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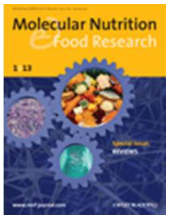

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Research Article

Pomegranate by-products in colorectal cancer chemoprevention: effects in *Apc*-mutated Pirc rats and mechanistic studies *in vitro* and *ex vivo*

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Key words: butyrate; colon carcinogenesis; MDF; pomegranate mesocarp; urolithin

Abbreviations: CRC, colorectal cancer; **MDF,** Multiple Depleted Foci; **PMD,** Pomegranate Mesocarp Decoction; **u-A,** urolithin-A; **SB,** sodium butyrate; **NM,** normal mucosa; **AD,** adenoma

Abstract

Scope: To investigate the effect of pomegranate mesocarp, a polyphenol-rich by-product of juice production, in colorectal cancer (CRC) chemoprevention.

Methods and Results: A mesocarp decoction (PMD) was administered for 6 weeks in the diet to Pirc rats, mutated in *Apc,* a key-gene in CRC. Multiple Depleted Foci (MDFs), as CRC biomarkers, were reduced in PMD-fed rats compared to Controls (MDF/colon: 34±4 vs 47±3, $P<0.05$). Apoptosis in MDFs from PMD-treated rats was increased compared to Controls (2.5±0.2 vs 1.6±0.2, $P<0.01$). To elucidate the involved mechanisms, two colon-relevant metabolites of the polyphenolic and fiber PMD components, urolithin-A (u-A) and sodium butyrate (SB), were tested alone or in combination *in vitro* (HT-29 cells), and *ex vivo* in adenoma (AD) and normal mucosa (NM) from Pirc rats. U-A 25 µM plus SB 2.5 mM (USB) caused a significant reduction in COX-2 expression compared to untreated controls (-74.5%, -76.5%, -69.02% in HT-29, AD, NM). USB also caused a strong increase in C-CASP-3 expression in HT-29 cells (10 times), in AD and NM (+74% and +69%).

Conclusion: These data indicate a chemiopreventive activity of PMD due, at least in part, to pro-apoptotic and anti-inflammatory action of its metabolites, that could be exploited in high-risk patients.

1 Introduction

Considering the significant impact of cancer in terms of human lives and economic cost (US$ 1.16 trillion in 2010, World Cancer Report 2014, http://www.who.int/mediacentre/factsheets/fs297/en/), there is a continuous interest in searching new mechanisms to be targeted, and in implementing preventive strategies such as chemoprevention, that is "*the use of natural, synthetic or biologic agents, able to delay, reverse or inhibit tumor progression"* [1]. Colorectal cancer (CRC), the second leading cause of cancer death in Europe (IARC, GLOBOCAN 2012 http://gco.iarc.fr/today/home), is strongly affected by lifestyle, particularly nutritional habits. CRC develops through a sequential multistep progression of epithelial cells initiated to a cancerous state with defined pre-cancerous intermediaries. Numerous trials document the ability of Non-Steroidal Anti Inflammatory Drugs (NSAIDs) to prevent CRC, but the chronic use of these drugs increases the risk of serious cardiovascular events, so that alternative strategies are needed [2, 3]. In the last thirty years, several studies focused on the effects of polyphenols from various sources (i.e. curcumin, resveratrol, green tea) on colorectal carcinogenesis *in vitro, in vivo*, and in some clinical trials, testing their synergistic effects in association with chemotherapeutic treatments or their preventive activity in subjects at high CRC risk [4] (https://clinicaltrials.gov/). Among fruits with a high polyphenolic content is pomegranate (*Punica granatum),* endowed with anti-oxidative and anti-inflammatory properties in *in vitro* studies, as well as anticancer activity *in vitro* and *in vivo* [5] [6] [7]. Pomegranate juice is commonly produced from arils or from the whole pressed fruit also containing peel and mesocarp [8]. The predominant phenolic component of pomegranate belongs to the ellagitannin family (ETs), among which punicalagin, showing pronounced anti-proliferative and anti-inflammatory activities in addition to anti-oxidative effects, is peculiar of this fruit [9] [10]. Several studies reported that total phenolic compounds (TPC) and punicalagin are more abundant in pomegranate mesocarp and peel than in arils and seeds, used for industrial juice production [11]. Beside polyphenols, the polysaccharide component, also present in mesocarp, has been suggested to exert anticancer activity [12] [13]. We recently demonstrated that the polysaccharide component of Wonderful pomegranate has prebiotic properties [14], which have in turn been associated with beneficial effects against colon carcinogenesis [15].

Taking into account these studies and the recent encouragement to the implementation of new approaches for *the efficient use of the huge biomass into a spectrum of bio-based products,* a concept named "biorefining" *(Sustainable development goals 09/2015, UN; Bioenergy task 42, IEA.* https://sustainabledevelopment.un.org/?menu=1300*,* http://www.iea-bioenergy.task42-biorefineries.com/en/ieabiorefinery.htm*)*, the use of fruit (including pomegranate) by-products in cosmetics and food industries is rising [16]. On the other hand, there are no data about the use of these by-products in medical preparations for therapeutic or preventive purposes.

The present study has been conceived to investigate the possibility of employing a pomegranate mesocarp decoction (PMD), characterized in polysaccharide and ellagitannin content, for CRC prevention. After its characterization, PMD was tested in a short-term (six weeks) *in vivo* experiment on Pirc male rats. Pirc rats are a genetic model of CRC bearing an heterozygote mutation in the *Apc* gene, that predisposes to spontaneous development of colon polyps as in Familial Adenomatous Polyposis (FAP) individuals [17]. Pirc rats also present microscopic pre-neoplastic lesions in the colon, called Multiple Depleted Foci (MDF), which represent the early step in the development of CRC and can thus be used as tumor biomarkers in chemoprevention studies [18] [19]. Moreover, to investigate the mechanisms involved in the effects observed *in vivo* at the level of early carcinogenesis phases, and to assess the capacity of PMD to influence carcinogenesis at more advanced phases, we studied *in vitro* (HT-29 colon cancer cells) and *ex vivo (*biopsies of normal mucosa (NM) and adenoma (AD) from Pirc rats), two main metabolites of PMD: urolithin-A (u-A) and sodium butyrate (SB). U-A is the main punicalagin metabolite in the colon [20] [21]; sodium butyrate (SB), one of the main short chain fatty acid (SCFA) produced by fermentation of the soluble fiber component in the colon, has anticancer activity *in vitro* and *in vivo* [22] [23].

2 Materials and Methods

Chemical reagents

Gentamicin, Hank's Balanced Salt Solution (HBSS), DTT, Thiazolyl Blue Tetrazolium Bromide (MTT), RIPA buffer and protease/phosphatase inhibitors were purchased from Sigma Aldrich (Milan, Italy). α+β punicalagins and ellagic acid were purchased from Merck. AIN76 diet components were purchased from

Piccioni (Milan, Italy). NucleoSpin® TriPrep kit for RNA extraction was purchased from Machery-Nagel GmbH & Co. KG (Duren, Germany). DMEM and penicillin-streptomycin solution were purchased from PAN Biotech (Aidenbach, Germany). Fetal Bovine Serum (FBS) was purchased from GIBCO, BRL (Rodano-Milan, Italy). Urolithin-A was purchased from Toronto Research Chemicals (Toronto, Canada). Sodium butyrate was purchased from Merck-Millipore (Vimodrone-Milan, Italy). MTS solution was purchased from Promega (Gessate, Italy). Protein assay was performed using DC Protein Assay kit purchased from Bio-Rad (Segrate-Milan, Italy).

2.1 Pomegranate Mesocarp Decoction preparation and characterization

Pomegranate ripe fruits of Wonderful variety cultivated in Puglia, Italy (harvested in 2015), were used to recover the mesocarp. A total amount of 18 kg of fresh fruits were manually treated to separate the mesocarp from the other parts of the fruit. The mesocarp (80% moisture) was then used to prepare a decoction through boiling in water for 1h (extractive ratio 1:40 w dried mesocarp/v). The sample was cooled, filtered and the solution freeze-dried, then ground and used as powder (PMD). The final yield was 75% of dried mesocarp. The total fat content was extracted by Soxhlet, and gravimetrically determined according to ISS protocol (1996/34); the protein content (PC) was evaluated by Kjeldhal method: PC (g/100g) = N*6.25, where N is total nitrogen. Lastly, dietary fiber analysis (both soluble and insoluble) was done according to AOAC method 991.43 (Determination of soluble, insoluble and total dietary fiber in foods and food products, final approval 1991).

The HPLC-DAD analysis was carried out using a Sinergi Fusion 150mm×2 mm i.d., 4µm, RP-18, column from Phenomenex (Bologna, Italy), with a flow rate of 0.2 mL/min. The mobile phase was constituted by A, acidified water by HCOOH (0.1% v/v) and B, acetonitrile. The linear multistep solvent gradient started from 95% A and reached 75 % A in 4 min, followed by a 4 min plateau; the next step was 6 min to reach 65 % A, and finally 2 min to reach 10 % A and 90% B, with a ri-equilibration time of 10 min and a total time of analysis of 26 min. The ellagitannins determination was performed using as external standards α+β punicalagins (at 380 nm, linearity range between 0.5-8 µg) and ellagic acid (at 370 nm, linearity range of 0.031–1.25 µg) obtaining calibration curves with R^2 0.998 and R^2 0.9995 respectively.

2.2 Animals and treatments

Pirc (F344/NTac-Apc^{am1137}) and wild type (*wt*) Fisher F344/NTac rats, originally obtained from Taconic (Taconic Farms, Hudson, NY, USA), were maintained in polyethylene cages and bred in CESAL (University of Florence, Italy) in accordance with the Commission for Animal Experimentation of the Italian Ministry of Health (Authorization number 323/2016-PR). The colony was maintained by mating heterozygote Pirc rats with *wt* rats and pups genotypized at 3 weeks of age [17]. To study the chemopreventive effect of PMD, male Pirc rats, aged 4 weeks, were randomly assigned to two groups: controls (n=10), fed with standard AIN76 diet, and PMD-treated (n=11) fed with the same AIN76 diet supplemented with 10000 ppm of PMD, corresponding, on the basis of the polyphenolic content of PMD, to a dose of 50 mg/kg/die of total polyphenolic compounds. Rats were sacrificed by CO_2 asphyxia after 6 weeks of treatment, in line with the experimental protocol approved by the Commission for Animal Experimentation of the Italian Ministry of Health.

2.3 Processing of colon, sample collection and determination of Multiple Depleted Foci (MDF)

At sacrifice, the entire intestine (from pylorus to anus) was dissected, flushed with cold saline and longitudinally opened. The apparently Normal Mucosa (NM) from the proximal portion of the colon was scraped and stored at - 80°C in RNAlater™ (RNA stabilization Reagent, Qiagen) for RNA and protein analyses. A small sample of NM from the medial portion (about 9 mm^2) was collected and fixed in 10% formalin solution to assess proliferative and apoptotic activity. The remaining colon and rectum were processed to determine the presence of MDF (number of MDF/colon) and their multiplicity (number of crypts/MDF) [18]. After the enumeration of MDFs, these were marked with permanent ink, dissected under microscope and then embedded in paraffin in such a way that crypts could be sectioned longitudinally (4 μm thick).

2.4 Cell proliferation and apoptosis in NM and MDF

To assess the proliferative activity in the morphologically normal mucosa (NM), Proliferating Cell Nuclear Antigen (PCNA) immunoreactivity was determined using a mouse monoclonal antibody (PC-10, Santa Cruz, CA, USA) at 1:1000 dilution. Proliferative activity was expressed as labelling index (LI): number of cells

positive to PCNA/cells scored x 100, evaluated in at least 15 full longitudinally sectioned crypts of the NM. Histological sections of the NM (4 μm thick) were also stained with hematoxylin-eosin to determine the number of apoptotic cells in at least 15 full entire longitudinally sectioned crypts, according to previous published procedures [24]. Apoptosis was also evaluated in histological sections of MDFs dissected as previously described [25].

2.5 Semi-quantitative RT-PCR

Gene expression was evaluated in the NM, taken at the sacrifice as described above. Total RNA extraction, reverse-transcription of 1 μg of total RNA and subsequent PCRs were performed as previously described [26]. For each target gene, the relative amount of mRNA in the samples was calculated as the ratio of each gene to β-actin mRNA (primers used are shown in Tab.1).

2.6 Western Blotting

Protein expression was evaluated in samples from *in vitro, in vivo* and *ex vivo* experiments. For HT-29 cells, 40 μL of RIPA-buffer supplemented with 1% protease inhibitors and 1% phosphatase inhibitors were added to each well, and the obtained protein solution was sonicated for 15''and centrifuged for 1' at 14000 rpm and 4°C. For colon samples, scraped NM in the *in vivo* experiments and *ex vivo* samples of adenoma and normal mucosa were homogenized in RIPA-buffer in the proportion of 8 μL/mg tissue for not more than 2', sonicated and centrifuged as above. Each supernatant was collected and the protein content was measured. For western blotting, 40 μg of protein extracts were used for each experimental point. Electrophoretic running, immunostaining, band acquisition and quantification were performed as previously described [27]. Each measured density was normalized by using the corresponding GAPDH density value.

The antibodies used were: COX-2 (160126 rabbit, Cayman Chemical), 1:200; PCNA (PC10: sc-56 mouse Santa Cruz Biotechnology, INC), 1:1000; C-CASP-3 (Asp175 Rabbit Cell Signaling), 1:1000; NOS2 (N-20 rabbit Santa Cruz Biotechnology, INC) 1:500; BAK (rabbit Anti-BAK, NT Millipore) 1:500; GAPDH (14C10 Rabbit mAb Cell Signaling) 1:3000; anti-rabbit IgG antibody (Cell Signaling), 1:4000 and anti-mouse IgG (Chemicon, Temecula, CA), 1:5000.

2.7 HT-29 cells culture and treatments

HT-29 cells (provided from ATCC) were grown in high glucose Dulbecco's Modified Eagle's Medium supplemented with 10% of fetal bovine serum, Pen-Strep (penicillin 10000 U/mL and streptomycin 10 mg/mL), incubated at 37°C in a cell incubator (5% CO_2) and used during the linear phase of growth. For performing experimental treatments, cells were plated in 12-MW (130000 cells/well) and treated starting on the following day with DMEM (control treatment) or: a) urolithin-A (3,8-diydroxybenzo(c)chromen-6-one, u-A) 0.1, 1, 10, 25, 50 and 100 µM; b) sodium butyrate (SB) 50 µM, 100 µM, 500 µM, 1 mM, 5 mM, 10 mM; c) u-A 25 µM plus SB (USB) 500 µM, 1 mM and 5 mM. Treatments lasted 24h or 72h at 37°C in a humidified atmosphere.

2.8 MTS viability assay on HT-29 cells

Cell proliferation assays were performed in 96-MW (8000 cells/well) after 72h treatments. At the end of this time, media were removed, cell monolayers were washed two times with 1 X PBS, and finally 100 µL/well of DMEM with 5% FBS and 20 µL of ready-to-use MTS solution were added. The measurement of absorbance at 490 nm was performed after 90' incubation at 37°C in 5% CO_2 humidified atmosphere.

2.9 *Ex vivo* short-term cultures and treatment

Pirc rats (n=4) at 8-months of age were sacrificed as described above. The colon was rinsed two times with PBS supplemented with Pen-Strep (penicillin 10000 U/mL and streptomycin 10 mg/mL) and gentamycin (10 µg/mL), then opened to collect samples of adenoma (AD) and apparently normal mucosa (NM). All samples were transferred in 1.5 mL tubes containing 40 mM DTT in HBSS, rinsed with HBSS supplemented with Pen-Strep and gentamycin 50 µg/mL (HBSS/PSG), and dissected under microscope. ADs and NMs were dissected into approximatively equal parts (weight range: 10-15 mg). Samples were then transferred in 48-MW containing DMEM (control) or DMEM containing u-A 25 µM plus SB 2.5 mM (USB) and maintained for 24 h in cell incubator at 37°C and 5% CO_2. All these procedures were performed within 1 h from sacrifice.

Cell viability of AD and NM samples was assessed in separate samples performing the MTT test at time zero (T_0) and after 24 h (T_{24}). Briefly, samples dissected under microscope were transferred in 2 mL tubes containing HBSS/PSG. Then, T_0 samples were transferred in 48-MW containing 200 µL of DMEM without

red-phenol supplemented with 1% FBS, Pen-Strep, 50 µg/mL gentamycin and 1 mg/mL of MTT and incubated for 3 h at 37°C in cell incubator. At the end of the incubation, samples were transferred in 2 mL tubes containing acidified isopropanol (isopropanol plus HCl 4 mM and 0.1% Nonidet-P40), weighted, and incubated at 37°C with shaking for 3 h to extract MTT. At the end of the extraction, they were centrifuged for 5' at 4°C and 1000 rpm. The T_{24} samples after dissection were transferred into 48-MW containing DMEM, incubated for 24 h and then subjected to the MTT test as described for the T_0 counterparts. For all samples, 200 µL of each supernatant were then transferred in 96-MW for measuring MTT absorbance at 570 nm in a plate reader spectrophotometer. All measures were performed in duplicate and the absorbance values (A) were normalized to the weight of the sample after MTT incubation (A/mg) [28]. Finally, the viability at 24 h was expressed as % of the corresponding T_0 counterpart.

2.10 Statistics

Differences between PMD group and controls in MDFs, immunological and morphological indexes and RT-PCR data were analyzed with t-test for unpaired samples. Data from MTS test and western blotting assay *in vitro* were analyzed by one-way ANOVA followed by Bonferroni's multiple range test, with GraphPad Prism 5.0 (GraphPad Software) as appropriate. Western blotting data from the *ex vivo* experiments were subjected to two-way ANOVA to take into account the effect of both treatment and tissue type (AD or NM).

3 Results

3.1 Composition of PMD

The proximate composition of the dried decoction resulted to be: proteins 1.9%, total sugar 0.4%, dietary fibers about 11% (soluble 10.9%, insoluble < 1%); fat was absent, as expected. The decoction contained also ellagitannins (151.47±3.42 mg/g) with a prevalence of $\alpha+\beta$ punicalagines (67.5±1.24 mg/g) and a minor content of ellagic acid and derivatives (21.7±0.35 mg/g).

3.2 Effect of PMD on colon tumorigenesis in Pirc rats

The mean weight of the rats at the beginning of the treatment (4 weeks of age) was 60 ± 3 g (means ± SE, n=21). At sacrifice, when the animals were 10 week-old, the mean weight was similar between controls and

PMD group (216 ± 11 g in controls (n= 10) vs 217 ± 6 g in PMD treated (n = 11), means ± SE), with no apparent signs of toxicity of the treatment.

The determination of pre-neoplastic lesions MDF in the two experimental groups showed that the number of MDF was significantly reduced in PMD-treated rats compared to controls (Fig. 1, panel a). Moreover, PMD-treated rats showed MDF with a lower multiplicity when compared to those in the control rats (Fig. 1, panel b).

3.3 Proliferation, apoptosis and inflammation in the colon mucosa of Pirc rats

To understand the molecular mechanisms of action underlying the protective effect of PMD, we determined the expression of inflammatory proteins, apoptosis and proliferation in the normal mucosa (NM) of both groups. Among the inflammatory genes, we measured by RT-PCR the mRNA expression of *S100-A9, Il-6, Il-1β, Nos-2* and *Cox-2* in NM samples. The results showed that none of these genes was influenced by PMD administration (data not shown). Protein levels evaluation (western blot) of NOS and COX-2 confirmed the gene expression data, indicating no difference between control and treated animals (data not shown).

Concerning the impact on proliferation, the labelling index (LI) in sections immunostained with PCNA, as well as PCNA immunoblotting in NM samples, showed that proliferative activity was not affected by PMD (Fig. 2 panels a and b).

Apoptosis determined in histological sections of NM based on nuclear morphology was also similar in the two groups (AI: 0.15 ± 0.03 and 0.21 ± 0.11 in controls and treated animals, respectively, means ± SE). In agreement with the histological analysis, western blot determination of CASP-3 and BAK proteins showed no statistically significant difference between control and treated groups (CASP-3/GAPDH: 8.93 ± 1.9 vs 10.07 ± 2.02; BAK/GAPDH: 0.51 ± 0.21 vs 1.10 ± 0.29 in controls (n=10) and in PMD treated (n=11) groups respectively, means ± SE). Apoptosis level was also determined in sections of dissected MDF. In this case, the apoptotic index (AI) resulted to be significantly higher in the MDFs from the PMD group compared to those in controls (Fig. 2, panel c).

3.4 Effect of PMD on HT-29 colon cancer cells viability

Based on the protective effects observed *in vivo* on microscopic pre-neoplastic lesions (MDFs), we also evaluated the effects of pomegranate products in more advanced steps of colon carcinogenesis, such as macroscopic adenomas from older Pirc rats and human colon cancer cell lines. For these *in vitro* and *ex vivo* experiments, two main colon-relevant metabolites of the polyphenolic and fiber PMD components, urolithin-A (u-A) and sodium butyrate (SB), were used. First, we investigated the effect of different doses of u-A and SB on the viability of HT-29 cells upon 72 h exposure (Fig. 3, panels a and b). The IC_{50} for u-A resulted to be 43.9 µM , and that of SB 3 mM. The combination of u-A and SB was also tested (Fig. 3 panel b, dotted line): in the presence of a fixed concentration of u-A (25 µM, inducing a 25% reduction in cell viability) the IC_{50} of SB was not statistically different from that of SB alone, indicating the absence of a synergistic effect. However, at SB concentrations lower than IC_{50}, the co-presence of u-A further reduced viability, (Fig. 3 panel b, compare dotted and continue lines), suggesting an additive type interaction between the two metabolites.

3.5 Proliferation, apoptosis and inflammation in HT-29 cells treated with urolithin-A, Sodium Butyrate, or both

The effect of urolithin-A and Sodium Butyrate on markers of proliferation, apoptosis and inflammation was assessed in HT-29 cells by means of western blotting. Cells were treated for 24 or 72 h with u-A 25 µM (u-A) corresponding to 25% cell viability reduction as described above, SB 2.5 mM (SB) or with a combination of u-A 25 µM and SB 2.5 mM (USB).

Compared to the respective controls, the expression of the proliferation marker PCNA was significantly reduced by SB after 24 h, while u-A and USB reduced it slightly, but not significantly. At 72 h the untreated cells showed a slight reduction in proliferation compared to 24 h, and the USB combination brought about a further significant decrease compared to respective 72 h control (Fig. 3, panel c). As for apoptosis, activated Caspase-3 (C-CASP3) expression was strongly increased by the combination of u-A and SB by 24h; during the following 48h, the levels in treated cells were still higher compared to the respective controls, although the differences were no longer significant (Fig. 3, panel d).

Interestingly, we also observed that in cells treated for 72 h, the expression of the inflammatory markers iNOS and COX-2 was reduced by all the treatments (fig. 3, panels e and f respectively), with the USB treatment being the most effective (-79.7% and -74.5% compared to respective controls for iNOS and COX-2 respectively, $p<0.01$). Notably, in the untreated 72 h controls, iNOS and COX-2 protein levels were higher compared to 24 h controls; no significant effects of the treatments were observed at 24 h.

3.6 Effect of PMD on proliferation, apoptosis and inflammation in biopsies from Pirc adenomas and normal mucosa

Colon adenoma (AD) and normal mucosa (NM) samples from Pirc rats were used for short term (24h) *ex vivo* experiments, in which the combination of u-A and SB treatment (USB), being the most effective in the *in vitro* experiments, was tested at the same concentration used in HT-29 cells. Viability of the samples, assessed with the MTT method at time 0 (control) and after 24 h of incubation, was 75% and 50% of the corresponding values at 0 time for NM and AD respectively.

The effect of USB treatment on proliferation, evaluated by means of PCNA western blot, was slight and non-significant both in AD and in NM samples (Fig. 4, panel a). On the contrary, USB showed a prominent pro-apoptotic effect in AD, as indicated by increased C-CASP-3 and BAK expression (Fig. 4, panels b and c, respectively) compared to untreated controls; a significant increase in these two pro-apoptotic proteins was also observed in the NM. Finally, a marked anti-inflammatory effect on both AD and NM samples treated with USB was observed (Fig. 4, panel d): COX-2 protein expression was decreased of about 77% in AD and 69% in NM. Interestingly, the two-way ANOVA analysis on these data showed that, as expected, basal proliferative activity and apoptosis level were higher in the adenoma tissue compared to the normal mucosa.

4 Discussion

The majority of the published studies on the potential beneficial and chemopreventive effects of pomegranate focused on the juice obtained either from whole fruits or arils [29]. Despite the fact that peel and mesocarp also contain polyphenols, to which the beneficial effects of pomegranate are ascribed [30], only few studies investigated the effects of peel [31] and no studies, to our knowledge, focused on mesocarp. In the present study we evaluated the possibility of employing a decoction obtained from

pomegranate mesocarp (PMD), a juice production by-product, as a source of molecules with chemopreventive activity in CRC. The decoction we used was characterized by a high content in ellagitannins (about 15% w/w), and a moderate content in soluble fibers, providing a manageable powder with low hygroscopicity. Fibers from different vegetables can act as a vehicle for polyphenols in the colon improving their bioavailability, particularly after the fermentation of the substrate [32]. Accordingly, our preliminary experiments on the advanced M-Shime® gastrosimulator pointed out a beneficial effect of PMD, mainly in terms of short chain fatty acids (SCFA) production by the human microbiota (manuscript in preparation), suggesting prebiotic properties associated with low CRC risk [15]. PMD was first tested in a short-term *in vivo* experiment, at a dose corresponding to a daily intake of 50 mg/kg of polyphenols, on Pirc male rats. Pirc rat, with a germ line mutation in the *Apc* gene, is a suitable model for CRC chemopreventive studies [33], as it spontaneously develops colon adenomas and, at young age, microscopic pre-neoplastic lesions (MDFs) that can be used as an end-point in short-term chemopreventive studies [34]. Our results clearly show that both the number of MDFs and their size in terms of crypts forming each MDF (multiplicity index) were significantly reduced by PMD treatment, suggesting that indeed PMD is able to reduce colorectal tumorigenesis. In the normal mucosa of PMD-treated rats, only slight effects on proliferation, apoptosis and inflammation were observed compared to controls. On the contrary, in MDFs from the PMD group, an increase in apoptosis was observed, suggesting that the observed chemopreventive effect may be due to increased apoptosis in these lesions, leading to elimination of precancerous cells, as also suggested by the lower multiplicity of MDF in the PMD-group. Increased apoptosis in tumours and preneoplastic lesions was previously observed in animals treated with compounds showing preventive activity [35].

To elucidate the molecular mechanisms involved in the protective effects observed *in vivo,* and to verify the efficacy of PMD treatment at more advanced carcinogenesis phases (i.e. in cancer cells and in macroscopic adenomas), we tested the effect of two colon-relevant metabolites of the polyphenolic and fiber components of PMD: urolithin-A (u-A) and sodium butyrate (SB) respectively [20] [23]. These were assessed *in vitro* on HT-29 human carcinoma cell line, bearing an *Apc* gene mutation. U-A and SB alone demonstrated inhibitory effects on cell viability with an IC_{50} (43.9 μM and 3 mM respectively) in line with those reported previously

[6] [36]. We also wanted to evaluate the effect of the combination of these two metabolites, and combining 25 µM u-A (corresponding *per se* to a 25% reduction in cell viability) with various concentration of SB, we found an additive effect on cell viability reduction. We then focused on the combination of u-A and SB (USB) using 2.5 mM of SB and 25 µM of u-A, concentrations that can actually be reached in the colon [23] [21]. The effect on HT-29 cells were tested after 24 and 72 h of incubation. Western blotting analyses of several protein markers involved in proliferative, inflammatory and apoptotic mechanisms showed that USB treatment was capable of affecting these parameters in a more pronounced manner than single treatments. In fact, USB treatment brought about the strongest reduction in proliferative activity (PCNA reduction) at 72 h and the strongest apoptotic induction (C-CASP-3 increase) at 24h. Besides, although u-A and SB alone were both able to lower iNOS and COX-2 after 72 h treatment, in line with previously reported data [37] [38], the anti-inflammatory effects of the combination were again more pronounced. The efficacy demonstrated by USB in this human cancer cell line, encouraged us to verify the efficacy of the combined treatment on macroscopic lesions of Pirc rats. For this purpose, and to reduce the number of experimental animals, we performed *ex vivo* experiments, in which small samples of adenomas (ADs) and NMs of eight-month-old Pirc rats were collected and maintained in culture for 24h with or without USB at the same concentrations tested in HT-29 cells. In the control samples, the MTT method showed that the tissue was still viable at 24 h. Furthermore, PCNA expression (proliferative activity) and cleaved CASP-3 and BAK expression (apoptosis) were significantly higher in AD compared to NM, in agreement with many studies [39] [24] documenting higher proliferation and apoptosis in AD compared to NM. As a whole, these results indicate that this *ex-vivo* system is quite reliable. The treatment with USB increased apoptosis, as measured by BAK and C-CASP-3 protein level, in both AD and NM, compared to untreated controls. A pro-apoptotic effect of PMD was also observed *in vivo* in MDF from PMD-treated rats, while in the NM *in vivo*, this effect was not detected. A significant reduction in COX-2 expression was brought about by USB in *ex vivo*-treated AD and NM samples; regarding the NM, again this result is at variance with the *in vivo* studies were COX-2 expression was not varied. The discrepancies observed between the *in vivo* and the *ex vivo* results for apoptosis and COX-2 in NM might be due to modifications associated with the incubation in an oxygen-rich

environment, which might sensitize cells to apoptosis and shift them towards an inflammatory phenotype. Thus, the expression of COX-2 in the NM *ex-vivo* might be higher than in physiological conditions and a reduction by PMD could be more easily observed. Finally, regarding proliferation, a slight effect of USB was observed on AD, while PCNA expression in NM was not affected by USB treatment, in agreement with what observed in the *in vivo* experiments.

Concluding Remarks. In conclusion, these results show that PMD is able to significantly reduce tumorigenesis in a robust model of carcinogenesis *in vivo*, with a mechanism of action that involves an increase in apoptosis in the preneoplastic lesions MDFs. PMD is obtained by a green and simple process, an added value to the use of by-products of juice production. The particular combination of ellagitannins (up to 15%) and the pool of fermentable polysaccharides (about 10%) of PMD may increase the production of bioactive metabolites, such as urolithins and butyrate, in the colon. Indeed, the results of parallel *in vitro* and *ex vivo* experiments clearly indicate that urolithin-A and sodium butyrate, and especially their combination, exert significant anti-inflammatory and pro-apoptotic effects both on human colon cancer cells and on adenomas of Pirc rats. All together, these data suggest that the chemopreventive effect of PMD may be due, at least in part, to the pro-apoptotic and anti-inflammatory effects of its colon metabolites. Finally, this study indicates the potential value of these by-products as a source of bioactive molecules, that could be exploited for CRC prevention in high-risk subjects.

Author's contribution

KT carried out the *in vitro* and *ex vivo* experiments, she also drafted the manuscript together with LG and GC. APF carried out the carcinogenesis experiment and gene expression analysis. AR and IS carried out part of the carcinogenesis experiments and the gene expression and histochemistry experiments. MK prepared the decoction of pomegranate mesocarp and carried out the relative chemical analyses together with NM. LG performed the *in vitro* experiments. GC determined apoptosis and proliferation in immunohistochemistry. GC, LG and NM conceived, designed and supervised the work. All authors have read and approved the manuscript.

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Conflict of interest. The Authors have declared no conflicts of interest.

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Table 1: primers used for the amplification of different genes by RT-PCRs

Gene	Forward	Reverse
β-actin	5'-ACCACAGCTGAGAGGGAAAT-3'	5'-AGAGGTCTTTACGGATGTCAAC-3'
IL-6	5'-TCTCTCCGCAAGAGACTTCC-3'	5'-TCTTGGTCCTTAGCCACTCC-3'
IL-1β	5'-TGACCCATGTGAGCTGAAAG-3'	5'-AACTATGTCCCGACCATTGC-3'
S100A9	5'-GCACGAGCTCCTTAGCTTTG-3'	5'-GACTTGGTTGGGCAGATGTT-3'
NOS-2	5'-GCCTAGTCAACTACAAGCCCC-3'	5'-CCTGGGGTTTTCTCCACGTT-3'
COX-2	5'-ACGTGTTGACGTCCAGATCA-3'	5'-GGCCCTGGTGTAGTAGGAGA-3'

Figure Legends

Figure 1. Pre-neoplastic lesions: MDF/colon (panel A) and crypts/MDF (panel B) in Pirc rats fed with AIN76 (CTRL) or AIN76 diet supplemented with 50mg/kg/die of PMD (means ± SE); controls: n=10, PMD-treated: n= 11. * $p<0.05$ significantly different from controls (Student *t*-test).

Figure 2. Effects of PMD in the Pirc colon: proliferation activity evaluated as LI in histological sections immunostained with a PCNA-antibody and PCNA protein expression by western blot (panels A and B respectively) in NM of Pirc rats treated with PMD. Panel c: Apoptotic Index in MDFs from controls and PMD group. Bars are means + SE; controls: n=10, PMD-treated: n= 11. ** $p < 0.01$ significantly different from controls (Student *t*-test).

Figure 3. Effects of PMD on HT-29 cells: panel A: concentration-response curve of u-A (log IC_{50} = -4.358, R^2 = 0.8274); panel B: concentration-response curves of SB (log IC_{50} = -2.511, R^2 = 0.9224) and SB plus u-A 25 µM (dotted line, logIC_{50} = -2.405, R^2 = 0.9275); incubation time: 72 h. Panels C to F: relative protein expression of PCNA, C-CASP-3, iNOS and COX-2 respectively, after 24 and 72 h treatment with u-A 25 µM (u-A), SB 2.5 mM (SB) or u-A 25 µM plus SB 2.5 mM (USB) compared to the respective control. Bars are means + SE; *: $p< 0.05$, **: $p < 0.01$, ***: $p < 0.001$ significantly different from respective controls. §: $p < 0.05$, §§: $p < 0.01$ significantly different from 24 h control (one-way ANOVA).

Figure 4. Effects of PMD in Pirc adenomas and NM biopsies *ex vivo*: relative expression of PCNA, C-CASP-3, BAK and COX-2 (panels A, B, C and D, respectively) in control and USB-treated AD and NM samples after 24 h in culture. §, §§, §§§: $p<0.05$, <0.01, < 0.001, respectively, comparing AD vs NM (two way ANOVA). *, **, ***: $p < 0.05$, < 0.01, < 0.001, respectively, comparing treated vs untreated samples. (two-way ANOVA). Bars are means + SE.

Figures

Figure 1

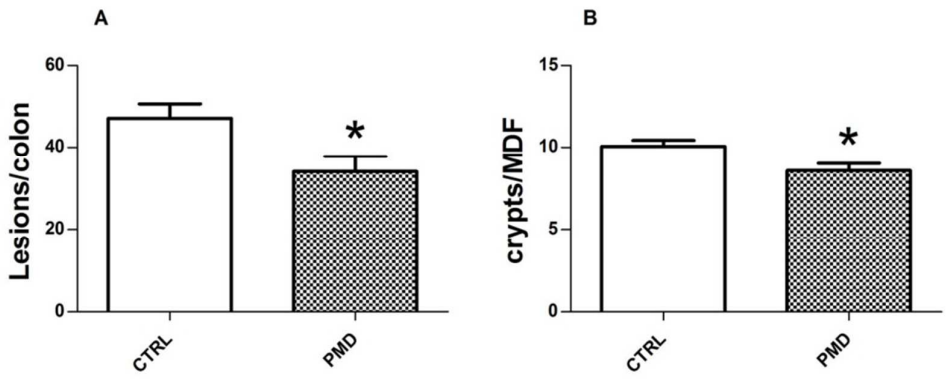


Figure 2

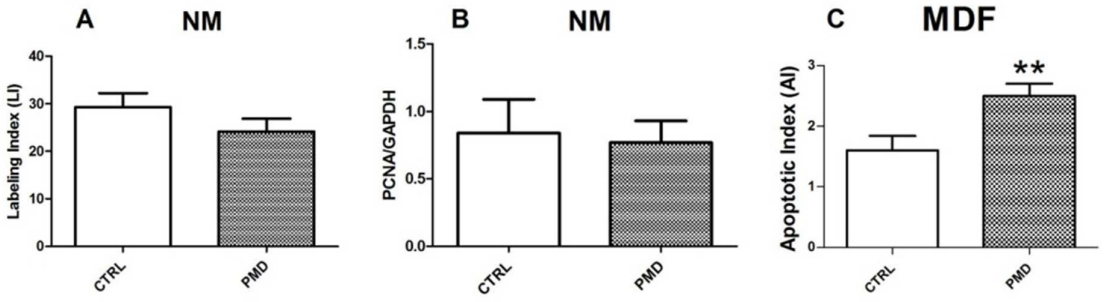


Figure 3

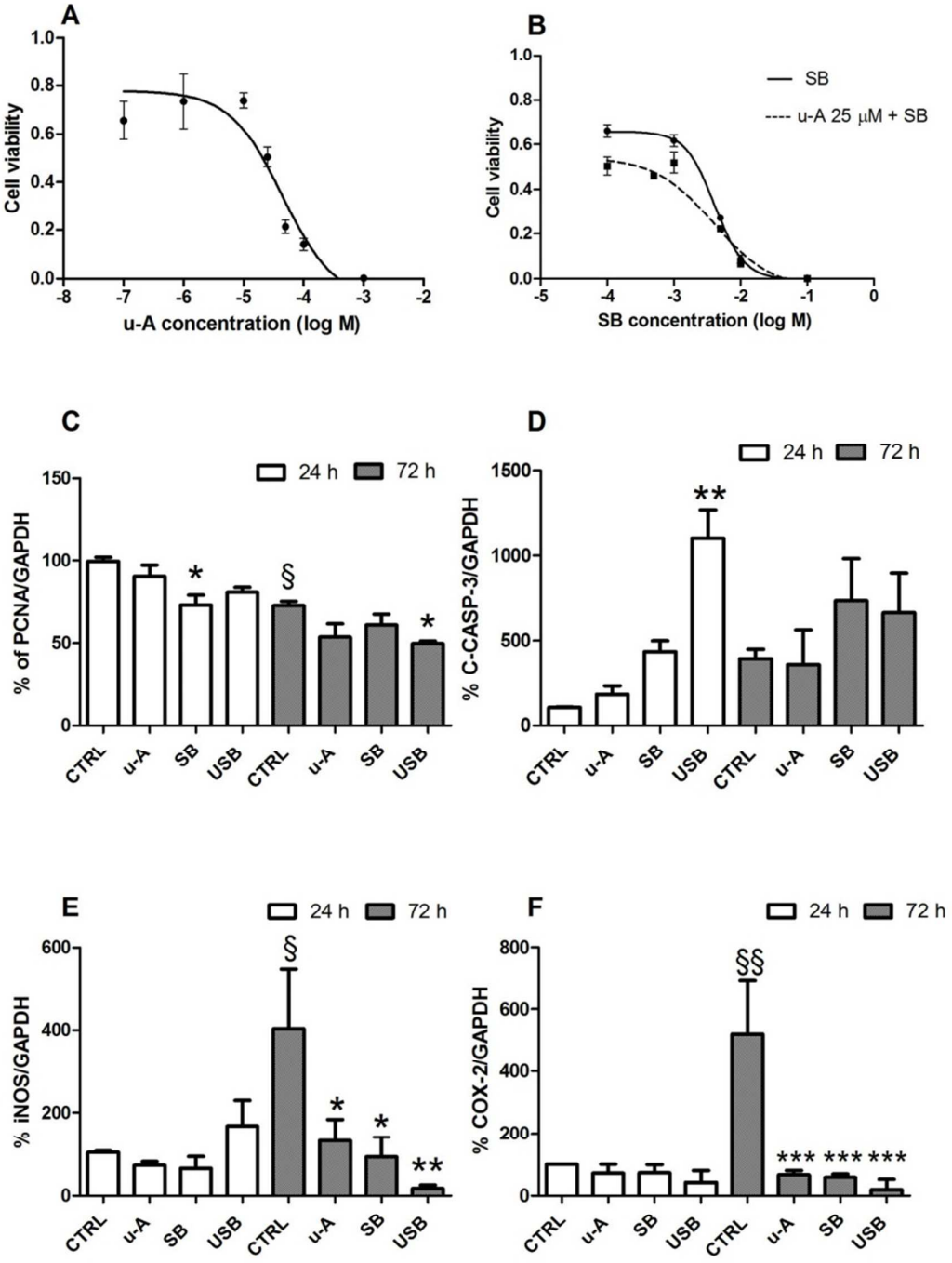
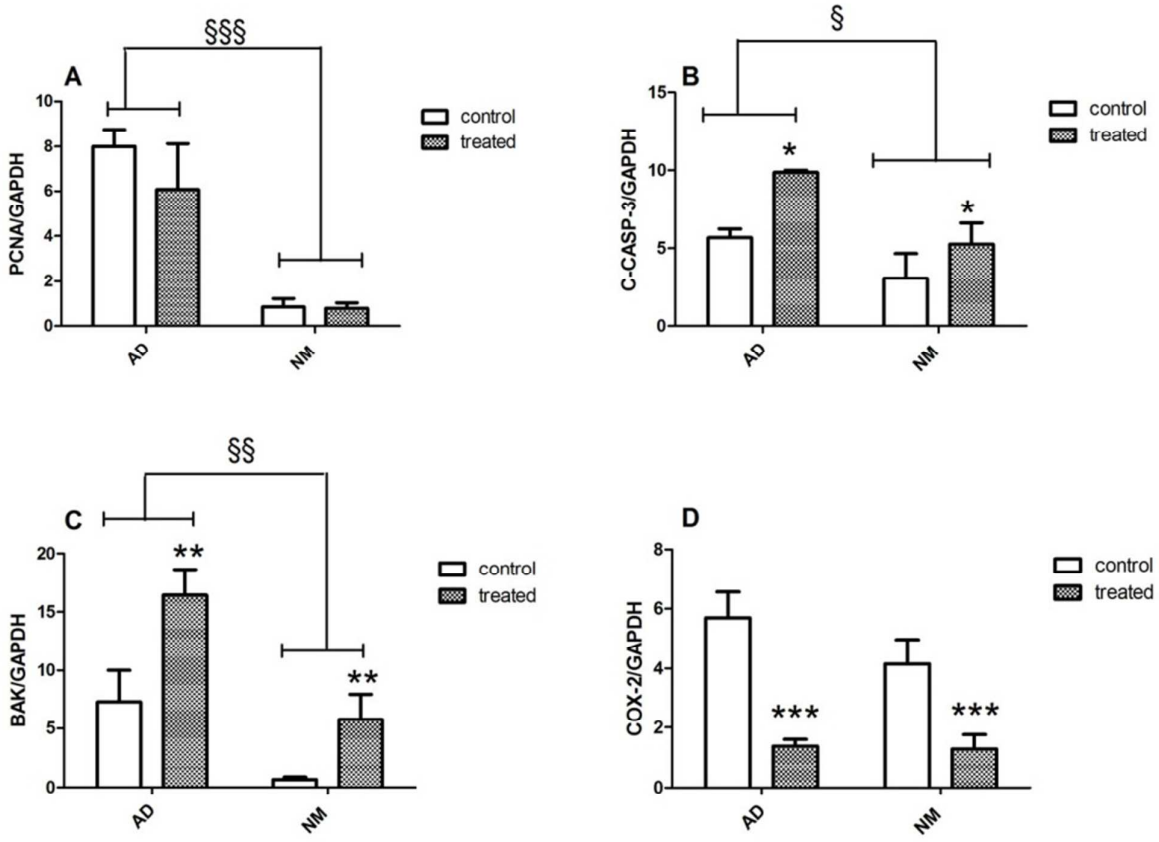

A
Cell viability
u-A concentration (log M)
B
SB
u-A 25 μM + SB
Cell viability
SB concentration (log M)
C
24 h
72 h
% of PCNA/GAPDH
CTRL
u-A
SB
USB
D
24 h
72 h
% C-CASP-3/GAPDH
E
24 h
72 h
% iNOS/GAPDH
F
24 h
72 h
% COX-2/GAPDH

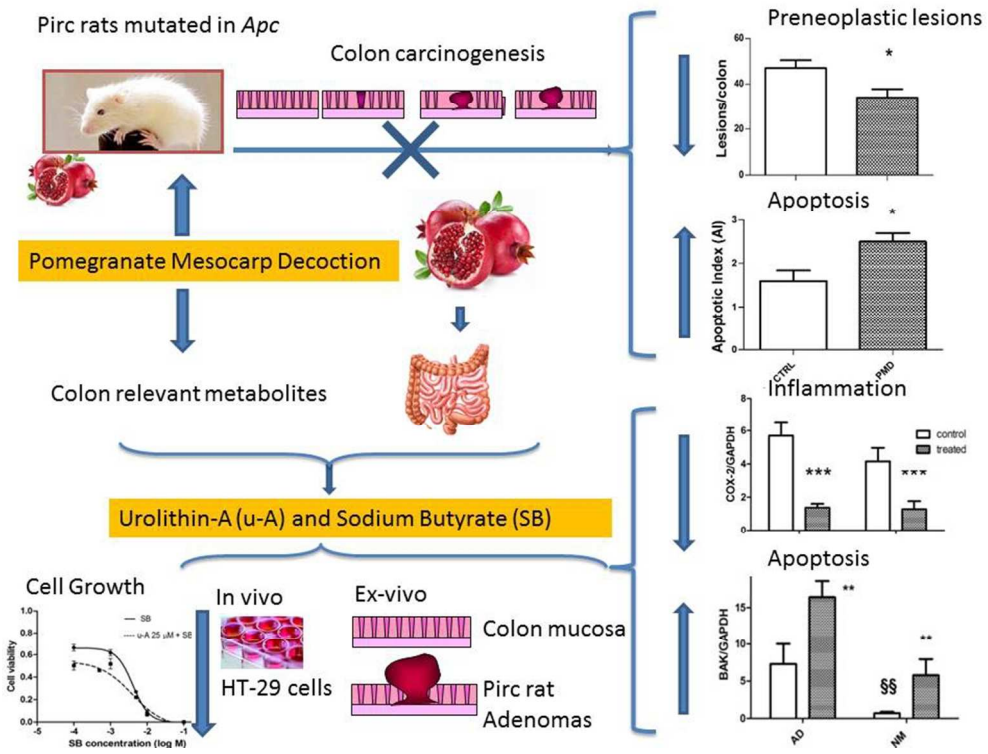
Figure 4



Pomegranate by-products in colorectal cancer chemoprevention: effects in *Apc*-mutated Pirc rats and mechanistic studies *in vitro* and *ex vivo*

Tortora et al.

Pomegranate mesocarp (PMD), a polyphenol-rich by-product of juice production, reduces colon carcinogenesis in *Apc*-mutated rats and increases apoptosis (cell death) in precancerous lesions. Two main metabolites of PMD: urolithin-A and butyrate, alone or in combination, reduce inflammation and increase apoptosis in a cancer cell line or in adenoma and normal mucosa from rats. The data indicate a preventive activity of PMD that could be exploited in patients at high risk for colon cancer.



254x190mm (96 x 96 DPI)