FOOD & FUNCTION

Marie Ménard apples with high polyphenol content and a low-fat diet reduce 1,2-dimethylhydrazine-induced colon carcinogenesis in rats: Effects on inflammation and apoptosis

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Inflammation may increase cancer risk, therefore, we studied whether polyphenol-rich Marie Ménard (MM) apples with reported anti-inflammatory activity prevent 1,2-dimethylhydrazine (DMH)-induced colon carcinogenesis in rats and, likewise whether high-fat (HF) diet promoting carcinogenesis, may affect inflammation. DMH-induced rats were fed for 15 weeks with: an HF diet (23% corn oil w/w); an HF diet containing 7.6% w/w lyophilized MM (apple diet (AD)); a low-fat (LF) diet and an HF diet containing piroxicam (PXC) (0.01% w/w) as control. Mucin depleted foci (MDF), precancerous lesions in the colon, were dramatically reduced in the AD, LF, and PXC groups compared with the HF. Peritoneal macrophage activation, an index of systemic inflammation, was significantly decreased in the AD, LF, and PXC groups. TNF-α, iNOS, IL-1β, IL-6 m-RNA expression in the colon, as well as CD68 cells and plasmatic PGE2 were lower in the AD, but not in the LF group. Apoptosis in the MDF of both the AD and LF-fed rats was significantly higher than in HF rats. In conclusion, AD has a strong chemopreventive effect, reducing inflammation, and increasing apoptosis, while the chemopreventive effect of the LF diet seems mediated mainly by increased apoptosis in MDF.

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Plant-rich foods may decrease colon cancer risk [1] affecting inflammation, colon proliferation, and apoptosis [1–3]. Inflammation has been linked to cancer risk since nonsteroidal anti-inflammatory drugs reduce carcinogenesis, whereas inflammatory bowel diseases (IBDs) increase the risk [1, 2]. A low-grade inflammation might increase carcinogenesis also in the sporadic colon cancer [2]. Moreover, high-fat/high-calorie (HF) diets increasing cancer risk [4] have been suggested to cause low-grade systemic and perhaps colonic inflammation [1, 2]. Diet may also affect carcinogenesis by varying colon proliferation and apoptosis [5]. Previous studies suggest that apples may have anti-inflammatory activity [6, 7] and our recent study showed that high-polyphenol apples (Marie Ménard (MM)) reduce inflammation in a genetic model of IBD [8]. Experimental studies have provided contrasting results on the effect of apples on colon cancer, with both protective and detrimental effects having been reported [7, 9–13]. Given these considerations, we thought it of interest to test whether a diet containing MM apples, which reduces inflammation [8] may affect colon carcinogenesis in 1,2-dimethylhydrazine (DMH)-induced rats, determining mucin depleted foci (MDF) precancerous lesions of the colon as cancer end points [14]. Likewise, because it could be possible that HF diet may affect colon-systemic inflammation we also tested this hypothesis. Accordingly, F344 male rats were induced with DMH and one week later allocated to the following diets (see experimental protocol in Supporting Information): (i) an HF diet (23% w/w corn oil); (ii) the same HF diet containing 7.6% w/w lyophilized MM apples (apple

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Abbreviations: AD, apple diet; **DMH**, 1,2-dimethylhydrazine; **HF**, high-fat; **IBD**, inflammatory bowel disease; **LF**, low-fat; **MDF**, mucin depleted foci; **MM**, Marie Ménard; **PXC**, piroxicam

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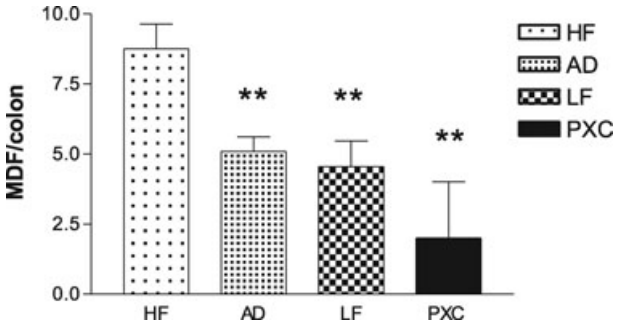

Figure 1. MDF per colon in the different groups. Bars: means ± SE. $n = 12$, 12, 9, and 4 in HF, AD, LF, and PXC groups, respectively. $^{**}p < 0.01$ compared to HF.

diet (AD) group) [8]; (iii) a low-fat (LF) diet (with the same composition of the AIN-76 diet); and (iv) an HF diet supplemented with piroxicam (PXC) 0.01% w/w as a positive control for chemoprevention [14]. At sacrifice (15 weeks after DMH), the number of MDF/colon in the AD group was about half when compared to the HF diet (Fig. 1). Similarly, LF and PXC diets showed a strong chemopreventive effect when compared with the HF diet, in line with previous results [4, 14]. RT-qPCR experiments (see protocol in Supporting Information) measuring the expression of proinflammatory genes in the mucosa of the medial colon, showed that TNF-α and iNOS were significantly down-regulated in AD-fed rats (Figs. 2A, B). Similarly, the expression of IL-6 and IL-1β tended to be lower ($P = 0.054$ and 0.12 for IL-6 and IL-1β, respectively) in the AD compared with the HF group (Figs. 2C, D). No significant variations were observed with the LF diet since only a slight reduction in IL-6, IL-1β, and TNF-α was observed. COX2 expression was not varied (Fig. 2E). CD68 expression, as a measure of macrophage infiltration in the mucosa was tested with immunohistochemistry (IH; see protocol in Supporting Information) showing that rats fed the AD had fewer CD68 positive cells compared with the HF diet (borderline significant with $p = 0.06$; Supporting Information Fig. S1), while the LF diet did not induce significant changes. Myeloperoxydase activity in the colonic mucosa was not varied among the groups (data not shown). The production of NO and H_2O_2 by peritoneal macrophages

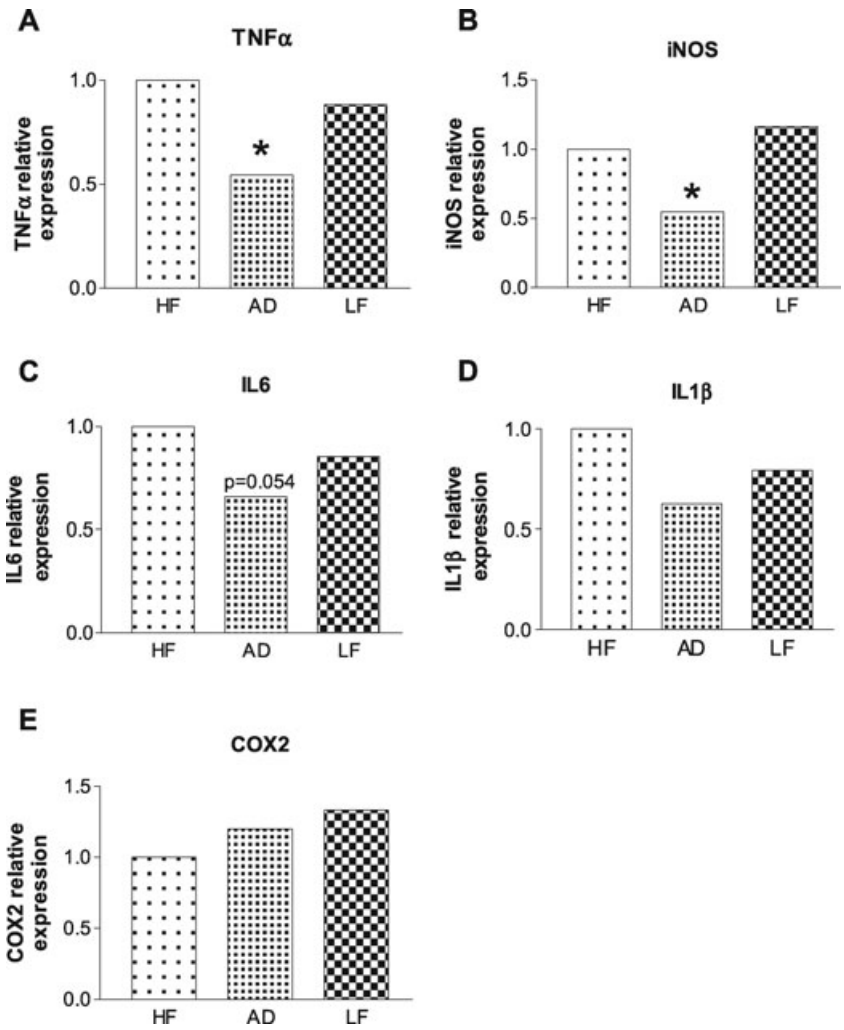

Figure 2. Expression of proinflammatory genes in the colon of the different groups. Bars: mean relative expression (fold change) compared to HF for each gene. $n = 10$, 9, and 7 in HF, AD, and LF groups, respectively. $^{*}p < 0.05$ as described in Supporting Information.

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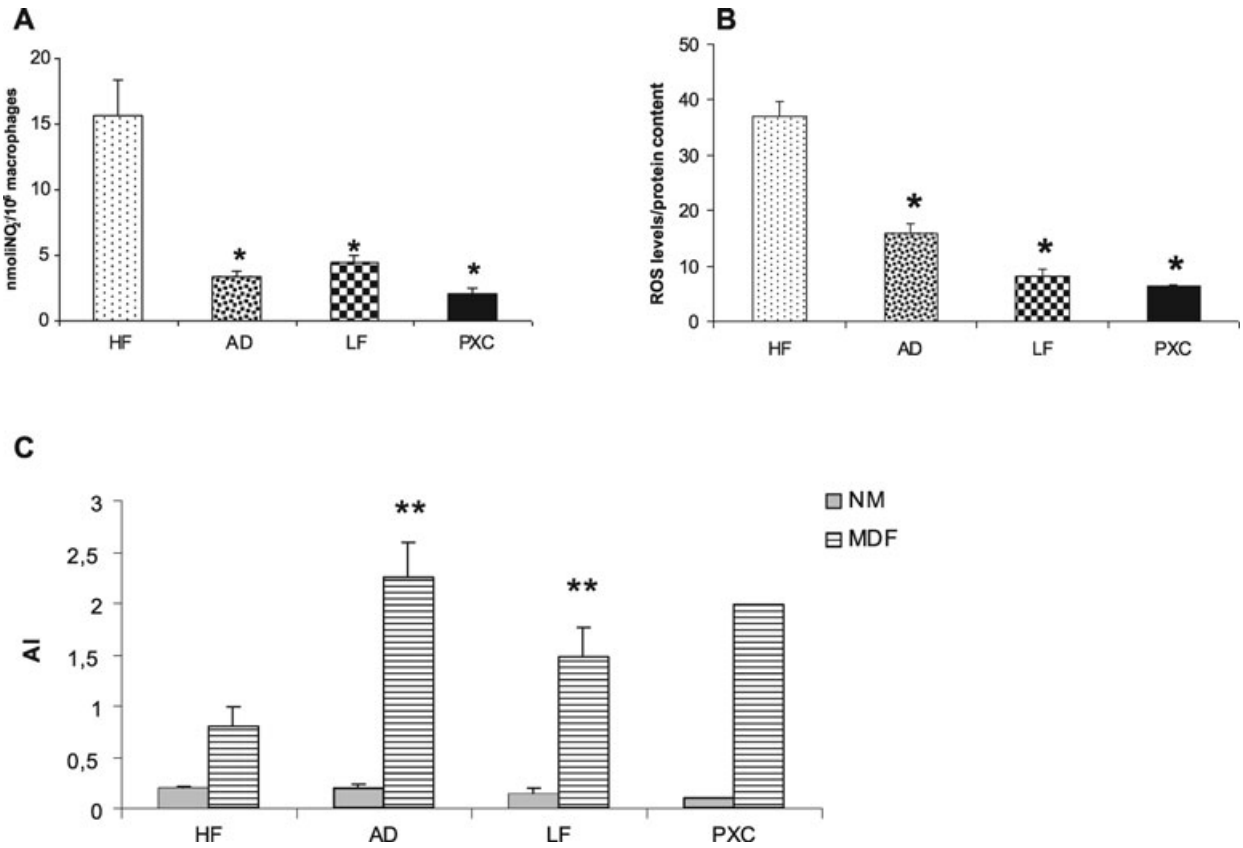

Figure 3. (A) NO secretion in thioglycollate-elicited peritoneal macrophages from HF, AD, LF, and PXC rats. Bars: means ±SE ($n = 8$, 7, 9, and 3 determinations from different groups, respectively). (B) ROS production in thioglycollate-elicited peritoneal macrophages from HF, AD, LF, and PXC rats. Bars: means ± SE ($n = 5$, 6, 6, and 3 determinations from different groups, respectively). *$p < 0.05$ as described in Supporting Information. (C) Apoptotic index (AI) in normal mucosa (NM, gray bars) and MDF (striped bars). Bars: means ± SE. For NM, $n = 7$, 8, 6, and 2 in HF, AD, LF, and PXC groups, respectively. For MDF, $n = 10$, 7, 8, and 2 in HF, AD, LF, and PXC groups, respectively. *$p < 0.05$ compared to HF.

was evaluated as a measure of systemic inflammation (see protocol in Supporting Information). In AD, LF, and PXC groups the production of these metabolites was significantly lower than in the HF group (Figs. 3A, B). PGE_2 plasma concentration was significantly lower in the AD compared with the HF diet, while the LF group was unvaried (Supporting Information Fig. S2).

Colon proliferation, measured determining proliferating cell nuclear antigen (PCNA) expression (see IH protocol in Supporting Information), was similar among dietary groups (LI values: 42.9 ±2.05, 43.2 ±2.7, and 44.5 ±2.2, in the HF, AD, and LF diets, respectively, means ± SE).

Apoptosis (see experimental protocol in Supporting Information) in the normal mucosa was, as expected, very low and did not differ among the groups (Fig. 3C). On the contrary, apoptosis in the MDF from the rats fed the AD and LF diets was significantly higher than that in the rats fed the HF diet (Fig. 3C and Supporting Information Fig. S3).

Protective effects of apple or apple components have been reported in epidemiological and experimental studies [7, 9–13, 15, 16]. However, although unpublished results from our group indicate that MM apples do not affect carcinogenesis when given to non-induced rats, apple proanthocyanidins have been reported to increase aberrant crypt foci (ACF) in carcinogen-induced rats [9]. Moreover, an increased small intestinal carcinogenesis in Apc Min/+ mice has been reported [10]. Some of these inconsistencies could be due to the experimental models used. Accordingly, Apc Min/+ mice [10, 11] develop tumors especially in the small intestine, while widely used ACF have also been questioned as cancer endpoints [17] and other lesions (β-catenin accumulated crypts or MDF) have been proposed as more reliable cancer biomarkers [17]. Therefore, our results showing a reduction in MDF indicate that MM may effectively reduce colon carcinogenesis. Moreover, different apple components and not the whole fruit have been tested previously. We used a lyophilized prepared from whole apples, also considering, that the consumption of whole fruits as opposed to extracts or supplements has been recently encouraged for chemoprevention [18].

Regarding inflammation, we found that AD diet was associated with both decreased systemic and colonic inflammation, confirming previous reports on the anti-inflammatory

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activity of some apple varieties [6–8, 19]. Importantly, this is the first demonstration of the anti-inflammatory effect of apples in a model of sporadic colon cancer in which inflammation is not as overt as in other experimental models (i.e. HLAB27, dextran sodium sulphate-induced colitis) histologically. Regarding fat, it has been reported that rodents fed an LF diet showed lower systemic inflammation and oxidative stress markers compared to animals fed HF or a western-style diet [20, 21]; however, some of these effects were no longer evident after 6 months of feeding [21]. Our results did not show significant effects of fat on colon inflammation, although the observed effects on peritoneal macrophages suggest that dietary fat may affect carcinogenesis with systemic actions.

Previous studies on apples and colon proliferation in vivo have shown either a decrease [7], an increase [10], or a null effect in the normal mucosa of rats not treated with carcinogens [12]. Regarding fat, although some studies demonstrated an increase in colon proliferation, especially when fat is administered as a bolus [22], it has also been reported that high fat, despite its detrimental effects on colon carcinogenesis, does not affect proliferation [23], a result that also supported by our data.

Cell lines exposed to apple components in vitro have been shown to undergo apoptosis [7, 24]; however, no previous study evaluated the effect of apples in vivo. Increased apoptosis in precancerous lesions can contribute to the elimination of damaged cells with potentially carcinogenic mutations [25], and it is possible, at least regarding the AD group, that the effect on apoptosis results from lower inflammation, which at least in cell systems, hampers apoptosis [26]. Interestingly, rats on an LF diet showed high apoptosis in the MDF. Although a similar effect has been reported in colonic tumors of LF-fed rats, our results is the first demonstration that this effect occurs in the initial phases of carcinogenesis, perhaps explaining why a LF diet results in a lower number of tumors.

In conclusion, we have demonstrated, in a validated model of colon carcinogenesis, a strong chemopreventive effect of MM apples through both anti-inflammatory and proapoptotic mechanisms. Our results also confirm the strong protective effect of a low-fat diet that increases apoptosis in MDF, while anti-inflammatory activity in the colon seems to play a minor role as a mechanism of action of this diet.

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References

[1] World Cancer Research Fund and American Institute for Cancer Research. *Food Nutrition, Physical Activity and the Prevention of Cancer: a Global Perspective*. 2007, Washington, DC, USA: AICR.

[2] Rhodes J. M., Campbell B. J, Inflammation and colorectal cancer: IBD-associated and sporadic cancer compared. *Trends Mol. Med.* 2002, *8*, 10–16.

[3] Warren, C.A., Paulhill, K. J., Davidson, L. A., Lupton, J. R. et al., Quercetin may suppress rat aberrant crypt foci formation by suppressing inflammatory mediators that influence proliferation and apoptosis. *J. Nutr.* 2009, *139*, 101–105.

[4] Reddy, B. S., Maeura, Y., Tumor promotion by dietary fat in azoxymethane-induced colon carcinogenesis in female F344 rats: influence of amount and source of dietary fat. *J. Natl. Cancer Inst.* 1984, *72*, 745–750.

[5] Risio, M., Lipkin, M., Newmark, H., Yang, K. et al., Apoptosis, cell replication, and Western-style diet-induced tumorigenesis in mouse colon. *Cancer Res.* 1996, *56*, 4910–4916.

[6] Jung, M., Triebel, S., Anke, T., Richling, E. et al., Influence of apple polyphenols on inflammatory gene expression. *Mol. Nutr. Food Res.* 2009, *53*, 1263–1280.

[7] Gerhauser, C., Cancer chemopreventive potential of apples, apple juice, and apple components. *Planta Med.* 2008, *74*, 1608–1624.

[8] Castagnini, C., Luceri, C., Toti, S., Bigagli, E. et al., Reduction of colonic inflammation in HLA-B27 transgenic rats by feeding Marie Menard apples, rich in polyphenols. *Br. J. Nutr.* 2009, *102*, 1620–1628.

[9] Lhoste, E.F., Bruneau, A., Bensaada, M., Cherbuy, C. et al., Apple proanthocyanidins do not reduce the induction of preneoplastic lesions in the colon of rats associated with human microbiota. *J. Agric. Food Chem.* 2010, *58*, 4120–4125.

[10] Mandir, N., Englyst, H., Goodlad, R. A., Resistant carbohydrates stimulate cell proliferation and crypt fission in wild-type mice and in the Apc mouse model of intestinal cancer, association with enhanced polyp development. *Br. J. Nutr.* 2008, *100*, 711–721.

[11] Fini, L., Piazzi, G., Daoud, Y., Selgrad, M. et al., Chemoprevention of intestinal polyps in ApcMin/+ mice fed with western or balanced diets by drinking annurca apple polyphenol extract. *Cancer Prev. Res.* 2011, *4*, 907–915.

[12] Barth, S. W., Fahndrich, C., Bub, A., Dietrich, H. et al., Cloudy apple juice decreases DNA damage, hyperproliferation and aberrant crypt foci development in the distal colon of DMH-initiated rats. *Carcinogenesis* 2005, *26*, 1414–1421.

[13] Gosse, F., Guyot, S., Roussi, S., Lobstein, A. et al., Chemopreventive properties of apple procyanidins on human colon cancer-derived metastatic SW620 cells and in a rat model of colon carcinogenesis. *Carcinogenesis* 2005, *26*, 1291–1295.

[14] Femia, A. P., Dolara, P., Caderni, G., Mucin-depleted foci (MDF) in the colon of rats treated with azoxymethane (AOM) are useful biomarkers for colon carcinogenesis. *Carcinogenesis* 2004, *25*, 277–281.

[15] Jedrychowski, W., Maugeri, U., Popiela, T., Kulig, J. et al., Case-control study on beneficial effect of regular consumption of apples on colorectal cancer risk in a population with relatively low intake of fruits and vegetables. *Eur. J. Cancer Prev.* 2010, *19*, 42–47.

[16] Veeriah, S., Hofmann, T., Glei, M., Dietrich, H. et al., Apple polyphenols and products formed in the gut differently

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inhibit survival of human cell lines derived from colon adenoma (LT97) and carcinoma (HT29). *J. Agric. Food Chem.* 2007, *55*, 2892–2900.

[17] Mori, H., Hata, K., Yamada, Y., Kuno, T. et al., Significance and role of early-lesions in experimental colorectal carcinogenesis. *Chem. Biol. Interact* 2005, *155*, 1–9.

[18] Brower, V., An apple a day may be safer than vitamins. *J. Natl. Cancer Inst.* 2008, *100*, 770–772.

[19] Liu, L., Li, Y. H., Niu, Y. B., Sun, Y. et al., An apple oligogalactan prevents against inflammation and carcinogenesis by targeting LPS/TLR4/NF-kB pathway in a mouse model of colitis-associated colon cancer. *Carcinogenesis* 2010, *31*, 1822–1832.

[20] Baltgalvis, K. A., Berger, F. G., Pena, M. M., Davis, J. M. et al., The interaction of a high-fat diet and regular moderate intensity exercise on intestinal polyp development in Apc Min/+ mice. *Cancer Prev. Res.* 2009, *2*, 641–649.

[21] Erdelyi, I., Levenkova, N., Lin, E. Y., Pinto, J. T. et al., Western-style diets induce oxidative stress and dysregulate immune responses in the colon in a mouse model of sporadic colon cancer. *J. Nutr.* 2009, *139*, 2072–2078.

[22] Caderni, G., Stuart, E. W., Bruce, W. R., Dietary factors affecting the proliferation of epithelial cells in the colon of the mouse. *Nutr. Cancer* 1988, *11*, 147–153.

[23] Steinbach, G., Kumar, S. P., Reddy, B. S., Lipkin, M. et al., Effects of caloric restriction and dietary fat on epithelial cell proliferation in rat colon. *Cancer Res.* 1993, *53*, 2745–2749.

[24] Kern, M., Pahlke, G., Balavenkatraman, K. K., Böhmer, F. D. et al., Apple polyphenols affect protein kinase C activity and the onset of apoptosis in human colon carcinoma cells. *J. Agric. Food Chem.* 2007, *55*, 4999–5006.

[25] Samaha, H. S., Hamid, R., el-Bayoumy, K., Rao, C. V. et al., The role of apoptosis in the modulation of colon carcinogenesis by dietary fat and by the organoselenium compound 1,4-phenylenebis(methylene)selenocyanate. *Cancer Epidemiol. Biomarkers Prev.* 1997, *6*, 699–704.

[26] Kundu, J. K., Surh, Y. J., Inflammation: gearing the journey to cancer. *Mutat. Res.* 2008, *659*, 15–30.

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