Dietary Extra-Virgin Olive Oil Polyphenols Do Not Attenuate Colon Inflammation in Transgenic HLAB-27 Rats but Exert Hypcholesterolemic Effects through the Modulation of HMGCR and PPAR-α Gene Expression in the Liver

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Abstract

Background: Human studies have demonstrated that olive oil phenolic compounds reduce inflammatory markers associated with chronic diseases. Objectives: To explore the anti-inflammatory effects of extra-virgin olive oil polyphenols in an experimental model of inflammatory bowel disease (IBD). Methods: HLA-B27 transgenic rats were fed an AIN-76 diet containing 10% corn oil (CO) or extra-virgin olive oil with high (EVOO) or low phenolic content (ROO) for 3 months. Wild-type rats (WT) were fed the CO diet. Results: CO-fed HLA-B27 animals developed intestinal inflammation characterized by diarrhea, increased myeloperoxidase activity, and mucosal injury. None of these parameters were influenced by EVOO. Gene expression profiling indicated that pro-inflammatory pathways were upregulated in the colon mucosa of CO-fed HLA-B27 rats compared to WT, and this was further confirmed by RT-PCR for the iNOS, TNFα, and IL1β genes. EVOO significantly reduced TNFα gene expression in the colon mucosa and decreased total cholesterol blood levels compared to CO HLA-B27 rats (89.43 ± 3.66 vs. 111.5 ± 8.10 mg/dL, p < 0.05). This latter effect with EVOO was associated with reduced HMGCR and increased PPAR-α hepatic gene expression, compared to ROO. Conclusion: These data indicate that olive oil polyphenols do not control colon inflammation in HLA-B27 transgenic rats but exert a positive effect on blood lipids by reducing total cholesterol levels. This preliminary result suggests the need to explore the efficacy of EVOO rich in polyphenols as a complementary strategy for managing hypercholesterolemia and to potentially limit statin-associated myotoxicity.

Introduction

Human studies have demonstrated that olive oil phenolic compounds exert health-promoting effects and reduce inflammatory markers associated with chronic diseases [1, 2]. In particular, oleuropein, oleocanthal, hy-
droxytyrosol, and tyrosol are inhibitors of oxidative damage and inflammation in a variety of experimental systems [3]. Hydroxytyrosol is a potent radical scavenger and a chelator of metals like iron [4]; it reduces platelet aggregation and has anti-inflammatory effects through cyclooxygenase (COX) inhibition [5]. Oleocanthal, responsible for the pungent taste of new olive oil, exhibits potency similar to nonsteroidal anti-inflammatory drugs in inhibiting both COX-1 and COX-2 [6]. Oleuropein is also a radical scavenger and a LDL oxidation blocker [7].

We have recently reported that the anti-inflammatory effects of hydroxytyrosol were detected at pharmacologically relevant concentrations and involved decreased nitric oxide (NO) and PGE2 production and NRF2 and miR-146a modulation [8]. Recent studies have also indicated that the beneficial effects of the phenolic compounds from extra-virgin olive oil (EVOO) may be related to their impact on the genome, since they modify the expression of genes involved in many cellular processes and in particular oxidative stress, inflammation, DNA repair, and metabolism pathways [3, 9]. We reported that EVOO rich in phenols prevented the age-associated cognitive decline in rats by modulating the expression of genes and microRNAs involved in neuronal function [10] and that resveratrol counteracted features of cellular senescence such as oxidative stress and inflammation [11].

Although multiple sources of evidence support the anti-inflammatory effect of olive oil and its phenolic compounds [3], their beneficial effects in inflammatory bowel disease (IBD) have been almost exclusively studied in animal models of chemically induced colitis and involved the reduction of COX-2 and iNOS expression [12, 13]. However, such animal models do not accurately reflect the human disease pathogenesis [14]. HLA-B27 rats expressing the human β2-microglobulin and the histocompatibility gene HLA-B27, spontaneously develop immune-mediated intestinal chronic inflammation and are recognized as an important animal model of IBD [14, 15]. The inflammatory cytokine pattern of HLA-B27 transgenic rats (T helper [Th]1>Th2 cytokine expression) corresponds to that observed in Crohn’s disease, while the histological features of their colitis closely resemble those of ulcerative colitis [14, 15].

In the present paper, we investigated for the first time whether EVOO rich in polyphenols may control colon inflammation in a genetic model of IBD. As a control, we used an identical olive oil depleted of most phenolic compounds.

### Materials and Methods

#### Animals and Diets

The experimental protocol was designed in conformity with the recommendations of the European Economic Community (86/609/CEE) for the care and the use of laboratory animals and was approved by the animal care Committee of the University of Florence (Florence, Italy) in compliance with the legislative decree number 116 27/01/1992.

Dietary components (Piccioni, Gessate, Milan, Italy) were based on the AIN76 diet, modified to contain 10% fat w/w. EVOO was purchased from Cipolloni and Petesse S.p.A. (Foligno, Perugia, Italy). The analysis of the phenolic content and the preparation of the rectified olive oil (ROO), devoid of phenolic compounds, was performed in the laboratory of Prof. Servili, Department of Food Sciences, University of Perugia, Italy, as previously reported [16].

Male HLA-B27 transgenic rats, aged 6–8 weeks (Taconic Laboratory, Germantown, NY, USA), were maintained in specific pathogen-free conditions and divided into: corn oil (CO) diet group (n = 6), EVOO diet group (n = 7), or ROO diet group (n = 7). A fourth group of animals was composed of F344 wild-type rats (WT, n = 6) fed the CO diet. All animals had free access to water and were fed ad libitum. Food intake of rats was assessed by placing the animals in metabolic cages for 1 day at the end of the second month of treatment (week 8). The calculated average food consumption was 15 g of diet/day, with no differences observed between WT and HLA-B2 animals.

The diet composition (in g/100 g of diet) was: 10 g fat, 43.5 g sucrose, 20 g casein, 14.8 g corn starch, 6 g cellulose, 4 g AIN 76 Mineral mix, 1.2 g AIN 76 Vitamin mix, 0.3 g methionine, and 0.2 g choline. CO groups were fed a diet in which the lipid component was provided by CO, the EVOO group by an EVOO rich in phenols (718.8 mg of total phenols/kg of olive oil resulting in a daily dose of 4.3 mg/kg/body weight [bw]), and the ROO group by the same EVOO deprived of phenolic compounds (9.3 mg of total phenols/kg of olive oil, resulting in a daily dose of 0.056 mg/kg/bw) but retaining other minor components such as α-tocopherol (Table 1). After 12 weeks, the animals were sacrificed. For each animal, the last part of the distal colon was fixed in buffered formalin for histological analysis. The remaining part of the distal colon was scraped with a glass slide to isolate the mucosal layer and stored at –80°C for DNA extraction and biochemical determinations, or in RNA later (Qiagen, Milan, Italy) for RNA analyses.

#### Blood Biochemistry

Blood samples were collected from all animals prior to scheduled necropsy. The blood was collected in EDTA tubes and was centrifuged at 2,000 g for 10 min at room temperature to prepare plasma. The levels of total cholesterol and triglycerides in the rat plasma were determined using enzyme methods with commercial kits (Reflotron® Triglycerides and Cholesterol, Roche) according to the manufacturers’ protocols.

#### Colon Inflammation: Histological Grading of Colitis, Fecal Water Content, and Myeloperoxidase Activity

The histological evaluations were made by observing the whole tissue sections along the entire colon length (from caecum to rectum), expressing the score as a mean between regions with different damage levels in order to obtain an overall rating of each ani-
Table 1. Phenolic components of the two extra-virgin olive oils

| Component                        | EVOO  | ROO   |
|----------------------------------|-------|-------|
| Hydroxytyrosol (3,4-DHPEA)       | 15.0±0.5 | –     |
| Tyrosol (p-HPEA)                 | 11.0±0.1 | 0.1±0.01 |
| Oleocanthal (p-HPEA-EDA)         | 81.9±1.1 | 1.7±0.01 |
| (p)-1-Acetoxyphinoresinol        | 17.0±0.0 | 1.5±0.01 |
| 3,4-DHPEA-EDA                    | 346.7±3.5 | 1.1±0.02 |
| (p)-Pinoresinol                  | 32.3±0.1 | 2.0±0.02 |
| 3,4-DHPEA-EDA (oleuropein aglycone) | 214.9±2.1 | 2.9±0.03 |
| Total phenols                    | 718.8±7.4 | 9.3±0.1 |
| α-Tocopherol                     | 131.2±1.1 | 129.1±1.1 |

Values indicate mg/kg of olive oil. EVOO, phenol-rich olive oil; ROO, phenol-deprived olive oil; the phenolic content is the mean value of three independent determinations ± standard deviation.

Oxidative DNA Damage

Oxidative DNA damage in the colon mucosa was assessed using two methods: (1) the comet assay on samples treated with a bacterial repair enzyme (formamidopyrimidine glycosylase, gift of A.R. Collins, University of Oslo, Norway) [19], and (2) the HPLC determination of 8-hydroxy-2-deoxyguanosine (8-OHdG) levels [20].

RT-PCR and Microarray Analysis

Total RNA was extracted using the RNEasy Mini kit plus (Qiagen, Milan, Italy). We performed RT-PCR analysis of the expression of a number of proinflammatory genes (COX2, iNOS, IL1β, TNFa, and IFNγ) in the colon mucosa and of lipid metabolism-related genes (HMGCR, CYP7A1, PPAR-α, and APOCIII) in the liver, as previously reported [21].

Microarray gene expression analyses were performed using the Agilent Whole Rat Genome Microarray Kit 4x44K (Agilent Technologies, Palo Alto, CA, USA) using a two-color Agilent microarray protocol in which pools of RNA extracted from the colon mucosa from each experimental group (WT [n = 6], EVOO [n = 7], and ROO [n = 7]) were contrasted with a reference RNA obtained by pooling equal amount of RNA samples extracted from the colon mucosa of CO rats (n = 6) (Two-Color Microarray-Based Gene Expression Analysis version 5.7). By pooling the RNA samples, we aimed to highlight the primary changes in colon mucosa gene expression profiles in each experimental group. The pooling approach is often used to reduce the complexity of analysis of transcript profiling and has been reported in other studies [22, 23]. Images were scanned with a Genepix 4000B microarray scanner at 5-μm resolution (Axon Instruments, Foster City, CA, USA). Image analysis and initial quality control were performed using Agilent Feature Extraction Software v9.5. Quality criteria included a minimal spot size, a median/mean ratio of at least 0.9 for each spot, nonsaturated intensity for both channels, a signal well above background, and a minimal signal intensity for at least one channel.

Genes of interest were those that exhibited a PValueLogRatio (statistical significance on the LogRatio per each gene between red and green channels) of < 0.05. Functional analysis was performed using the pathway visualization and analysis tool PathVisio (https://www.pathvisio.org/downloads) and the latest PathVisio release. The microarray data sets supporting the results of this article are available in the MIAME public database ArrayExpress repository (http://www.ebi.ac.uk/arrayexpress/) (accession No. MTAB-6218).

Statistical Analyses

Data were analyzed by unpaired t test (genotype effects, WT vs. CO) and by one-way ANOVA and Tukey’s multiple comparisons test (diet effect on HLA-B27 groups). All analyses were carried out using GraphPad Prism 5.0 and STATGRAPHICS Centurion XVI. Results are expressed as means ± standard error; p < 0.05 was considered statistically significant.

Results

Survival, Physical Examinations, Body Weights

All animals survived until the sacrifice. All experimental diets were well tolerated, and at the end of the study period, notwithstanding the growth curves which indicated a lower final body weight of CO compared to WT (p < 0.05), the body-weight gains were similar. No differences were observed between the three HLA-B27 rat groups (Fig. 1). The excretion of dark-red feces was not observed, but compared with WT rats, the degree of diarrhea (evaluated as percentage of fecal water content) was increased in CO rats (p < 0.01 vs. WT; Table 2). No differences were observed between CO, ROO, and EVOO groups.

Oxidative Damage and Inflammation in the Colon Mucosa

Oxidative DNA damage, as determined by 8-OHdG levels and by Comet Assay with formamidopyrimidine glycosylase, was not increased in the colon mucosa of
HLA-B27 rats compared to WT and was similar among HLA-B27 rats receiving different types of oils (Table 2). MPO activity was higher in the colon mucosa from CO rats compared to WT (\( p < 0.01 \)), but no differences were observed between HLA-B27 rats fed the different oils (Table 2). Histological examination performed to assess the colitis score also revealed signs of inflammation and mild mucosal injury in CO rats (\( p < 0.05 \) vs. WT; Table 2). No effect of the different types of oils was detected (Table 2), as depicted in representative images (Fig. 2).

| Table 2. Markers of inflammation, oxidative stress, and gene expression in the colon mucosa of WT and HLA-B27 rats fed CO, ROO, and EVOO diets |
|---------------------------------------------------------------|
| **Markers of colon inflammation**                             |
| Fecal water, %                                                |
| Colitis score                                                 |
| MPO activity, U/mg of tissue                                  |
| **Oxidative stress markers**                                  |
| FPG sites                                                     |
| 8-OHdG × 10^{-6} dG                                           |
| **Gene expression in the colon mucosa (RT-PCR)**              |
| TNFa                                                          |
| iNOS                                                          |
| IFN\( \gamma \)                                               |
| IL1\( \beta \)                                               |
| COX2                                                          |
| Data are expressed as mean ± SEM; \* \( p < 0.05 \); ** \( p < 0.01 \); *** \( p < 0.001 \) vs. CO by t test. \# \( p < 0.05 \) vs. CO by one-way ANOVA and Tukey’s multiple comparisons test. FPG, formamidopyrimidine glycosylase; MPO, myeloperoxidase. |

RT-PCR Results and Gene Expression Profiling in the Colon Mucosa

RT-PCR data indicated that the expression of proinflammatory genes such as COX-2, iNOS, TNFa, and IL1\( \beta \) was significantly higher in CO rats compared to WT (\( p < 0.001 \)), but no differences were observed between HLA-B27 rats fed different types of oils (Table 2). Only the expression of TNFa was slightly, but significantly (\( p < 0.05 \)), reduced in the EVOO group compared to the CO group (Table 2). To identify the main effects of the
dietary treatments on global expression profiles of the colon mucosa, we performed a microarray analysis. These results highlighted that HLA-B27 rats fed the CO diet compared to WT rats showed an upregulation of genes associated with inflammatory response as well as T-cell, TGF-B, IL1, and IL2 receptor pathways. This analysis also identified the inflammatory response pathway to be significantly downregulated in both EVOO and ROO groups compared to HLA-B27 CO-fed rats (Fig. 3). Table 3 shows the fold change values obtained with microarrays that correspond to the five genes analyzed by RT-PCR.

Effects on Blood Lipids (Cholesterol and Triglycerides)
Total cholesterol blood levels were significantly lower only in HLA-B27 rats fed the EVOO diet compared to those fed CO (p < 0.05), whereas triglycerides were unchanged (Fig. 4a, b).

Expression of Hepatic Genes Related to Lipid Metabolism
The hepatic expression of HMGCR, CYP7A1, PPAR-α, and APOCIII genes, which encode key enzymes involved in cholesterol synthesis and lipid metabolism, were also

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Fig. 2. Representative images of colon histological samples: (a) WT; (b) HLA-B27-CO; (c) HLA-B27-EVOO; (d) HLA-B27-ROO. a Some normal crypts (black asterisks) and a few lymphocytes located around crypts in the connective tissue (black arrow) are visible. b–d An almost complete disarray of the colon structure is evident: very few residual crypts (black arrowheads) and rich inflammatory infiltrate that replaces the mucosa (black points). ×200 magnification. Scale bar, 100 µm.
investigated. Interestingly, rats fed EVOO showed lower expression of HMGCR and increased expression of PPAR-α compared to rats fed the ROO diet (Fig. 4c, d).

**Discussion**

IBD is a common inflammatory disorder of the gastrointestinal tract, with an increasing prevalence both in Western and developing countries [24]. Current therapies for IBD include mesalazine, immunosuppressive drugs, corticosteroids, and anti TNF-α, which are not devoid of serious side effects; therefore, alternative and safer therapeutic strategies for IBD management are needed [25]. Several nutraceuticals and phytochemicals are under investigation for their effects on IBD; however, despite some of them showing positive effects [26], strong evidence of effective protection is lacking [27, 28].

To explore whether dietary polyphenols from EVOO may be a useful strategy to control chronic colon inflammation, we used HLA-B27 rats as a genetic animal model of chronic colitis. These rats revealed several features that are also found in human IBD, such as reduced body weight, diarrhea, increased expression of proinflammatory genes such as COX-2, iNOS, TNFα, and IL1β in the colon mucosa, MPO activity, intestinal mucosal inflammation, and injury. In contrast, as demonstrated by other researchers [29], the development of colitis in HLA-B27 was not associated with an increase in oxidative DNA damage.

Functional analysis performed on microarray data revealed an overall downregulation of genes involved in the inflammatory response in WT rats, as well as in both EVOO and ROO rats, compared to the HLA-B27 rats fed the CO diet. However, RT-PCR results indicated that EVOO and ROO did not reduce the expression of the proinflammatory genes COX-2, iNOS, and IL1β compared to CO-fed HLA-B27 rats, and only a small but significant effect of EVOO was observed on the expression of TNFα. However, neither diarrhea nor MPO activity were changed in EVOO-fed rats compared to CO-fed rats. Histological analyses did not show any beneficial effects of EVOO on crypt erosion and inflammatory infiltrate.

These results indicate that EVOO rich in polyphenols did not exert beneficial effects on the HLA-B27 rat model of chronic colitis. In contrast, in a DSS-induced chronic colitis in mice, Sánchez-Fidalgo et al. [13] reported that a diet containing 10% of EVOO enriched with a polyphenol extract reduced histopathological colitis scores and inflammatory markers. They also demonstrated that dietary EVOO and EVOO enriched with hydroxytyrosol...
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**Fig. 4.** Blood total cholesterol (a) and triglyceride levels (b) in the four experimental groups. RT-PCR results of HMGCR (c) and PPAR-α expression (d) in the liver of the four experimental groups. Treatments with the same letter are significantly different. (a, *p* < 0.05; b, *p* < 0.01) by one-way ANOVA and Tukey’s multiple comparisons test.

**Table 3.** Data from microarray results, relative to the five genes analyzed by RT-PCR

| Probe name | Gene name | Systematic name | Description | EVOO | ROO | WT   |
|------------|-----------|-----------------|-------------|------|-----|------|
| A_43_P11513 | TNFα      | NM_012675       | *Rattus norvegicus* tumor necrosis factor (TNF superfamily, member 2) (Tnf), mRNA [NM_012675] | 1.3  | 2.33 | –1.78 |
| A_43_P13320 | IFNγ      | NM_138880       | *Rattus norvegicus* interferon gamma (Ifng), mRNA [NM_138880] | 1.77 | 1.35 | –1.10 |
| A_44_P278593 | COX-2    | NM_017232       | *Rattus norvegicus* prostaglandin-endoperoxide synthase 2 (Ptgs2), mRNA [NM_017232] | –1.17 | 1.00 | –1.75 |
| A_44_P472989 | COX-2    | NM_017233       | *Rattus norvegicus* prostaglandin-endoperoxide synthase 2 (Ptgs2), mRNA [NM_017232] | –1.04 | 1.00 | –1.25 |
| A_44_P545686 | iNOs     | NM_012611       | *Rattus norvegicus* nitric oxide synthase 2, inducible (Nos2), mRNA [NM_012611] | 1.46 | 1.03 | –4.44 |
| A_44_P230320 | IL1β     | NM_031512       | *Rattus norvegicus* interleukin 1 beta (Il1b), mRNA [NM_031512] | 1.11 | 1.22 | –5.31 |
| A_43_P14911 | IL1β     | NM_031512       | *Rattus norvegicus* interleukin 1 beta (Il1b), mRNA [NM_031512] | 1.07 | 1.16 | –2.68 |

Data are fold changes versus HLA-B27-CO, used as relative control group. For COX-2 and IL1β genes, the array contains two different probes.
reduced the severity of chronic colonic damage induced by an acute exposure to DSS [12]. Similarly, Takashima et al. [30] reported that a 5% olive oil-rich diet attenuated inflammation in DSS-induced colitis in rats. In our study, EVOO contained 718 mg/kg of oil of total phenols, resulting in a daily dose of 4.3 mg/kg/bw. This level is similar to that reached by Sánchez-Fidalgo et al. [13] with an EVOO enriched with a polyphenol extract (3.5 mg/kg/bw). However, in our EVOO, the concentration of hydroxytyrosol was 15 mg/kg of oil, resulting in a daily dose of only 90 µg/kg bw. This dose is much lower than that provided in the study of Sánchez-Fidalgo et al. [13], which we calculated to be approximately 400 µg/kg/bw. In another study from Sánchez-Fidalgo et al. [12], the daily dose of hydroxytyrosol in the hydroxytyrosol-enriched EVOO group was even higher (5 mg/kg/bw). In both studies by Sánchez-Fidalgo et al. [12, 13], hydroxytyrosol or a polyphenol extract was added to olive oil, whereas in the present study, the amount of phenols in the EVOO was that naturally occurring in the olive oil. The composition of phenolic compounds in EVOO is extremely variable due to the combination of agronomic and technological aspects such as olive cultivars, ripeness, mechanical extraction process, and storage conditions. The amount of phenols in the EVOO used in the present study was relatively high (718 mg/kg) when compared to a large number of commercial EVOO. Indeed, Servili et al. [31] reported that the phenolic composition of commercial EVOO may vary from 133.5 to 950.5 mg/kg of oil.

The differences in experimental models between the present study and these previous studies (which used a model resembling an acute colitis) may account for these different results. However, by using the same HLA-B27 rat model of chronic colitis, we previously reported that Marie Ménard apples, a variety rich in proanthocyanidins, were able to control inflammation [17], demonstrating that a dietary intervention may be effective in modulating a genetically driven chronic colitis. Marie Ménard apple supplementation also downregulated genes associated with the inflammatory response and several cytokine and integrin-mediated cell adhesion pathways [17]. However, in that previous study, the Marie Ménard apple-based diet provided approximately 125 mg/kg/bw of proanthocyanidins, which is considerably higher than the phenolic content provided by the EVOO diet (4.3 mg/kg/bw) in the present study. In addition to quantitative differences in the polyphenolic content, there are also qualitative differences that can change the bioavailability of these compounds and differentially modulate local and systemic effects. In particular, polyphenols with a high polymeric degree such as proanthocyanidins are not absorbed and likely exert local effects on the colon, whereas single molecules such as tyrosol and hydroxytyrosol are absorbed and may reach the systemic circulation and the liver to potentially exert metabolic effects.

Since gene expression analyses also suggested effects of ROO and EVOO on the cholesterol biosynthesis pathway, we measured plasma cholesterol concentrations. We observed a positive effect of EVOO-based diet that was further investigated by examining the hepatic expression of HMGCR, CYP7A1, PPAR-α, and APOCIII genes which all encode for key enzymes that regulate cholesterol synthesis and lipid metabolism. Interestingly, only rats fed EVOO showed a significantly lower hepatic expression of HMGCR, the gene encoding for the rate-limiting enzyme for cholesterol synthesis, and increased expression of PPAR-α, a nuclear receptor with a key role in lipid metabolism and inflammatory response [32]. Taken together, this suggests that these effects were related to the phenolic content of EVOO rather than the fatty acid composition of olive oil since no changes in gene expression were observed in ROO rats. Accordingly, the upregulation of PPAR-α and downregulation of HMGCR have been previously observed in animals treated with polyphenols [33–35].

In conclusion, we demonstrated that although EVOO rich in polyphenols positively modulated genes and pathways involved in the inflammatory process, this was not enough to control intestinal inflammation. However, since the current gold standard pharmacological strategy to treat hypercholesterolemia is based on the use of statins, a class of HMGCR inhibitors with important side effects such as myalgia, myopathy, and liver damage [36], the efficacy of EVOO rich in polyphenols deserves to be explored as a potential safer complementary strategy for managing hypercholesterolemia.

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Statement of Ethics

The experimental protocol was designed in conformity with the recommendations of the European Economic Community (86/609/CEE) for the care and the use of laboratory animals and was approved by the animal care Committee of the University of Florence (Florence, Italy) in compliance with the legislative decree No. 116 27/01/1992.
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Disclosure Statement

The authors have no conflicts of interest to declare.

Author Contributions

E.B. and C.L. carried out in vivo experiments and wrote the manuscript. C.L. performed microarray experiments; S.T. performed statistical analysis; M.L. and E.B. performed 8-OHdG determinations; L.G. performed Comet assay. L.C. carried out histopathological analysis; M.D. performed gene expression analysis. C.L. conceived and designed the work. All authors read and approved the final version of the manuscript.

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