

ORIGINAL ARTICLE

Chemoprevention of inflammation-related colorectal cancer by silymarin-, acetyl-11-keto-beta-boswellic acid-, curcumin- and maltodextrin-enriched dietetic formulation in animal model

Bruna Girardi^{1,†}, Mariabeatrice Principi^{2,†}, Maria Pricci¹, Floriana Giorgio¹, Andrea Iannone², Giuseppe Losurdo^{2,*}, Enzo Ierardi², Alfredo Di Leo^{2,*} and Michele Barone²

¹THD SpA, Correggio, Reggio Emilia, Italy and ²Gastroenterology Section, Department of Emergency and Organ Transplantation, University of Bari, Bari, Italy

*To whom correspondence should be addressed. Tel: +39 080 5592577; Fax: +39 080 5593088; Email: alfredo.dileo@uniba.it

[†]These authors contributed equally to this work.

Abstract

On the basis of preliminary *in vitro* experience, we assessed whether an enriched nutritional formulation with estrogen receptor (ER)-beta agonist and anti-inflammatory properties may prevent inflammation-associated colorectal cancer (CRC) in an animal model. Study sample enclosed 110 C57BL/6J male mice. Forty underwent dietary supplement safety assessment (20 standard diet and 20 enriched formulation). Seventy were treated with azoxymethane (AOM)/dextran sulfate sodium and divided into two groups: 35 received standard diet and 35 enriched formulation (curcumin, boswellic acids, silymarin and maltodextrins). Miniature colonoscopy demonstrated colitis and solid lesion development in five mice/group 100 days after first AOM injection. Mice were killed after 10 days. In each group, four subgroups received intraperitoneal bromodeoxyuridine (BrdU) injection at 24th/48th/72nd/96th hour before killing. Anti-inflammatory effect and chemoprevention were evaluated by lesion number/size, histological inflammation/dysplasia/neoplasia assessment, pro-inflammatory cytokine messenger RNA (mRNA), ER-beta/ER-alpha/BrdU immunohistochemistry and TUNEL immunofluorescence. Standard formulation assumption was associated with colon shortening compared with enriched one ($P = 0.04$), which reduced solid lesion number and size ($P < 0.001$ for both), histological inflammation score ($P = 0.04$), pro-inflammatory cytokine mRNA expression ($P < 0.001$), number of low-grade dysplasia (LGD; $P = 0.03$) and high-grade dysplasia ($P < 0.001$) areas. CRC was observed in 69.6% in standard and 23.5% in enriched formulation assuming animals ($P < 0.001$). Enriched formulation induced lower ER-alpha expression in CRC ($P < 0.001$) and higher ER-beta expression in LGD ($P < 0.001$) being associated to higher epithelial turnover (BrdU; $P < 0.001$) in normal mucosa and increased apoptosis in LGD and CRC ($P < 0.001$ for both). Our results are promising for a successful anti-inflammatory and chemopreventive effect of enriched formulation in CRC arising from inflamed tissue.

Introduction

Colorectal cancer (CRC) is the final outcome of a multi-step process that, in most cases, goes through the adenoma–carcinoma sequence pathway (1,2). The final evolution into cancer is mainly the consequence of three different pathways:

- Long-standing inflammation of the colon, such as ulcerative colitis (inflammatory model of CRC)
- Congenital genetic alterations leading to adenoma–carcinoma onset, such as familial adenomatous polyposis and Lynch syndrome (inherited model of CRC)
- Sporadic recurrent adenomas (sporadic CRC)

Received: April 20, 2018; Revised: July 19, 2018; Accepted: July 31, 2018

© The Author(s) 2018. Published by Oxford University Press. All rights reserved. For Permissions, please email: journals.permissions@oup.com.

Abbreviations

AOM	azoxy methane
AKBA	acetyl-11-keto-beta-boswellic acid
BrdU	bromodeoxyuridine
CRC	colorectal cancer
DSS	dextran sulfate sodium
ER	estrogen receptor
HGD	high-grade dysplasia
LGD	low-grade dysplasia
LI	labelling index
TUNEL	terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling

Since the discovery of estrogen receptors (ERs) in neoplastic colonic tissue, several epidemiological and clinical studies supported the hypothesis of a protective role of these hormones against the development of colorectal malignancy, thus suggesting their potential use in the prevention of CRC (3–7). ERs show two main forms: ER-alpha and ER-beta. The first mainly drives the estrogen-mediated proliferative activity, whereas the second carries out the opposite effect. Indeed, ER-beta, the prevalent form in the gut, acts as the most suitable mediator of estrogenic antiproliferative action in this site (8–10).

Some substances of plant origin exert an effect in reducing intestinal neoplasia development in animal models through ER-beta agonist effect, such as silymarin, a phytoestrogen compound derived from milk thistle (*Silybum marianum*) (11–14). *Boswellia serrata* is a plant with anti-inflammatory properties. Interestingly, acetyl-11-keto-beta-boswellic acid (AKBA), a component of the gum resin of *B. serrata*, has been recognized as a promising agent for the prevention of intestinal tumorigenesis in a mouse model of inherited carcinogenesis, i.e. adenomatous polyposis coli multiple intestinal neoplasia (min) animals (15,16). Curcumin, derived from the root of *Curcuma longa*, has shown anti-inflammatory and antineoplastic activity by the modulation of several important molecular targets, such as transcription factors, enzymes, cell cycle proteins, cytokines, receptors and adhesion molecules (17,18). Maltodextrins are polymers derived from starch hydrolysis, used as dietary supplement to satisfy the energetic demands of the body under intense exercise. Interestingly, they have been shown to exert an anti-oxidative activity (19).

The hypothesis of this study was that the combinations of phytochemicals may exert beneficial health effects beyond what provided by a single phytochemical (20,21). For this purpose, we preliminarily compared *in vitro* the effect of each component of the nutritional combination with the complete mixture on cultured cancer cell proliferation. Therefore, dietary component amounts were administered *in vivo* below the maximum order of magnitude of the effective concentrations of the single substances in animals. The formulation was based on bioavailability data obtained by the daily intake amount of the components achieving high plasma concentrations and total intestinal absorption (22).

On these bases, we performed this study using a well-standardized animal model in which intestinal carcinoma is induced by a combination of a carcinogen [azoxy methane (AOM)] plus an inflammatory agent [dextran sulfate sodium (DSS)], namely the AOM/DSS model (23–26). The endpoint was to assess whether an enriched nutritional formulation based on the combination of silymarin, AKBA and curcumin (enriched dietary supplement) may exert an inhibitory activity on colonic carcinogenesis (19). In addition, the anti-inflammatory properties of enriched dietary supplement were estimated.

Materials and methods

Animals

For this study, 110 C57BL/6J male mice (age 8 weeks and body weight 22 g) were purchased from Charles River (Calco, Italy). They were kept under controlled conditions of temperature, air and light (from 7 a.m. to 7 p.m.) and received food and water *ad libitum*. Animals did not receive any surgical or hormonal manipulation but were kept anatomically and physiologically intact. All animals received care in compliance with the *Guide for the Care and Use of Laboratory Animals* (27). The study protocol was approved by the local ethics committee for animal experimentation (University of Bari, Italy, no. 6/12).

Dietary features

Of the 110 animals, 40 (20 receiving standard diet and 20 modified diet—enriched formulation, Correggio, Italy) were used for the preliminary assessment of the safety of dietary supplement. The remaining 70 animals (35 receiving standard diet and 35 modified diet—enriched formulation, Correggio, Italy) were used for the evaluation of anti-inflammatory and chemoprevention effect of enriched formulation. The feeding procedures, used in both phases of the study, were as follows:

- Standard diet (Harlan Teklad Rodent diet): 18.5% proteins, 3% oils and fats, 6% fibers, 7% crude ash and 65.5% of non-nitrogenous compounds—wheat, maize, toasted soybean meal, corn gluten feed, wheat straw, fish meal, lucerne meal, mineral dicalcium phosphate, calcium carbonate, sodium chloride, whey powder, soybean oil, yeast and hazelnut skins, poly-vitamin complex (Mucedola srl, Settimo Milanese, Milan, Italy).
- Modified diet (enriched formulation): the standard diet was enriched by a formulation containing silymarin (4 g %), AKBA (3 g %), curcumin (2 g %), maltodextrins (69.553 g %) and excipients (soluble fibres 16.667 g %, citric acid 1 g %, silicon dioxide 1 g %, lignans 0.5 g %, sucralose 0.280 g % and orange flavour 2 g %) and was administered at the cumulative dose of 22.4 mg/100 g of body weight.

The enrichment dietetic formulation was established taking into consideration the following:

- Mice with a body weight of 20–40 g usually eat about 5 g of daily food amount (16.7 g/100 g of body weight) (28).
- Daily intake was 0.892 mg of silymarin, 0.672 mg of AKBA and 0.448 mg of curcumin per 100 g of body weight for animal. In detail, these amounts were administered below the maximum order of magnitude of the effective concentrations of the single substances against cancer in animals (29,30).
- Our supposition was that these substances could exert beneficial effects beyond what provided by the single phytochemical.

Safety test of enriched formulation

The following parameters were monitored within a 24-week period: viability/mortality, body weight, daily assumed food amount and welfare status. At the end of survey period, animals were killed and abdominal organs were examined by two expert pathologists in blind; in addition, the whole colon was histologically evaluated.

Anti-inflammatory and chemoprevention assessment of enriched formulation

As reported, 70 animals underwent AOM/DSS treatment and were divided into two groups: 35 of them were fed a standard diet and 35 by the modified (enriched formulation) diet.

All received the carcinogenic treatment (23–26). In detail, AOM (Sigma-Aldrich Chemie GmbH, Germany) was administered by intraperitoneal injection at the dose of 10 mg/kg of body weight at the 10th week of age and 5 mg/kg at the 12th week. Moreover, DSS (molecular weight 40 000; Sigma-Aldrich Chemie GmbH) was added at the concentration of 2% ad

libitum in drinking water for three cycles of 7 days. The first dose was given one week after the initial AOM injection, the second dose was added in water after the successive AOM administration and the third dose 7 days after the end of second DSS assumption.

Endoscopic procedure

The endoscopic evaluation was performed 100 days after the first AOM injection to verify the development of colitis and solid lesions in five mice per group. The endoscopic observation was performed using a specifically developed high-resolution miniature video endoscopic system (COLOVIEW miniendoscopic system; Karl Storz, Tuttlingen, Germany). Before the examination, animals were fasted for 3 days, and received polyethylene glycol intestinal washing (3 g in 200 ml drinking water). The dosage was obtained adapting the human dose for colonoscopy preparation to mice weight (macrogol 3 g, simethicone 0.29 g, sodium sulphate 0.09 g, sodium bicarbonate 0.07 g and potassium chloride 0.04 g). Then, they were anaesthetized using intraperitoneal injection of tiletamine–zolazepam 250 mg/5 ml water at the dose of 20 mg/kg of body weight plus xylazine 200 mg/1 ml water at the dose of 10 mg/kg of body weight, diluted in saline solution (NaCl 0.9%). The endoscopic procedure was observed on a colour monitor and digitally recorded on a memory card (model 8402 ZX; Karl Storz). In addition, we computed an endoscopic score (range 0–5), which has been validated for murine models of inflammatory cancer (31).

Animal killing and autoptic tissue collection

All mice were killed 110 days after the first AOM injection. All animals received intraperitoneal administration of bromodeoxyuridine (BrdU, 50 mg/kg). In both groups, mice were divided into four subgroups and killed under metaphane anaesthesia at 24th, 48th, 72th or 96th hour after the injection. The colon from each animal was collected, washed and dissected along the longitudinal axis to evaluate both macroscopic and microscopic inflammatory and neoplastic lesions along the whole organ. After the macroscopic evaluation, the colon was fixed in 10% buffered formalin and embedded in paraffin. Colon samples from each animal were frozen at -80°C for molecular biology analysis.

Histological evaluation

Sections (4 μm thick) were sequentially stained with haematoxylin and eosin for histological examination. Inflammation score (range 0–5) was evaluated according to Yu et al. (32). For each animal, the number of low-grade dysplasia (LGD) and high-grade dysplasia (HGD) and CRC areas were recorded and expressed as mean \pm SD/animal. All tissues were examined by two expert pathologists in blind. In detail, dysplasia is defined by the reduction/disappearance of goblet cells and the presence of some major pathological microscopic changes: anisocytosis (cells of unequal size), poikilocytosis (abnormally shaped cells), nuclear hyperchromatism (excessive pigmentation) and the presence of mitotic figures (unusual number of cells that are dividing). Moreover, a glandular architecture derangement could be observed. LGD is indicated by a relatively preserved glandular architecture with a minimal distortion and stratified atypical pencil-shaped nuclei mainly limited to the basal portion of the cells. The degree of cytological and architectural transformation is markedly more advanced in HGD, which is characterized by the complete loss of nuclear polarity. Despite the high risk of neoplastic evolution, differently from CRC, HGD has not yet spread and is isolated within the basal membrane (33).

Real-time polymerase chain reaction assay of cytokine mucosal expression

This method was used to assess the anti-inflammatory effect of enriched formulation on frozen colonic mucosal samples. Therefore, pro-inflammatory cytokine (interferon gamma, interleukin-6 and tumour necrosis factor alpha) gene expression was assessed by real-time polymerase chain reaction (RT-PCR). This assay was performed by quantifying molecular transcription levels, thus estimating the amount of the mRNA codifying for their synthesis expressed by a numerical value represented by the fold-change compared with control. RNA was extracted from five sections of 10 μm , using QIAgen RNA mini kit (QIAGEN GmbH, Germany). Two-step reverse transcription PCR was performed using first-strand complementary DNA with a final concentration of $1\times$ TaqMan gene expression assay, i.e. tumour necrosis factor

alpha and glyceraldehyde 3-phosphate-dehydrogenase (Applied Biosystems, Foster City, CA). The final reaction volume was 25 μl and analysed in triplicate (all experiments were repeated twice). A non-template control (RNAse-free water) was included on every plate. A further validation of our method was performed by enclosing in each assay fresh samples from at least three healthy mouse colonic mucosa. Specific thermal cycler conditions were used by RT-PCR system (Applied Biosystems). A standard curve plus validation experiment were performed for each primer/probe set. A series of six serial dilutions (20 to 0.1 ng/ μl) of tissue cDNA were used as a template.

ER-beta and ER-alpha nuclear expression

ER-beta and ER-alpha nuclear expression was assessed in colonic epithelial cells. For this purpose, an anti-ER-beta monoclonal 'in home-made' chicken (courtesy Prof. J.A.Gustafsson, Houston, TX) and an anti-ER-alpha polyclonal mouse (Santa Cruz, CA) antibody were used. Briefly, after antigen retrieval (shaking conditions in phosphate buffered saline with TWEEN 0.025% followed by enzymatic unmasking—proteolytic enzyme for auto-stainer, Dako, Copenhagen, Denmark) for ER-beta and by microwave irradiation in citric buffer at pH 6.0 for ER-alpha, slides were incubated with primary antibodies overnight at 4°C . Both antibodies were diluted 1:50 with phosphate-buffered saline. After washing in phosphate-buffered saline, samples were incubated for 20 min (two steps) at room temperature with a horseradish peroxidase anti-mouse/anti-chicken polymer-based visualization kit (Biocare Medical, Concord, CA); 3,3'-diaminobenzidine tetrahydrochloride (Vector Laboratories, Burlingame, CA) was used as chromogen, and Harris haematoxylin (Sigma, Italy) for nuclear counterstain. As suggested by the manufacturer, sections from normal large bowel and human breast cancer tissue specimens were used as positive controls for ER-beta and ER-alpha expression, respectively.

Epithelial proliferation evaluation by BrdU immunostaining

BrdU immunostaining was performed using the BrdU Labeling and Detection kit II (Roche Diagnostics, Mannheim, Germany) following the manufacturer's instructions. Immunohistochemical analysis was performed in the normal colon near the neoplastic lesions. For the analysis, 10 well-oriented crypts and 10 randomly fields were selected. DNA synthesis in the cells, their proliferation in the crypts and migration towards mucosal-free surface were evaluated, using the highest labelled cell within the crypt as marker of the percentage of covered axis (34). The analysis was performed in four subgroups in both enriched and standard diet in relation to the time of killing after BrdU injection (i.e. 24, 48, 72 and 96 h).

Apoptosis evaluation by TUNEL immunofluorescence

Apoptosis was studied using the terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) method (In Situ Cell Death Detection Kit; Roche Diagnostics). In brief, after de-waxing, sections were treated with 0.1 M citrate buffer (pH 6.0) cooled to an internal temperature of subboiling in the microwave 350 W for 10 min. Successively, the slides were incubated with TUNEL probe at 37°C for 1 h. After washing, the sections were counterstained with TO-PRO-3 iodide diluted 1:3000 (Invitrogen Molecular Probes). All sections were observed with confocal microscopy magnification.

Immunohistochemical evaluation methods

All sections were observed at $\times 400$ magnification. At least 10 well-oriented crypts were considered for the count of positive cells. Results were expressed as labelling index (LI), i.e. percentage of positive cells. For each animal, the cell count was carried out in the areas of CRC, HGD, LGD and normal tissue. Because of the large size of surgical resection, each sample contained all the four mentioned pictures. The evaluation was performed by two expert observers in a blinded approach.

Statistical analysis

The comparison of continuous variables in the two groups (enriched and standard diet) was performed by Student's t-test for unpaired data. The comparison of ER-alpha, ER-beta and TUNEL expression in normal mucosa, LGD, HGD and CRC, was performed using one-way analysis of variance corrected by Bonferroni's test. Fisher's exact test and chi-squared

test for trend were used for the analysis of categorical data. The level of statistical significance was established at $P < 0.05$ (two tails). The statistical analysis was performed using the statistical software GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA).

Results

Preliminary *in vitro* study

We previously *in vitro* compared the effect of each component of the nutritional combination with the complete mixture on cultured colonic cancer cell proliferation. Each substance (silymarin, AKBA and curcumin) demonstrated a significant antiproliferative effect on colonic cancer cultured cells compared with a control sample. In addition, the antiproliferative effect of the combination of the three substances was significantly higher than single concentration and double composition (Appendix 1, Supplementary Figure 1, available at *Carcinogenesis* Online).

Safety test

In the safety preliminary test, no enriched diet-related adverse event or mortality was observed. No difference between the two groups was found in body weight, daily assumed food amount and welfare status. In detail, no statistically significant difference was observed in total body weight (in grams; mean \pm SD: 32.00 ± 1.41 for enriched and 31.78 ± 1.28 for standard diet group; $P = 0.72$) at the time of killing. Daily food assumption reflected what estimated (16.7 g/100 g of body weight), when the amount of the provisions before and after 24 h was weighted and divided for the number of animals (five) occupying a single cage. At the time of killing, liver weight did not differ between the two groups (g/100 g of body weight: 4.00 ± 1.27 for enriched diet and 4.26 ± 0.6 for standard diet; $P = 0.75$). In Supplementary Figure 2, available at *Carcinogenesis* Online, we reported liver pictures from both groups demonstrating the absence of organ injury.

Anti-inflammatory and chemoprevention assessment of enriched formulation

Macroscopic findings

Supplementary Figure 3, available at *Carcinogenesis* Online, shows endoscopic picture of polypoid lesions in both groups. Moreover, the same illustration demonstrates that the endoscopic score was higher in the standard diet compared with the enriched diet group (3.1 ± 1.2 versus 2.2 ± 1.2 , respectively; $P = 0.03$).

At autopsy examination, in the standard diet group, the total length of the colon was shortened compared with the enriched diet group (82.3 ± 0.4 versus 90.8 ± 0.5 mm, respectively; $P = 0.04$).

As reported in Figure 1A, there was a significant difference in the total number of solid lesions (mean \pm SD/animal) observed

in the colon at the moment of killing (6.0 ± 1.9 in the standard versus 2.1 ± 1.8 in the enriched diet group; $P < 0.001$). In the standard diet group, lesion size was >7 mm in 35/150 (23.3%), 4–7 mm in 70/150 (46.6%) and <3 mm in 45/150 (30.1%) of the total number of solid lesions. In the enriched diet group, the lesion size was >7 mm in 1/53 (1.8%), 4–7 mm in 16/53 (30.3%) and <3 mm in 36/53 (67.9%) of the total number of lesions. In addition, mean size of polyps was higher in the standard than enriched diet group (5.3 ± 1.9 versus 3.8 ± 1.7 mm, respectively; $P < 0.001$).

Microscopic findings

As shown in Figure 1B, there was a significant difference in the histological score of inflammation, evaluated according to Yu et al. (range 0–5), between the two groups (4.4 ± 0.2 points in the standard and 1.8 ± 0.3 points in the enriched diet group; $P = 0.04$).

The mean number of LGD areas per mouse was 3.5 ± 0.8 in the standard and 2.6 ± 0.5 in the enriched diet group ($P = 0.03$). The number of HGD areas was 1.8 ± 0.2 in the standard and 0.3 ± 0.2 in the enriched diet group ($P < 0.001$). The number of CRC areas was 1.8 ± 0.4 in the standard and 0.4 ± 0.2 in the enriched diet group ($P = 0.006$). Results are summarized in Figure 2A–C.

CRC was observed in 23/33 (69.6%) of the standard and 8/34 (23.5%) of the enriched diet group ($P < 0.001$).

Representative pictures of LGD, HGD and CRC are shown in Supplementary Figure 4A, B and C, respectively, available at *Carcinogenesis* Online.

Pro-inflammatory cytokine expression

Pro-inflammatory cytokine mRNA expression was significantly higher in the standard than in the enriched diet group. In detail, interferon gamma showed a value of 6.8 ± 2.3 (standard) versus 3.6 ± 2.8 (enriched diet) fold-change ($P < 0.001$); interleukin-6 showed a value of 14.0 ± 5.4 (standard) versus 4.0 ± 0.4 (enriched diet) fold-change ($P < 0.001$); tumour necrosis factor-alpha showed a value of 27.1 ± 6.7 (standard) versus 5.0 ± 0.4 (enriched diet) fold-change ($P < 0.001$). Such findings are summarized in Supplementary Figure 5, available at *Carcinogenesis* Online, where real-time PCR representative pictures have been reported.

Estrogen receptors

Immunohistochemical ER-alpha and ER-beta expression is reported in Figure 3. For ER-alpha LI values, statistical analysis showed: Normal tissue (N) = LGD < HGD < CRC in the standard ($P < 0.001$; Figure 3A) and N = LGD < HGD = CRC in the enriched diet group ($P < 0.001$; Figure 3B). For ER-beta LI values, statistical analysis showed: N > LGD > HGD = CRC in the standard ($P < 0.001$; Figure 3C) and N = LGD > HGD > CRC in the enriched

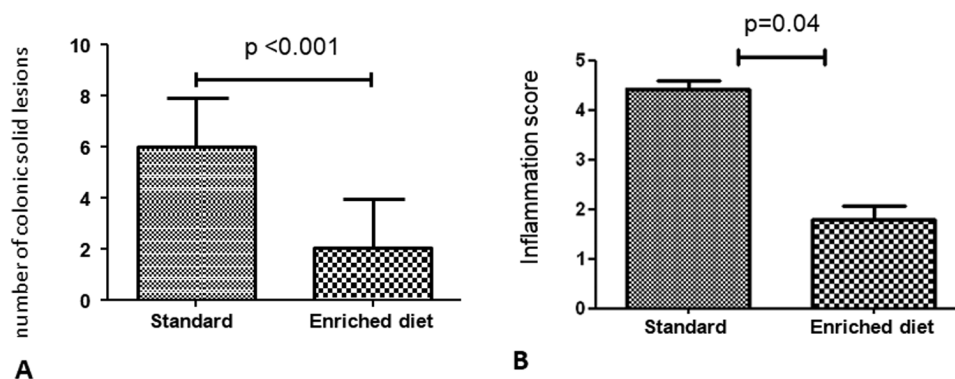


Figure 1. (A) Total number of colonic solid lesions at autopsy (mean \pm SD/animal); (B) histological inflammation score according to Yu et al.

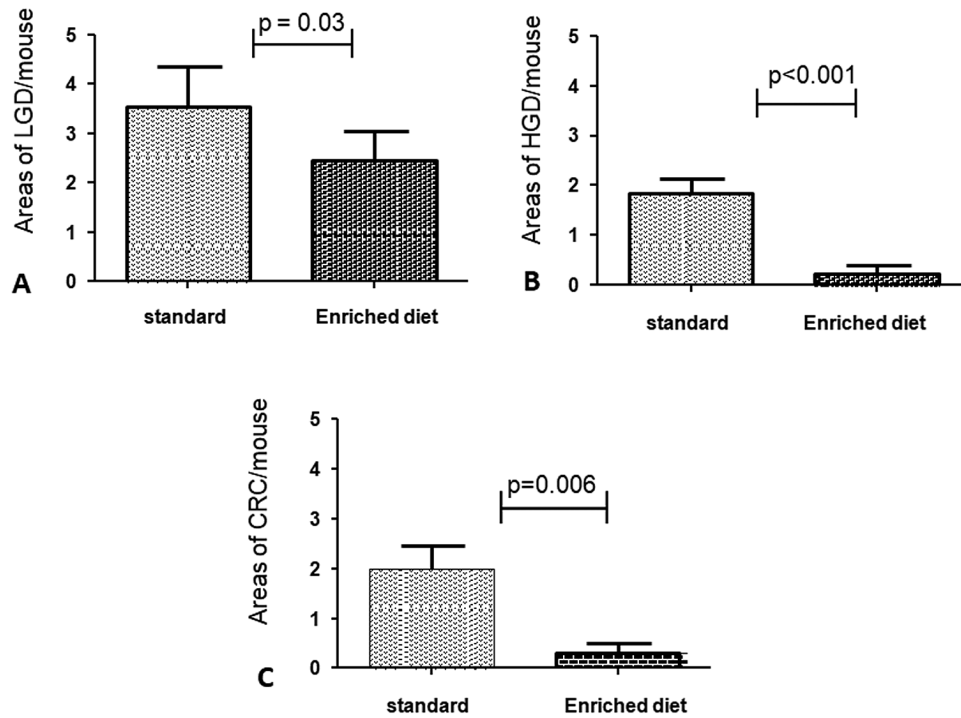


Figure 2. Mean number \pm SD/mouse of areas of microscopic pre-neoplastic and neoplastic lesions. CRC, colorectal carcinoma; HGD, high-grade dysplasia; LGD, low-grade dysplasia.

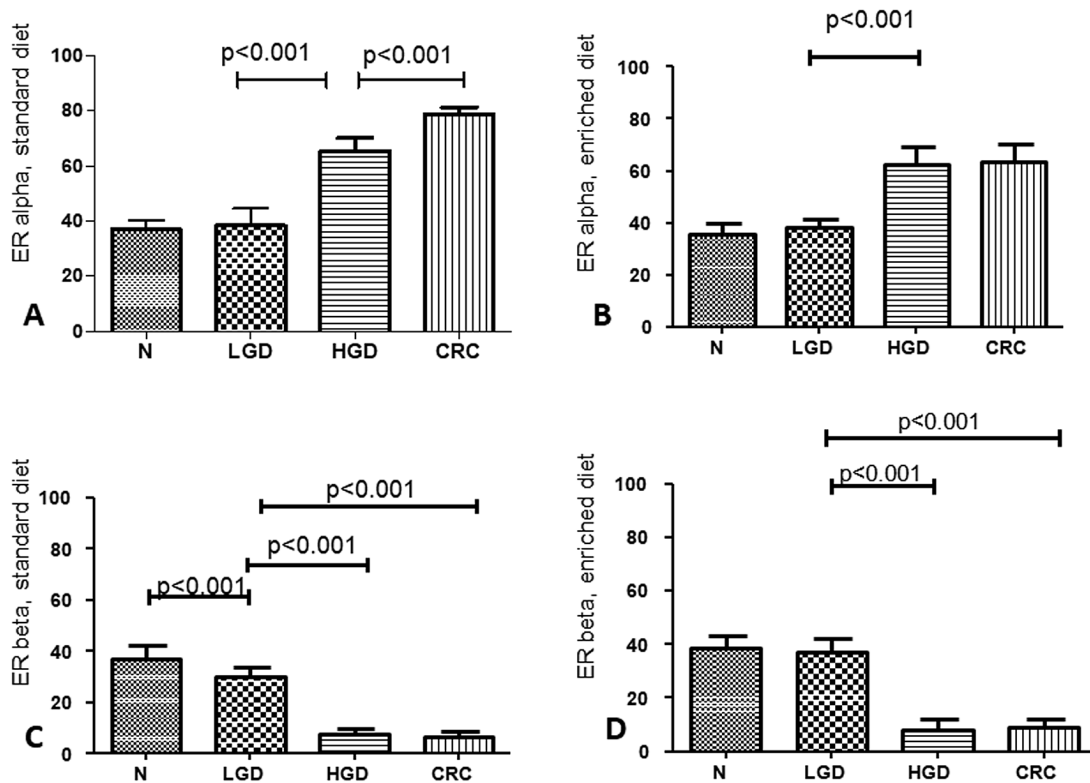


Figure 3. (A) Estrogen receptor (ER)-alpha immunohistochemical expression in the standard group; (B) ER-alpha immunohistochemical expression in the enriched diet group; (C) ER-beta immunohistochemical expression in the standard group; (D) ER-beta immunohistochemical expression in the enriched diet group.

diet group ($P < 0.001$; Figure 3D). Interestingly, enriched dietary supplementation induced a lower ER-alpha expression in CRC ($P < 0.001$, Figure 4A) and a higher ER-beta expression in LGD

areas ($P < 0.001$, Figure 4B) compared with standard diet. Figure 4 clearly shows the different ER-beta immunohistochemical expression in LGD between standard and enriched diet group.

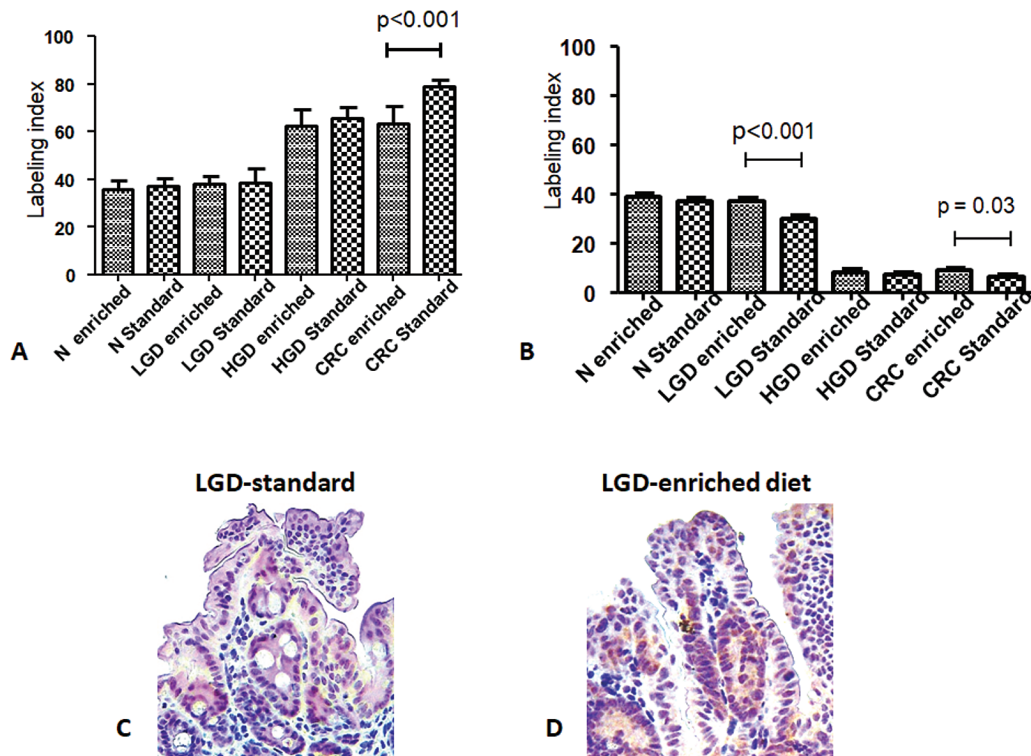


Figure 4. (A) Comparison of estrogen receptor (ER)-alpha immunohistochemical expression between the enriched and standard diet groups; (B) comparison of ER-beta immunohistochemical expression between the enriched- and standard diet groups; immunohistochemical ER-beta staining in areas of low-grade dysplasia in standard (C) and enriched (D) diet group (positive nuclei are stained brownish and negative ones counterstained blue, magnification: $\times 400$).

Epithelial DNA synthesis, proliferation and migration

In both standard and enriched diet groups, we observed in normal mucosa that positive cells were confined in the lower half of the crypts after 24 h, whereas a progression towards the upper half was observed after 48 h. In addition, in the standard but not in the enriched diet group, we found still few positive cells near the free surface at 72nd and 96th h.

Figure 5 illustrates the percentage of crypt axis covered by the highest labelled cell at the 48th hour after injection. This parameter was significantly higher in the enriched than in the standard diet group ($80.6\% \pm 5.1\%$ versus $61.7\% \pm 5.2\%$, respectively; $P < 0.001$).

In LGD areas of the two groups, we found positive cells in both lower and upper half even at 24th hour. The same picture was seen after 48, 72 and 96 h with a decreasing LI.

In HGD and CRC, the complete derangement of the crypt architecture did not allow to evaluate the position of BrdU-positive cells.

Epithelial apoptosis

Immunofluorescent TUNEL expression is reported in Figure 6. Statistical analysis showed: $N < LGD > HGD = CRC$ in the standard ($P < 0.001$) and $N < LGD > HGD > CRC$ in the enriched diet group ($P < 0.001$). Moreover, a significant higher level of epithelial apoptosis was observed in the enriched compared with the standard diet group in LGD and CRC areas.

Discussion

The aim of this study was to assess whether an experimental diet formulation, enriched with silymarin, AKBA and curcumin, could exert a protective role on colonic solid lesion development in a mouse model of colitis-associated CRC. The hypothesis of

this study was that the combinations of phytochemicals may exert beneficial health effects beyond what provided by a single phytochemical (20,21). For this purpose, we preliminarily proved *in vitro* the effect of each component of the nutritional combination with the complete mixture on cultured cancer cell proliferation, demonstrating that the antiproliferative effect of the combination of the three substances was significantly higher than the single concentration and the double composition. We selected the AOM/DSS model because it mimics the stages of colon carcinogenesis, observed in the inflammatory bowel disease-related CRC. This murine model supports the hypothesis that chronic inflammation in inflammatory bowel disease plays a critical role in epithelial malignancy development in the human large bowel (35–39). AOM treatment followed by repeated cycles of DSS is described to result in the onset of chronic inflammation followed by the development of CRC in a very high percentage of treated mice (23–26).

A first relevant result of our study was the finding of a marked anti-inflammatory effect of the modified diet, since enriched diet group showed a reduced inflammatory histological score as well as an impressive decrease of mucosal pro-inflammatory cytokine mRNA expression. Moreover, we observed in standard diet group a significant shortening of the colon presumably as a result of long-term active inflammation and consequent fibrosis, despite this feature was not demonstrated by a specific histological staining. However, this study could not clarify whether the anti-inflammatory effect of enriched diet was due to an interaction with DSS, being both substances orally administered, or to an effect of dietary formulation on inflamed intestinal mucosa. On the other hand, it is possible that anti-inflammatory effect of enriched dietary supplementation could have affected the subsequent development of dysplastic and neoplastic lesions. Indeed, we found colonic

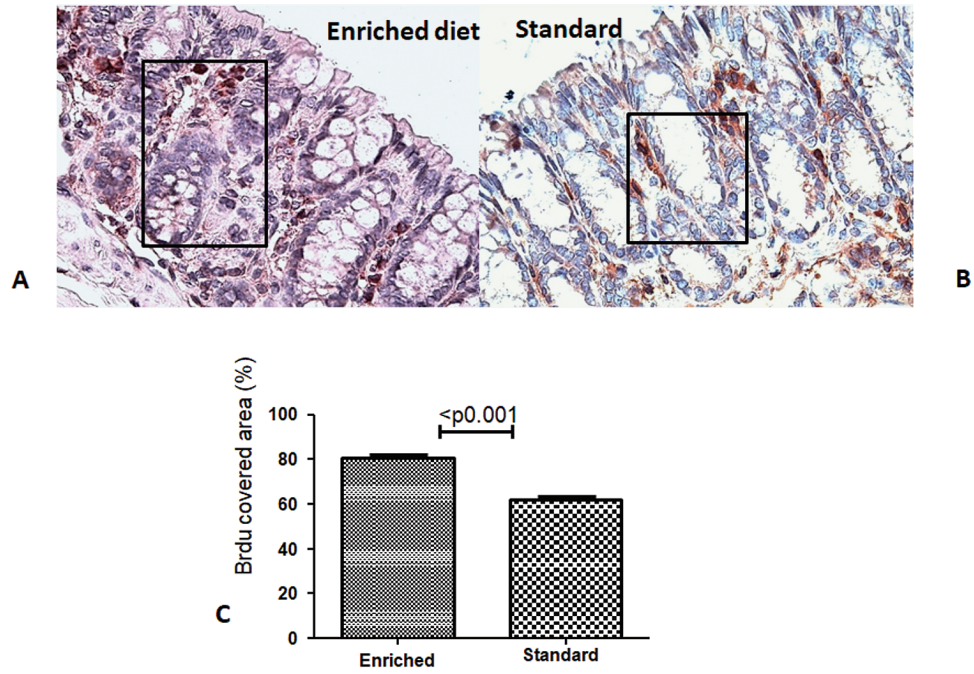


Figure 5. Percentage of crypt axis covered by the highest bromodeoxyuridine (BrdU) labelled cell at the 48th hour after injection in normal colonic mucosa. Positive cells are stained red-brownish and negative ones blue (magnification: $\times 400$).

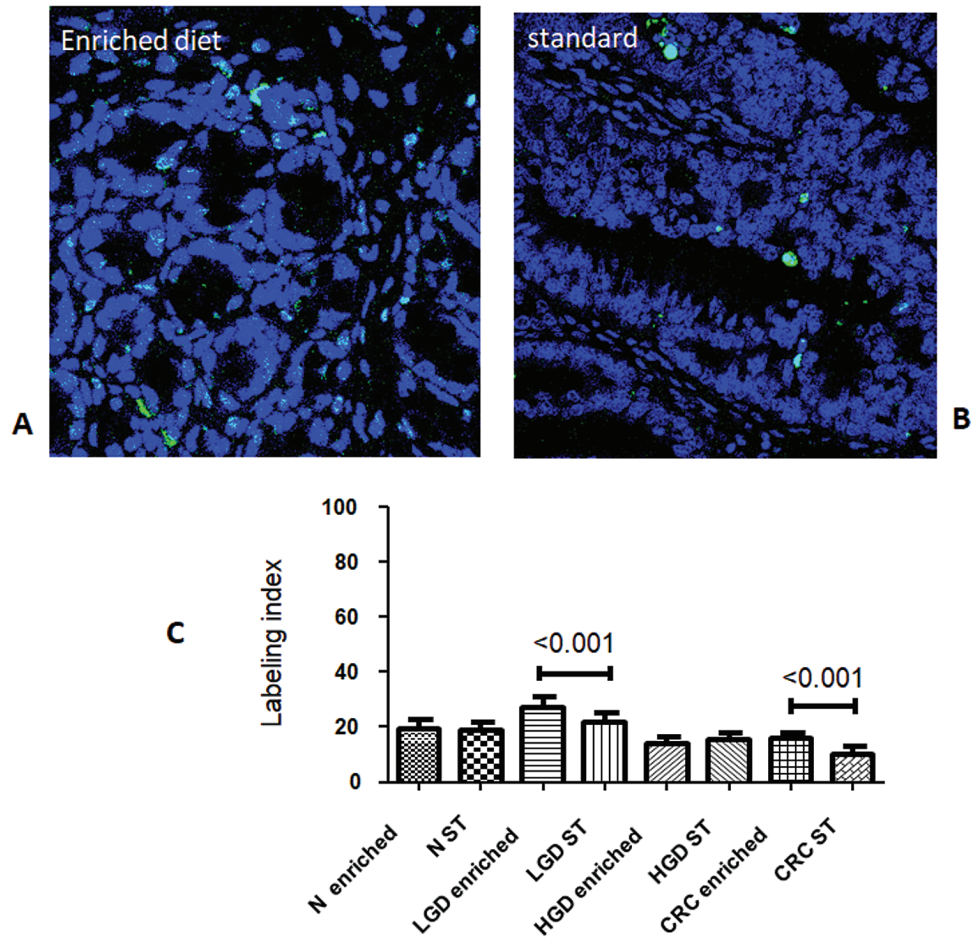


Figure 6. Immunofluorescent TUNEL expression in low-grade dysplasia areas of both standard and enriched diet group. Positive cells are stained green and negative ones blue (magnification: $\times 600$).

carcinomas in 70.1% of the animals receiving a standard diet, whereas the enriched diet group showed a marked decrease in the number, size and volume of all colonic solid lesions. Further, a small percentage (23.1%) developed carcinomas, whereas only dysplastic changes were observed in the remaining animals.

The antineoplastic effect of our dietary treatment was also related to the modulation of ERs. As expected, ER-alpha showed a massive expression in HGD and CRC areas. Moreover, in the same districts, ER-beta demonstrated a progressive decrease with the sequence of carcinogenesis, in accordance with previous evidence from literature in both sporadic and inflammatory bowel-related CRC (40–43). Interestingly, enriched diet group showed a higher expression of ER-beta receptors than the standard diet group in LGD. This result is in agreement with Calabrese et al. (44), although observed in a different model of intestinal carcinogenesis (familial adenomatous polyposis). In this study, an increase in ER-beta immunohistochemical signal was found in polyps showing LGD after a treatment of 3 months with phytoestrogens, anti-inflammatory substances and insoluble indigestible fibres. Therefore, this stage could represent the checkpoint for the neoplastic progression and ER-beta agonist effect of enriched formulation may increase apoptotic process, thus slowing the progression to carcinoma. At this regard, the highest TUNEL expression was observed in LGD areas compared with normal and HGD/CRC districts in both the standard and enriched diet group. Additionally, enriched diet induced a significantly higher expression of both ER-beta and TUNEL in LGD compared with standard one. This finding strongly agrees with what we demonstrated previously, i.e. a co-localization in colonic cells of ER-beta and caspase 3, which represents a well-known early marker of programmed cellular death (45,46).

A further relevant finding of our study concerns the effect of enriched diet on epithelial renewal (i.e. proliferation in crypts and migration to free surface) evaluated by BrdU immunohistochemical staining. In both the standard and enriched diet groups, we observed in normal mucosa that positive cells were confined in the usual site of epithelial proliferation (i.e. the lower half of the crypt) 24 h after injection. In addition, a progression towards the upper half was observed after 48 h. At this time, we found that the percentage of crypt axis covered by the highest labelled cell was significantly higher in the enriched than in the standard diet group. This result may suggest that enriched diet induces an acceleration of epithelial cell migration along the crypts. In agreement with this finding, in the standard but not in the enriched diet group, we found still some positive cells near the free surface at 72nd and 96th hour.

The renewal time of epithelial cells of colorectal tract is estimated 5–7 days (47) and encloses proliferation by stem cells in the lowest part of crypts with successive migration towards upper districts and differentiation until programmed death. Therefore, we may hypothesize that epithelial migration in normal tissue is faster in the enriched compared with the standard diet group and a reduced cellular half-life decreases the time of exposure of DNA synthesizing cells to mutation risk due to both intrinsic and extrinsic factors.

In conclusion, the results of this study appear to be promising for an effective anti-inflammatory and chemopreventive effect of enriched diet in the course of colonic carcinogenesis arising from inflamed tissue (48). Possible pathways of carcinogenesis prevention by enriched diet induced could be

- i. the marked reduction in inflammatory process;
- ii. the increase in ER-beta receptors and apoptosis especially in LGD (the early stage of adenoma–carcinoma sequence);

- iii. the raised epithelial renewal rate associated to a reduced exposure time to genetic mutants.

Supplementary material

Supplementary data are available at *Carcinogenesis* online.

Funding

THD SpA, Correggio, Reggio Emilia, Italy.

Conflict of Interest Statement: A.D.L. is an advisory board member of THD SpA. F.G., B.G. and M.P. are employees of THD SpA. All the other authors declare no financial support or conflict of interest.

References

1. Tomlinson, I. et al. (1997) Molecular genetics of colon cancer. *Cancer Metastasis Rev.*, 16, 67–79.
2. Iannone, A. et al. (2016) Stool investigations for colorectal cancer screening: from Occult Blood Test to DNA Analysis. *J. Gastrointest. Cancer*, 47, 143–151.
3. Williams, C. et al. (2016) Estrogen receptor beta as target for colorectal cancer prevention. *Cancer Lett.*, 372, 48–56.
4. Niv, Y. (2015) Estrogen receptor β expression and colorectal cancer: a systematic review and meta-analysis. *Eur. J. Gastroenterol. Hepatol.*, 27, 1438–1442.
5. Barzi, A. et al. (2013) Molecular pathways: estrogen pathway in colorectal cancer. *Clin. Cancer Res.*, 19, 5842–5848.
6. Lechner, D. et al. (2005) Phytoestrogens and colorectal cancer prevention. *Vitam. Horm.*, 70, 169–198.
7. Principi, M. et al. (2015) Prevention of colorectal adenomas. *Colorectal Dis.*, 17(suppl. 1), 20–24.
8. Mosselman, S. et al. (1996) ER beta: identification and characterization of a novel human estrogen receptor. *FEBS Lett.*, 392, 49–53.
9. Bardin, A. et al. (2004) Loss of ERbeta expression as a common step in estrogen-dependent tumor progression. *Endocr. Relat. Cancer*, 11, 537–551.
10. Barone, M. et al. (2008) Estrogens, phytoestrogens and colorectal neoproliferative lesions. *Genes Nutr.*, 3, 7–13.
11. Barone, M. et al. (2012) Dietary, endocrine, and metabolic factors in the development of colorectal cancer. *J. Gastrointest. Cancer*, 43, 13–19.
12. Barone, M. et al. (2014) Olive oil and omega-3 polyunsaturated fatty acids suppress intestinal polyp growth by modulating the apoptotic process in ApcMin/+ mice. *Carcinogenesis*, 35, 1613–1619.
13. Seidlová-Wuttke, D. et al. (2003) Silymarin is a selective estrogen receptor beta (ERbeta) agonist and has estrogenic effects in the metaphysis of the femur but no or antiestrogenic effects in the uterus of ovariectomized (ovx) rats. *J. Steroid Biochem. Mol. Biol.*, 86, 179–188.
14. Barone, M. et al. (2010) Dietary-induced ERbeta upregulation counteracts intestinal neoplasia development in intact male ApcMin/+ mice. *Carcinogenesis*, 31, 269–274.
15. Liu, H.P. et al. (2013) Chemoprevention of intestinal adenomatous polyposis by acetyl-11-keto-beta-boswellic acid in APC(Min/+) mice. *Int. J. Cancer*, 132, 2667–2681.
16. Wang, R. et al. (2014) The comparative study of acetyl-11-keto-beta-boswellic acid (AKBA) and aspirin in the prevention of intestinal adenomatous polyposis in APC(Min/+) mice. *Drug Discov. Ther.*, 8, 25–32.
17. Shishodia, S. et al. (2005) Curcumin: getting back to the roots. *Ann. N. Y. Acad. Sci.*, 1056, 206–217.
18. Imran, M. et al. (2016). Curcumin, anticancer and antitumor perspectives: a comprehensive review. *Crit. Rev. Food Sci. Nutr.*, 2016, 22, 1–23.
19. Rossi, R.E. et al. (2016) The role of dietary supplements in inflammatory bowel disease: a systematic review. *Eur. J. Gastroenterol. Hepatol.*, 28, 1357–1364.
20. Pesakhov, S. et al. (2010) Distinct combinatorial effects of the plant polyphenols curcumin, carnosic acid, and silibinin on proliferation and apoptosis in acute myeloid leukemia cells. *Nutr. Cancer*, 62, 811–824.

21. Cheung, K.L. et al. (2009) Synergistic effect of combination of phenethyl isothiocyanate and sulforaphane or curcumin and sulforaphane in the inhibition of inflammation. *Pharm. Res.*, 26, 224–231.
22. Kidd, P.M. (2009) Bioavailability and activity of phytosome complexes from botanical polyphenols: the silymarin, curcumin, green tea, and grape seed extracts. *Altern. Med. Rev.*, 14, 226–246.
23. Ikeda, I. et al. (2007) 5-aminosalicylic acid given in the remission stage of colitis suppresses colitis-associated cancer in a mouse colitis model. *Clin. Cancer Res.*, 13, 6527–6531.
24. Stolfi, C. et al. (2011) Involvement of interleukin-21 in the regulation of colitis-associated colon cancer. *J. Exp. Med.*, 208, 2279–2290.
25. Tanaka, T. et al. (2003) A novel inflammation-related mouse colon carcinogenesis model induced by azoxymethane and dextran sodium sulfate. *Cancer Sci.*, 94, 965–973.
26. Rosenberg, D.W. et al. (2009) Mouse models for the study of colon carcinogenesis. *Carcinogenesis*, 30, 183–196.
27. National Research Council (US) Committee for the Update of the Guide for the Care and Use of Laboratory Animals (2011) *Guide for the Care and Use of Laboratory Animals*, 8th edn. National Academies Press, Washington, DC.
28. Italian Association for Laboratory Animal Science (November 2012): <https://www.aisal.org/corsi-aisal/corso-di-formazione-in-scienza-degli-animali-da-laboratorio/> (7 July 2018, date last accessed).
29. Zaidi, S.N.F. et al. (2017) Prevention of liver cirrhosis by Silymarin. *Pak. J. Pharm. Sci.*, 30, 1203–1211.
30. Hatcher, H. et al. (2008) Curcumin: from ancient medicine to current clinical trials. *Cell. Mol. Life Sci.*, 65, 1631–1652.
31. Becker, C. et al. (2005) *In vivo* imaging of colitis and colon cancer development in mice using high resolution chromoendoscopy. *Gut*, 54, 950–954.
32. Yu, C. et al. (2014) Rac1 signaling regulates neutrophil-dependent tissue damage in experimental colitis. *Eur. J. Pharmacol.*, 741, 90–96.
33. Levin, B. et al.; American Cancer Society Colorectal Cancer Advisory Group; US Multi-Society Task Force; American College of Radiology Colon Cancer Committee. (2008) Screening and surveillance for the early detection of colorectal cancer and adenomatous polyps, 2008: a joint guideline from the American Cancer Society, the US Multi-Society Task Force on Colorectal Cancer, and the American College of Radiology. *Gastroenterology*, 134, 1570–1595.
34. Javid, S.H. et al. (2005) Modulation of tumor formation and intestinal cell migration by estrogens in the *Apc(Min/+)* mouse model of colorectal cancer. *Carcinogenesis*, 26, 587–595.
35. Dulai, P.S. et al. (2016) Colorectal cancer and dysplasia in Inflammatory Bowel Disease: a review of disease epidemiology, pathophysiology, and management. *Cancer Prev. Res. (Phila.)*, 9, 887–894.
36. Axelrad, J.E. et al. (2016) Inflammatory bowel disease and cancer: the role of inflammation, immunosuppression, and cancer treatment. *World J. Gastroenterol.*, 22, 4794–4801.
37. Fries, W. et al. (2017). Disease patterns in late-onset ulcerative colitis: results from the IG-IBD “AGED study”. *Dig. Liver Dis.*, 49, 17–23.
38. Ierardi, E. et al. (2001) Epithelial proliferation and ras p21 oncoprotein expression in rectal mucosa of patients with ulcerative colitis. *Dig. Dis. Sci.*, 46, 1083–1087.
39. Biancone, L. et al.; Italian Group for the study of Inflammatory Bowel Disease. (2016) Inflammatory Bowel Disease phenotype as risk factor for cancer in a prospective multicentre nested case-control IG-IBD study. *J. Crohns. Colitis*, 10, 913–924.
40. Principi, M. et al. (2012) Estrogen receptors expression in long-lasting ulcerative pancolitis with and without dysplasia: a preliminary report. *Scand. J. Gastroenterol.*, 47, 1253–1254.
41. Principi, M. et al. (2015) The sharp decline of beta estrogen receptors expression in long-lasting ulcerative-associated carcinoma. *Scand. J. Gastroenterol.*, 50, 1002–1010.
42. Di Leo, A. et al. (2008) ER-beta expression in large bowel adenomas: implications in colon carcinogenesis. *Dig. Liver Dis.*, 40, 260–266.
43. Barone, M. et al. (2010) ERβ expression in normal, adenomatous and carcinomatous tissues of patients with familial adenomatous polyposis. *Scand. J. Gastroenterol.*, 45, 1320–1328.
44. Calabrese, C. et al. (2016) Can supplementation of phytoestrogens/insoluble fibers help the management of duodenal polyps in familial adenomatous polyposis? *Carcinogenesis*, 37, 600–606.
45. Principi, M. et al. (2013) Phytoestrogens/insoluble fibers and colonic estrogen receptor β: randomized, double-blind, placebo-controlled study. *World J. Gastroenterol.*, 19, 4325–4333.
46. Di Leo, A. et al. (2016) Epithelial turnover in duodenal familial adenomatous polyposis: a possible role for estrogen receptors? *World J. Gastroenterol.*, 22, 3202–3211.
47. Parris, A. et al. (2015) A human colonic crypt culture system to study regulation of stem cell-driven tissue renewal and physiological function. *Methods Mol. Biol.*, 1212, 141–161.
48. Principi, M. et al. (2014) Ulcerative colitis: from inflammation to cancer. Do estrogen receptors have a role? *World J. Gastroenterol.*, 20, 11496–11504.