
31 132 asphyxiation in a random order at day 96 to 98. Colons were removed and fixed in 10%
32 133 buffered formalin (Sigma Chemical) between two sheets of filter paper with a blinding
33 134 code. ACF and MDF were scored. Faecal water samples were analyzed for haem,
34 135 TBARS, cytotoxicity and ATNC. Urine samples were analyzed for DHN-MA.

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44 136 *Animal diets*

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46 137 The type of meat and the additives that were given to groups of rats during the
47 138 short-term and carcinogenesis studies are listed in the first column of Tables 1 and 2,
48 139 respectively.

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51 140

52 141 Pork meat was cured in a specialized workshop by IFIP-*Institut du Porc* (14-day study) and
53 142 in a ham factory by Fleury Michon (Pouzauges-France) (100-day study). Meat was given
54 143 as such (moist piece) to the rats, because freeze-drying boosts peroxidation of fat in meat
55 144 (34). The experimental cured meat, which was similar to air-exposed picnic ham and
56 145 called dark cooked meat with nitrite, oxidized (DCNO), was chosen because it promotes

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2 146 carcinogenesis in rats (10). DCNO was made from *Musculus vastus intermedius*, cured
3 147 with 2.19 g salt with 0.6% sodium nitrite (131 ppm NaNO₂), and 1.4 g sodium erythorbate
4 148 (an ascorbate isomer) per 100 g meat. DCNO was then heated at 70°C for 3 hour in
5 149 vacuum-sealed plastic bags in a water bath. The final product contained 12 mg haem
6 150 iron/kg, 71 mg sodium nitrite/kg, and 500 mg ascorbate/kg. One group of rats was given
7 151 an erythorbate-free DCNO. The processed meat was divided into 1.3 cm thick slices of
8 152 300g, that were stored separately at - 20°C in air-tight plastic bags with low-oxygen
9 153 permeability (14 day study) or under CO₂/N₂ 50/50 atmosphere to avoid further fat
10 154 oxidation (100 day study). Before being given to rats, each slice was exposed to air for five
11 155 days in a dark refrigerator (4°C), then cut in ten 30 g portions that were given to rats at
12 156 5:00 p.m. for 14 or 100 days. A low-calcium powdered diet (35) was given in a separated
13 157 feeder, 7.6 g/d/rat, so that each rat would eat roughly half meat / half powder (dry matter).
14 158 This modified AIN76 diet was prepared by UPAE (INRA, Jouy, France) as follows
15 159 (g/100g): sucrose, 59.5; corn starch, 15.0; cellulose, 12.5; AIN76 mineral mix without
16 160 calcium, 8.7; AIN76 vitamin mix, 2.5; methionine, 0.75; calcium phosphate, 0.52; choline
17 161 bitartrate, 0.5. Safflower oil (5g) was mixed with 100g powder to provide polyunsaturated
18 162 fatty acids (MP Biomedicals, Illkirch, France).

19
20 163 Six polyphenols-rich plant extracts were added to DCNO during the curing process,
21 164 at a concentration recommended by the supplier: white grape extract (NutriPhy® white
22 165 grape 100, 72% of total polyphenols, CHR Hansen, Hoersholm, Denmark; 0.055% w/w in
23 166 DCNO), carnosic acid (Stabilenhance® OSR5 extracted from rosemary leaves, 10%
24 167 carnosic acid, Naturex, Avignon, France; 1% w/w in DCNO), and a water soluble rosemary
25 168 extract, containing 7% of rosmarinic acid (Stabilenhance® WSR6, Naturex; 0.66% w/w in
26 169 DCNO), red wine concentrate, 10% of total polyphenols (Avvinr9005®, Diana Naturals,
27 170 Antrain, France; 2% w/w in DCNO), pomegranate extract, 12 % ellagic acid (Naturex,
28 171 Ultimate Botanical Benefits; 0.6% w/w in DCNO), green tea extract, 98% of total
29 172 polyphenols (Naturex; 0.08% w/w in DCNO). Polyphenol data were given by the suppliers
30 173 and the composition of extracts was not determined more precisely in this pilot study.
31 174 Another group was given DCNO supplemented with α-tocopherol (Covitol®, Nutrition &
32 175 Health, Cognis, BASF; 0.045%): this fat-soluble antioxidant agent suppresses MDF in
33 176 carcinogen-induced rats and was used as a positive control for protection (7). A last group
34 177 of rat given DCNO without sodium erythorbate was added to the carcinogenesis study.

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36 179 *Meat composition*

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2 180 Processed meat was analyzed by Lareal (Vannes, France, laboratory specialized in
3 181 physico-chemical and microbiological analyzes) for total iron, total pigments, nitrosylated
4 182 pigments (36). Hexanal, a marker of secondary products of lipid peroxidation was
5 183 analyzed by Lareal by gas chromatography of the headspace of the sample dispersed in
6 184 phosphate buffer at 37°C, with solid-phase micro-extraction fiber. Trolox equivalent
7 185 antioxidative capacity (TEAC-1), malondialdehyde (MDA by HPLC), and TBARS (after
8 186 acidic extraction) were measured by ADIV (Clermont-Ferrand, France). The Oxygen
9 187 radical absorbance capacity (ORAC) was measured by Naturex (Avignon, France). Two
10 188 measures per processed meat batch were done.
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190 **Fecal and urinary measures**

191 *Analysis of haem, thiobarbituric acid reactive substances in, and cytotoxicity of faecal*
192 *water, and 1,4-dihydroxynonane mercapturic acid in urine*

193 Faecal pellets were collected under each cage for 24h, at day 11 of the short-term study
194 and days 88-91 of the carcinogenesis study. TBARS value was used as a global measure
195 of lipid peroxidation endproducts. Faecal water was prepared, and haem and TBARS were
196 measured in faecal water exactly as previously described (7) except that 1 mL of distilled
197 water was added to 0.42g of crushed fresh faeces, but not to 0.3g of dried faeces. 1,4-
198 Dihydroxynonane mercapturic acid (DHN-MA) is the main urinary metabolite of 4-
199 hydroxynonenal, which is a major toxic end product of endogenous fat peroxidation (16).
200 The 24-hour urine was collected under each metabolic cage, at day 11 of the short-term
201 study and days 74 to 76 of the carcinogenesis study. DHN-MA assay was done (n= 5 to 8
202 for the 14-day study and n=10 to 26 for the 100-day study) as previously described (7). To
203 determine cytotoxicity of fecal water (n=6), the 3(4,5-dimethylthiazol-2-yl)-2,5-
204 diphenyltetrazolium bromide (MTT) assay was used on a cancerous mouse colonic
205 epithelial cell line, CMT93 (European Collection of Animal Cultures), as previously
206 described (7).

207 *ATNC analysis*

208 ATNC were analyzed using a modification of the method previously used (37), using a
209 CLD88 Exhalyzer (Ecomedics, Duernten, Switzerland). Sulfamic acid solution (500 µl, 5%)
210 was added to 100 µl of faecal water to remove nitrite and samples were injected into a
211 purged vessel kept at 60°C and filled with a standard tri-iodide reagent (38 mg I₂ was
212 added to a solution of 108 mg KI in 1 ml water; to this mixture, 13.5 ml glacial acetic acid
213 was added) to determine total ATNC. To determine mercury(II) stable compounds, 100 µl

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2 214 10 mM aqueous HgCl₂ was added prior to analysis; to determine mercury(II) and
3 215 ferricyanide stable compounds, 100 µl each of 10 mM aqueous HgCl₂ and 10 mM
4 216 aqueous K₃Fe(CN)₆ solution were added prior to analysis. Nitrosothiols were determined
5 217 as the difference between total ATNC and mercury(II) stable ATNC; Nitrosyl iron was
6 218 determined as difference between mercury(II) stable ATNC and mercury(II) and
7 219 K₃Fe(CN)₆ stable compounds. Data are concentrations (in µM), measured in triplicate in
8 220 100 µL of each sample.
9 221

222

222 **ACF and MDF assays**

16 223 ACF and MDF were scored by a single observer blinded for the origin of the colon, exactly
17 224 as described previously (7). Number of lesions and number of crypts per lesions (i.e. size
18 225 of ACF and MDF) were numbered.
19 226

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227 **Statistical methods**

22 228 Results were analyzed using Systat 10 software for Windows, and all data were reported
23 229 as mean ± SD (except Fig.1B). Values were considered firstly using one-way ANOVA. If a
24 230 significant difference was found between all groups (P < 0.05), comparison of each
25 231 experimental group with the control group was made using Dunnett's test. For ORAC
26 232 analysis, data show results of two measures per processed meat batch, but Student t test
27 233 statistics could be done because the within-pair correlation was high, however, P values
28 234 should be taken cautiously (38).
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236

236 **Results**

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237 **Fourteen-day study: Effect of plant extracts on peroxidation biomarkers**

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238 ***Fecal and urinary fat peroxidation biomarkers***

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40 240 Dietary DCNO cured meat increases the number of carcinogen-induced precancerous
41 241 lesions, and urinary and faecal water peroxidation biomarkers in rats (10, 14). These early
42 242 peroxidation biomarkers were thus measured here, because they correlate with haem-
43 243 induced promotion of colon carcinogenesis (8, 39). Extracts of pomegranate, red wine,
44 244 white grape, green tea, rosemary and carnosic acid and α-tocopherol were added to
45 245 DCNO before being fed to rats for 14 days. As shown in Table 1, faecal water from rats
46 246 given DCNO added with pomegranate, red wine or white grape extracts contained half
47 247 TBARS than control rats given DCNO. In contrast, faecal water from rats given DCNO plus
48 248 carnosic acid contained surprisingly twice more TBARS than controls. All tested extracts
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2 250 led to some reduction in urinary DHN-MA but only rosemary extract significantly decreased
3 251 the excretion of this 4-hydroxynonenal metabolite. All the tested plant extracts reduced
4 252 fecal haem excretion in DCNO-fed rats, except for α -tocopherol and rosemary extract.
5 253 Lastly, addition of plant extracts in DCNO did not affect the cytotoxicity associated with
6 254 DCNO consumption.

10 255

11 256 ***Choice of polyphenol additives for the carcinogenesis study***

12 257 Pomegranate, red wine, and white grape extracts that decreased TBARS in fecal water of
13 258 DCNO-fed rats (Table 1) were chosen to be tested in the carcinogenesis study, because
14 259 our starting hypothesis was that polyphenols would exert their protective action by
15 260 inhibiting lipid peroxidation (25, 40). We also chose to test carnosic acid, a common
16 261 additive to brine in Europe, because it surprisingly increased TBARS in faecal water. In
17 262 addition we tested α -tocopherol as a protection control because it suppresses cured-meat
18 263 promotion in rats (7). Lastly, a special DCNO meat, cured without erythorbate, was given
19 264 to a group of rat to test the effect of this common additive.

26 265

27 266 ***Carcinogenesis study: effect of plant extracts***

28 267

29 268 ***General observation***

30 269 All rats survived and were healthy, except rats given carnosic acid that had diarrhea. Moist
31 270 meat and powdered diet were given to the rats in separated feeders: the relative intake of
32 271 meat and of powder that was 48:52 (dry weight) on day 18 of the study slowly changed to
33 272 39:61 on day 82. The final body weight of rats was 343 ± 19 g without significant difference
34 273 between groups except rats fed cured meat plus carnosic acid (321 ± 16 g, $P < 0.05$). Rats in
35 274 this group ate and drank less than the rats in others groups: their average food intake per
36 275 day was 12 ± 1 g compared with 13 ± 1 g in other groups ($P < 0.05$). Water intake was
37 276 reduced in rat fed carnosic acid and increased in rat fed α -tocopherol or white grape
38 277 extract, compared with the other groups (full data not shown, $P < 0.0001$).

46 278

47 279 ***Quantification of ACF and MDF***

48 280 A DCNO-based diet increases the number of MDF and ACF in the colon of carcinogen-
49 281 injected rats, in comparison with a no-meat control diet (7, 10). The DCNO diet was thus
50 282 chosen as a promoting control to test potentially protective plant extracts. At the doses
51 283 tested, all plant extracts decreased the number of MDF per colon in comparison with
52 284 DCNO diet, but only α -tocopherol, pomegranate and red wine extracts led to a significant
53 285 protection (Fig.1B). Number of ACF was not different between groups, nor the MDF and
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2 286 ACF multiplicity (Table 3). Surprisingly, the removal of erythorbate from DCNO curing
3 287 brine led to a significant reduction in the number of colonic MDF. Mean number of large
4 288 ACF or of large MDF, with 4 or more crypts per foci, was similar in all dietary groups. In an
5 289 attempt to explain the observed protection, diets, fecal water, and urine were analyzed for
6 290 lipoperoxydes and nitroso-compounds.
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10 291

11 292 ***Meat analyses***

12 293 Trolox, MDA, TBARS, ORAC, hexanal and nitrosylated haem were analysed but only
13 294 ORAC, hexanal and nitrosylated haem values were reported here, because they are not
14 295 significantly affected by the modification of process of meats. ORAC is a measure of
15 296 antioxidant power. As expected, all tested plant extracts increased cured meat ORAC
16 297 value 2-3 times, and suppressed hexanal production, a measure of meat peroxidation
17 298 (Table 4). Haem and NO from nitrite can form nitrosyl haem (19), that might be the
18 299 promoting factor in cured meat (8). The tested plant extracts did not change much
19 300 nitrosylated haem concentration in meat, except red wine that increased it (Table 4).
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28 302 ***Fecal and urinary fat peroxidation biomarkers***

29 303 Faecal water from rats fed DCNO added with pomegranate, red wine or white grape
30 304 extracts, or α -tocopherol contained 1.5 to 2 times less TBARS, and about 1.5 times less
31 305 haem than faecal water from rats fed DCNO alone (Table 2). Pomegranate extract,
32 306 carnosic acid and α -tocopherol also decreased urinary DHN-MA, a metabolite of 4-
33 307 hydroxy-nonenal. Carnosic acid that significantly increased faecal TBARS in first study
34 308 (Table 1) tended to increase it in this second study (not significant). Addition of plant
35 309 extracts had not modified the cytotoxicity of fecal water in DCNO group except for carnosic
36 310 acid that induces a significant increase in faecal water cytotoxicity (Table 2). Surprisingly,
37 311 the absence of erythorbate in DCNO significantly decreased faecal TBARS and haem
38 312 compared with erythorbate-supplemented DCNO, without modification of cytotoxic activity
39 313 of fecal water (Table 2).
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50 315 ***Faecal Nitroso Compounds***

51 316 Apparent total nitroso compounds (ATNC) concentration in faecal samples was reduced
52 317 by the addition of a plant extract to the curing brine of DCNO (Fig. 1A). The reduction was
53 318 more than a three-fold (except white grape). Significance could not be formally established
54 319 since only one value was obtained per group because faeces from all rats in one dietary
55 320 group had been pooled, so results were interpreted with caution. "ATNC" are a complex
56 321 mixture of nitrite-derived products, and the ATNC composition was not identical in the
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2 322 faeces from different groups. Faecal ATNC from rats fed DCNO cured meat plus carnosic
3 323 acid or white grape extract were made of 100% nitrosyl iron (Fig.1A). In contrast, faecal
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5 324 ATNC from rats fed DCNO with pomegranate or red wine extracts were 100% nitrosothiols
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7 325 (data not shown). Tocopherol fully suppressed nitrosation, but the removal of erythorbate
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9 326 from DCNO curing brine led to a fifty percent increase in faecal ATNC, no nitrosyl iron
10 327 being detected (Fig.1A).
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12 328

13 14 329 **Discussion**

15
16 330 This study shows that polyphenol-rich plant extracts can inhibit the promotion of
17 331 colonic mucin depleted foci by cured meat that had been demonstrated repeatedly in this
18
19 332 model (7-10): dried red wine and pomegranate extract suppressed cured meat-induced
20 333 colon tumorigenesis promotion as well as α -tocopherol, while white grape extract and
21 334 carnosic acid extracted from rosemary did not. Promotion was evidenced on a surrogate
22 335 endpoint biomarker, mucin depleted foci. MDF, formed by dysplastic crypts devoid of
23 336 mucin, have been identified in the colon of humans at high risk for colon cancer (41). Like
24 337 tumours, MDF harbour mutations in genes affecting colon carcinogenesis (Apc and K-ras)
25 338 and show Wnt signalling activation (42), a dramatic reduction of MUC2 expression (43),
26 339 and a strong activation of the inflammatory process (44), all features suggesting that MDF
27 340 are precancerous. Several rodents studies suggest that MDF are better predictors of
28 341 colorectal cancer than ACF are (45), and respond more consistently than ACF to
29 342 promotion by red and processed meat and by dietary haem (9, 20, 39) this is why we
30 343 focused on MDF data.
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34 345 The promotion of colon carcinogenesis by fresh, moist cured meat (DCNO) in rats
35 346 has been associated with increased fecal nitroso-compound (ATNC) concentrations and
36 347 increased fecal biomarkers of fat peroxidation (TBARS) (10). Hence, we chose to use
37 348 DNCO to identify prevention strategies aiming at normalizing fecal biomarkers. But the
38 349 prediction of cancer-promoting properties in food by simple chemical analysis would be a
39 350 great step toward cancer prevention. Unfortunately, no correlation was seen between
40 351 cured meat composition and the number of MDF: neither hexanal, ORAC, nitrosylated
41 352 haem, or any other meat component was associated with MDF promotion. This supports
42 353 the hypothesis that CRC promotion by processed meat is not directly due to a factor
43 354 present in food. Meat-induced endogenous factors would thus promote MDF, e.g.,
44 355 aldehydes or N-nitroso compounds (6). Our starting hypothesis was that lipid peroxidation
45 356 endproducts would promote carcinogenesis, and that polyphenols would decrease luminal
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2 357 macromolecules and peroxidation of fatty acids, and they can bind iron, thus reducing
3 358 catalytic properties of haem (46). The measurement of ORAC is commonly used to study
4 359 the radical-scavenging ability of polyphenols. Here, the tested plant extracts doubled or
5 360 tripled the ORAC of meat (Table 4). In addition, faecal excretion of haem iron was reduced
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7 361 in rats given polyphenol-supplemented meat (Table 2). However, neither the antioxidant
8 362 effect nor the reduced fecal haem iron was linked with MDF reduction: for instance
9 363 carnosic acid tripled ORAC value in meat but did not reduce MDF number in rats (Fig.1B).
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16 365 Our previous studies strongly suggest that faecal aldehydes collectively measured by
17 366 the TBARS assay would participate to carcinogenesis promotion in meat-fed rats (8, 39).
18 367 Present data support this hypothesis, since all plant extracts that decreased MDF number
19 368 significantly reduced faecal TBARS concentration. In this way, previous *in vitro* data of our
20 369 team allowed to propose that a premalignant cell selection by heme-induced aldehydes
21 370 explains the heme-induced promotion of MDF (20). Thus limitation of peroxydation and
22 371 aldehydes formation by antioxidant could explain the protective effect by limitation of the
23 372 selection of preneoplastic cells. In addition, carnosic acid that increased TBARS did not
24 373 reduce MDF number (Tables 1 and 4, fig.1B). In contrast, white grape extract reduced
25 374 faecal TBARS but had no effect on MDF number. This discrepant group may suggest that
26 375 TBARS are not the only parameter involved in CRC promotion.
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36 377 Although data on the NOCs were obtained on faecal pools, our results support the
37 378 hypothesis of Cross *et al* on the role of endogenous ATNC in the promotion of CRC by
38 379 processed meat. The presence of nitrosyl iron in faeces, but not the other types of ATNC,
39 380 was associated with the promotion of CRC by cured meat (Fig.1). Several human studies
40 381 strongly suggest that the formation of ATNC can explain the positive links between
41 382 processed meat intake and CRC (10, 11, 47). Here, α -tocopherol fully inhibited the
42 383 formation of faecal ATCN and suppressed MDF promotion in rats. Similarly, the reduction
43 384 of MDF by red wine and pomegranate extracts was associated with reduced faecal ATNC
44 385 and lack of nitrosyl iron in faeces (Fig.1). Nitrosyl iron was indeed the only faecal marker
45 386 that was consistently associated with MDF promotion. However, no dose-response
46 387 relationship was seen, since white grape and carnosic acid that boosted nitrosyl iron
47 388 formation did not increased the MDF number over the control number (fig.1). We
48 389 nevertheless suggest that nitrosyl iron might be used as a short-term biomarker to screen
49 390 additives added to cured meat to reduce cancer risk.
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2 392 To test the hypothesis that nitrosation can explain promotion, an artificial model of
3 393 cured meat was made with no erythorbate. Currently all commercial processed meats
4 394 contain erythorbate. Indeed, this additive is usually added to brine during the curing
5 395 process to increase nitrosylation and to block nitrosation (19). As expected, faecal ATNC
6 396 value was higher in rats given erythorbate-free DCNO than in control rats given DCNO
7 397 with erythorbate (+55%, Fig.1A). Surprisingly, this ATNC increase was associated with a
8 398 decrease in the number of MDF per colon, which shows that all ATNC types do not
9 399 promote tumorigenesis. However, no nitrosyl iron was detected rats given erythorbate-free
10 400 DCNO (Fig.1A), as already observed by Hotter, Zhou and Mirvish (Abstract B-111,
11 401 Frontiers in Cancer prevention, Am. Ass. Cancer Res., Boston, Oct. 2011). We thus
12 402 suggest a central role of luminal nitrosyl iron in the promotion of colorectal tumorigenesis
13 403 by cured meat, associated with a minor role of luminal aldehydes. This contradicts Hogg's
14 404 hypothesis that the sequestration of the "nitrosating potential" of diet as nitrosyl iron is a
15 405 protective mechanism (48). In contrast, it supports Kuhnle's hypothesis that nitrosyl haem
16 406 may cause the formation of DNA adduct O6-carboxymethyl guanine in colonic cells (49),
17 407 which are found in stools of volunteers given red meat (37) with a stimulating effect of
18 408 haem-iron on adduct production during in vitro fermentation of meat (50). The evidence
19 409 presented here is weak however, since no statistics could be done on ATNC data, and
20 410 because no dose-effect relation was seen between nitrosyl haem excretion and MDF
21 411 numbers (Fig. 1).

22 412 Major weaknesses of this study are the pooling of faecal samples before ATNC
23 413 analysis, and the lack of a no-meat arm. Hence no statistics could be done on ATNC data:
24 414 significance of the three-fold reduction in total ATNC by plant extracts, and of full
25 415 suppression of nitrosyl-iron by three of the extracts is unknown. Also, the present study
26 416 was not designed to confirm MDF promotion by cured meat (DCNO). This promoting effect
27 417 had repeatedly been shown in the same model (7-10), but could not be tested again in the
28 418 present study.

29 419 The finding that it is possible to counteract the cancer promoting effect of processed
30 420 meat by adding selected plant extracts into the meat should have consequences on public
31 421 health and on dietary recommendations. The World Cancer Research Fund's advice to
32 422 avoid processed meat may be updated with the advice that any cured meat meal should
33 423 also include a polyphenol- or tocopherol-rich plant food. Despite recommendations,
34 424 individuals, particularly those in low socio-economic groups, consume large amounts of
35 425 processed meat. These people are at a higher risk of CRC, early disability and death. We
36 426 suggest the meat industry should use specific protective plant-based additives during the

1 427 curing process, as this could reduce cancer risk in all consumers. Making safer meat
2 428 products might be a better approach than banning meat (6, 7).
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7 430 **Conclusions**

8 431 This study shows that the incorporation of polyphenol-rich plant extracts (pomegranate or
9 432 red wine) or of α -tocopherol inhibited the promoting effect of cured meat on preneoplastic
10 433 lesions in carcinogen-induced rats. If these results were confirmed in volunteers' study,
11 434 these agents might be added to meat during the curing process to make functional
12 435 processed meat. This study represents an informative starting point, however future
13 436 research should address dose dependence and potential efficacy of modified meats that
14 437 might induce effects ranging from protection, lack of protection to possible cancer-
15 438 promoting effect at other doses. The use of the protective agents would reduce colorectal
16 439 cancer risk compared with processed meat. This study also shows that faecal excretion of
17 440 a specific class of nitroso-compounds, nitrosyl iron, was associated with tumorigenesis
18 441 promotion by cured meat.
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30 443 **List of abbreviations:** ACF: Aberrant Crypt Foci; ATNC: Apparent Total N-nitroso
31 444 Compounds; CRC: colorectal cancer; DCNO: Dark meat, Cooked, cured with sodium
32 445 Nitrite, Oxidized by air; DHN-MA: DiHydroxyNonane Mercapturic Acid; MDF: Mucin
33 446 Depleted Foci; TBARs: Thiobarbituric Acid Reactive Substances; ORAC: Oxygen Radical
34 447 Absorbance Capacity;
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40 449 **Competing interests**

41 450 G. Nassy and J.L. Vendeuvre were employed by the *Institut Français du Porc* (IFIP).
42 451 N.Bastide, N.Naud, S.Taché, F.Guéraud, D.Hobbs, G.Kuhnle, D.Corpet, F. Pierre: No
43 452 conflicts of interest.
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47 454 **Authors' contributions**

48 455 NMB and NN contributed equally to this work; FHFP, DEC, GN and JLV designed
49 456 research; NMB, NN, ST, FG, DAH, and GCK conducted research; NMB, DEC and FHFP
50 457 analyzed data and wrote the paper; DEC and FHFP had primary responsibility for final
51 458 content. All authors have read and approved the final manuscript.
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4 464 *memoriam*” of J.L. Vendevre.
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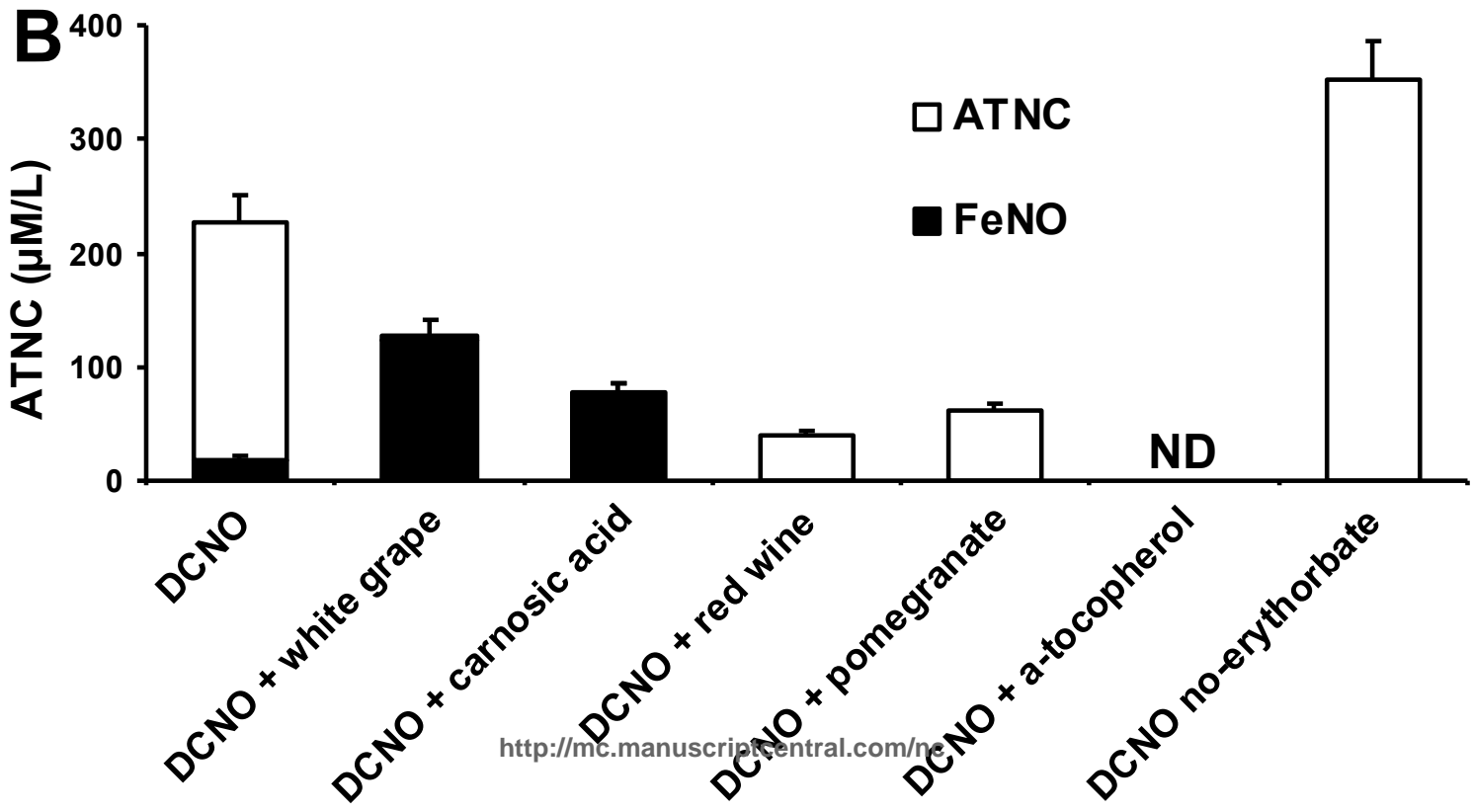
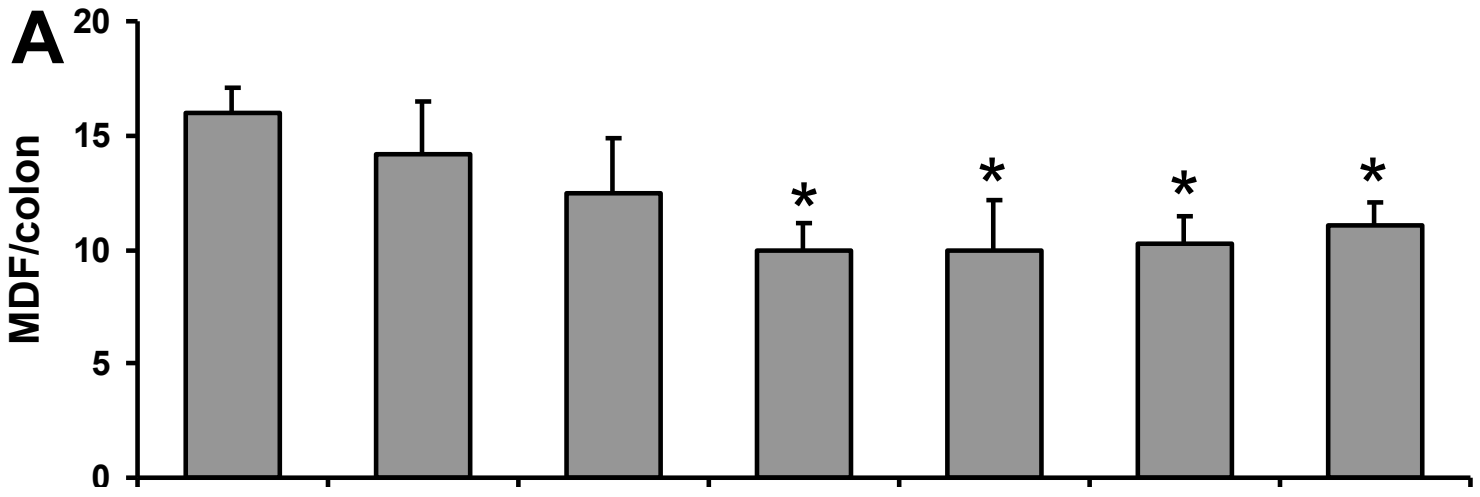
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4 **Figure 1: Faecal excretion of nitroso compounds and promotion of**
5 **preneoplastic lesions in the colon of rats.**
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7 A: Mucin Depleted Foci (MDF) in the colon of azoxymethane-initiated rats given cured
8 meat added with plant extracts for 100 days. Values are mean \pm SEM (same data in
9 Table 3). * Significantly different from DCNO by Dunnett's t test.
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12 B: Apparent Total N-nitroso Compounds (ATNC) and nitrosyl iron (FeNO) values
13 were obtained on pooled faecal samples from all rats in one group: error bars show
14 analytical SD. ND: not detected.
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Table 1. Faecal and urinary biomarkers in rats given cured meat with plant extracts during the fourteen-day study

Diet	N. of rats	Haem in FW (nmol/24h)	TBARS in FW (Eq. MDA nmol/24h)	Urinary DHN-MA (ng/24h)	Cytotoxicity of FW (% of dead cells)
DCNO	8	279±90	138±46	410±210	22±8
DCNO + White grape	5	116±54**	77±23*	287±89	23±24
DCNO + Carnosic acid	5	140±72**	273±81***	258±83	29±13
DCNO + Rosemary	5	196±104	144±18	222±30*	20±19
DCNO + Red wine	5	174±70*	68±38**	262±53	35±11
DCNO + Pomegranate	5	97±38***	64±35**	307±225	37±5
DCNO + Green tea	5	150±56*	87±21	288±128	35±13
DCNO + α -tocopherol	5	193±130	98±56	370±194	12±16

Footnotes

FW: faecal water. TBARS: thiobarbituric acid reactive substances; MDA: malondialdehyde; DHN-MA: dihydroxynonene mercapturic acid; DCNO: dark meat, cooked, cured with sodium nitrite, oxidized by air.

Significantly different from DCNO by the Dunnett's t test: * P < 0.05; ** P < 0.01; *** P < 0.001.

Table 2. Effect of cured meat diets on fecal and urinary biomarkers in carcinogen-initiated rats 80 days after an azoxymethane injection

Diet	N. of rats	Haem in FW (nmol/24h)	TBARS in FW (Eq. MDA nmol/24h)	Urinary DHN-MA (ng/24h)	Cytotoxicity of FW (% of dead cells)
DCNO	26	238±84	153±16	243±99	52±20
DCNO + White grape	10	145±40***	99±19***	235±80	57±32
DCNO + Carnosic acid	10	135±61***	164±39	122±34***	100±1***
DCNO + Rosemary	10	172±51**	76±11***	191±60	59±16
DCNO + Red wine	10	162±55**	85±11***	172±64**	66±17
DCNO + Pomegranate	10	135±41***	120±19**	124±44***	46±20
DCNO + Green tea	10	162±46**	132±14**	257±90	47±23

Footnotes

FW: faecal water. TBARS: thiobarbituric acid reactive substances; MDA: malondialdehyde; DHN-MA: dihydroxynonene mercapturic acid; DCNO: dark meat, cooked, cured with sodium nitrite, oxidized by air.

Significantly different from DCNO by the Dunnett's t test: * P < 0.05; ** P < 0.01; *** P < 0.001.

Table 3 Preneoplastic lesions (ACF and MDF) in the colon of rats fed cured meat added with plant extracts for 98 d, 105 d after an azoxymethane injection¹

Diet	No. of rats	MDF/Colon		Crypt/MDF		ACF/colon		Crypt/ACF	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD
DCNO	26	16.0	5.8	2.6	0.8	106	22	3.4	0.2
DCNO + White grape	10	14.2	7.3	2.3	0.2	105	14	3.4	0.2
DCNO + Carnosic acid	10	12.5	7.4	2.6	0.7	95	17	3.5	0.2
DCNO + Red wine	10	10.0	3.5**	2.6	0.5	108	19	3.4	0.3
DCNO + Pomegranate	10	10.0	6.8**	2.3	0.4	108	28	3.6	0.2
DCNO + α -tocopherol	10	10.3	3.6**	2.4	0.4	99	21	3.4	0.3
DCNO without erythorbate	10	11.1	3.1*	2.6	0.5	103	24	3.5	0.3

¹ ACF: aberrant crypt foci. MDF: mucin depleted foci. Other notes: see Table 1.

Table 4 Processed meat analysis: Antioxidant activity, nitrosyl heme and hexanal concentrations after air exposure for five days at 4°C in cured meat added with plant extracts¹

Processed meat	Oxygen radical absorbance capacity (Trolox eq.µmol/100g)	Nitrosylated Heme (mg/kg)	Hexanal (mg/kg)
DCNO	10.4 - 11.1 ²	97 - 100	5-6
DCNO + White grape	25.8 - 27.3 **	97 - 101	1
DCNO + Carnosic acid	30.7 - 31.2 **	95 - 101	<1-1
DCNO + Red wine	22.0 - 27.0 *	123 - 138 *	<1-1
DCNO + Pomegranate	20.1 - 20.5 **	108 - 108	<1
DCNO + α-tocopherol	12.4 - 12.7 *	91 - 94	<1-1
DCNO without erythorbate	9.8 - 11.2	96 - 101	3-8

¹ Plant extracts were added during the curing process to DCNO (dark meat, cured with sodium nitrite, cooked and oxidized).

² Data show results of two measures per processed meat batch. Details: see Materials and Methods. * $p < 0.05$, $p < 0.003$ ** compared with DCNO group. Student t test statistics could be done because the within-pair correlation was high, however, P values should be taken cautiously (38)

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3 Dr. Fabrice PIERRE
4 National Institute of Agronomic Research
5 UMR INRA 1331 ToxAlim
6 180 chemin de Tournefeuille
7 31027 Toulouse, FRANCE
8 email: fabrice.pierre@toulouse.inra.fr
9 Tel: 33 582 066 370
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13 **to L.A. Cohen**
14 **Editor of Nutrition and Cancer**
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17 Dear Editor,
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19
20 Please find in the attached file a revised manuscript N&C-01-16-2624.R2, entitled " Red wine
21 and pomegranate extracts suppress cured meat promotion of colonic mucin-depleted foci in
22 carcinogen-induced rats" we resubmit for publication to Nutrition and Cancer: An
23 International Journal.
24

25 All referee comments were considered, and the revised MS was changed accordingly, as
26 detailed in the letter below, and highlighted "red" in the revised MS.
27

28 My co-authors and I hope that the revised manuscript will now be suitable for publication in
29 Nutrition and Cancer. We thank you for your editorial services.
30

31 Yours sincerely
32

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34 Fabrice Pierre
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40 TOULOUSE, 10-02-2016
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3 **Please find below our propositions of modifications of the MS**
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5

6 **Reviewer: 1**

7 Administrative change made. Thank you.

8 => *We thank the reviewer for his review that has improved the MS*
9

10 **Reviewer: 2**

11 Suggest a further minor revision to the last sentence of the Abstract:

12 At optimised concentrations, the incorporation of these plant extracts in cured meat might reduce the risk of
13 colorectal cancer associated with processed meat consumption.

14 => *We agree with the reviewer #2 and have modify the end of the abstract*

15 => *And we thank the reader for his review that has improved the MS*
16
17

18 **Reviewer: 3**

19 No additional comments

20 => *We thank the reader for his review that has improved the MS*
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