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In vitro and *in vivo Apc*-mutated models to study colon carcinogenesis and nutraceutical chemopreventive products

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Research is formalized curiosity. It is poking and prying with a purpose.

Zora Neale Hurston

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List of abbreviations

AD: Adenoma
ACF: Aberrant Crypt Foci
AI: Apoptotic Index
Apc: Adenomatous Polyposis Coli
BJe: Bergamot Juice Extract
CAFs: Cancer Associated Fibroblasts
CRC: Colorectal Cancer
ECM: Extracellular Matrix
FAP: Familial Adenomatous Polyposis
FDB Fast Digest Buffer
ICF: Immunocytofluorescence
LI: Labelling Index
MDF: Mucin Depleted Foci
NM: Normal Mucosa
NSAID: Non-Steroidal Anti-Inflammatory Drug
PCF/y: Pirc Colon Fibroblasts From Young Animal
PCF/o: Pirc Colon Fibroblasts From Old Animal
PMD: Pomegranate Mesocarp Decoction
QoL: Quality Of Life
SB: Sodium Butyrate
α-SMA: α-Smooth Muscle Actin
USB: Urolithin-A Plus Sodium Butyrate
WCF/y: WT Colon Fibroblasts From Young Animal
WCF/o WT Colon Fibroblasts From Old Animal
u-A: Urolithin-A

Abstract

Colorectal cancer (CRC) is the third most common cancer in Western Countries and one of the leading causes of mortality (WHO). CRC is a multistep process involving hyperproliferation of normal *epithelial* cells, due to an *Apc* gene mutation, and proceeding through adenomas and adenocarcinomas development due to progressive accumulation of mutations on oncogenes and oncosuppressor genes. These mutations can be due, among others, to increased genomic instability, oxidative stress, and epigenetic changes. Among this last one, DNA methylation changes in CpG islands are involved in 18% of CRC cases (Gallois et al. 2016). Moreover, progress and invasion of CRC are also stimulated by the microenvironment: Cancer Associated Fibroblasts (CAFs) are its main component, responsible for the epithelial-mesenchymal transition and inflammation promotion (Wang et al. 2017). A wide range of data are present in literature about CAFs role in colon carcinogenesis: nevertheless, the link between *Apc* mutation and colon tissue microenvironment has not been clearly defined yet.

Three aims have characterized this PhD project: first, the study of natural chemopreventive strategies capable of interrupting CRC carcinogenesis with associated very low toxicity. Indeed, the use of NSAIDs, showed to be a promising chemopreventive strategy in the past decades, is limited by their possible side effects: instead, the use of natural products could allow to overtake this limitation and be useful for secondary and tertiary prevention in individuals at high risk of CRC development. Three different natural compounds/products (a bergamot juice extract from endocarp (BJe), morin, and a pomegranate mesocarp decoction (PMD)) were tested *in vivo* in the Pirc rat model (F344/NTac-Apc^{am1137}), bearing an *Apc* gene mutation and spontaneously developing colorectal polyps (Amos-Landgraf et al. 2007) and microscopic preneoplastic lesions (MDFs, Femia et al. 2015) in the normal mucosa (NM): MDFs are also considered useful endpoint for short-term chemopreventive studies. In addition, these *in vivo* experiments were supported by *in vitro* tests, aimed to explain the molecular mechanisms of the 3 tested compounds. The second aim was the study of the link between *Apc* gene mutation and tissue microenvironment at very early stages of colon carcinogenesis, addressing the role of colon fibroblasts within tissue microenvironment, at a stage in which colon carcinogenesis is already ongoing but no macroscopic lesions can be found. We evaluated the effects of this mutation on colon fibroblasts phenotype in established primary cultures from the colon of Pirc and F344-Wt age-matched (one month) rats. The third aim was to add knowledge on the role of *Apc* mutation on DNA stability at early stages of colon carcinogenesis, with the use of the COMET assay to evaluate DNA strand breaks and oxidative damage in both cultured colon fibroblasts of Pirc and Wt

rats and in apparently morphological normal mucosa samples of Pirc and Wt rats aged one month. In addition, we set up a modification of the method, aiming at evaluating the global DNA methylation status.

The effects of the treatments tested in Pirc rats were evaluated assessing the number of MDFs, apoptosis and proliferation both histologically and measuring relevant genes and/or proteins expression. Established Pirc and Wt colon fibroblasts primary cultures were characterized for their proliferative and phenotypic profile: both inflammatory and senescence-associated markers were evaluated with immunochemical and cytochemical assays.

Concerning BJe, morin and PMD tested *in vivo,* each of them demonstrated to be capable of perturbing colon carcinogenesis as suggested by the reduction in MDFs number and size, possibly through a combination of pro-apoptotic and pro-inflammatory actions as suggested by *in vitro* experiments and also in an *ex-vivo* model of adenoma (for PMD study) from Pirc rats (Tortora et al. 2018). The novelty of these studies was represented by either the use of non-canonical sources of natural compounds (fruit by-products) or by the possibility to target an oncoprotein (LMW-PTP, low molecular weight phospho-tyrosine phosphatase) consequently enhancing the therapeutic response (study on morin). These data support the idea that a combination of natural compounds acting synergistically with each other, and possibly with drugs, can represent a promising secondary and tertiary chemopreventive strategy.

The data obtained on colon fibroblasts mutated in *Apc* suggest that this mutation determines the development of a pro-inflammatory phenotype in this tissue microenvironment component, which might be involved in the creation of a pro-tumorigenic environment favoring micro and macro pre-neoplastic lesion development. Moreover, data obtained with the COMET assay on colon fibroblasts and NMs from one month-old Pirc and Wt rats showed a lower level of oxidative damage in Pirc compared to Wt animals: also based on previous data from this lab (Femia et al. 2015), we speculate that *Apc* mutation could account for a selective advantage for carcinogenesis development through an increase in anti-oxidants defenses, as reported in the literature (Ogasawara and Zhang 2008).

Finally, we succeeded in the development of a methyl sensitive COMET assay, as proven by the reliability of the methylation changes observed after hypo- and hypermethylating stimuli in two different kinds of normal colon cell lines (epithelial and fibroblasts): this method will be used in the future to address the link between *Apc* gene and methylation.

Preface

Cancer: a worldwide challenge

Almost each family in West countries and emerging states has a relative who has faced, or is facing, a battle against cancer: this is not an overstatement if it is considered that in 2016, US President Obama announced the creation of a task force on cancer, defining the cure of the disease as a new "moonshot". Clearly, epidemiological statistics strongly support this scenario: in 2012, cancer was the 2nd leading cause of death in the world accounting for 8.2 milions of death, with a perspective of new cancer cases diagnosed estimated to pass from 14.1 milions in 2012 to 21.6 milions by 2030 (http://www.who.int/cancer/media/news/cancer-prevention-resolution/en/).

Hence, these numbers will be translated in the next decades, into high expenditures for cancer diagnosis, treatments and hospitalization, making cancer a public health, social and economic emergency to contrast. For this reasons, the awareness about the key role that prevention can represent in this multifactorial challenge, is raising worldwide.

The World Health Organization (WHO) estimates that half of the cancer cases can be prevented by risk factors reduction, which, together with early diagnosis and appropriate treatments, can reduce mortality and improve the quality of life (QoL). WHO resolution on *Cancer prevention and control in the context of an integrated approach* (WHA A70/A/CONF./9 , May 25th- 2017) encourages all Member States to:

- *Implement scale up national cancer prevention and control plans;*
- *Develop strategies for cancer prevention and control taking into account cancer-risk factors;*
- *Promote the primary prevention of cancers;*
- *Promote cancer research to improve the evidence base for cancer prevention and control including, on health outcomes, quality of life and cost-effectiveness;*
- *Promote access to comprehensive and cost-effective prevention, treatment and care for the integrated management of cancer.*

The points listed above, are only part of the 22 points which are then followed by the request of WHO Director-General "*to establish and implement comprehensive cancer prevention and control programs and […] to make an investment case for cancer prevention and control*".

Regarding the European Union (EU), the European Commission is supporting several projects related to health promotion and implementation of strategies for primary and secondary prevention. Primary prevention consists in identifying the major health determinants (i.e. smoking or dietary habits) and modify them in a healthy manner, it is considered the most cost-effective and long-term strategy for reducing the burden of diseases. This concept has been recently enforced in 2017 by the European Commission within the "*Final report of the expert group on quality of life (QoL) indicators*" (*https://publications.europa.eu/en/publication-detail/-/publication/1c2fee3e-15d5-11e7-808e-01aa75ed71a1/language-en*), in which health status is one of the nine determinants of QoL. The indicators of health status include factors resulting from lifestyles that are:

- Body Mass Index (BMI);
- Tobacco consumption;
- Alcohol consumption;
- Physical inactivity;
- Fruits and vegetables consumption

Secondary prevention consists of population-based screening programs, aiming at reducing mortality thanks to an early cancer detection. These efforts seem to have produced good results in terms of assuring healthy long lives to the population: an European report dated 2014, showed an increase in survivorship after 5 years since cancer diagnosis across Europe, so reflecting the advances in cancer management including capillary screenings and improved treatments (http://ec.europa.eu/eurostat/statistics-explained/index.php/Cancer_statistics_-_specific_cancers). In addition to screening programs, chemoprevention that is the use of a compound for extended period of time without significant side effects in high risk individuals, is going to play a key role within secondary prevention of cancer diseases, in line with the evidences existing for cardiovascular ones: indeed, the use of drugs to lower cholesterol synthesis or blood pressure has led to a steady fall in heart disease in the last three decades (Hansson, 2005).

Introduction

1 Colorectal cancer: what's going on?

As mentioned above, cancer accounted for nearly 26% of all causes of deaths in EU in 2013, with men more affected than women (726500 vs 570300 deaths respectively). Unfortunately, this percentage increases when the attention is pointed out on the population under 65 years of age, reaching the EU average of 37% with a peak of 46% in Italy. The main fatal cancer is lung cancer, followed by colorectal, breast, pancreas and prostate (Eurostat data, February 3rd – 2016. http://ec.europa.eu/eurostat).

In 2018, colorectal cancer (CRC) has been estimated to account for 177400 deaths in the EU (12% of all cancer related deaths), although the rate has been declining since 2012.

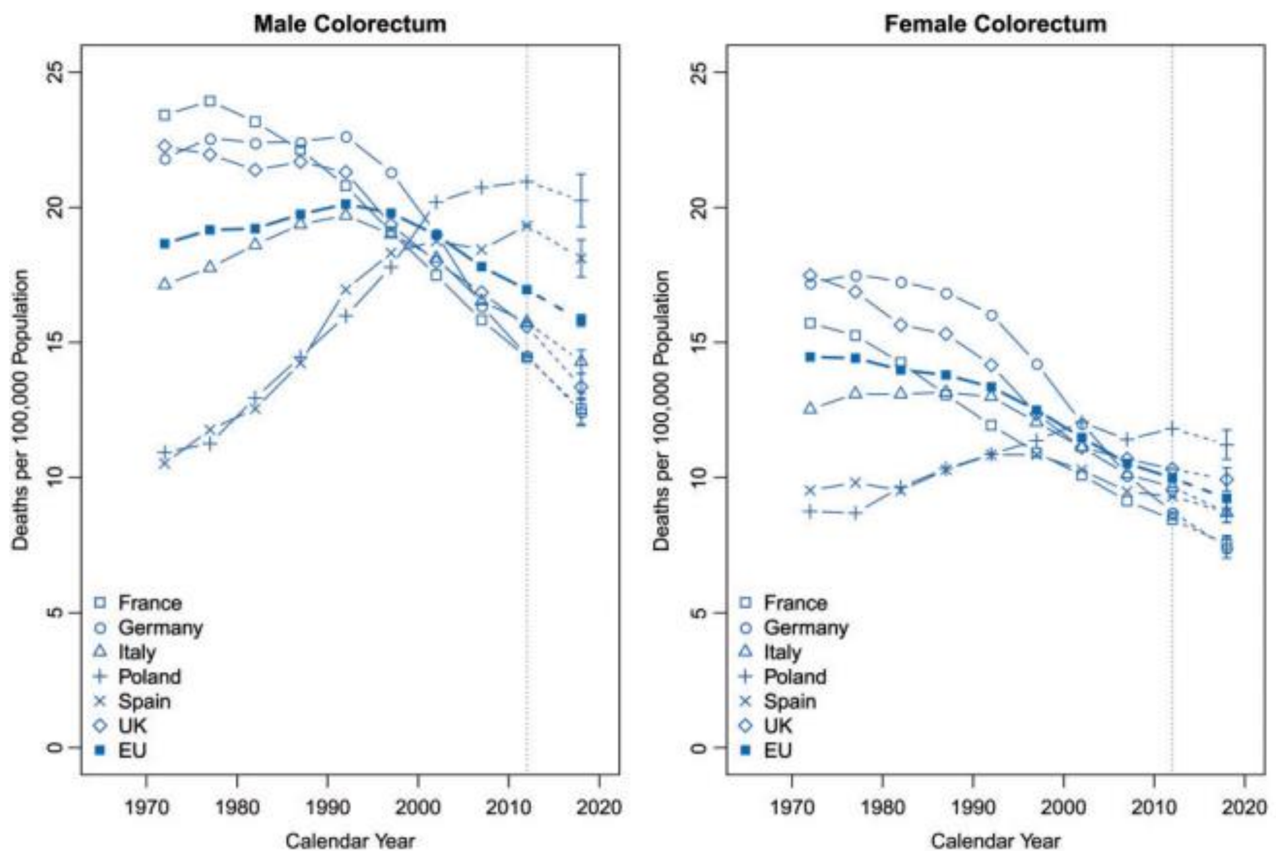

Fig.1. Age-standardized (world population) colorectal cancer mortality rate trends for men and women in quinquennia from 1970–1974 to 2005–2009 plus the year 2012 and predicted rates for 2018 with 95% PIs for colorectal cancer in studied countries and the EU as a whole (Malvezzi *et al*, 2018).

Apart from Spain and Poland, as shown in fig.1, in the last two decades the mortality due to CRC is decreased in both male and female individuals, and female show lower rates: this trend is also followed by age-related CRC death rates (fig. 2). The main cause of mortality among CRC patients is represented by metastases: 25% and 50% of CRC patients have a distant metastases at the time of diagnosis or will develop it, respectively. Frequently, metastases cannot be surgically removed and commonly develop in the liver, lung and peritoneum.

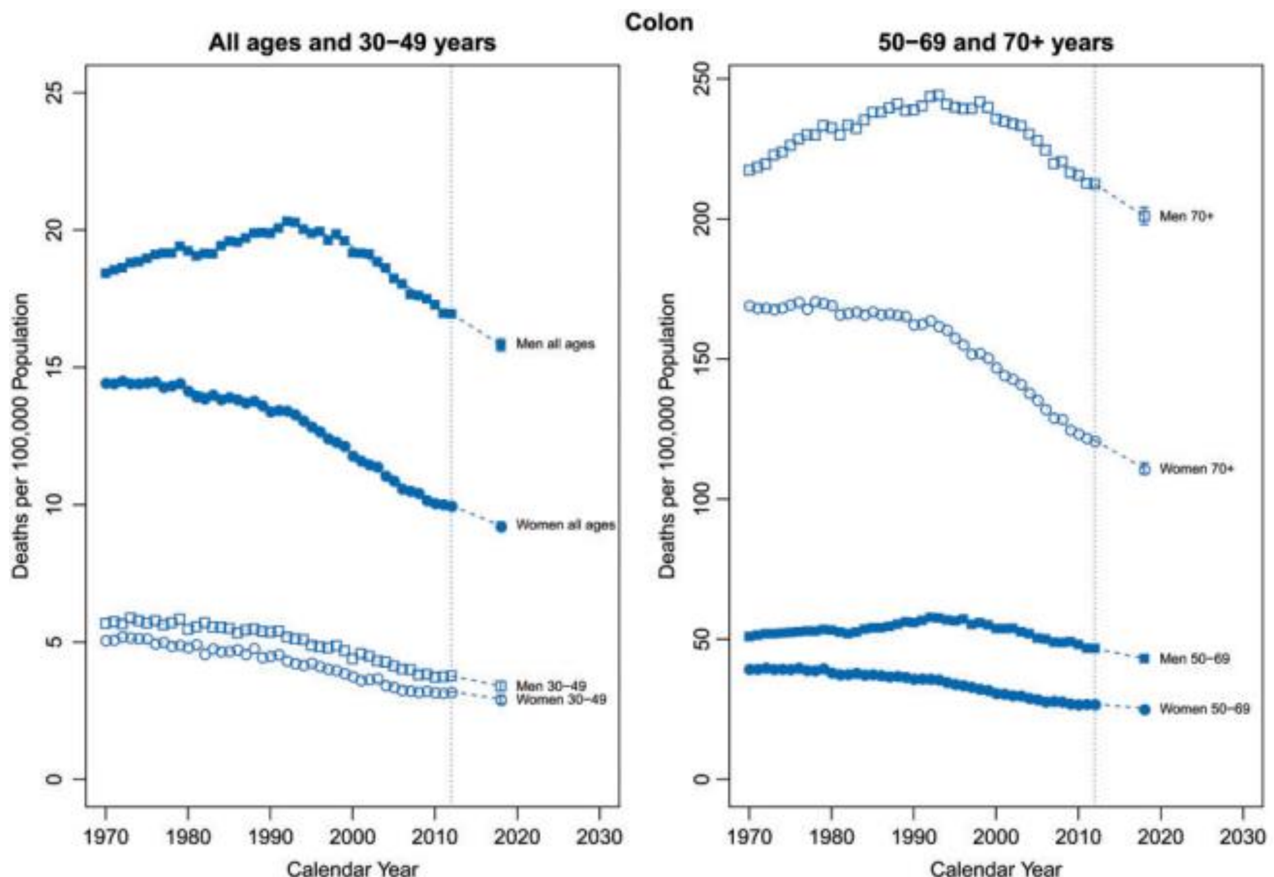

Fig. 2. Annual colorectal cancer age-standardized (world population) death rates in the EU per 100000 for all-ages, 30–49, 50–69, and 70+ years age groups from 1970 to 2012, the resulting join point regression models, and predicted rates for the year 2018 with 95% PIs. On the left, men and women at all-ages (full squares and full circles, respectively) and at 30–49 years (empty squares and empty circles), on the right, men and women at 50–69 years (full squares and full circles), and at 70+ years (empty squares and empty circles) (Malvezzi *et al*, 2018).

The reduction observed can be addressed in part to an increased awareness, among the general population, of the benefits achieved by following healthy diet habits and lifestyles: these can represent a risk factor for CRC insurgence comprehending tobacco and alcohol consumption, sedentariness, obesity, diabetes and diets rich in processed red meat with a low intake of vegetables and fibers (Turati *et al*, 2017). The decreased mortality rate can also be attributed to the screening program implemented in the over 50-years population, although with differences among EU countries, a factor that may influence single state trends (Malvezzi *et al*, 2018). Unfortunately, there is an increase in early-onset CRC,

meaning CRCs occurring under 50 years of age with no identifiable inherited gene involved in the carcinogenesis process (Dienstmann *et al*, 2017).

As outlined above, primary prevention points its basis on lifestyle habits that can be modified by the population, among which diet is considered the most important factor influencing colorectal carcinogenesis.

Epidemiological data have always represented the starting point to examine in depth the role that some foods, nutrients or other lifestyle components (non-related to diet, i.e. smoking) could exert on diseases risk. Regarding CRC, an example of the usefulness of epidemiological data is represented by the assessment of the link between red meat consumption and CRC risk. In 2015, 22 scientists from ten countries were designated by the International Agency for Research on Cancer (IARC) to evaluate the carcinogenicity of red meat consumption, both processed or not. Having examined 800 epidemiological studies, the Working Group concluded that processed meat consumption can be classified as "*carcinogenic to humans on the basis of sufficient evidence for colorectal cancer*". They also classified red meat (unprocessed) consumption as "*probably carcinogenic to humans*" (http://monographs.iarc.fr/).

Additionally, population-based studies have showed the ability of both macro and micro-nutrients, derived from fruit and vegetables, to reduce cancer risk (Surh, 2003). Indeed, fruit and vegetable are rich in fibers which have been demonstrated to reduce fecal transit time, dilute and bind carcinogens, adsorb heterocyclic amines and stimulate bacterial anaerobic fermentation so increasing the production of short chain fatty acids (SCFA) such as acetate, propionate and butyrate which inhibit carcinogenesis (Harris *et al*, 1996; Rieger *et al*, 1999; Michels *et al*, 2000; Bingham *et al*, 2003; Peters *et al*, 2003; Scharlau *et al*, 2009; Van Duijnhoven *et al*, 2009). These clinical evidences have been driving health organizations worldwide to recommend a healthy dietary habit in order to reduce the risk of cancer, CRC in particular. In addition to the reduction in processed red meat intake and the consumption of five portions/day of fruit and vegetables, those recommendations also include other evidence-based points, as reported by the World Cancer Research Fund in the report on "Diet nutrition, physical activity and colorectal cancer" in 2018 (World Cancer Research Fund/American Institute for Cancer Research. Continuous Update Project Expert Report 2018., 2018) (fig. 3).

2017	DIET, NUTRITION, PHYSICAL ACTIVITY AND COLORECTAL CANCER 2017		
		DECREASES RISK	INCREASES RISK
STRONG EVIDENCE	Convincing	Physical activity[1,2]	Processed meat[3] Alcoholic drinks[4] Body fatness[5] Adult attained height[6]
	Probable	Wholegrains Foods containing dietary fibre[7] Dairy products[8] Calcium supplements[9]	Red meat[10]
LIMITED EVIDENCE	Limited – suggestive	Foods containing vitamin C[11] Fish Vitamin D[12] Multivitamin supplements[13]	Low intakes of non-starchy vegetables[14] Low intakes of fruits[14] Foods containing haem iron[15]
	Limited – no conclusion	Cereals (grains) and their products; potatoes; animal fat; poultry; shellfish and other seafood; fatty acid composition; cholesterol; dietary n-3 fatty acid from fish; legumes; garlic; non-dairy sources of calcium; foods containing added sugars; sugar (sucrose); coffee; tea; caffeine; carbohydrate; total fat; starch; glycaemic load; glycaemic index; folate; vitamin A; vitamin B6; vitamin E; selenium; low fat; methionine; beta-carotene; alpha-carotene; lycopene; retinol; energy intake; meal frequency; dietary pattern	
STRONG EVIDENCE	Substantial effect on risk unlikely		

Fig. 3. Summary of strength of evidence on diet and lifestyle factors and the risk of developing CRC (adapted from the report "Diet nutrition, physical activity and colorectal cancer", WCRF 2018).

1.1 Cancer chemoprevention: an old concept but still promising

The popular quote "preventing is better than treating" is at the base of the continuous increasing interest in the field of cancer prevention exhibited by both economic and scientific communities worldwide. "*Chemoprevention is the use of natural, synthetic (laboratory-made), or biologic (living source) agents, able to delay, reverse or inhibit tumor progression*": this is the concept originally defined by Sporn and Newton in 1979 (Sporn, M. B., Newton, D. L., 1979); accordingly, a chemopreventive agent is able to avoid cancer insurgence or progression with concomitant low toxic effects. This strategy could be particularly relevant for high risk individuals, like people extensively exposed to carcinogens (i.e. tobacco, UV radiation) with a family history of neoplasia or an inherited cancer, or people with a previous neoplasia. The concept that is emerging is the interference on cancer progression, i.e. blocking the growth and invasion of tumor mass as early as possible: this is leading to continuous research on both new molecules or existing

drugs capable of perturbing pathways involved in growth, angiogenesis and metastasis development (Albini *et al*, 2016). According to Lee Wattenberg (Wattenberg, 1985) chemopreventive agents can be divided into: blocking and suppressing agents. The first group consists of molecules that are capable of protecting DNA from mutations and genomic instability: these events, that prompt cancer insurgence, can be due to chemical carcinogens, endogenous oxygen and nitrogen radicals, chronic inflammatory responses, altered DNA methylation and deacetylation (Hauser & Jung, 2008).

The second one includes compounds which succeed in inhibiting pathways involved in tumor progression, such as NFkB pathways (Karin, 2006), cell metabolism, drug resistance, and angiogenesis.

The study of a compound supposed to be endowed of cancer chemopreventive activities, as suggested by epidemiological data, is characterized by extensive preclinical studies both *in vitro* and *in vivo*, with concentrations and doses relevant to human carcinogenesis. During these studies, the effects of the compound on important cellular processes are investigated with mechanistic assays *in vitro.* Meantime, *in vivo* studies, in addition to giving pharmacokinetics and safety information, aim at exploring the prevention of tumor burden using end points that occur earlier than cancer (i.e. considering the number of preneoplastic lesions) or its progression into malignancy. Aberrant crypt foci (ACF) and adenomas in colon and rectum, or mammographic density in the breast are examples of these end-points. Moreover, efforts for the identification of new biomarkers associated with carcinogenesis are investigated during preclinical studies and then in clinical trials and observational studies: variations in blood or urine levels of proteins or smaller molecules (i.e. miRNA, circulating DNA fragments) suggesting carcinogenesis progression, can be easily used as reliable end-points in chemopreventive clinical-trials. Indeed, use of cancer incidence as end-point for chemopreventive trials means that ten years or more are necessary to obtain significantly different disease rate for an active agent tested: this is the main reason why research on carcinogenesis biomarkers keeps going on (Steward & Brown, 2013).

“The Breast Cancer Prevention Trial” (BCPT) can be considered the first chemopreventive trial in the cancer field which demonstrated the efficacy of tamoxifen intake for five years in women at increased risk of breast cancer, to reduce both invasive and non-invasive disease incidence (Fisher *et al*, 2005). This study was followed by two additional trials that confirmed the data and surprisingly demonstrated an even more protective effect of tamoxifen intake for ten years with no increased toxicity (Cuzick *et al*, 2007).

Negative trials can also be found in literature, an example is represented by the "α-tocopherol, β-carotene prevention study group" (1994): in this trial, 29133 smoker men were randomized to one of the two cited molecules, or to a combination of both or to placebo, with lung cancer incidence as end-point. The results showed an 18% increased incidence of lung cancer and 8% overall mortality in the β-carotene arm (Albanes *et al*, 1996): these increments were particularly observed in modest alcohol consuming men smoking more than 20 cigarettes daily, so suggesting that heavy smoking could be associated with the side effects observed with high β-carotene intake (Goralczyk, 2009). Another example is the "Selenium and vitamin E Cancer Prevention Trial" (Lippman *et al*, 2009): 35534 men were enrolled to receive selenium, vitamin E, both or placebo. After an interim analysis the study was suspended and a later report showed a significant increased risk of prostate cancer among the vitamin E arms (alone and in combination with selenium). Interestingly, the analysis of two smallest previous studies on selenium which had suggested its positive effects on carcinogenesis, clarified the results obtained from the former: baseline levels of selenium in blood were strictly linked to the outcomes of the supplementation with selenium (Duffield-Lillico *et al*, 2002), suggesting that a natural compound can exert or not preventive effects depending on, but not limited to, its levels in the body.

These negative trials underline the importance of the dose tested also when considering natural compounds. In fact it is plausible that those negative results could be related to a supra-physiological concentration reached in participants that might have been harmful. All considering, bioavailability and pharmacokinetics of phytochemicals should be assayed carefully before being tested in a clinical trial.

Nevertheless, the benefits derived by the approved use of tamoxifen or raloxifen and anti-HPV vaccine to prevent recurrence or insurgence of cancer (breast and cervix respectively), represent the best examples of the power of cancer chemoprevention and justify the increasing investments in this research area in order to find preventive strategies for other types of cancer, particularly for high incidence ones (Wu *et al*, 2011).

1.1.1 CRC chemoprevention: history of NSAIDs use

Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) have been widely investigated *in vitro, in vivo*, in epidemiological and randomized clinical trials (RCTs) in the last decades, showing to be a promise for cancer risk reduction. The first study in animal models of induced tumorigenesis was published in 1981, when Goto and colleagues (Goto *et al*, 1981) found reduced tumor incidence in rats treated for 10 weeks with indomethacin compared to untreated animals, so suggesting

that this treatment could effectively reduce development of CRCs in high risk individuals. Inflammation is considered a cancer risk factor, especially for oesophageal cancer and CRC (Ulrich *et al*, 2006): briefly, the inflammatory response leads to the recruitment of leukocytes and mast cells to the site of damage, with a subsequent release of free radicals which in turn damage intracellular macromolecules, while prostaglandins release triggers proliferative responses in the damaged tissue (Coussens & Werb, 2002). Cyclooxygenase 1 and 2 (COX-1 and 2), constitutively activated and inducible respectively, are responsible for the production of prostaglandins in response to stimulatory events from growth factors and cytokines: high concentrations of prostaglandins were found in neoplastic tissues, especially in colon where this was associated to an up-regulation of COX-2 in carcinomas and adenomas (Kutchera *et al*, 1996; Mladenova *et al*, 2011).

The first epidemiological data on NSAIDs preventive use date 1988, when Kune et al. (Kune et al 1988) reported a decreased risk of CRC with the use of aspirin (inhibitor of both COX subtypes). More in detail, data were collected by questionnaires administered to 715 patients with a confirmed diagnosis of CRC (excluding patients with Familial Adenomatous Polyposis or ulcerative colitis) and to 727 controls: regarding the use of medicaments, a significantly reduced risk of CRC was associated with the use of aspirin but not with other NSAIDs. These data were supported by following case-control and prospective studies, enforcing the relation between the regular intake of aspirin or other NSAIDs and CRC risk reduction (Greenberg *et al*, 1993; Martínez *et al*, 1995; Freedman *et al*, 1998). However, the lack of a relationship between dose and duration of aspirin intake and benefits in those studies, drove the implementation of further trials aiming at investigating this issue. Sandler and colleagues (Sandler *et al*, 2003) succeeded in demonstrating that the use of 325 mg/day of aspirin among patients with primary colorectal adenomas or cancer, was able to reduce the development of further lesions, with an odds ratio of 0.65 when the aspirin group was compared to placebo. In this trial, patients with FAP (Familial Adenomatous Polyposis) and inflammatory bowel disease were excluded, as were individuals with documented contraindications to the use of aspirin like peptic ulcer disease, aspirin sensitivity, or bleeding diathesis. However, it has to be considered that the chronic use of NSAIDs is known to induce gastrointestinal bleeding and renal toxicity, so suggesting their use under accurate medical surveillance. This suggestion has also a validity in the case of COXib use for cancer chemoprevention: despite of their COX-2 specific inhibitory activity, which makes them better tolerated than aspirin from the point of view of gastric toxicity, their chronic use has showed to increase adverse effects on both coagulation and cardiovascular toxicity (Nussmeier *et al*, 2005). Nevertheless, COXibs received in 2000 the approval from EMA and FDA for their use in FAP patients for the reduction of intestinal polyps: this

was the consequence of a trial involving 77 FAP patients who took 800 mg/day of COXibs for 6 months which determined a 28% reduction in polyps number and 30% reduction of polyp burden (Steinbach *et al*, 2000). Accordingly with “ESMO Clinical Practice Guidelines for familial risk-colorectal cancer” (Balmaña *et al*, 2013), COXibs can be considered as an adjuvant treatment when adenoma recurrence is detected after surgery but “caution is warranted” due to their cardiovascular side effects. In this guideline, also sulindac (a NSAID) is indicated for chemoprevention in FAP individuals: indeed, in 1983 Waddel and colleagues (Waddell *et al*, 1989) were the first to observe that rectal polyps developed after colectomy in a small group (4 individuals) of FAP patients, completely disappeared after sulindac treatment. Following small clinical trials (Spagnesi *et al*, 1994) and studies *in vitro* and *in vivo* (Boon *et al*, 2004; Femia *et al*, 2015b) have confirmed the promising chemopreventive use of sulindac, also in association with target therapy in FAP patients: in fact, sulindac plus erlotinib significantly reduced the tumor burden after 6 months of treatment compared to sulindac plus placebo in a phase II clinical trial (Samadder *et al*, 2018). Regarding aspirin, its use does not seem to have any effect in FAP patients: in a trial involving 227 FAP patients with an average age of 18 years, who did not undergo colectomy yet, the administration of 600 mg/day aspirin for 17 months was able to reduce the diameter but not the number of polyps compared to untreated individuals (Burn *et al*, 2011).

A recent systematic review and network meta-analysis best summarized the state of the art on CRC prevention with NSAIDs, leading to consider the preventive use of either aspirin or non-aspirin NSAIDs in a subset of individuals (Dulai *et al*, 2016) for short-term prevention. More in detail, non-aspirin NSAIDs could be used in people with a low baseline risk of cardiovascular disease and a moderate-high risk of CRC, while low-dose aspirin, due to its higher risk:benefit profile, could be used for secondary prevention in patients with previous low or high risk of CRC (or any other neoplasia). However, these are suggestions and not definitive recommendations on the use of this class of drugs for CRC prevention.

1.1.2 CRC prevention with phytochemicals

As previously illustrated, diet is a well-established determinant of colorectal cancer and considering that fruit and vegetables, daily intakes of which are strongly recommended, are rich in different kind of molecules, it is quite clear why a wide literature exists on food molecules and gastrointestinal cancers, among which a wide portion is accounted for by CRC. Indeed, more than half of cancer therapeutics approved by regulatory entities are natural compound derivatives, compounds based on or mimicking a natural product, or a natural product itself (Mann, 2002). Worthy of note is the

interest around phytochemicals, that are the non-nutrient compounds present in fruit and vegetables, which have been largely studied in *in vitro* CRC models (Aggarwal *et al*, 2013): these molecules have different kind of targets which can be up or down-regulated. Up-regulated targets are transcriptional factors, ligands, cell membrane proteins, tumor suppressors, enzymes involved in different pathways (i.e. proliferation, oxidative stress response) and pro-apoptotic proteins; on the other hand, down-regulated targets are represented by receptors, kinases, metastatic proteins, inflammatory factors, transcriptional factors, cyclins, stem cell markers.

Among the hundreds of molecules that have been tested for CRC preventive purpose both *in vitro* and *in vivo,* the most promising so far, that have been also tested in clinical trials, are listed below:

- ✓ *Curcumin (Curcuma longa)* is one of the most studied natural products in the cancer prevention field, particularly for gastrointestinal cancer. Curcumin is able to down-regulate, among others, NFkB, COX-2, IL-6 and TNF-α (Singh & Aggarwal, 1995; Bharti *et al*, 2003; Rao, 2007), and it has been found to be safe also at high doses (Lao *et al*, 2006). Some studies demonstrated the efficacy of curcumin in association with *piperine* (Shoba *et al*, 1998) and *quercetin* (Cruz-Correa *et al*, 2006): *piperine (Black pepper)* seems to enhance curcumin bioavailability; instead *quercetin*, also known for its cell-growth inhibitory activity *in vitro* and *in vivo* (Hosokawa *et al*, 1990; Newmark & Rupertc, 1993) was administered daily with curcumin for up to 9 months to five FAP patients with a prior colectomy, showing a decrease in number and size of adenomas at six months. Another study evaluated the preventive activity of a daily dose of curcumin (2 g or 4 g) administered for 30 days to 44 smokers with at least eight ACFs on screening colonoscopy: the higher dose was capable of reducing ACFs number without reducing proliferative index in normal mucosa (Carroll *et al*, 2011). In a wider study, the effect of curcumin (360 mg orally, 3 times/day) on body weight loss, TNF-α levels, and tumor cell apoptosis was assayed in 106 CRC patients monitored up to 30 days, finding an increased p53 expression in tumor tissue, a decreased loss of weight and decreased serum TNF-α levels so leading the authors to support the beneficial effect of curcumin on CRC patients' health (He *et al*, 2011).

- ✓ *Gingerol (Zingiber officinale)* has shown to be a potent anti-oxidant, anti-inflammatory and immune-stimulant, and these actions are thought to contribute to its cancer-preventive activity (Lee *et al*, 2008). In a randomized clinical trial RCT (Citronberg *et al*, 2013), 20 people at increased risk of CRC were randomized to 2 g of ginger

or placebo daily for 28 days: the results suggested a reduction in proliferation and increase in apoptosis and differentiation especially in the differentiation zone of colon crypts.

- ✓ *Resveratrol:* it is present in more than 70 plants (e.g. blueberries, raspberries, peanuts, grape). It interacts with cytochrome P450, downregulates COX-2 and NFkB and acts synergistically with 5-FU and oxaliplatin (Fulda & Debatin, 2004; Subramanian *et al*, 2010). It has also been studied in clinical trials on patients with CRC: the first one showed the inhibition of the Wnt pathway in the normal mucosa of CRC patients treated for 14 days with 80 g of a resveratrol containing grape powder, but not in cancer tissue (Nguyen *et al*, 2009). Following studies suggested that resveratrol can be a suitable chemopreventive agent at a dose of not higher than 1 g/day (Patel *et al*, 2010; Delmas *et al*, 2013) in order to avoid gastrointestinal side effects. Recently, a phase I trial investigated the effect of micronized resveratrol given at 5 g/day for 14 days to CRC patients with hepatic metastases: interestingly, serum levels of resveratrol were 3.6 fold increase compared to previous studies investigating non-micronized resveratrol, and were well tolerated by patients. Moreover, resveratrol was found in hepatic metastases concomitantly with a 39% significant increase in an apoptotic marker (Howells *et al*, 2011).

- ✓ *Epigallocatechin-3-gallate (EGCG)* is the main polyphenol present in green tea leaves. Concerning CRC, it is capable of inhibiting the activation of NFkB pathway leading to increased expression of caspase-7 and decreased expression of MMP-9 *in vitro,* so blocking migration and proliferation (Zhou *et al*, 2012). Moreover, it also contributes to DNA methyltransferase 3 (DNMT3) and histone deacetylase (HDAC) degradation (Singh *et al*, 2011). 176 patients who had undergone a polypectomy were enrolled in a randomized placebo-controlled trial testing the preventive activity on metachronous adenomas of a green tea extract at a dose of 0.9 g/day for 12 months: in the treated group, both incidence of metachronous adenomas and the number of relapsed adenomas were significantly lower than placebo arm (Shin *et al*, 2018). Other trials are still recruiting individuals or are completed but results have not been published yet.

- ✓ *Silymarin (Silybum marianum)* is present in the seeds of milk thistle. It is a complex of flavonolignans and flavonoids: silybinin, silydianin, silychristin belong to the first class, taxifolin and quercetin to the second (Abenavoli *et al*, 2010). Silymarin has demonstrated anti-cancer effects *in vitro* on several epithelial cancer cell lines including colon. It is also able to delay the development of adenomas in dimethylhydrazine (DMH) or

azoxymethane (AOM) induced carcinogenesis in rats and reduce the number of ACF (Kohno *et al*, 2002; Volate *et al*, 2005). Several mechanisms seem to be involved in the preventive action of silymarin: a recent study of Khorsandi et al. (Khorsandi *et al*, 2017) on the HT-29 colon cell line suggests that multiple pathways are involved in the inhibition of cell growth exerted by silymarin, including autophagy, while Barone and colleagues (Barone *et al*, 2010) demonstrated that the reduction of intestinal neoplasm insurgence in *Apc Min* mouse could be linked to the induction of the estrogen receptor beta (ERβ) pathway. In a clinical trial on FAP patients with ileal pouch-anal anastomosis and treated for 3 months with an oral silymarin formulation (Eviendep®), the number and size of duodenal adenomas were reduced of 32% and 51% respectively (Calabrese *et al*, 2013). Silymarin has also been investigated in association with sulindac to develop a device like a silicon-based vector with two aims: specifically targeting neoplastic cells and consequently, after its internalization only in tumor cells, reducing NSAID adverse effects (Scavo *et al*, 2015); results have not been published yet. Moreover, a phase IV trial is recruiting patients with metastatic CRC undergoing FOLFIRI chemotherapy, to assess the efficacy of silymarin to reduce side effects due to the adjuvant therapy (https://clinicaltrials.gov/).

✓ *Ellagitannins (ETs):* are phenolic compounds endowed with a high radical scavenging activity. Strawberries, raspberries, muscadin grape, walnuts and pomegranate contain a peculiar ETs: i.e. *punicalagin* is an ET peculiar of pomegranate. Both punicalagin and its principal colon metabolite urolithin-A (Cerdá *et al*, 2005; Espín *et al*, 2007; Nunez-Sanchez *et al*, 2015), have been tested *in vitro* for their anti-inflammatory, anti-oxidant and anti-proliferative activity in colon cancer cell lines (Boateng *et al*, 2007; Kasimsetty *et al*, 2010). Although non-edible parts of pomegranate, such as the peel and the mesocarp, are rich in punicalagin (Orgil *et al*, 2014), pomegranate juice obtained from arils has been largely tested in *in vivo* CRC models showing to be endowed of preventive properties (Kohno *et al*, 2004; Boateng *et al*, 2007; Larrosa *et al*, 2010; Banerjee *et al*, 2013). A pilot RCT on CRC patients who were treated with 900 mg/day of a pomegranate extract, reported the presence of punicalagin metabolites in plasma, serum, urine, normal and malignant colon tissues after 15 days of treatment (Nuñez-Sánchez *et al*, 2017) enforcing the idea that the modulation of gene expression observed in normal and cancer tissues of the enrolled patients could be attributed to its metabolites instead of punicalagin *per se*.

1.2 Colorectal carcinogenesis

Colon (large intestine) epithelium is made of functional structures called crypts made of a single layer of polarized cells. A longitudinal section of a colon crypt can be described as follows: starting from the top facing the lumen of the colon, polarized enterocytes (also named colonocytes) are the absorptive cellular component. Then, secretory cells (goblet cells) located in the middle of the crypt are responsible for mucins secretion while hormones are secreted in the lower third of the crypt by enteroendocrine cells. The stem cell niche occupies the bottom of the crypt: cell differentiation proceeds during migration of cells from stem niche towards the top of the crypt, where the differentiation process is completed. In physiological conditions, differentiated cells are continuously replaced: senescent colonocytes are removed into the lumen and replaced by new ones every 24 h (Barrasa *et al*, 2013).

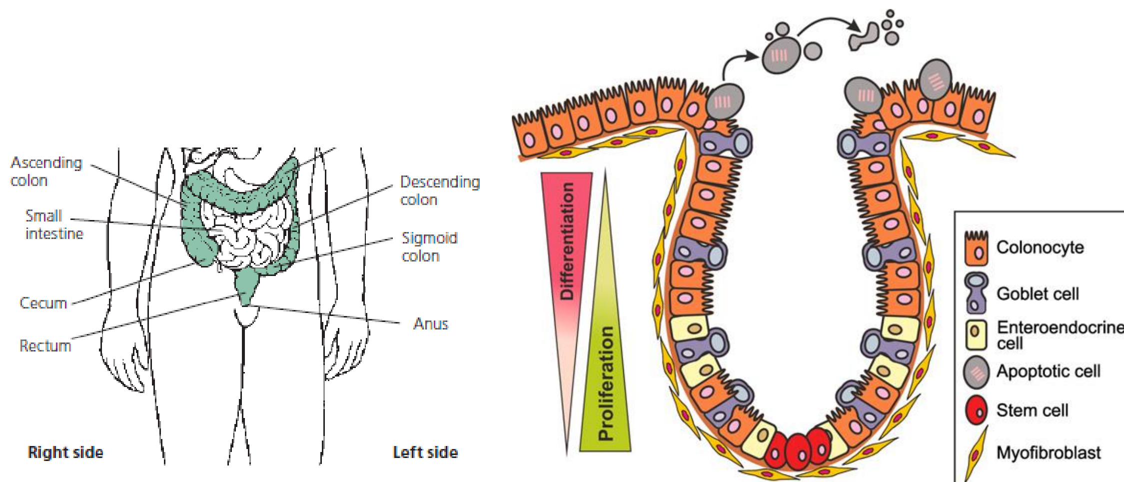

Fig.4. Colon organization. On the left side, colon anatomy (adapted from Colorectal Cancer Facts and Figures 2014-2016). On the right side, diagram showing a schematic view of a colon crypt (from Barrasa *et al*, 2013).

Clinically, CRCs are subdivided in proximal (or right-sided) when they originate in the region between the cecum and transverse colon, and distal (or left-sided) in case they originate within the descending colon and rectum. Some researchers tried in the past to find an association between prognosis and tumor location, but failed to demonstrate it (Testa *et al*, 2018).

1.2.1 Apc: the leader gene of colorectal carcinogenesis

Apc gene mutation (Adenomatous Polyposis Coli, localized on chromosome 5q21) is the key initial player in both sporadic and genetic CRC. *Apc* gene encodes for a 310 kDa protein (2843 amino-acids length) consisting of several

domains that determine the different mechanisms in which it is involved, some of them still partially or nearly clear or undefined. Starting from the N-terminus and moving towards the C-terminus of the protein we encounter the following domains (fig. 5) (Fodde *et al*, 2001b):

- the Armadillo domain, a Drosophila homologous region, involved in the Wingless/Wnt signaling pathway;
- ~3 interspersed 15 aa repeats in the region comprised between residues 1020-1169 considered β-catenin binding sites;
- the DNA Repair Inhibitory Domain (DRI-domain) between amino-acid residues 1245-1262;
- ~7 interspersed 20 aa repeats between the residues 1342-2075 which mediate *Apc* downregulation of β-catenin together with other molecules forming the destruction complex (see below for details); in this region also three interspersed SAMP repeats (Ser-Ala-Met-Pro) allowing the interaction with axin/conductin are present;
- the Mutation Cluster Region (MCR) between amino-acids 1284-1580, where the majority of *Apc* mutation (both in CRC and FAP) arises;
- a microtubule binding site comprised between residues 2219-2580;
- an EB/RP binding site: this family of proteins interact with microtubules.

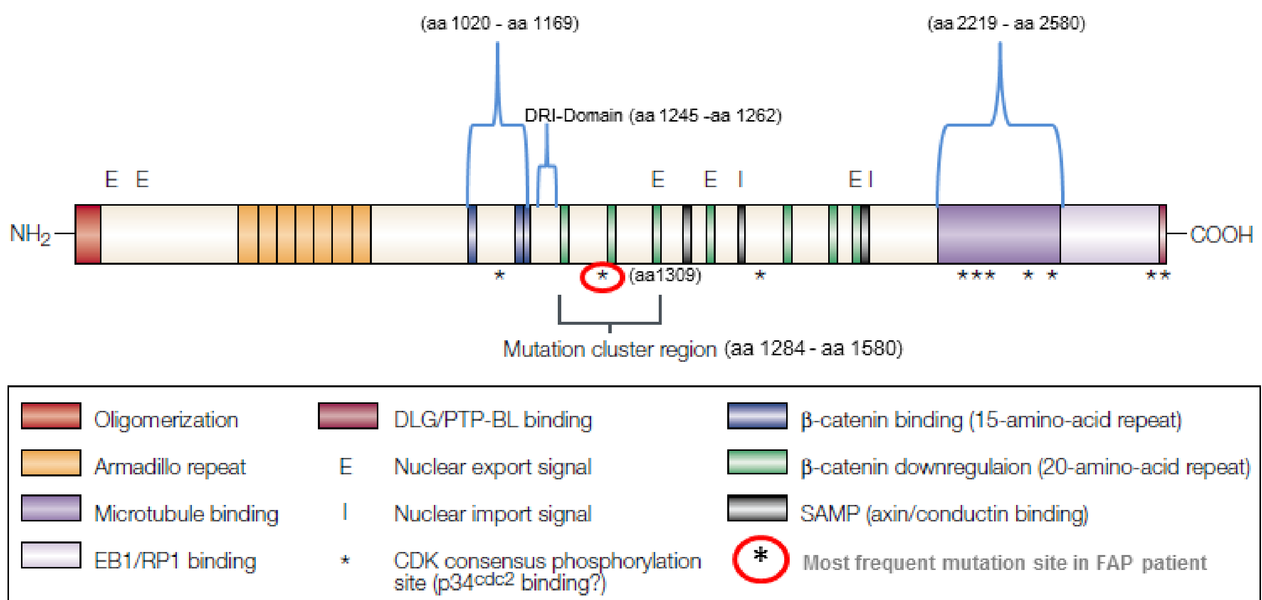

Fig.5. Apc human protein and its multiple functional domains (adapted from Fodde et al, 2001).

These regions account for the different functions in which Apc protein is involved: control of the WNT signal transduction pathway, cell adhesion, migration, apoptosis, chromosomal segregation at mitosis, inhibitory interaction with some components of the Base Excision Repair system (BER). Moreover, three Nuclear Export Signals and two Nuclear Import Signals (NES and NIS respectively) are interspersed along the protein.

Mutations in this gene can be detected in early preneoplastic lesions in the colon, Mucin Depleted Foci (MDFs) (Femia *et al*, 2005) and in a subtype (the flat dysplastic one) of Aberrant Crypt Foci (ACFs) (Paulsen *et al*, 2006), so supporting the role of *Apc* as tumorigenesis leading gene both in sporadic CRC and FAP patients.

As previously mentioned, this gene is also mutated in genetic syndromes which increase the risk of CRC development as FAP (Familial Adenomatous Polyposis). These patients harbor a heterozygote germ-line mutation in *Apc*: indeed, an homozygote germ-line mutation in this gene is incompatible with life. This heterozygosity is not supposed to last for long, particularly at the intestinal level: the loss of heterozygosity (LOH) of a tumor suppressor gene, as for *Apc*, is the so-called "second hit" postulated by Knudson in his "Two hit-model" (Hutchinson, 2001). This simple model supposes that two mutations, temporally separated and independent, in both alleles of a tumor-suppressor gene are needed to achieve its full loss of function: in agreement with this model, both alleles of the *Apc* gene are mutated at early stages of tumor development. Taking into account studies of Lamlum et al., Smits et al., and Albuquerque et al. (Lamlum *et al*, 2000; Smits *et al*, 2000; Albuquerque *et al*, 2002), it has been supposed that cancer cells tend to avoid excessive β-catenin accumulation in the nucleus, that has been demonstrated to induce apoptosis (Kim *et al*, 2000), due to retention of 1 or 2 β-catenin down regulating binding sites (20 aa repeats). More in detail, considering that each *Apc* allele has 7 β-catenin downregulating binding sites (fig. 5 green bars), the number of sites retained by the germline inherited allele will influence the somatic mutation in the MCR of the second one: this will lead to the retention of at least one downregulating site in the translated protein (fig. 6) (Fodde *et al*, 2001). Although the mechanism and regulation of this hypothetical retention have not been explained yet, undoubtedly the loss of β-catenin regulation due to *Apc* mutation allows the initial clonal expansion of cancer colon cells. The majority of the germline *Apc* mutations falls frequently into the MCR domain (aa 1309) or less frequently just before it (aa 1069), and because these mutations cause stop codons, they determine the production of a truncated protein retaining all or only one β-catenin binding site respectively. The truncated Apc forms retain all the functional domains before the truncation site, while those at the C-terminus are lost (Fodde *et al*, 2001).

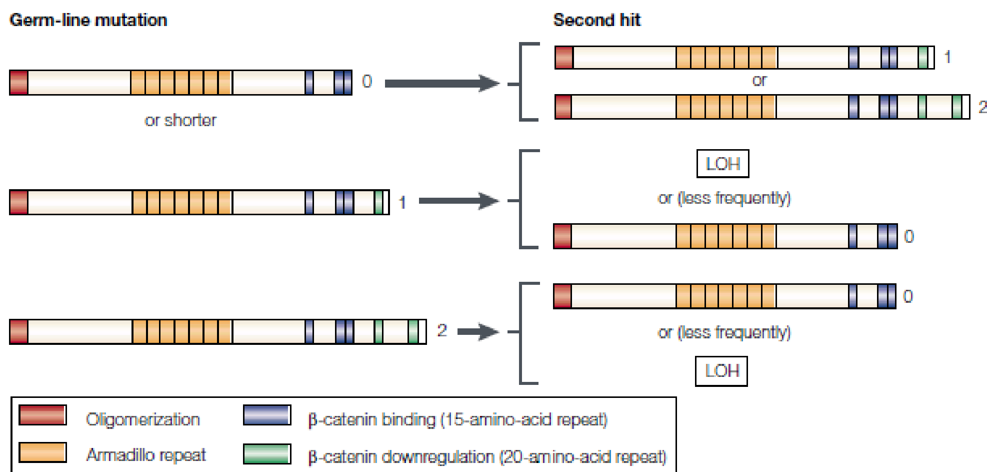

Fig. 6. The second hit model applied to Apc protein. In this model, at least one β-catenin downregulating binding site is retained in cells with a germline mutation in *Apc* (from Fodde et al, 2001).

1.2.2 N-terminus linked Apc functions

As first reported in early 2000 by two independent studies (Rosin-Arbesfeld *et al*, 2000; Henderson & Fagotto, 2002), Apc shuttles to the nucleus due to the NES sequences; surprisingly, the second study demonstrated that the truncated form moves more effectively than the full-length.

Apc could interact through its N-terminus with a nucleoporin or other nuclear membrane proteins to bind directly the nucleus to the centrosome as illustrated by Collin et al. (Collin *et al*, 2008); moreover, it seems to localize at the nuclear pore maybe regulating transport through it (Markiewicz *et al*, 2006).

The most interesting function of Apc N-terminus is the ability to block the Base Excision Repair systems, both the Single Nucleotide (SN-BER) and the Long Patch (LP-BER) one. This block occurs through interaction of the DRI-domain of the Apc protein with Pol- β, which exerts a 5'-deoxirybose phosphate lyase activity in both pathways (Narayan *et al*, 2005; Balusu *et al*, 2007). BER systems are important for the repair of a-basic DNA sites that can be caused by alkylating agents; a-basic sites can lead in turn to nucleotide transversions and transitions during DNA replication if not repaired. Thus, the inhibition of BER systems by Apc could have a dual function: on one hand, it could increase the efficacy of chemotherapeutic alkylating agents, like 5-FU, in cancer cells, determining accumulation of DNA damage and instability leading to apoptosis. This is the reason why the possibility to block BER pathways has been exploited recently as a

chemotherapeutic target for cancer cell death induction (Narayan *et al*, 2005). On the other hand, the inhibition of this repair pathway in normal cells, could increase their susceptibility to carcinogens, promoting carcinogenesis.

Additionally, it has been reported that Apc localizes at DNA-DSB (Double Strand Break) sites, recruiting DNA-PK (DNA-dependent protein kinase) which phosphorylates H2AX histone leading to γ-H2AX foci: these foci are markers of DNA-DSB and are the sites where dedicated DNA repair systems are recruited (Brocardo *et al*, 2011). Kouzmenko and colleagues (Kouzmenko *et al*, 2008) showed that this interaction could be mediated by Apc C-terminus domain mapping in the region comprised between aa 1441-2077, so suggesting a lack of this interaction in Apc mutated cells which could contribute to colorectal carcinogenesis.

1.2.3 C-terminus linked Apc functions

<u>Wnt signaling</u>

Full length Apc protein is generally present in the cytoplasm where it forms, together with axin, conductin and the glycogen synthase kinase 3β (GSK3β), a destruction complex for β-catenin in excess in the cytoplasm. GSK3β is thought to be responsible for β-catenin phosphorylation in absence of activating stimuli, which allows its degradation in the proteasome complex: this process permits to maintain the balance between free β-catenin molecules in the cytoplasm and the ones forming adherent junctions (fig. 7). Meanwhile, as a consequence of the absence of β-catenin in the nucleus, a complex made of TCF/LEF (T-cell factor/lymphoid enhancer factor) and TLE/Groucho (transducing-like enhancer protein) recruits histone deacethylases (HDACs) to repress target genes. When Wnt binds Frizzled receptors (Fzd) and LRP co-receptors, these last ones are phosphorylated and recruit Dishevelled (Dvl) proteins to the cell membrane (Zhan *et al*, 2017). After Dvl proteins polymerization and activation, they inactivate the destruction complex so allowing the release of β-catenin from it and its translocation into the nucleus where it functions as co-activator with the transcription factor TCF-LEF and switching on the transcription of various genes such as *c-Myc*, *Axin-2*, *Lgr5* and *cyclin D1* (Phelps *et al*, 2009). In FAP and non-hereditary CRC patients, the Apc scaffolding function in the β-catenin destruction complex is lost: consequently, β-catenin is free to move to the nucleus in absence of any Wnt signals, promoting the transcription of genes involved in proliferation, differentiation, adhesion and migration.

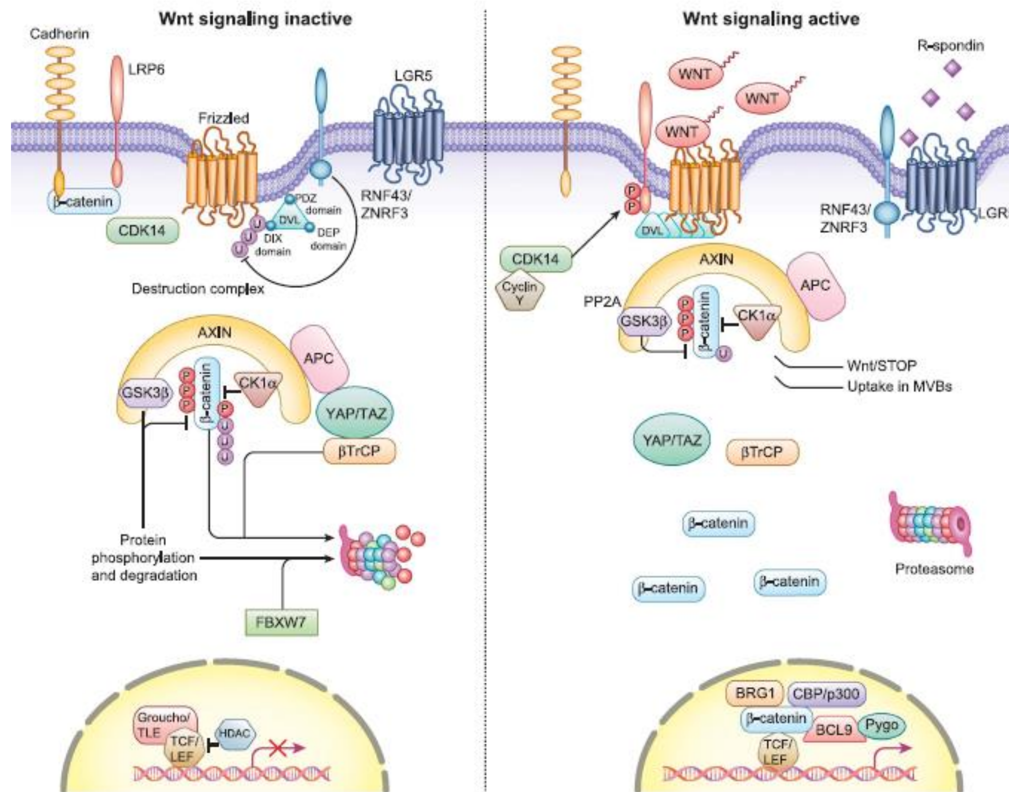

Fig.7. WNT signaling pathway (adapted from Zhan et al. 2017).

<u>Cytoskeletal scaffolding function and Chromosomal instability (CIN)</u>

Apc is also involved in the stabilization of the plus-ends of microtubules involved in the control of cell polarity during migration and microtubule attachment during mitosis. Concerning the first function, it has been shown that Apc accumulates in clusters beyond the microtubule plus-ends, driving the formation of membrane protrusions which allow cell movement (Reilein & Nelson, 2005). Moreover, Apc has been identified at adherent junctions and tight junctions where it interacts with actin filaments: so, the presence of a truncated form of Apc could permit a reduced cell adhesion and loss of polarity that confer aberrant migratory properties to colon cells (Brocardo *et al*, 2011).

Chromosomal abnormalities in terms of quantitative changes and structural rearrangements have been identified in *Apc* mutant cells leading to the hypothesis that Apc, interacting with EB1, could contribute to the proper attachment of the mitotic spindle to the chromosomes at the kinetochore, and could act as a checkpoint for the correct centrosome duplication during mitosis (Kaplan *et al*, 2001). The presence of an Apc defective protein could allow cells to bypass this checkpoint despite a failure in the kinetochore-microtubules attachment, leading to quantitative and qualitative chromosomal abnormalities (Green *et al*, 2005).

1.3 CRC molecular pathogenesis

Colon carcinogenesis is a slow multistep process: indeed, it has been estimated that 17 years are required for a benign adenoma to develop into a carcinoma, which in turn is capable of metastasizing within 2 years (Fodde *et al*, 2001). Due to its slow progression, colon carcinogenesis could be interrupted, hopefully permanently, at very early steps to avoid the insurgence of carcinoma: this justifies the increasing importance that chemoprevention has been acquiring in the field of CRCs.

Multiple molecular events are involved in CRC carcinogenesis: Vogelstein and colleagues were the pioneer of the theory on the progressive step-wise accumulation of genetic and epigenetic events that occur during the adenoma → adenocarcinoma transition (Vogelstein *et al*, 1988). These events can be divided into “driving” alterations and “promoting” ones: the former are responsible for the insurgence of small dysplastic focal lesions and their evolution into adenoma (benign lesion, also called polyp) so suggesting that tumor insurgence depends on the proliferation of a small mutated cell clone, likely located in the stem cell niche (Schepers & Clevers, 2012). More in detail, in a time period lasting 20-40 years, progression from microscopic preneoplastic lesions to adenoma and, in the end, to carcinoma seems to be driven by subsequent additional mutations in oncosuppressor (the earliest affecting *Apc,* then *p53* and *Smad4*) and oncogenes (mainly in *Kras* and *PIK3CA*), along with epigenetic changes (mainly methylation) that make the genome more instable: an instable genome makes the DNA more susceptible to accumulate mutations and abnormalities, so hastening carcinogenesis (Fearon & Vogelstein, 1990). These steps are shown in fig. 8 diagram.

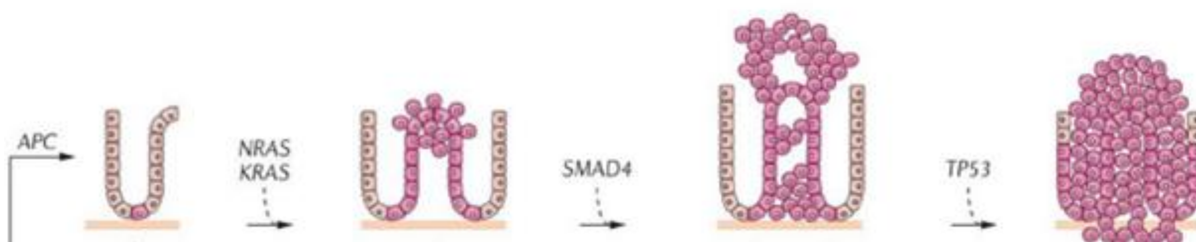

Fig.8. CRC carcinogenesis and principal mutated genes with push the process towards malignancy development (adapted from Kuipers *et al*, 2015).

Three kinds of small preneoplastic focal lesions have been identified in *in vivo* models of CRC carcinogenesis (fig. 9):

- Aberrant Crypt Foci (ACFs) were identified by Bird in 1989 and described as foci constituted by enlarged crypts (3-4 fold larger than a normal one), thick epithelial lining, increased pericryptic zone and oval lumen (Tudek *et al*, 1989). They can be visualized on formalin fixed whole colon samples stained with methylene blue. In clinical studies, where ACF have been observed in patients at risk, they have been further subdivided in 3 types: non-dysplastic, moderate dysplastic and severe dysplastic, based on histology. ACF are mainly found in the rectal and middle segment of colon and their number and size in the colon of carcinogen-induced animals is directly related to the dose and time from the exposure. Moreover, they have been identified also in humans (Roncucci *et al*, 1998).

- Mucin Depleted Foci (MDFs), were first identified by Caderni et al. (Caderni *et al*, 2003) in unsectioned colon tissues stained with high-iron diamine Alcian blue (HID-AB), a technique which highlights mucin production. These dysplastic lesions are characterized by scarce production of mucins, high expression of inflammatory markers (iNOS and COX-2) and mutation in *Apc* and β-*catenin, KRAS* mutation are less frequent. Constitutive Wnt-signaling, as observed in tumours, is present in these lesions (Femia *et al*, 2007, 2009). Their number and size are carcinogen dose- and time-dependent, and similarly to ACFs they can be found in human colon samples (Femia *et al*, 2008).

- β-catenin accumulated crypts (BCAC) identified by Yamada et al. are lesions composed by dysplastic crypts showing high amount of β-catenin in both cytoplasm and nuclei (Yamada *et al*, 2001); in this lesion Paneth cells can often be found (Mori *et al*, 2005). Up to now there are no data available about their identification in humans.

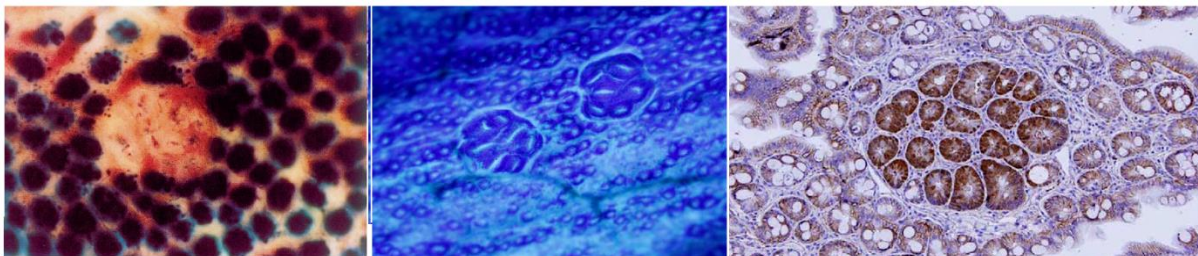

Fig.9. Examples of colon preneoplastic lesions: from the left side are shown in sequence an MDF (adapted from Femia et al. 2004), an ACF and a BCAC (both adapted from Mori et al, 2005).

Kras and *p53* mutations are considered as late events in CRC carcinogenesis, particularly involved in the transition from adenoma to carcinoma. *Kras* mutations cause a permanent activation of MAPK pathway that makes cells capable of avoiding apoptosis and proliferating continuously: tumors mutated in this gene are not sensitive to anti-EGFR therapy; loss of function of *p53* can further affect proliferation leading to loss of cell cycle control and invasion, induction of an inflammatory microenvironment and epithelial-mesenchymal transition (Kuipers *et al*, 2015).

Less frequent (<20%) mutations in CRCs are in *PI3KCA* and *Smad4*: mutations in the former (phosphoinositide-3-kinase), are nearly simultaneous to the *Apc* ones, which in turn activates AKT and mTOR pathways so further stimulating cell growth and metabolism; additionally, an increase in PGE2 synthesis follows *PI3KCA* mutation so contributing to the creation of an inflammatory tissue environment (Colussi *et al*, 2013; Testa *et al*, 2018). On the other hand, mutations in *Smad4,* a mediator of TGF-β pathway, inactivate this signaling pathway allowing cells to escape from apoptosis and blocking their differentiation, thus increasing their metastatic potential (Fumagalli *et al*, 2017).

Chromosomal instability (CIN) and microsatellite instability (MSI) are considered promoting events: these can lead to the accumulation of mutations in additional genes, leading to deregulation of key cellular mechanisms (i.e. proliferation, differentiation) and can promote the cross-talk between tumor cells and microenvironment allowing invasion and metastasis (Pino & Chung, 2010; Matano *et al*, 2015). Both pathways can be triggered by high cell division rates and oxidative DNA damage.

CIN (observed in 70% of CRC cases) arises from defects in chromosomal segregation and telomere instability that determine aneuploidy and loss of heterozygosity (LOH) so affecting alterations in SMAD receptor family, EGFR, VEGFR and in the DNA damage repair response (Dienstmann *et al*, 2017), in addition to the genes listed above. MSI (15% of cases) is due to mutation in genes involved in the DNA mismatch repair system (i.e. MLH1 and 3, MSH2), determining high CpG island methylation phenotype (CIMP) in multiple key genes (Vilar & Tabernero, 2013), high immune cells infiltration (mainly NK and T-helper1 cells) and mutations in *TGF-β* receptors, *BRAF*, *PTEN* and *ATM* (The Cancer Genome Atlas Network, 2013).

Large scale-data on CRC transcriptomics has led to the implementation of a consensus molecular classification of CRC in four classes, also named consensus molecular subtypes (CMS): tumors with MSI belong to the first group (CMS1, 14% of primary tumors), while tumors with CIN are subdivided in the other three groups (Dienstmann *et al*, 2017). CMS1 are hypermutated and hypermethylated tumors with high immune cells infiltration. The CMS3 subtype (representing 13%

of primary CR tumors) shows hypermutated "driving genes", low stromal infiltration, an intermediate level of hypermethylation and metabolic reprogramming. CMS2 subtypes (37% of all primary tumors diagnosed and having the best prognosis compared to the other classes) show higher expression of EGFR, HER2, IGF2 and *cyclins* which account for their sensitivity to targeted therapies. Finally, CMS4 tumors show the activation of pathways implicated in epithelial-mesenchymal transition (EMT, mainly driven by TGF-β activation) and stemness, resulting in high infiltrating rate of cancer associated fibroblasts (CAFs), inflammation and suppression of the immune response: consequently, CMS4 primary tumors are associated with poor prognosis and high risk of metastasis, accounting for 23% of all colonic primary tumors diagnosed (Sinicrope *et al*, 2015). Indeed, a retrospective analysis showed a poor prognosis for CRC patients displaying CMS4 subtype (Song *et al*, 2016); as a consequence, the inhibition of TGF-β signaling has been investigated in combination with canonical chemotherapy within the MoTriColor project (http://www.motricolor.eu/), in view of the possibility that tumor stroma may exert a considerable impact on clinical and biological CRC outcomes.

1.4 Genetic predisposition to CRC

Commonly, CRC cases are divided in sporadic and hereditary: the latter are due to inherited genetic factors (germline mutations) which increase the risk of CRC between 70-100%, accounting for about 5% of all cases and comprehending Lynch syndrome (hereditary non-polyposis colorectal cancer, HNPCC) and familial adenomatous polyposis (FAP) (Kanth *et al*, 2017). In both syndromes, germline mutations are of heterozygote type, meaning that all cells bear a wild-type copy of the gene until a second mutation on the other chromosome in a subset of cells, such as in colon epithelial cells, determines its complete loss (the two-hit theory of Knudson): this event promotes carcinogenesis initiation. However, the accumulation of mutations in additional genes, i.e. *Kras* or *p53* have also to occur in order to result into carcinoma development.

Lynch syndrome (4% of all hereditary CRC) is characterized by mutations in the mismatch repair genes (MMR) that increase the risk of CRC of 30-80% and predispose to at least seven other cancers including stomach, endometrium and ovaries. The germline mutation in a MMR gene (*Mlh1, Msh2, Msh6* or *Pms2*) is followed by a somatic mutation in the wild-type allele due to chromosomal deletion, mutation or CpG island methylation in the promoter region, triggering tumor progression (Liu *et al*, 2012). Individuals diagnosed for LS should undergo colonoscopy for surveillance purpose every 2 years starting at 20-25 years of age; additionally, women should undertake annual endometrial sampling and

transvaginal ultrasound during pre-menopausal period, and a prophylactic hysterectomy is advisable after child-bearing completion. Prophylactic colectomy in LS patients is not recommended (Balmaña *et al*, 2013).

FAP (Familial Adenomatous Polyposis) patients (≤ 1% of all CRCs) present a very early insurgence (late childhood or during adolescence) of hundreds to thousands of adenomas along the entire colon which must be surgically removed to avoid their transformation into malignancies: indeed, the risk of CRC reaches 100% if colectomy is not performed. FAP patients bear a germline heterozygote mutation in *Apc* which causing polyp formation along the intestine following somatic inactivation of the second allele: although the majority of FAP patients presents a family history of polyposis 25-30% of cases are due to *de novo Apc* mutation (Aretz *et al*, 2004). Depending on the site of mutation within the gene, extra-colonic features are associated with FAP, like a congenital hypertrophy of the retinal pigment epithelium, or skin fibromas (desmoid tumors) and epidermoid cysts: this last FAP variant has been often referred to as Gardner's syndrome (de la Chapelle, 2004). Desmoid tumors affect connective tissue in up to 20% of FAP patients: these solid tumors, usually benign, can also transform into invasive tumors; their progressive growth can exert a pressure on adjacent organs which can determine complications and even death (Fábio, 2014), accordingly, they are treated with NSAIDs, chemotherapy or surgery. Additionally, extra-colonic tumors in FAP patients can develop in the duodenum, stomach, thyroid, liver, and pancreas.

Colonoscopy is the recommended screening procedure, that should be performed every year since12 years of age or earlier and until an increased polyp burden or advanced polyp histology occur: at this point, a total colectomy with ileal-rectal anastomosis (IRA) or proctocolectomy with ileal-pouch-anal anastomosis (IPAA) are performed. However, patients will be under annual surveillance either after an IRA or IPAA: rectum is retained with IRA so it should be performed in patients without polyps in the rectum that consequently will undertake annual rectal surveillance; instead, IPAA patients are still at risk of adenomas in the ileum and in the anal transition zone so they will undertake endoscopic surveillance every year or less (Kanth *et al*, 2017). FAP patients have also to be kept under surveillance for the extra-colonic tumors mentioned above: the major cause of death in FAP patient who underwent colectomy is represented by duodenal carcinoma which occurs in more than 12% of FAP patients and for whom challenging and suboptimal surgical procedures are available (Heiskanen *et al*, 1999).

Hence, considering the short and long-term complications due to colectomy procedures and the need for continuous surveillance, prophylaxis for FAP patients could have a considerable impact on quality of life (QoL): this justifies the

efforts devoted to finding less invasive preventive approaches for these patients, aiming at delaying surgical interventions. NSAIDs have been widely investigated in chemopreventive clinical trials in FAP patients: Giardiello et al. (Giardiello *et al*, 1993) demonstrated a 56% and 65% reduction in number and diameter of adenomas in the colon of FAP patients respectively, after a treatment with sulindac 150 mg twice a day for 9 months; unfortunately, a re-growth of adenomas was observed 3 months after the interruption of the therapy.

1.5 Sporadic CRC cases

Sporadic CRCs are due to modifiable factors, that are lifestyles and exposition to mutagens (see par. 1) and non-modifiable factors, that comprehend genetic alterations, as illustrated above for familial CRC, and age: in fact, 70% of sporadic CRC arise over 65 years of age. Screening programs are conceived for apparently healthy population in order to detect a pre-cancer condition or a malignancy at very early-stage which can be treated with better prognosis. Regarding CRC screening programs, these are conceived for population over 50 years of age: the European Guidelines for quality assurance in CRC screening and diagnosis (Von Karsa *et al*, 2013), recommend to perform the faecal occult blood test (FOBT) or a faecal immunochemical test (FIT) in both sexes with a biannual frequency which should be followed by a colonoscopy or other endoscopic techniques, i.e. contrast (CT) colonoscopy, in case of positivity. Adherence to recommended screening test is of particular importance for CRC as it is mainly an asymptomatic cancer: alarm signals can be weight loss, abdominal pain, fatigue and anemia-related symptoms. Treatment of CRC depends on stage assessment at the diagnosis that is performed on polyp samples collected during colonoscopy, as briefly outlined below (Schmoll *et al*, 2012) :

<u>Stage 0 and I</u> are mainly treated with polypectomy and surgical resection plus anastomosis respectively without adjuvant therapy;

<u>Stage II</u> are treated by means of surgery as for stage I, followed by adjuvant therapy in high-risk patients;

<u>Stage III</u> is treated like stage II but adjuvant therapy is compulsory.

Depending on the location of the tumor, that is colon or rectum, surgical procedures are different, consisting in laparoscopy or open surgery for colon resection, and total mesorectal excision (TME) for rectum resections. Neoadjuvant

(pre-operative) treatments are recommended in case of advanced diagnosed CRCs and can be represented by chemotherapy alone or in combination with radiotherapy (Quirke *et al*, 2014).

Systemic adjuvant therapies (post-operative) consist of:

- FLOX: 5-fluorouracil (5-FU)/leucovorin (LV)
- FOLFOX4: 5-FU (intravenous)/LV/oxaliplatin
- FOLFIRI: 5-FU/LV/Irinotecan
- XELOX: capecitabine plus oxaliplatin

Although it is estimated that 30-50% of patients will undergo a relapse, CRC survivors represent the third largest group of long-term cancer survivors in Western countries (Labianca *et al*, 2010): survivorship is strictly dependent on adherence of patients to scheduled follow-ups and healthy lifestyles. CRC patients follow-ups consist of a colonoscopy 1 year after adenomas resection and then every 2-3 years in high-risk patients and up to 5 years in low-risk ones: moreover, in elderly population, comorbidity and possible complications during colonoscopy (bleeding, perforation) have to be taken into account accordingly to the European Society of Gastrointestinal Endoscopy (ESGE) guidelines (Mathus-Vliegen *et al*, 2013). Less invasive methods for CRC patients surveillance consist in measuring carcinoembryonic antigen (CEA) in plasma and in a CT scanning for metastases detection (Kuipers *et al*, 2015).

For metastatic disease presenting at diagnosis with resectable metastases, adjuvant chemotherapeutic regimens suggested are FOLFOX or FOLFIRI or the FOLFOXIRI (5-FU/LV/oxaliplatin/irinotecan) associated with targeted therapies. These are divided in three groups (Kuipers *et al*, 2015):

a) Anti-EGFR monoclonal antibodies (Cetuximab and Panitumumab);

b) Anti-VEGF-A monoclonal antibodies (Bevacizumab);

c) Anti-proangiogenic factors (Aflibercept) and multikinase inhibitors (Regorafenib)

Undoubtedly, surgical procedures and (chemo)radiotherapy for treating both sporadic and hereditary CRCs as illustrated above, are accompanied by more or less severe short or long-term complications which affect QoL and can, in some cases and particularly in elderly subjects, increase mortality risk. Complications due to systemic treatments can be

represented by: change in bowel movement, abdominal pain, fatigue, weight loss, diarrhea, alopecia, neutropenia, skin rushes, hypertension, cardiovascular events, loss of appetite and anemia. On the other hand, surgery complications can be represented by: abdominal scar herniation, wound dehiscence, fecal and urinary incontinence, sexual dysfunctions (Kuipers *et al*, 2015).

1.6 Inflammatory bowel diseases (IBDs) and CRC risk

Apart from lifestyle and genetic factors, IBDs such as Crohn's disease (CD) and ulcerative colitis (UC) are associated with increased risk of CRC, accounting for 1% of all cases. CRC carcinogenesis due to an IBD condition seems to follow a similar multistep progression as shown in sporadic cases, but with a somewhat different genetic pathway: two studies (Robles *et al*, 2016; Yaeger *et al*, 2016) have found that *Apc* mutation rate is lower in IBD-associated cancers than in sporadic CRC, and occurs later during carcinogenesis. Although this low mutation rate in *Apc*, the Wnt/β-catenin pathway is compromised because of mutations in other molecules involved in the activation of this pathway: these could explain the high nuclear β-catenin accumulation found in CRC associated to IBDs (Balzola & Bernstein, 2009). Consequently, CRC development associated to IBDs seems to proceed on a different multistage process, in which *Apc* mutation is the last step while MSI, methylation events, and mutations in *KRAS* and *TP53* are earlier events (Romano et al, 2016). The accumulation of these genetic alteration and genomic instabilities are triggered by the high proliferative rate of epithelial cells, which in turn is stimulated by the inflammatory acute response. Indeed, immune cells interact with fibroblasts and also with tissue stem cells through the release of cytokines, TNF-α TGF-β so promoting proliferation, expression of adhesion molecules and activation of pro-coagulant factors (Sakurai *et al*, 2014). These findings have suggested a model to explain inflammation-related CRC carcinogenesis in which chronic inflammatory stimuli and the inflamed tissue microenvironment act together to favor carcinogenesis: the former stimulate epithelial cells to continuously divide (increasing the risk of accumulating gene mutations) to repair the damaged mucosa; the latter provide favorable conditions for mutated cell clones expansion (Galandiuk *et al*, 2012).

1.7 Epigenetic alteration in CRC carcinogenesis: the role of DNA methylation

As previously illustrated, CRC can be classified in two groups showing either CIN or MSI pathway: the latter are hypermutated cancers associated with CpG Island Methylation Phenotype (CIMP), which is considered a unique subgroup of CRCs representing 15% of all cases. Methylation is the most common epigenetic modification of DNA: it does not affect nucleotide sequence but it modifies transcription (it allows gene silencing) and genome stability (allowing genomic transposition and/or inversion) (Gallois *et al*, 2016).

Methylation can occur either on hystone H3, through hystone methyl transferase (HMT), or on the 5-carbon of cytosine residues, producing 5-methyl-cytosine (5mC), through one of the five members of the DNA methyl transferase (DNMT) family. Cytosine methylation occurs at the level of the so called CpG islands, repetitions of the dinucleotide cytosine-guanine (CG) (between 200-500 times): the majority of CpG islands are found in gene promoters, mostly oncosuppressor genes, in a hypomethylated status allowing transcription; otherwise, they can also be found in the gene body or in large repetitive sequences (LINE, SINE, Alu elements) in a hypermethylated status blocking their transposition in the genome (Rhee *et al*, 2017).

Promoter methylation plays a critical role in the regulation of different processes, starting from embryonic development to tissue differentiation, and different methylation patterns can be found among human tissues. Both overall genome and promoter methylation changes are associated with carcinogenesis: many tumors show a direct relation between tumor stage and increased overall genome hypomethylation, together with hypermethylated promoters of tumor suppressor genes (Duthie, 2011). As previously mentioned, CRC progression is driven by the accumulation of mutations in key genes and by an increased genomic instability: both alterations can be due to CpG methylation changes (Porcellini *et al*, 2018). Indeed, hypomethylation occurring in extensive genomic regions is associated with high rate of inversions and transpositions of short and long sequences (SINE and LINE elements) that can determine activation of oncogenes such as *Kras*: recently, LINE-1 hypomethylation has been found in familiar CRCs so suggesting a possible heritability of this epigenetic modification (Ogino *et al*, 2013). Meanwhile, hypermethylation of promoters causes silencing of oncosuppressor genes as *Apc, Mgmt* (Rhee *et al*, 2017).

These changes occur very early, at the level of the preneoplastic lesions ACF, as recently illustrated by Hanley and colleagues (Hanley *et al*, 2017). Analyzing the methylation profiles of ACFs in stage III and IV CRCs in patients with BRAF and KRAS mutations, and normalizing the data on the respective normal mucosa samples, they focused on Differentially Methylated Regions (DMRs) defined as genomic regions showing ≥ 15% change in methylation compared with matched normal mucosa. Both ACFs and CRCs showed the highest percentage of DMRs located in the gene bodies, followed by intergenic regions and then promoters, the latter being hypermethylated in both lesions (72% and 95% of CRCs and ACFs respectively, compared to NM). Regarding gene bodies and intergenic regions, in ACFs these were hypermethylated compared to NM (61% and 57% respectively), while they were hypomethylated in advanced CRCs, leading to speculate that hypomethylation of these two regions could be a determinant for the transformation of ACFs into adenomas and adenocarcinomas. Additionally, it was also noticed that genes with a methylated promoter were silenced in CRCs but not in ACFs. In fact, considering 12 of these genes, they found that their promoters showed a methylation increase of 40% in CRCs and only 20% in ACFs compared with matched normal mucosa, suggesting that the extent of promoter methylation in preneoplastic lesions was not sufficient to alter gene expression, a limiting factor for the progression of some ACFs into malignancies. However, this study took into consideration only *Kras* mutated patients (a particular sub-type of CRC), so that the differences observed between CRCs and ACFs could be dependent on this factor. In addition, it has also to be considered that ACFs are a very heterogenous population of preneoplastic lesions and that their correlation with carcinogenesis has also been questioned.

Many studies have evaluated methylation changes during CRC progression in sporadic CRCs (Luo *et al*, 2014; O'Reilly *et al*, 2016), and others have focused on promoter methylation of key genes in familial CRCs, whereas few studies have investigated how methylation changes could affect tumor microenvironment in CRC. Indeed, an immunohistology assay on 2 adenomas and 6 adenocarcinomas compared with the respective normal mucosa from 8 patients, revealed a significant hypomethylation of CAFs (cancer-associated fibroblasts) in adenocarcinomas compared to normal mucosa but not in adenomas (Ling *et al*, 2016). This result, together with a previous *in vitro* study of the authors (unpublished) suggested that the continuous cross-talk between epithelial cancer cells and CAFs occurring during carcinogenesis, could affect methylation changes in the latter. Indeed, a normal colon fibroblast cell line (CCD-18Co) co-cultured with a colon adenocarcinoma cell line (HT-29) showed a time-dependent loss of global DNA methylation that might contribute to altered expression of pathways (see par.1.3) which in turn promote further CRC progression (Ling *et al*, 2016).

As epigenetic changes are potentially more easily reversible than genetic ones, molecules or drugs capable of perturbing methylation at a specific time point, could interfere with colon carcinogenesis.

1.8 Colon cancer epithelial cells are not alone: role of fibroblasts in carcinogenesis

In general, most of the efforts for an ever deeper understanding of carcinogenesis have focused on tumor cells, i.e. epithelial cells in the case of CRC, obviously considered the main actors of this process, and the most significant therapeutic target. However, in the last few decades (since 1980s) researchers began to consider that the stroma surrounding the tumor, the tumor microenvironment (TME), might actively participate in the carcinogenesis process (Durning & Schor, 1984; Schor *et al*, 1988).

The main cellular component of the stroma is represented by fibroblasts: they remodel the extracellular matrix (ECM) through the release of degradation enzymes (metalloproteinases, MMPs) and structural proteins (collagen, fibronectin, proteoglycans, laminins among others). In addition to the control of tissue homeostasis and integrity, fibroblasts express enzymes of the cytochrome P450 family, cytokines, chemokines and growth factors: these molecules are released in the ECM exerting effects also on epithelial cells, from which they are separated by the basement membrane (Kalluri, 2016).

Generally, in a normal tissue, fibroblasts are quiescent cells and exert the functions listed above only in case of tissue injury (the so-called wound healing response), when they became activated to myofibroblasts. Once the tissue has been repaired, myofibroblasts turn again to a quiescent status. Permanent fibroblast activation has been found in acute and chronic inflammation and tissue fibrosis (Micallef *et al*, 2012). In cancer, TME fibroblasts are constitutively activated and are called cancer-associated fibroblasts (CAFs).

CAFs are highly proliferative and secretory cells, with a multi-spindled morphology instead of single-spindle one as the quiescent counterpart; they are positive for fibroblast activating protein (FAP protein) and alpha-actin smooth-muscle (α-SMA), and are endowed with migratory ability. Considering the CAFs secretory phenotype, this is richer than that of myofibroblasts: TGF-β, HGF, FGF, NFkB, VEGF, PDGF, TNF-α, IL-6 and many other mediators are over-secreted by CAFs and account for the high impact exerted by these cells on tumor cell proliferation and invasiveness (Olumi *et al*, 1999; Orimo *et al*, 2005; Rajaram *et al*, 2013).

Moreover, changes in the ECM composition and enzymes expressed by CAFs (i.e. increased amount of collagen and expression of cytochrome P450 respectively), can create a physical barrier to drugs diffusion towards the tumor site, and allow tumor cells to tightly adhere to ECM in order to escape from chemotherapeutic drugs, a process named cell-adhesion mediated drug resistance (CAM-DR) (Damiano *et al*, 2001). This phenomenon could lead to the subsequent epithelial-mesenchymal transition (EMT) of cancer cells. Moreover, ECM remodeling and chemokines and cytokines release by CAFs create an immunosuppressive TME which functions as a barrier for T helper 1 cells (T_H1) and macrophages infiltration (Salmon & Donnadieu, 2012): these two types of immune cells are capable of eliminating tumor cells and immunotherapeutic strategies are based on their recruitment (fig.10).

TGF-β signals and epigenetic events partially stimulated by tumor cells seem to activate resident fibroblasts into CAFs in an irreversible manner, which in turn promote additional genetic and epigenetic mutations at the level of tumor cells so creating a complex network of signals supporting cancer growth and invasiveness (Mrazek *et al*, 2014).

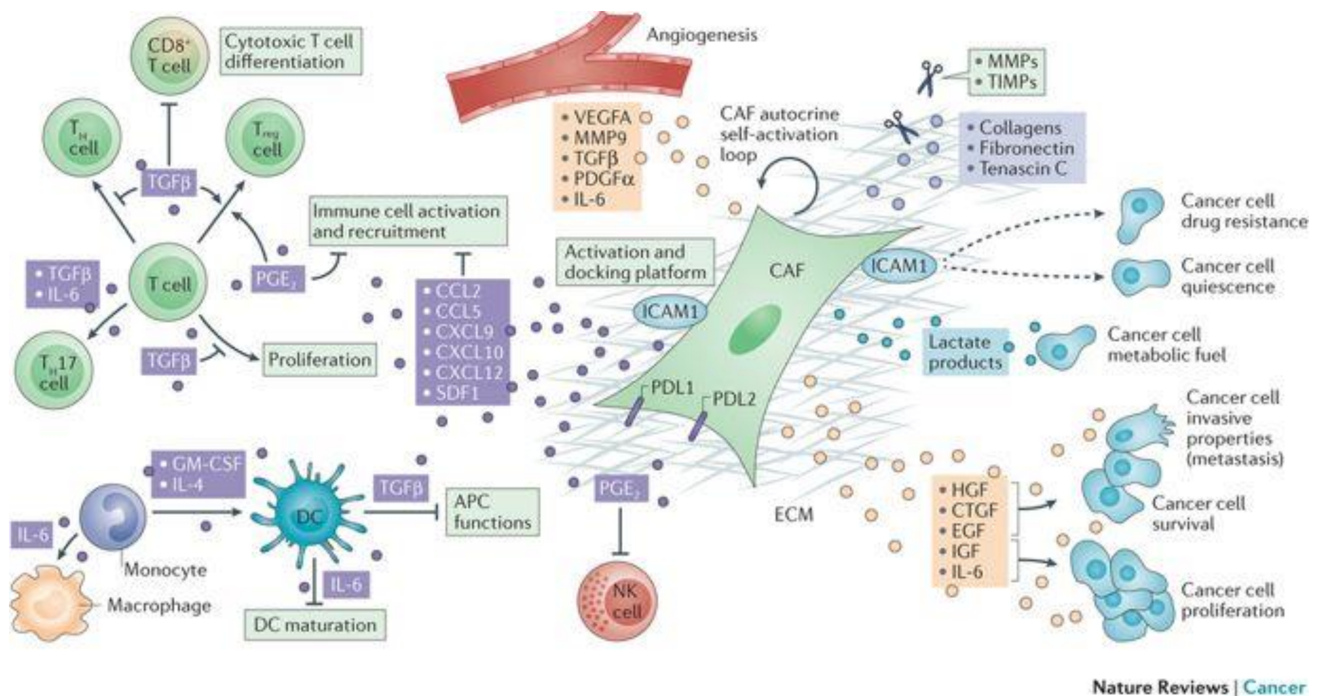

Fig.10. CAFs secretome and its multiple targets (Kalluri, 2016).

The increased appreciation of the role exerted by CAFs during carcinogenesis led investigators to make efforts for implementing new synergistic therapeutic strategies targeting both tumor cells and CAFs. The table below (tab. 1) shows molecules targeting CAFs that are used in ongoing clinical trials (Valkenburg *et al*, 2018): in addition to molecules

targeting CAFs markers or involved in cancer-CAFs signaling pathways (i.e. Fibroblast Activating Protein, FAP, and Fibroblast Growth Factor Receptor, FGFR, respectively), strategies to enable drug diffusion through ECM are also being investigated. Only FAP protein and TGF-β are currently investigated targets in CRC patients (tab. 1).

Tab.1: List of molecules and their respective tumor stroma targets investigated in both ongoing and concluded clinical trials. (adapted from Valkenburg et al, 2018)

Stroma targets	**Representative agents involved in cancer clinical trials**
CXCR4	Plerixafor (NCT01610999)
CYP3A4	Clarithromycin (NCT03043989) Itraconazole (NCT02157883)
FAK	Defectinib (NCT03287271)
FAP	Sibrotuzumab (NCT02198274) RO6874813 (NCT02558140)
Hyaluronic acid	PEGPH20 (NCT01453153)
TGF-β	Galunisertib (NCT02304419) Fresolimumab (NCT02581787)

1.8.1 CAFs and CRC

An interest on CAFs also arose in the field of CRC where several studies confirmed their involvement in the carcinogenesis process through the mechanisms listed in the previous paragraph. More in detail, CRC like other solid tumors is characterized by stromal infiltration due to inflammatory signaling and molecular cross-talk between cancer cells and stroma. Cancer cells are thought to release factors which create an inflamed stroma supporting their own survivorship; consequently, stroma cells, particularly CAFs, tend to promote proliferation and progression of colonic adenomas to carcinomas through some of the mechanisms listed above as shown in mouse models (Okada *et al*, 2000).

The possibility of a prognostic link between CAFs infiltration and CRC was raised in the 1990s: Hewitt and colleagues (Hewitt *et al*, 1991) demonstrated a higher immunoreactivity for collagenase enzyme and its specific TIMP in CRC

carcinomas than adenomas and normal mucosa, while Porte et al. stated the importance of the interaction between tumor cells and ECM, mediated by CAF released factors (in particular a MMP), in the initiation of cancer invasion and metastasis (Porte *et al*, 1995). More recently, Gong et al (Gong *et al*, 2013) assessed that TIMP-1 promotes accumulation of CAFs within colon cancer tissue supporting tumor growth *in vivo,* suggesting the potential of TIMP-1 as a therapeutic target, in addition to its prognostic value emerged in previous clinical trials (Eveno *et al*, 1995; Nakopoulou *et al*, 2002).

Based on the results of the VICTOR trial, showing that patients with primary tumors with high percentage of CAFs at diagnosis had a poor prognosis, a negative prognostic value has been associated to the presence of CAFs in CRCs, (Huijbers *et al*, 2013), suggesting that this parameter should be taken in account in addition to the canonical TNM classification and MSI assessment.

The first study indicating a role of fibroblasts in CRC is dated in 1977: studying cultured skin fibroblasts isolated from normal-appearing cutaneous biopsies of FAP individuals with Gardner's variant (susceptible of desmoid tumors development), Kopelovich demonstrated proliferative abnormalities and lack of contact inhibition, although they were not capable of forming palpable tumors in athymic mice (Kopelovich *et al*, 1985). However, when these skin fibroblasts were transformed by the Kirsten murine sarcoma virus, they showed to be 100- to 1.000-fold more susceptible to transformation than normal counterparts, suggesting an oncogenesis predisposition of these cells due to their autosomal dominant mutation. This study opened the way to further investigation about the role exerted by fibroblasts in desmoid tumors development in FAP patients (Rider *et al*, 1986; Clark *et al*, 1999; Kohler *et al*, 2009).

Paradoxically, it seems that CAFs would have some tumor-suppressor properties at the beginning of the carcinogenesis process, while their supportive role on tumor progression and metastasis could come up with time, due to the dynamic and continuous interaction with tumor cells (de Visser & Coussens, 2006). An interesting study suggesting this paradox, was conducted in 1994 by Lieubaue and colleagues (Lieubeau *et al*, 1994). Using two cell clones (PROb and REGb) originated by the same colonic adenoma of induced rats, but different in the tumorigenic potential when injected in syngeneic animals (i.e. originating tumors with metastatic potential or regressive tumors respectively), they tested the different action exerted by these two types of tumor cells on stromal fibroblast activity. An initial similarity in fibroblast properties and distribution between the tumors derived by the two injected clones was lost during tumor growth: indeed, both tumors were enriched in myofibroblasts one week after inoculation, but three weeks later, fibroblasts were no more

positive for α-SMA in REGb tumors while a strong staining was still observed in PROb tumors. This was linked to a higher expression of TGF-β in PROb tumor cells compared to REGb ones, showing that TGF-β signaling between tumor cells and fibroblasts could explain, at least in part, the different fibroblast activity in these two type of tumors, suggesting that fibroblasts can be differently activated depending on tumor cells secretome.

Additionally, there are several proofs supporting the TGF-β pathway as one of the leading mechanisms involved in metastases development in CRC patients (Welt *et al*, 1994; Calon *et al*, 2012): CAFs stimulated by TGF-β show up-regulation, among others, of Snail1 (a transcriptional factor involved in the EMT process) and FGF-1 (Henriksson *et al*, 2011; Herrera *et al*, 2014).

All the reported evidences about the interplay between CAFs and CRC did not take into account the possible influence on these mechanisms of a mutation on the *Apc* gene at the level of colonic fibroblasts: indeed, as illustrated above, *Apc* mutation influence was investigated in skin or desmoid tumor fibroblasts from hereditary CRC (FAP) patients, but not in the colon of this subjects.

A study by Vermulen and colleagues (Vermeulen *et al*, 2010) documented that CAF are able to induce a stemness phenotype in CRC. The authors investigated the heterogeneity in Wnt signaling in cancer stem cells (CSCs) showing that CSCs express different level of activation of Wnt pathway due to a difference in nuclear β-catenin accumulation, the regulation of which is dependent on both intrinsic tumor cell features and CAFs stimuli (Vermeulen *et al*, 2010). Moreover, Kramer et al. (Kramer *et al*, 2017) have demonstrated that CAFs bear an up-regulation of *Wnt2,* enabling them to stimulate both Wnt pathway in colon cancer cells wild-type for *Apc,* and an autocrine signaling inducing a pro-migratory and pro-inflammatory CAF phenotype.

Meanwhile, Patel and colleagues (Patel *et al*, 2011) compared the proteome of colon fibroblasts from FAP patients versus healthy controls. The results showed a higher expression, among others, of RSU1, involved in apoptosis resistance through an enhanced endogenous Ras activity, and DJ-1, whose up-regulation increases the oxidative-stress response. These FAP features could be considered the "first-hit" of the Knundson's mechanism (see par.1.2.1) due to the heterozygote germline *Apc* mutation.

Considering the wide range of activities that CAFs can control, a deeper comprehension of how Apc gene mutation affects them in the context of CRC is needed.

1.9 In vitro and in vivo CRC models

As previously illustrated, colon carcinogenesis is a multistep process that occurs in a wide temporal window as a consequence of the combination of altered cellular pathways, the involvement of non-epithelial tissue components (i.e. stromal cells and immune cells) and a cross-talk between them and epithelial cells. Multiple genomic and genetic alterations are behind these events but each of them occurs at a different time point of the carcinogenesis process, thus suggesting the possibility to interrupt this process before the final step, that is the adenocarcinoma development in which all previous alterations coexist. Studies on the molecular mechanisms underlying CRC carcinogenesis and the chemopreventive studies rely on *in vitro* and *in vivo* assays.

Regarding the former, historically they have been conducted on immortalized human epithelial cell lines derived from colon adenocarcinoma and normal tissues: much of what is known about CRC at the molecular level has been discovered using these models and they also represent a useful tool for *in vitro* drugs screening. These cell lines have also been employed for testing compounds supposed to have chemopreventive properties: the capability of killing and/or inducing apoptosis in these cancer cells has always been associated with chemopreventive activity, although some consider this association misleading (Ricciardiello *et al*, 2016). Nevertheless, as they originate from colon carcinomas, the final step of CRC carcinogenesis, they show constitutive overexpression of genes that might not be in such a state in premalignant cells (Fenton & Hord, 2006). Additionally, it has to be considered that the more passages cells perform, the less they resemble the original tissue from which they have been isolated: indeed, cell lines are the result of the selective pressure of *in vitro* growth (Marian, 2002), and they do not represent the variation and heterogeneity of cancers.

Adenoma cell lines and derived primary cultures are in principle more reliable for chemopreventive studies, although the number of the available cell lines is limited: LT97 cell line has been established at the end of the last century (Williams *et al*, 1990; Paraskeva *et al*, 1992) and is the only commercially available one. Instead, primary cultures are directly comparable with the tissue from which they have been collected via a biopsy. Depending on their features they can divide several times (passages) before undergoing replicative senescence and show less clonal selection than immortalized cell lines, being suitable for short-term functional analysis and screening of putative chemopreventive molecules (Rosin-Arbesfeld *et al*, 2000). Moreover, the usefulness of primary cultures for pre-screening of chemopreventive molecules is promising for the possibility to establish them from *in vivo* models of CRC, thus allowing a

better understanding of colon carcinogenesis and a more consistent explanation of the mechanisms underlying the effects of a tested treatment observed in the corresponding *in vivo* model (Ricciardiello *et al*, 2016).

An attempt to do this has been done by De Giovanni et al., who settled a so-called APC 10.1 cell line from *Apc Min* mouse adenomas (De Giovanni *et al*, 2004). Moreover, pre-screening on this kind of primary cultures could be helpful in choosing the most promising subset of molecules, to be tested *in vivo*, implementing the *3R principles*: "Reduction, Replacement and Refinement" of laboratory animals in scientific procedures.

However, due to the lack of signals from microenvironment, both immortalized epithelial cell lines and primary cultures growing as monolayers most likely do not respond to drugs or modulators like they would in physiological conditions (Pereira *et al*, 2016). Indeed, primary cultures of fibroblast isolated from different kinds of colon specimens and their co-culture with epithelial cells have uncovered the crucial role they play in tumorigenesis (Lotti *et al*, 2013; Chen *et al*, 2014; Knuchel *et al*, 2015).

This problem is also encountered with the use of 3D cultures, also called spheroids or organoids: this method represents a great promise for drug screening applied to personalized medicine especially in the oncology field (Mueller-Klieser, 1997). Their establishment is quite easy, as it does not require any particular instrument or stringent culture conditions, or a large number of cells: due to the fact that they originate from a patient tumor and that it is possible to obtain more than one spheroid from a single tissue sample, their use seems to be a promising tool for assigning the best treatment to each patient. However, their generation takes a about 10 days and no microenvironment component is present (Jeppesen *et al*, 2017). 3D models are often used in patient derived xenografts (PDX) for *in vivo* testing: a xenograft was first developed in 1980 and it is now widely used for drug screening, biomarker discovery and evaluation of treatment strategies (Hidalgo *et al*, 2014). This method consists in implanting a tumor sample or a generated spheroid from a patient, into immune-compromised mice: their use allows the possibility to set up co-clinical trials, i.e. treating xenografts in parallel with the patient, thus facilitating personalized therapy (Rubio-Viqueira & Hidalgo, 2009; Chen *et al*, 2012). However, this model presents some limitations depending on the characteristics of the tumor implanted: the rate of engraftment is quite variable, generation time of xenograft tumor is long and, as outlined before, the lack of host microenvironment is a limiting factor (Meijer *et al*, 2017).

Both these limitations are overcome by *ex vivo* cultures, consisting in short-term cultures of tissue explants (i.e. tumor biopsies) or slices, maintaining tissue architecture and all the different cell types which constitute it; moreover, special

culture conditions are not required, although a decrease in tissue viability is observed with time in culture. This model is considered reliable for functional assays and drug sensitivity testing as it is, up to now, the only one that retains tumor heterogeneity and tumor-stromal interaction (Meijer *et al*, 2017).

Concerning the study of CRC carcinogenesis *in vivo*, this was possible in the last 80 years by the induction of tumor burden in rodent colon. The pioneer study dates 1941 when Lorenz and colleagues succeeded in inducing intestinal tumorigenesis in mice fed with 1,2,5,6 dibenzanthracene. However, the most used carcinogens to induce colon tumorigenesis were 1,2-dimethylhydrazine (DMH) or azoxymethane (AOM): in both cases, colon tumors exhibit the same pathological features of the human counterpart. Nonetheless, AOM was preferred to DMH for its greater stability and potency in dosing solution (Rosenberg *et al*, 2009). Studies of therapeutics, different diets and chemopreventive strategies have been tested on DMH or AOM inducible rodent models (Bull *et al*, 1979; Dolara *et al*, 1993; Femia, Angelo Pietro *et al*, 2004) and they have been also useful for the characterization of colon lesions (Pretlow *et al*, 1992; Caderni *et al*, 1995; Luceri *et al*, 2000). However, the limits of these methods are the long latency along with incomplete penetrance and low multiplicity (Amos-Landgraf *et al*, 2007). For this reason, the technological advances which allow to manipulate gene sequences have permitted the development of more suitable models: the target gene of this manipulation has been *Apc* for its high frequency of mutations in both FAP and sporadic CRCs.

The first developed genetic model of CRC has been the multiple intestinal neoplasia (Min) mouse (C57BL/6J): this model bears a germline point mutation at codon 850 of the *Apc* gene, which causes the expression of a truncated protein as observed in FAP patients (Moser *et al*, 1990). Although it has been widely used, the Min mouse presents some important limitations: it develops polyps in the small intestine (> 100) than in colon (0-2): nevertheless, these last ones do not evolve into invasive adenocarcinoma (Young *et al*, 2013). Although it is possible to increase tumorigenesis through carcinogen treatment in this model, this would be time and resources consuming (Irving *et al*, 2014). Recently, this model has been further modified by Sødring and colleagues (Sødring *et al*, 2016): A/J Min/+ mouse develops an equal number of adenomas both in the small intestine and in the colon, and this number increases with age. Furthermore, in line with the classic Min mouse model, it develops a subtype of preneoplastic lesion known as “flat ACF”, whose number decreases with age.

Another recent *in vivo* model that better mimics what happens in human FAP patients is the “Polyposis In the Rat Colon”, Pirc rat. The F344 rat strain has been genetically modified by Amos-Landgraf and colleagues (Amos-Landgraf *et al*,

2007) to harbor a germline mutation at the codon 1139 (FAP patients harbor mutations either in position 1061 or 1309) leading to the transcription of an Apc truncated protein as in Min mice, but this is the only feature they share. In fact, the Pirc rat begins to develop tumors early in the colon, more in male than female, with 71% displaying a constant growth and associated co-morbidities as bleeding, anemia and intestinal blockage that lead to death at 11 months of age (Irving *et al*, 2014). Moreover, MDFs can be found along Pirc colon mucosa since a very young age (one month) and increase progressively with time, along with high proliferative activity and resistance to apoptosis in the normal mucosa (Femia *et al*, 2015), so leading to consider it a model which best mimic FAP patients (Ricciardiello *et al*, 2016). Indeed, as reported in literature, the use of this model is recently rising for different kind of studies within CRC: chemopreventive studies (Irving *et al*, 2014; Femia *et al*, 2015b), for the study of metabolic reprogramming and colon microbiota (Ericsson *et al*, 2015; Cruz *et al*, 2017) and for addressing processes involved in both FAP and sporadic CRCs (Ertem *et al*, 2017; Okonkwo *et al*, 2018).

Aims

The aims of this PhD project can be subdivided in three parallel research lines.

The first aim was to study new chemopreventive strategies capable of interrupting CRC carcinogenesis with very low toxicity. In our idea these could unveil to be promising for primary, secondary and tertiary prevention, so targeting high risk individuals like FAP patients and individuals with a previous history of cancer or at increased risk of relapse. Indeed, accordingly with ESMO clinical guidelines (Balmaña *et al*, 2013) the use of sulindac or celecoxib can be considered for secondary prevention in FAP patients, but "caution is warranted" due to their cardiovascular side effects; meanwhile, there are no chemopreventive options but only surgical ones for primary prevention in these individuals. Thus, there is a need to develop primary preventive strategies which could improve the quality of life of these subjects. Three different natural compounds/products were tested *in vivo* in the Pirc rat model (F344/NTac-Apc^{am1137}) and were supported by *in vitro* experiments in order to explain their molecular mechanisms.

The second aim was to add knowledge on the effects of the germline *Apc* gene mutation on tissue microenvironment. In particular, we evaluated the effects of this mutation on colon fibroblasts phenotype (bearing a mutated allele) at very early stage of colon carcinogenesis. This could give new suggestions about the role of colon fibroblasts, and of tissue microenvironment, in the apparently morphological normal mucosa, where "apparent" means that no macroscopic lesion can be found although the carcinogenesis process is already ongoing. To address this question, primary cultures from colon of Pirc and F344-Wt age matched rats were established and characterized.The third aim was to contribute to a deeper understanding of the influence of *Apc* mutation on DNA stability at early stages of colon carcinogenesis. DNA stability can be compromised by different mechanisms, including oxidative stress and epigenetic changes. To study DNA damage, we employed the COMET assay: this method allows an easy and fast evaluation of the amount of basal DNA strand breaks on different kind of samples, such as tissue samples and blood samples. Moreover, with little modifications, it can be used to assess other types of damage, such as oxidative or UV ones. Consequently, the COMET assay was used to assess basal and oxidative DNA damage in both cultured colon fibroblasts of Pirc and Wt rats and in apparently morphological normal mucosa samples of Pirc and Wt rats aged one month. In addition, we set up a modification of the method, aiming at evaluating the global DNA methylation status in mutated colon mucosa and in Pirc fibroblasts: this latter part of the project was performed in the laboratory of prof. Duthie S. at the Robert Gordon University (Aberdeen, UK).

Materials and Methods

In vivo and *ex-vivo* experiments and colon fibroblasts primary cultures were performed using the colon carcinogenesis model Pirc rat (F344/NTac-Apc^{am1137}) and wild type (Wt) Fisher F344/NTac rats were originally obtained by Taconic (Taconic Farms, Hudson, NY, USA) and bred in CESAL (University of Florence, Italy) in accordance with the Commission for Animal Experimentation of the Italian Ministry of Health. The colony is maintained by mating heterozygous Pirc rats with Wt and pups genotyped at 3 weeks of age according to Amos-Landgraf et al. (Amos-Landgraf *et al*, 2007). Briefly, small tail samples from pups are subjected overnight to an enzymatic degradation with 600 U/ml of proteinase K (ThermoFisher Scientific Inc.) in a sterile Eppendorf containing a lysis solution (5mM EDTA, 0.2% SDS, 0.2M NaCl, 10% Tris-HCl, pH 8) at 56°C and with shacking at 300 rpm. Then, samples are centrifuged for 15' at 14000 rpm and 2 µl of each lysate are subjected to PCR reaction: only a fragment of *Apc* gene containing the single nucleotide mutation (position 1139) is amplified (primer Fw: 5'-GGAAGACGACTATGAAGATGG-3'; primer Rv: 5'-TGCCCTGTACTGATGGAG-3'); meantime, a Pirc DNA sample isolated in the past years, is also amplified to function as positive control. Finally, amplified fragments are subjected to enzymatic cut with fast digest restriction enzyme NHE I (ThermoFIsher Scientific Inc) at 37°C plus shacking at 600 rpm, followed by electrophoresis in 2.5% agarose gel: indeed, the enzyme will cut amplified fragments only in the presence of the single nucleotide mutation so that Pirc genotype will be detected as two bands instead of one (Wt genotype) on agarose gel (fig.11).

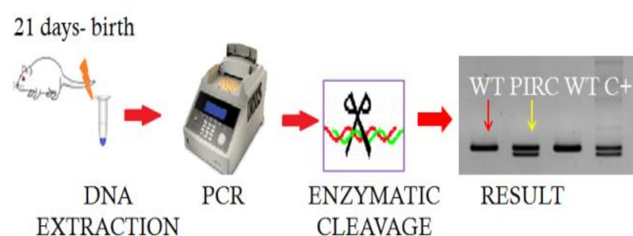

Fig.11. Schematic representation of rats genotyping process performed at 3 weeks of age. WT & PIRC: wild type and *Apc* mutated (Pirc) genotype respectively; C+: positive control (*Apc* mutated genotype from a Pirc rat).

3.1 In vivo: short-term chemopreventive studies with natural compounds

In vivo short-term (6 or 12 weeks) chemopreventive studies were performed in Pirc male rats of one month of age: each study consisted of two arms, treated and untreated (control) animals, both subjected to the same experimental procedures including monitoring of health status and body weight during the treatments. For each study, rats were

maintained in polyethylene cages under an experimental protocol approved by the Commission for Animal Experimentation of the Italian Ministry of Health; moreover, the same untreated animal was used for different determinations, when possible, in order to minimize the number of used animals. At the end of the treatments, animals were euthanized and samples collected (see below). Untreated animals, controls, were always fed with AIN-76 (purchased from Piccioni, Milan, Italy): this is a standard diet suitable for rats and mice formulated by the American Institute for Nutrition to be used in biomedical research; ingredients are listed in Table 2.

Tab.2. AIN-76 diet formula.

Formula	**g/Kg**
Casein	200
DL-Methionine	3
Sucrose	500
Corn starch	150
Corn oil	50
Cellulose	50
Mineral mix	35
Vitamin mix	10
Choline bitartrate	2

Treated animals were fed with AIN-76 diet supplemented with one of the compounds listed below:

- Bergamot (*Citrus bergamia)* juice extract (BJe): 12 weeks study. The juice obtained from bergamot fruits cultivated in Calabria (Italy) was transformed into powder and qualitatively characterized by high performance liquid chromatography system (HPLC), showing to be rich in neohesperidin, naringin, melitidin, hesperetin, neoeriocitrin and naringenin (98.31, 92.68, 68.42, 56.74, 48.16, 36.14 mg/g respectively). All these preliminary phases of the study were performed by the laboratory of prof. Navarra (University of Messina, Italy) who also

carried RT-PCR analysis on colonic samples. Two doses of BJe were tested and compared to controls (n= 10): 35 mg/Kg/die of body weight (BW) (n=10) and 70 mg/Kg/die BW (n=7).

- Morin: 6 weeks study. This is a polyphenolic compound (3,5,7,2',4'-pentahydroxyflavone), originally isolated from members of the *Moraceae* family, present in figs, mulberry, strawberries and endowed of antitumor activity *in vitro* (Hyun *et al*, 2015; Li *et al*, 2017; Lori *et al*, 2018a). Interestingly, morin seems to target the oncogenic low-molecular weight phosphotyrosine phosphatase (LMW-PTP) (Lori *et al*, 2018a): this small enzyme (18 kDa) is up-regulated in several cancer cells and associated with poor prognosis (Malentacchi *et al*, 2005). Morin (Sigma-Aldrich) was added to AIN-76 to provide treated animals (n=9, as for controls) with a dose of 50 mg/Kg/die of BW. *In vitro* experiments aiming at addressing the effects of the treatment observed *in vivo*, were performed in prof. Raugei's laboratory (University of Florence, Italy). Moreover, preliminary tests were performed in order to assess enzyme expression in the NM of Pirc rats and apoptosis response after a short pre-treatment with morin: for the former purpose, Pirc rats and Wt were sacrificed at 8 weeks of age and NM was scraped and stored in RNA-later™ (Qiagen) (n= 7 and 9 for Wt and Pirc rats, respectively). For the latter, 8 Pirc rats (aged 9 months) were randomly allocated to: Controls (4 rats), fed the standard AIN-76 diet and Morin-treated (4 rats), fed for one week the same AIN-76 diet supplemented with Morin to provide a dose of 50 mg/kg (body weight). The last day of the treatment, rats were treated (s.c.) with 5-FU (50mg/kg) and sacrificed 24 h later.

- Pomegranate mesocarp decoction (PMD): 6 weeks study. Pomegranate mesocarps, a by-product of pomegranate juice production, were recovered manually, then they were used to prepare a decoction which was finally transformed into powder and chemically characterized in the laboratory of prof. Mulinacci (University of Florence, Italy). PMD resulted to be composed by: proteins 1.9%, total sugar 0.4%, dietary fibers about 11% (soluble 10.9%, insoluble < 1%), ellagitannins (151.47±3.42 mg/g) with a prevalence of α+β punicalagins (67.5±1.24 mg/g) and a minor content of ellagic acid and derivatives (21.7±0.35 mg/g); fat was absent. A total of 21 animals were used (11 treated and 10 controls): PMD was added to AIN-76 diet at a dose of 50 mg/Kg/die of total phenolic compounds. *In vitro* and *ex-vivo* studies were performed in our laboratory in order to explain mechanisms underlying the effects observed in treated animals.

All supplemented diets were prepared weekly adjusting the quantity of the compound to be added on the basis of BW determined each week; furthermore, doses were chosen on the basis of previous carcinogenesis studies with polyphenols from various sources performed by Caderni et al. (Caderni *et al*, 2000).

3.1.1 Sample collection from colon and determination of MDFs

After euthanasia, the entire intestine was dissected, flushed with cold saline and 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 subsequent gene expression and protein analysis (this last one was performed only for PMD and morin treatments). From the medial portion of the NM, a small sample (about 9 mm^2) was collected, fixed overnight in 10% formalin solution, dehydrated and finally embedded in paraffin to assess proliferative and apoptotic activity; tumors were also collected for both purposes, if present. The remaining colon and rectum were entirely fixed in 10% formalin for 12 h and stained with High-Iron Diamine-Alcian Blue (HID-AB) solution without embedding in paraffin: the colons were observed under microscope (40 x magnification) to determine the number of MDF (number of MDF/colon) and their multiplicity (number of crypts forming each MDF) accordingly to Caderni et al. (Caderni *et al*, 2003). Furthermore, for the PMD experiment, proliferation and apoptosis was assessed also in MDFs: in this case, MDFs were dissected under microscope and then embedded in paraffin in such a way that crypts could be sectioned longitudinally.

3.1.2 Immunohistochemistry assays and apoptosis on colon tissue samples

For all the tested treatments, 4 μ thick sections of morphologically NMs and tumors or MDFs (only in case of PMD) were used to determine proliferative activity and apoptosis. The former was carried out by assessing the Proliferative Cell Nuclear Antigen (PCNA) via immunohistochemistry. Tissue sections were incubated at RT for 2 h with PCNA mouse monoclonal antibody (PC-10, Santa Cruz, CA, USA) at 1:1000 dilution and proliferative activity was expressed as labelling index (LI): the number of cells positive to PCNA/ scored cells x 100 (Femia *et al*, 2015), evaluated in at least 15 full longitudinally sectioned crypts, considering either NM or MDF, and in 600 cells in case of tumors.

Regarding apoptosis, this was evaluated on tissues sections via hematoxylin-eosin (H&E) staining: cells showing shrinkage, loss of normal contact with adjacent cells, chromatin condensation or formation of round or oval nuclear

fragments were counted in at least 15 full entire longitudinally sectioned crypts and expressed as apoptotic index (AI) defined as apoptotic cells/crypt (considering NM and MDF samples). In tumors, the AI was quantified as the number of apoptotic cells/ scored area with the ACT-2U software program at a magnification of 1000x according to Femia et al. (Femia *et al*, 2012).

In the experiment with BJe, also COX-2 (160126 rabbit, Cayman Chemical) at 1:100 dilution and CD68 1:200 dilution (mouse anti-rat CD68, AbD Serotec) were determined and expressed as number of labelled cells/ scored area as illustrated for the AI determination.

3.1.3 Semi-quantitative reverse transcriptase-PCR

Gene expression was evaluated in the NM, taken at sacrifice as described above, for the PMD experiment. Total RNA extraction was performed using NucleoSpin® TriPrep kit accordingly to the manufacturer (Machery-Nagel GmbH & Co. KG Duren, Germany); reverse-transcription of 1 µg of total RNA and subsequent PCRs were performed as previously described (Femia *et al*, 2009). 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 listed in table 3.

Tab. 3 List of primers used for gene expression assay in the PMD experiment.

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’

3.1.4 In vitro: study of the mechanisms of action of the treatments tested in vivo

As outlined before, *in vitro* studies were performed aiming at investigating the possible mechanisms of action targeted by the treatment tested. Regarding BJe and morin experiments, *in vitro* studies were performed in the laboratories of prof. Navarra (University of Messina, Italy) and Raugei (University of Florence, Italy), respectively: the results obtained from them will be illustrated in the "Results" chapter together with our *in vivo* results.

Concerning PMD treatment, both colon carcinoma cell lines and *ex-vivo* model were used: the latter, established from Pirc adenomas (ADs) and Normal Mucosa (NMs), was conceived with the aim of assessing the effects of the treatment on a model of the intermediate step of colon carcinogenesis. The MTS viability test and western blotting (WB) were used to study the mechanisms involved in PMD effects.

3.1.5 Colon cancer cell line used for PMD experiment

HT-29 cell line (provided from ATCC) was grown in high glucose DMEM and RPMI1640 Medium respectively, both supplemented with 10% of fetal bovine serum, PenStrep (penicillin 100 U/ml and streptomycin 0.1 mg/ml), maintained at 37°C in a cell incubator (5% CO_2) and used during the linear phase of growth. For experimental treatments, lasting 24 h or 72 h, cells were plated in 12-MW (130000 cells/well) and treated starting on the following day with or without (control animals) the following compounds:

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 or 50 μM plus SB 500 μM, 1 mM and 5 mM (USB).

u-A and SB, punicalagin and soluble fibers colon metabolites respectively, were purchased from Toronto Research Chemicals (Toronto, Canada) and Merck-Millipore (Vimodrone-Milan, Italy).

3.1.6 HT-29 viability after 72 h treatments

Viability of the cell lines at the end of 72 h treatments was assessed by means of the MTS test (Promega, Gessate, Italy). Accordingly with the manufacturer instructions, 8000 cells/well were seeded in 96-MW and treated for 72 h with one of the treatments listed in the previous paragraph. At the end, media were removed, cell monolayers were washed two times with 1 X PBS, and 100 µL/well of DMEM or RPMI 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.

3.1.7 Ex-vivo cultures for PMD mechanistic study

In order to elucidate the mechanisms involved in the effects observed in PMD treated rats, 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 100 U/mL and streptomycin 0.1 mg/mL) and gentamycin (50 µg/mL), then opened to collect samples of ADs and apparently NMs. 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 approximately equal parts (weight range: 10-15 mg). Samples were then transferred in 48-MW containing DMEM (control) or DMEM containing 25 µM u-A plus 2.5 mM SB (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 ADs and NMs samples was assessed in separate samples performing the MTT test (Sigma Aldrich, Milan, Italy) at time zero (T0) and after 24 h (T24). Briefly, samples dissected under microscope 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 MTT and incubated for 3 h at 37°C in cell incubator (T0 assessment). 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. Subsequently, they were centrifuged for 5' at 4°C and 1000 rpm. The T24 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 T0 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) (Castagnoli *et al*, 2003). Finally, the viability at 24 h was expressed as % of the corresponding T0 counterpart.

3.1.8 Western blotting assay on in vitro, in vivo and ex vivo samples from PMD experiment

Protein extraction was performed with RIPA buffer solution supplemented with 1% protease inhibitors and 1% phosphatase inhibitors (Sigma Aldrich, Milan, Italy): for cultured cell lines, 40 μL of this mix 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 no more than 2', sonicated and centrifuged as above. Each supernatant was collected and the protein content was measured accordingly to DC Protein Assay kit instructions (Bio-Rad, Segrate-Milan, Italy). 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 (Pitozzi *et al*, 2013). 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 HRP-linked antibody (#7074 Cell Signaling), 1:4000; anti-mouse IgG- peroxidase (A9044 Sigma-Aldrich, Milan, Italy), 1:5000.

3.2 Pirc & Wt colon fibroblast primary cultures: establishment and characterization

Colon fibroblasts (CF) were isolated from Pirc (PCF) and Wt (WCF) rats of one and ten months of age (young and old). The entire colons were washed and two/three samples from medial NM of approximatively 2 cm^2 each were collected as illustrated for the *ex-vivo* experiment. Within 1 h since collection, samples were washed 3 times with HBSS/PSG under sterile atmosphere, quickly cut into small pieces (~3 mm^2) in a Petri dish and then transferred in sterile Eppendorf for

enzymatic digestion (30’ in a water bath at 37°C under shacking) with 1 mg/ml collagenase II (Sigma Aldrich, Milan, Italy) dissolved in advanced DMEM plus 1% antibiotic-antimycotic solution (both purchased from GIBCO), and 3 mM $CaCl_2$. Digested samples (which should have been capable of easily pass through a 10 ml pipette) were recovered, transferred in new tubes containing advanced DMEM supplemented with 20% FBS, 1% antibiotic-antimycotic solution, 1% L-glutamine and then centrifuged 3 times at 1200 rcf for 5’ at RT, discarding the supernatant each time. Finally, fragments were resuspended in the same media used for centrifugations, seeded in a 6-MW plate and incubated in a humidified atmosphere in presence of 5% CO_2. Plates were daily checked for contamination and media was partially changed every two days until groups of cells were visible (within 7 days) in proximity of tissue fragments. These were completely removed as soon as 50% of well surface was covered by cells: in fact, the permanence of colon tissue fragments up to the confluence demonstrated to be a favoring factor for bacterial contamination. Moreover, FBS supplementation was decreased from 20% to 10% at the first subculture (occurred within 10 days from the seeding). Established cultures were checked for mycoplasma contamination via PCR and used in the log phase of growth for characterization experiments.

3.2.1 Cell growth

Differences in cell growth capability between PCF and WCF (young and old) were assessed performing MTS viability test. Cells were seeded in 96-MW (3000 cells/well) and viability was measured as described previously (see par. 3.1.6) at 3 time points: 24, 48 and 72 h after seeding (referred to as T_0, T_{24} and T_{48} respectively). Different adhesion potential was evaluated considering absorbance values measured at T_0 .

3.2.2 Response to different stimuli in vitro

PCF and WCF were seeded in 6-MW plate (50000 cells/well) and treated for 24hs with advanced DMEM supplemented with 1% FBS, 1% L-Glutamine, 1% antibiotic-antimycotic and: a) 10 µg/ml LPS; b) 20 ng/ml TNF-α; or c) 10 ng/ml TGF-β. Treatments started when cells were at 70% confluence; at the end of the treatments, proteins were collected and specific protein expression was measured by mean of western blotting as illustrated above. Antibodies anti-NFkB, anti-GAPDH, anti-IgG HRP-linked and anti-COX-2 were used at the same concentrations described above (see par. 3.1.8), while anti α-SMA antibody (ab5694, Abcam) was used at 1:500 dilution.

3.2.3 Il-6 ELISA assay

Media from the treatments illustrated in the previous paragraph were collected, centrifuged for 5' at 1200 rcf and supernatants aliquoted and stored at -20°C until use. Then they were used for IL-6 measurement by ELISA according to the kit manufacturer instructions (Rat IL-6 Mini ABTS ELISA Development kit, Peprotech).

3.2.4 Immunocytofluorescence (ICF)

For immunofluorescence assay (ICF), cells were seeded (10000 cells/well) onto round glass coverslips (previously washed in 100% EtOH, dried and UV sterilized) in 24-MW plates and cultured until 70% confluency, with or without treatment (par. 3.2.2). Cells were then washed in 1X PBS, fixed in 5% paraformaldehyde, then a-specific sites were blocked for 20' at RT with 3% Bovine Serum Albumin (BSA) and 0.1% Triton in PBS. After that, slides were incubated overnight at 4°C under slight shacking with primary antibody diluted in PBS containing 1.5% BSA and 0.1% Triton. After removal of the primary antibodies, coverslips were washed three times with 1X PBS, incubated at RT in the dark for 2 h with secondary antibodies diluted as the primary, washed again and then incubated with 5 μg/ml DAPI solution for 30' at RT in the dark. Finally, coverslips were washed again and mounted on a drop of 70% glycerol solution in PBS on microscope slides, and analyzed under a Bio-Rad MRC 1024 ES Confocal Laser Scanning Microscope (Bio-Rad, Hercules, CA) equipped with a 15 mW Krypton/Argon laser source for fluorescence measurements. Acquired images were analyzed with ImageJ software. Antibodies used were: anti-collagen I antibody (ab34710, Abcam) at 1:500 dilution; anti phospho-histone H2Ax (#2577, Cell Signaling) at 1:100 dilution; NFkB (the same used for western blotting) at 1:100 dilution; anti-rabbit IgG (Alexa Fluo 594) at 1:500 dilution.

3.2.5 Comet assay

The single cell gel electrophoresis (SCGE) method, known as COMET assay, was performed in order to assess basal levels of DNA breaks and DNA base oxidation in PCF and WCF. The solutions used are listed in Table 5. Cultured cells were detached by trypsin incubation at 37°C and 5% CO_2 for not more than 5' and counted in Burker chamber. Then, 20 x 10^4 cells were embedded in 70 μl of 0.75% low melting point agarose (LMA) and layered on pre-coated (1% normal melting agarose, NMA) microscope slides (2 gels/slide), covered with 22x22 mm coverslips and let solidify for 10' at 4°C; for each sample, 3 slides were prepared. Then, coverslips were removed and slides were transferred in a dark chamber

containing lysis solution, at 4°C for at least 1 h; after that, two slides/sample were washed in an enzyme wash buffer (EWB) and incubated with 30 µl/gel of either enzyme reaction buffer solution (EB) or FPG (formamidopyrimidine glycosylase from *E. coli*) enzyme at 1:1000 dilution in EB: incubation lasted 45' at 37°C in a dark and humidified box. Following this step, all the slides (both incubated and not), were transferred into the electrophoresis tank and covered by electrophoresis buffer: slides were laid in the tank for 40' at 4°C in the dark for DNA unwinding, before starting the run which lasted 30' at 25 V. At completion of electrophoresis, slides were washed in two changes of neutralizing buffer and one more time in distilled water, and placed O.N. in the dark at RT to let them dry. On the following day, they were stained with 100 µl of Sybr Gold (SYBR™ GOLD Nucleic acid gel stain, Invitrogen) diluted 1:10000 in 10 mM TE buffer and covered with 24x50 mm coverslips. Slides were finally observed under a fluorescent microscope connected to a camera at 40 x magnification: 100 cells/slide were scored with the "Comet Assay IV" software (Perceptive Instruments, UK).

Tab. 4. Composition of the solutions used for the COMET assay.

Solution	Formula
Lysis	2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris-HCl, pH 10.0
EWB (filtered)	2.5 M NaCl, 10 mM Na_2EDTA, 10 mM Tris-HCl, pH 7.4
EB (filtered)	40 mM HEPES, 100 mM KCl, 0.5 mM EDTA, 0.2 mg/ml BSA, pH 8.0
Electrophoresis solution	300 mM NaOH, 1 mM Na_2EDTA
Neutralising buffer	0.4 M Tris-HCl, pH 7.5

The COMET assay was also used for assessing DNA damage (both basal and oxidative) on scraped NMs samples of Pirc and Wt rats aged one month. Animals were euthanized and colon opened and flushed with sterile saline solution, then NM from each animal was scraped, transferred in a tissue homogenizer provided with a dedicated pestle which does not damage nuclei and containing Merchant solution (0.14 M NaCl, 1.47 mM KH_2PO_4, 2.7 mM KCl, 8.1 mM

Na_2HPO_4, 10 mM NaEDTA, pH 7.4). After homogenization, nuclei were recovered by centrifugation at 250 g for 5' and re-suspended in 1% LMA. The assay was then performed as described above.

3.2.6 Senescence associated Β-galactosidase expression

As for ICF assay, cells were seeded on coverslips in 24-MW plates and fixed with a 3% formaldehyde solution. Then, they were incubated in the dark at 37°C for 5 h with the following staining solution made in distilled water: 1 mg/ml X-Gal (5-bromo-4-chloro-3-indolyl P3-D-galactoside), 40 mM citric acid/sodium phosphate at pH 6.0, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl, 2 mM $MgCl_2$, according to Dimri et al. (Dimri *et al*, 1995). At least 6 microscopic fields/slide were captured (20x magnification) with an optical microscope (Leica DM IL Led Tissue Culture Microscope). Quantification of the staining intensity was expressed as the mean grey value calculated with the use of ImageJ software.

3.3 Methylation sensitive comet assay

Two normal human colon cell lines were used for setting up and validating the methyl sensitive COMET assay: NCM460 (Normal Colon Mucosa epithelial cells obtained through a MTA that prof. Duthie had with InCell Corporation LLC) and CCD-18Co (normal human colon fibroblast, obtained from ATCC). Both cell lines were cultured in DMEM high glucose (4.5 g/L) supplemented with 1% NEAA, 1% Pen-Strep solution and 10% FBS (all purchased from BioConcept Inc.). Preliminary experiments were performed to establish the more suitable culture condition, especially in case of NCM460 for which few data are available in literature. CCD-18Co and NCM460 were sub-cultured when at full confluence or at 70% of confluence, respectively.

The standard COMET assay (as described in par. 3.3.5), was used for testing four different incubation times (15', 30', 45', 60') with the Fast Digest buffer (FDB) alone or in combination with either HpaII or MspI (purchased from Thermo Fisher Scientific Inc.) on both cell lines. Both enzymes recognize the same sequence within CpG islands, but only *MspI* is capable of cutting it in case they are methylated.

For performing the experiments, $25x10^4$ cells were embedded in 1% LMA, put on pre-coated 1% NMA slides (2 gels/slide), covered with 18x18 mm coverslips and then transferred at 4°C for 15' to allow solidification of gels. After removal of the coverslips, slides were dived into lysis solution for at least 1h. Then, slides were firstly washed 3 times for

5' at 4°C with 1X PBS, then 30 µl master mix (MM) were put on each gel, covered with 22x22 mm coverslips and transferred inside a moist chamber (to avoid gel drying) in an incubator settled on 37°C. Thirty microliters of MM were constituted by 10X FD-buffer diluted to a final concentration of 1X in RNAse free water alone or in combination with 1% of enzyme accordingly to the manufacturer instructions. Control slides (no incubation with either FDB alone or enzyme) remained in the lysis solution at 4°C until unwinding was performed for all the slides. A visual scoring system was used for measuring DNA breaks.

3.3.1 Validation of the method

Hypo- and hypermethylation treatments were performed on NCM460 and CCD-18Co cells cultured in A52 and DMEM respectively (BioConcept Inc.), both at low glucose concentration (1.0 g/L), in order to validate the method. These experiments were performed in 6-well plates, seeding 10 x 10^4 cells/well. Hypermethylation was achieved by treating cells for 24 h with a 500 µM $NiCl_2$ solution (Perotti et al. 2015); the induction of a hypomethylated status was obtained through folic acid depletion for 48 h or 7 DIC (days in culture). Folic acid treated cells (FA+) were cultured in the respective media supplemented with 4 µg/mL of folic acid. The same concentration of FA was also added to both treated and untreated $NiCl_2$ +/- cells. Global DNA methylation was calculated by the percentage ratio between the two separate enzyme digestions (HpaII/MspI) after subtraction of the FDB score: so, the value obtained was inversely related to the number of methylated sites.

3.4 Statistics

Differences in MDFs, tumors, immunological and morphological indexes and RT-PCR data between treated groups and controls were analyzed with the Student *t*-test and presented as means ± SE. Data from *in vitro* and *ex-vivo* experiments were analyzed by one and two-way ANOVA respectively (in this last case to consider the effect of both treatment and tissue type, AD or NM), followed by Bonferroni's multiple range test, with GraphPad Prism 5.0 (GraphPad Software) as appropriate.

Regarding experiments on colon fibroblasts primary cultures, data from MTS, Comet assay, western blotting and ELISA were analyzed by two-way ANOVA (GraphPad Prism 5.0) in order to consider the effect of both the specific variable associated to the assay (time, incubation with FPG enzyme, and treatments respectively), and the genotype of the cells (PCF vs WCF).

Overlapping coefficient and integrated density on NFkB and γ-H2Ax images were calculated with ImageJ software, followed by analysis with the Student t-test for unpaired measures to compare PCF and WCF: data are presented as means ± SE.

Mean grey value on β-galactosidase images was calculated with ImageJ software, and data were analyzed by two-way ANOVA to account for both passages in culture and genotype (PCF vs WCF).

Data from Comet assay on NM samples were analyzed by two-way ANOVA in order to consider both the genotype of the animals (Pirc or Wt) and FPG enzyme incubation.

Data from methyl-sensitive COMET assay were analyzed by one-way ANOVA.

Results

4.1 In vivo: studies of CRC chemoprevention with natural compounds on Pirc rat, Apc mutated

Regarding the results obtained in *in vivo* short-term chemopreventive studies with natural compounds, toxicity of BJe, morin and PMD treatments was evaluated monitoring animals body weight weekly. At the beginning of the treatments, Pirc male rats of one month of age had a weight of 60 ± 2 g (mean ± SE, n=68).

4.1.1 BJe effects on tumorigenesis

At the end of the twelve weeks treatment, no sign of toxicity was found comparing body weights of controls with either 35 mg/kg or 70 mg/kg dose (mean values ± SE were: 313 ± 11 g, 311 ± 6 g and 296 ± 5 g, in Controls, BJe 35 mg/kg and BJe 70 mg/kg groups, respectively). Since rats had 4 months of age at the end of the treatment, tumors could be detected in the colon and also in the small intestine at the moment of sample collection: as shown in fig.12, in the 70 mg/kg group, the number of total tumors (considering both sites) was significantly lower ($p < 0.05$) compared with controls, and the same trend was observed considering the two sites separately. The same trend was partially observed in the 35 mg/kg group: however, no effect was found considering the number of tumors in the colon compared with the control, and no statistical significance was reached considering either the total number of tumors or the tumors in the small intestine only. Despite of this, effects on preneoplastic lesions were observed even with the lower 35 mg/kg dose: in fact, MDFs number in the colon was statistically lower in both groups compared with controls ($p < 0.05$ and 0.001 in 35 mg/kg and 70 mg/kg group respectively).

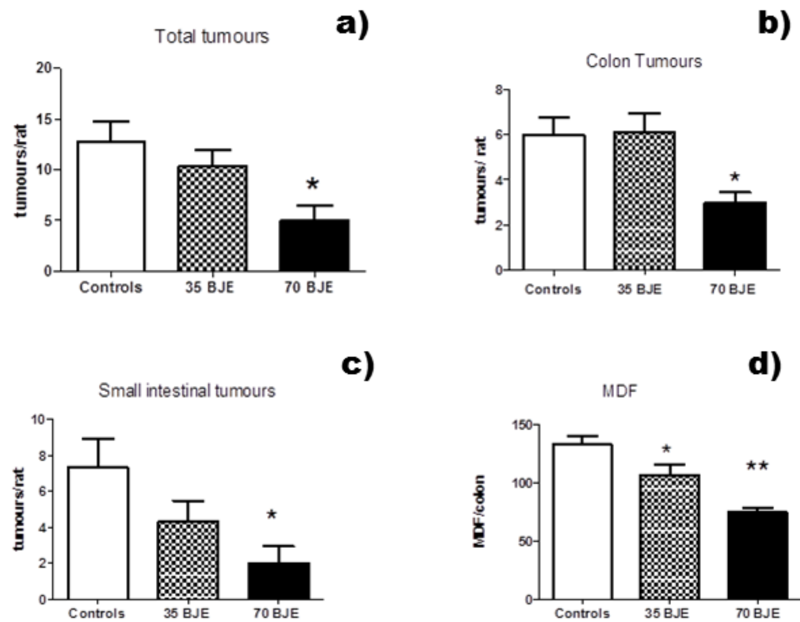

Fig. 12. Intestinal tumorigenesis in Pirc rats treated for 12 weeks with a control diet or with the same diet supplemented with BJe at 35 mg/kg or 70 mg/kg (body weight). Panel a: intestinal tumors, panel b: colon tumors; panel c: small intestine tumors; panel d: MDFs in the colon. Data are mean values + SE. * p <0.05, ** p <0.01 compared with controls.

Apoptosis and proliferation were histologically determined in the tumors: a significantly higher number of apoptotic cells (fig. 13 a) was found in the lesions from 70 mg/kg BJe group compared to controls (p <0.05); meanwhile, the proliferative activity, as labelling index LI (cells positive for PCNA/crypts) in the tumors was not affected by the treatments (46.3 ± 3.9 (n=8) and 44.3± 4.6 (n=6 in controls and BJe treated groups respectively), and the same result was obtained in the NM ((32.8 ± 3.9 and 31.5 ± 4.3 in Controls and BJe treated rats respectively).

These data obtained by immunohistochemistry assays, were in line with mRNA expression data obtained by the group of prof. Navarra (University of Messina): expression of genes involved in proliferation and apoptosis showed to be affected by the treatment with 70 mg/kg of BJe in tumors but not in the NM (data on NM not shown). More in detail, *p53* expression was significantly up-regulated in tumors of treated animals (1.6 fold) while *survivin* and *p21* (anti-apoptotic) were significantly down-regulated (0.5-fold and 0.35-fold respectively).

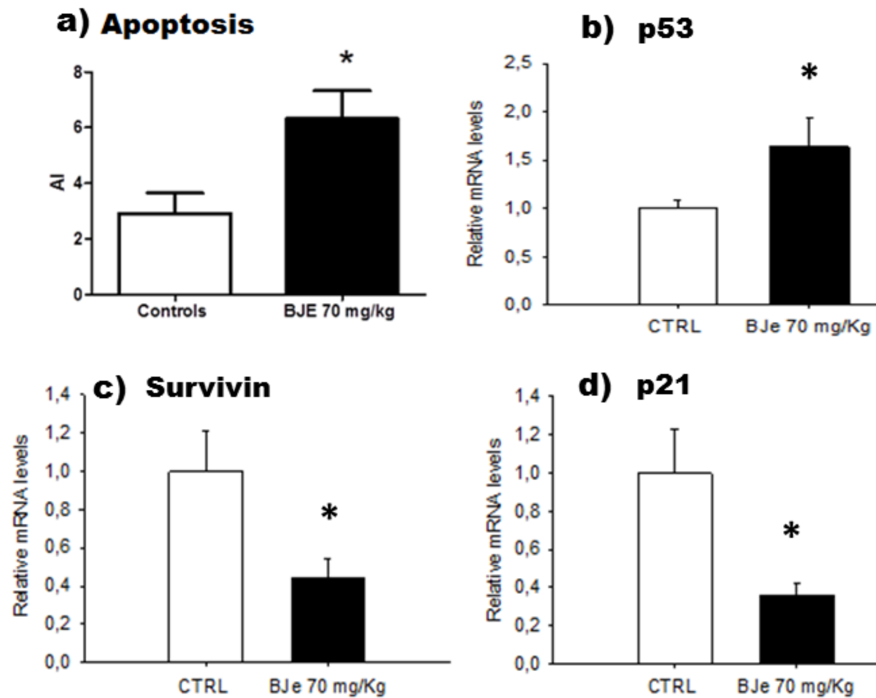

Fig.13. Effects of 12 weeks treatment with Bje at a dose of 70 mg/Kg on tumors of Pirc rats: Panel a: apoptotic index (AI) in colon tumors from Control or treated rats (dose of 70 mg/kg BJe); data are mean values + SE (n= 7 and 6, for tumors in Control and BJe group, respectively). Panels b, c, d: gene expression profile of *p53*, *survivin* and *p21* respectively, in colon tumors from Controls or from rats treated with 70 mg/kg of BJe. Results from real-time PCR are expressed as relative fold change detected in treated animals compared to the untreated, after normalization against β-Actin used as internal control. Values are mean + SE (n= 7 and 6, for tumors in Control and BJe 70 mg/kg group, respectively). * $p < 0.05$ compared with controls.

4.1.1.2 BJe effects on inflammation

Aiming at explaining the mechanisms of action of BJe, the expression of CD68 (marker of macrophages) and COX-2 was first assessed on NM histological sections: regarding the former, no differences were observed between controls and treated animals, while COX-2 was slightly less expressed in BJe group than controls, although the difference was not statistically significant (data not shown). These data are in agreement with gene expression results: indeed, considering NM samples no differences were observed in the expression of *Cox-2*, *iNos*, *Il-1β* and *Il-6* genes (data not shown); on the other hand, the same genes were significantly down-regulated (between 0.5-0.3 fold) in tumors from BJe animals compared to controls (fig.14).

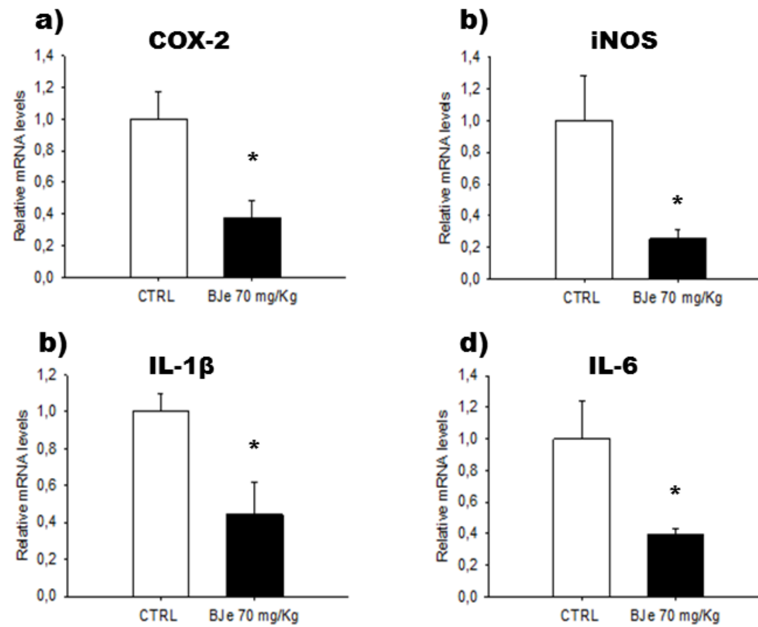

Fig.14. mRNA levels of inflammation-related genes in colon tumors from Controls or from rats treated with 70 mg/kg of BJe. Results from real-time PCR are expressed as relative fold change detected in BJe-treated animals compared to Controls, after normalization against *β-Actin* used as endogenous control. Values are mean + SE (n= 7 and 6, for Control and BJe 70 mg/kg group, respectively). * p <0.05 compared with controls.

4.1.2 Preliminary experiments with morin on Pirc rats: LMW-PTP expression and resistance to 5-FU

Since morin has been demonstrated to target the expression of LMW-PTP *in vitro* by the group of prof. Raugei (University of Florence), preliminary experiments were performed to verify the level of enzyme expression in Pirc rats compared with wt. Firstly, enzyme expression was assessed in the NM of 2 age months Wt and Pirc male rats and in the macroscopic lesions (tumors) of Pirc male rats of 11 months of age. Western blotting analysis performed by the research group of prof. Raugei (University of Florence) highlighted a surprisingly higher expression of this enzyme in the NM of Pirc rats compared to Wt. Interestingly, the comparison between NM and tumors in Pirc rats resulted in a higher expression of LMW-PTP in tumors than in the NM, so suggesting a strong link between the enhanced expression of this enzyme and carcinogenesis (fig. 15 a).

As a second step, we tried to verify the possibility of enhancing the sensitivity to 5-FU in Pirc rats with a pre-treatment with morin. Indeed, a previous characterization of the Pirc rat model suggested that NM of this CRC model was resistant to DMH, a specific colon carcinogenesis compared to Wt (Femia *et al*, 2015) and we confirmed in this study the Pirc rats are also resistant to 5-FU: accordingly, the apoptotic index (AI) was significantly lower ($p < 0.05$) in the NM of Pirc

rats compared to Wt treated with a single s.c. dose of 5-FU (50 mg/kg) and euthanized 24 h later (fig. 15 b). Subsequently, Pirc and Wt rats (8 months of age) were fed with AIN-76 supplemented (treated group) or not (control group) with morin at a dose of 50 mg/kg BW for one week before a s.c. single dose injection of 5-FU (50 mg/kg) and euthanized 24 h later. As fig. 15 c shows, AI in Pirc rats treated with morin was higher compared to untreated rats and LMW-PTP expression was statistically lower in pre-treated Pirc rats than in untreated controls (fig. 15 d). In the NM of Wt rats, which showed to be sensitive to 5-FU as described above (considering the AI, fig. 15 b), both parameters did not show any change in the treated group (data not shown).

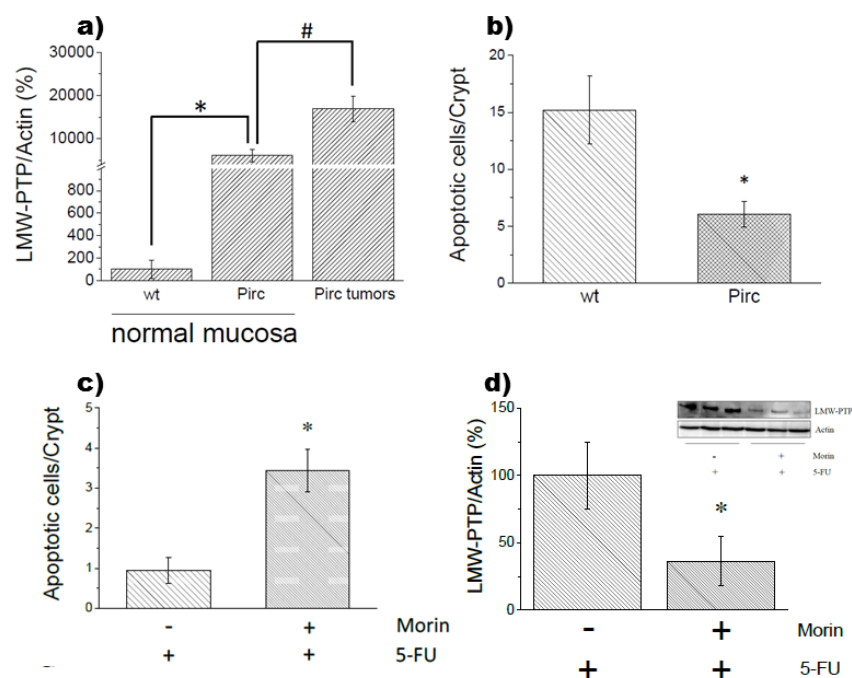

Fig.15. Expression of LMW-PTP and response to 5-FU. Panel a: Expression levels of LMW-PTP in normal mucosa of Wt and Pirc rats and in colon tumors from Pirc rats (data are means ± SE, n = 9, and 7, for the normal mucosa of wt and Pirc rats, respectively and n = 7 for colon tumor s; all data were normalized with respect to the values in the Wt normal mucosa. * p <0.01; # p <0.05). Panel b: Apoptotic cells/crypt (AI) in the NM of Wt and Pirc rats treated with 5-FU (50 mg/Kg) and euthanized 24 h later (data are means ± SE, n = 4 rats in both groups, * p <0.05). Panel c and d: AI measured in the NM of Pirc rats treated (AIN76 + morin at a dose of 50 mg/kg) and untreated (AIN76) for one week before receiving a single dose (50 mg/kg) of 5-FU (c) and expression levels of LMW-PTP (d) measured by western blotting (data are means ± SE, n = 4 rats in both groups, p < 0.05).

4.1.2.1 Effects of 6 weeks morin treatment

The dose of 50 mg/kg of morin, which showed to be capable of restoring 5-FU sensitivity in Pirc rats as explained in the previous paragraph, was chosen for the chemoprevention study. At the end of a 6 weeks treatment, Pirc male rats of 10 weeks of age did not present any sign of toxicity considering the BW of the treated group compared to the untreated

one. A significantly lower number of MDFs in morin-treated rats compared to control was found (fig. 16 a), concomitant with a decrease in LMW-PTP expression in the NM (fig. 16 b). In addition, a few macroscopic tumors were also present in these rats (2.3±0.8 and 1.3±0.5 in controls and morin-treated rats, respectively): however, AI in the tumors dissected from treated and control rats, was significantly higher in the former than in the latter (fig. 16 c). On the contrary, basal levels of apoptosis in the normal mucosa were not affected by the treatment with morin (apoptotic cells/crypt are 0.25 ± 0.04 and 0.24 ± 0.03 in controls and morin-treated rats, respectively; means ± SE, n=8 in both groups).

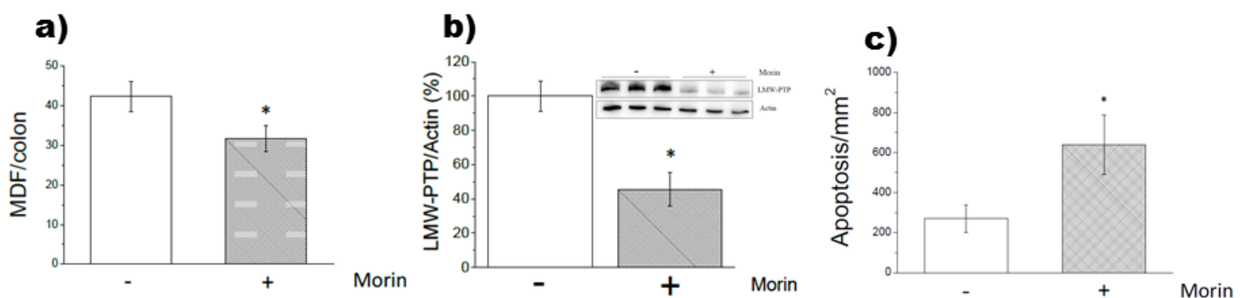

Fig. 16. Effects of a 6-weeks treatment with morin on Pirc rat carcinogenesis. Panel a and b: MDFs and LMW-PTP expression levels respectively, in the colon of Pirc rats fed for six weeks with the AIN-76 standard diet or the same AIN-76 diet supplemented with morin (50 mg/kg). Each bar represents the mean ± SE (n = 9 in both groups, * $p < 0.05$). Panel c: AI in the tumors of Pirc rats treated for 6 weeks with morin or with a standard diet. Bars are mean values ± SE (Control group, n= 6; morin group n = 5). * $p < 0.05$.

4.1.3 PMD: effects of a pomegranate by-product in vivo

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 sign of toxicity of the treatment.

4.1.3.1 Effects of PMD on MDFs

The number of the preneoplastic lesions MDFs was significantly reduced (p= 0.02) in PMD-treated rats compared to controls (fig. 17 A). Moreover, PMD-treated rats showed MDFs with a significantly lower multiplicity (i.e. formed by a lower number of crypts) when compared to those in the control rats (p=0.03) (fig. 17 B). Accordingly, there was a significant (p <0.01) increase in apoptotic index in MDFs dissected from PMD-treated rats compared to untreated ones (fig. 17 C).

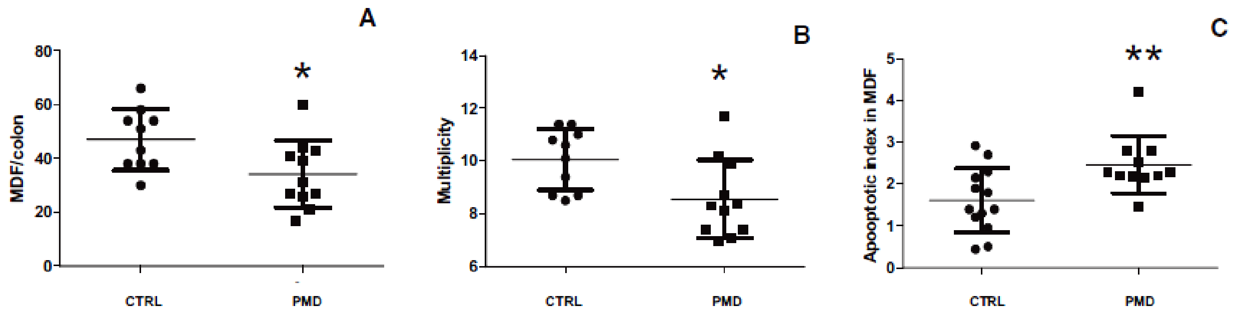

Fig.17. MDFs in the colon of PMD treated rats. MDF/colon (panel A) and crypts/MDF (panel B) in Pirc rats fed with AIN76 (CTRL) or AIN76 diet supplemented with 50 mg/kg/die of PMD (means ± SE); controls: n=10, PMD-treated: n= 11. * $p < 0.05$ significantly different from controls.

4.1.3.2 Effects of PMD on NM

In order to explain the mechanisms of action of PMD observed in the treated animals (see previous paragraph), we determined the gene expression of inflammatory proteins, apoptosis and proliferation in the normal mucosa (NM) of both experimental 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), in line with protein levels evaluation of NOS and COX-2 (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 the proliferative activity was not affected by PMD (fig. 18 A), and apoptosis determined in histological sections of NM 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 C-CASP-3 and BAK proteins (fig. 18 B) 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).

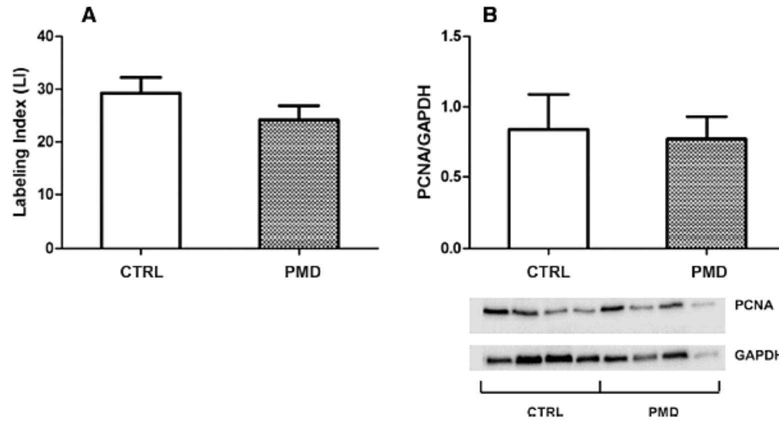

Fig. 18. Effects of PMD in the Pirc colon. Proliferation activity evaluated as LI in histological sections immunostained with a PCNA-antibody and PCNA protein expression by western blot (panels A and B respectively) in NM of Pirc rats treated with PMD. Bars are means + SE; controls: n=10, PMD-treated: n= 11.

4.1.3.3 Effect of pomegranate metabolites on HT-29 cell line proliferation

Based on the protective effects observed *in vivo* on microscopic preneoplastic lesions (MDFs), we also evaluated the effects of pomegranate products in more advanced steps of colon carcinogenesis in vitro and ex-vivo, i.e. human colon cancer cell lines and macroscopic adenomas from older Pirc rats respectively. For both 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. 19 A and B): this cell line is mutated in *Apc* as the *in vivo* model used. 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. 19 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 the IC_{50}, the co-presence of u-A further reduced viability, suggesting an additive type interaction between the two metabolites.

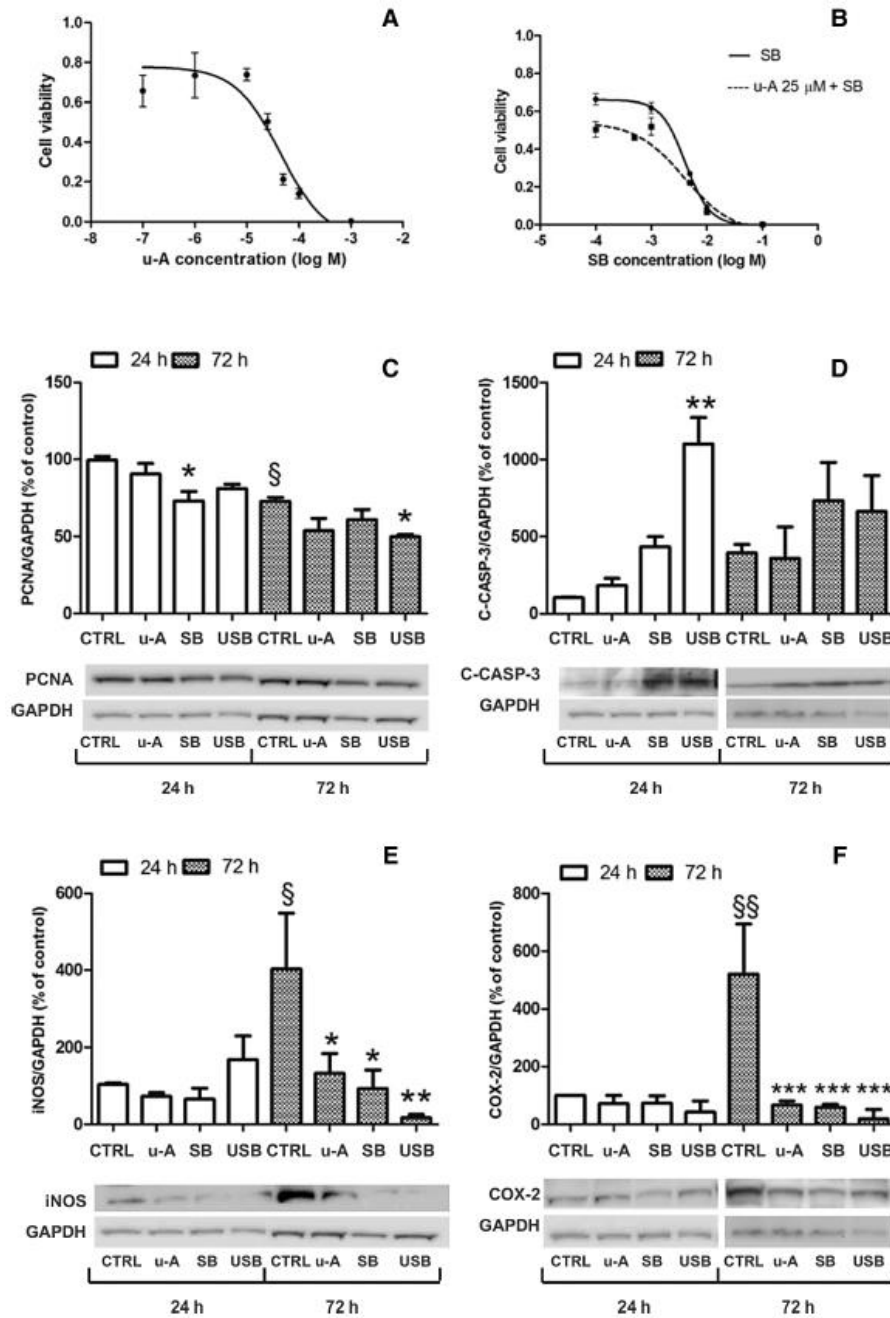

Fig. 19. Effects of PMD colon metabolites on HT-29 CRC cell line mutated in *Apc.* Panel A: concentration-response curve of u-A (log IC_{50} = -4.358, R^2 = 0.8274); panel B: concentration-response curves of SB (log IC_{50} = -2.511, R^2 = 0.9224) and SB plus u-A 25 µM (dotted line, logIC_{50} = -2.405, 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.

4.1.3.4 Urolithin-a, sodium butyrate, or combination treatments effects on HT-29 cell line

The effect of urolithin-A and Sodium Butyrate on markers of proliferation, apoptosis and inflammation was assessed in both cell lines 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).

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 a stronger effect was observed: 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. 19 C). As for apoptosis, activated Caspase-3 (C-CASP3) expression was strongly increased by the combination of u-A and SB by 24 h; during the following 48 h, the levels in treated cells were still higher compared to the respective controls, although the differences were no longer significant (fig. 19 D).

Interestingly, in cells treated for 72 h, the expression of the inflammatory markers iNOS and COX-2 was reduced by all the treatments (fig. 19 E and F respectively), with the most pronounced effect observed with USB treatment (-79.7% and -74.5% compared to respective controls for iNOS and COX-2 respectively, $p < 0.01$). No significant effects of the treatments were observed at 24 h.

4.1.3.5 Effect of pomegranate metabolites on biopsies from Pirc AD and NM samples ex-vivo

Colon adenoma (AD) and normal mucosa (NM) samples from Pirc rats were used for short term (24 h) 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, as this cell line bears an *Apc* gene mutation as Pirc rats. Viability of the samples, assessed with the MTT method at time 0 (control) and after 24hs 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. 20 A). On the contrary, USB showed a pro-apoptotic effect in AD, as indicated by increased C-CASP-3 and BAK expression (fig. 20 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. 20 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.

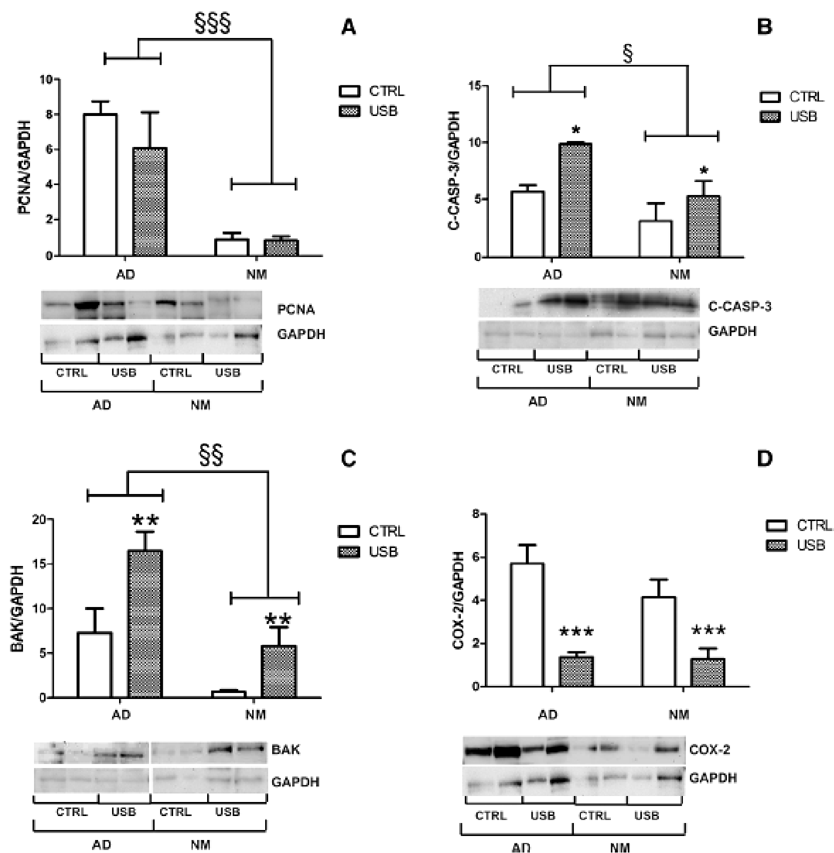

Fig. 20. Effects of PMD in Pirc adenomas and NM biopsies *ex vivo*: relative expression of PCNA, C-CASP-3, BAK and COX-2 (panels A, B, C and D, respectively) in control and USB-treated AD and NM samples after 24 h in culture. §, §§, §§§ p <0.05, <0.01, <0.001 respectively, comparing AD vs NM. *, **, *** p <0.05, <0.01, <0.001 respectively, comparing treated vs untreated samples). Bars are means ± SE.

4.2 Primary cultures of colon fibroblasts for the study of tumor microenvironment

These experiments were performed to address the link between *Apc* gene mutation and the role played by colon tissue environment in carcinogenesis since very early phases. For this reason, colon fibroblasts were isolated from the *in vivo* model used for chemopreventive studies: indeed, Pirc rat bears a germline heterozygote *Apc* mutation, so allowing the possibility of studying its influence on colon environment before the development of macroscopic lesions.

4.2.1 Characterization of colon fibroblasts isolated from Pirc (PCF) and Wt (WCF) rats

Colon fibroblast primary cultures were established from 3 matched couples of male Pirc and Wt of one month of age (young rats, referred to as PCF/y and WCF/y respectively). Preliminary MTS and ICF data were also obtained on cultures established from 1 male Pirc and 1 male Wt rat of 11 month of age (old rats, referred to as PCF/o and WCF/o respectively). Aliquots of freshly isolated cells (p0) were stored in liquid nitrogen, and the same was done for subsequent passages: no differences were observed in the use of revived cells. To confirm the isolation of fibroblast cells, an ICF of collagen 1A chain (colA1) was performed on colon cells isolated from Wt and Pirc rats. Hela cells were used as negative control (fig.21).

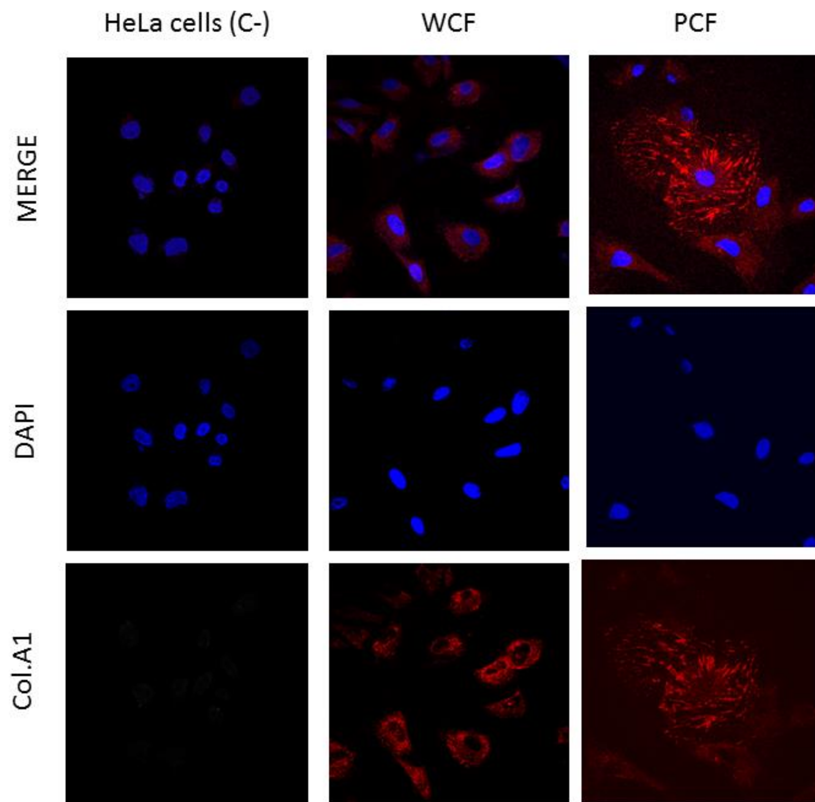

Fig.21. Fibroblast cell identity verification. Representative images of collagen chain A1 (ColA1) ICF on isolated colon cells from Wt and Pirc rats and on epithelial Hela cells as negative control. The images clearly show collagen immunoreactivity for both Wt (WCF) and Pirc (PCF) colon samples. 40x magnification.

4.2.2 Proliferative activity and senescent phenotype

Proliferative activity of PCF and WCF, measured by MTS cell viability test, was assessed on PCF/y and WCF/y between 0 and 6 passages in culture (p) and between p7-9: the reason of this choice is that a reduction in mitosis counts was observed in p7 cells, more pronounced in WCF/y than PCF/y, so leading us to consider them separately (data not shown). For both the subgroups of experiments, preliminary MTS tests were performed seeding 3000, 6000 or 9000 cells/well in order to find the suboptimal starting condition which allowed to outline differences between WCF/y and PCF/y (data not shown): 3000 cells/well were seeded in the first subgroup ($0 \leq p \leq 6$) experiments and 6000 for the second ($7 \leq p \leq 9$). As shown in fig. 22 a, the PCF/y population almost duplicated at T24 and further increases at T48 (202.5±8.8 and 346.6±22 respectively) highlighting a significant higher proliferative activity compared to WCF/y at both time points ($p<0.05$ and $p<0.001$, n=5). However, both WCF/y and PCF/y slow done at increased passages in culture (89.83 ± 10.81 vs 108.16 ± 8.63 WCF/y vs PCF/y and 119 ± 8.30 vs 128.67 ± 8.70 WCF/y vs PCF/y at T24 and T48 respectively, n=3), suggesting that they are subjected to *in vitro* replicative senescence: nevertheless, PCF/y still show a tendency

towards higher proliferative rate compared to WCF/y (not statistically significant, fig. 22 b). Interestingly, preliminary MTS assays performed on PCF/o and WCF/o between passage 1-3, outlined a significantly higher proliferative rate of the former compared to the latter (fig. 22 c) as observed with their young counterparts (167.33 ± 9.58 vs 117.50 ± 5.51 in PCF/o vs WCF/o and 192.23 ± 39.87 vs 140.67 ± 30.08 in PCF/o vs WCF/o at T24 and T48 respectively, n=2).

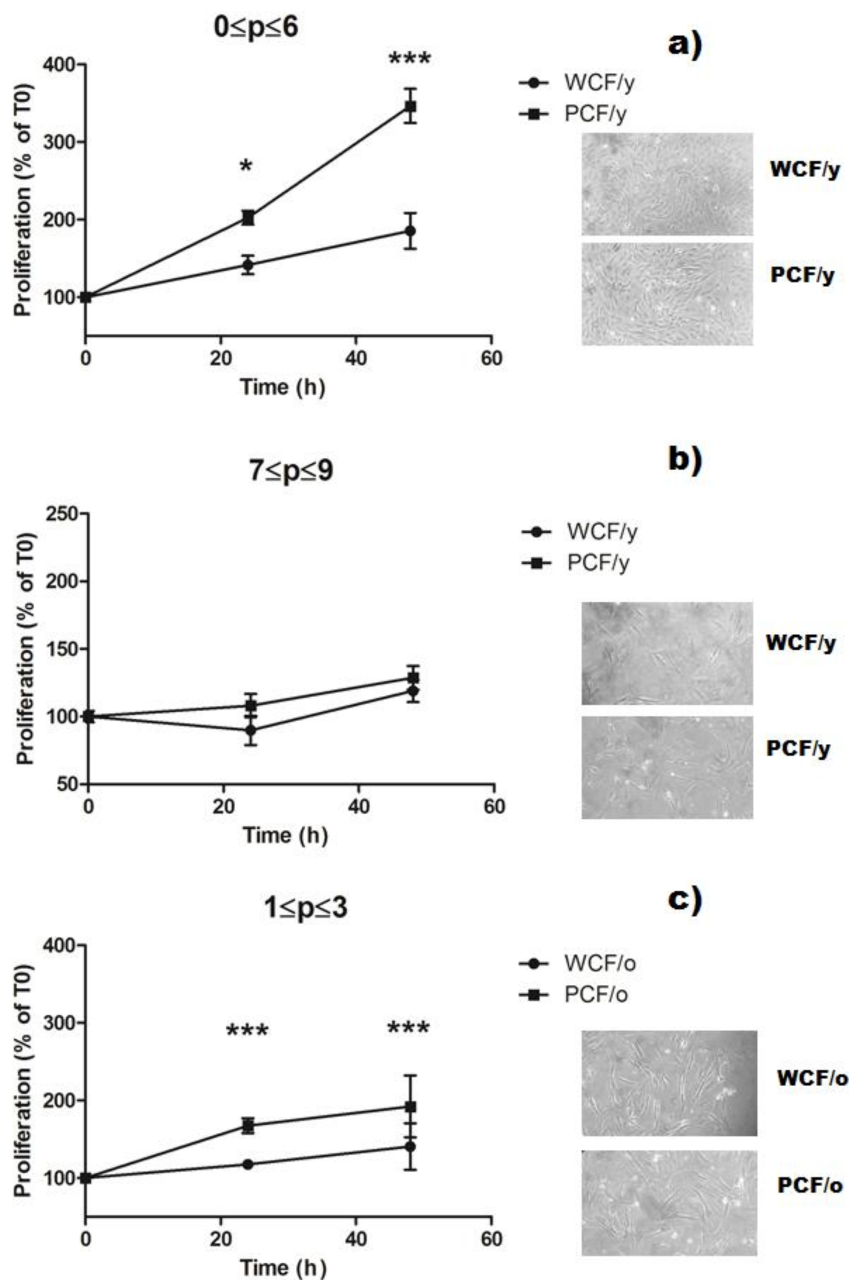

Fig. 22. Proliferative activity of PCF and WCF evaluated by MTS assay at time 24 (T24) and 48 h (T48). Panel a: proliferative activity assessed in WCF/y and WCF/y between passages (p) 1 to 6 (mean ± SE, n= 5 independent experiments on three couples of matched WCF/y and PCF/y). Panel b: proliferative activity between p7-9 (mean ± SE, n= 3 independent experiments on three couples of matched WCF/y and PCF/y). Panel c: proliferative activity of one couple of matched WCF/o and PCF/o between p1-3 (mean ± SE, n= 2 independent experiments). * $p < 0.05$; *** $p < 0.001$: Statistics obtained comparing PCF/y vs WCF/y or PCF/o vs WCF/o at matched time points. Representative images (20x magnification) of matched WCF/y - PCF/y at p2, p9 and WCF/o - PCF/o at p1 are on the right side of panels a, b and c respectively.

The decreased proliferative rate observed with increased passages in culture could be due to the onset of a replicative senescent phenotype. To verify this hypothesis, we assessed the expression of β-galactosidase in both WCF/y and PCF/y: as shown in the fig. 23 (a to f), both cell types showed a linear increase in the staining, concomitant with increased passages in culture. However, it is interesting to note that the staining level was higher in WCF/y compared to PCF counterpart, as also confirmed by the quantitative analysis (fig. 23 g) of the staining intensity ($p < 0.05$).

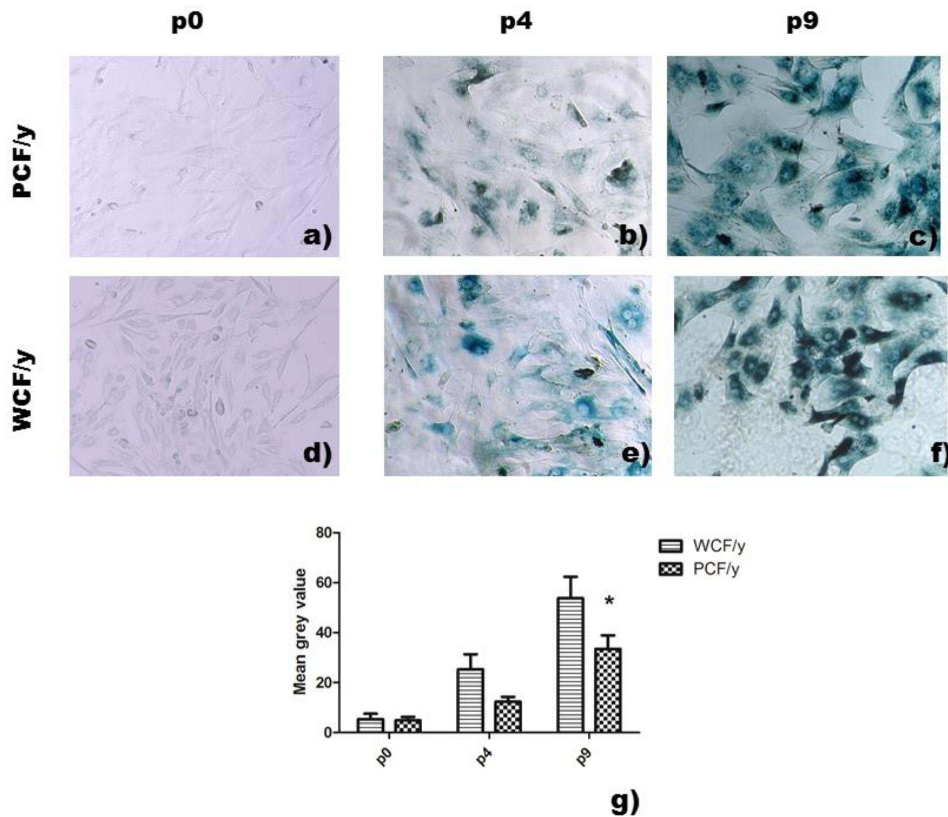

Fig. 23. *In vitro* replicative senescence of WCF/y and PCF/y. From panel a to f: representative images (20x magnification) of PCF/y (a to c) and WCF/y (d to f) stained for β-galactosidase at passages 0, 4 and 9. Panel g: mean grey value calculated on at least 5 microscopic fields/passage for both WCF/y and PCF/y (mean ± SE, n= 6 for both group at p0, n= 12 for both group at p4 and n=20 for both group at p9. * $p < 0.05$.).

In addition, freshly isolated (p0) PCF/o and WCF/o were not positive for the β-galactosidase staining as shown in fig. 24.

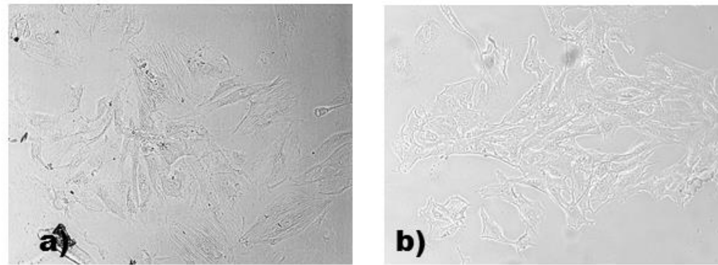

Fig. 24. Senescence associated β-galactosidase staining in colon fibroblasts prepared from old animals. The senescence associated β-galactosidase staining was performed on WCF/o and PCF/o at p0 (panel a and b respectively).

4.2.3 NFκB nuclear translocation

Considering that an inflamed microenvironment promotes carcinogenesis, we assessed whether PCF cells, particularly the ones isolated from young animals, showed activation of inflammatory pathways, the most studied of which is the NFkB signaling pathway. To this aim, NFkB ICF was performed on PCF/y and WCF/y at different passages and image analysis carried out as described in the Methods. Based on the values of the overlapping coefficient (comprised between 0 and 1 meaning 0% and 100% overlapping of antigen signal, red, and nuclear DAPI, blue), we found that mutated fibroblasts from Pirc rats (PCF/y) had higher levels of NFkB nuclear translocation compared to WCF/y (0.794 ± 0.013 n=12 vs 0.543 ± 0.071 n=9 respectively, $p < 0.001$) leading us to speculate that this pathway could be over-activated in mutated cells (fig.25 a-b). This difference cannot be attributed to a different NFkB expression level between Pirc and Wt cells: indeed, the total amount of NFkB protein does not show differences between PCF/y and WCF/y, as assessed by western blotting (fig. 25 c).

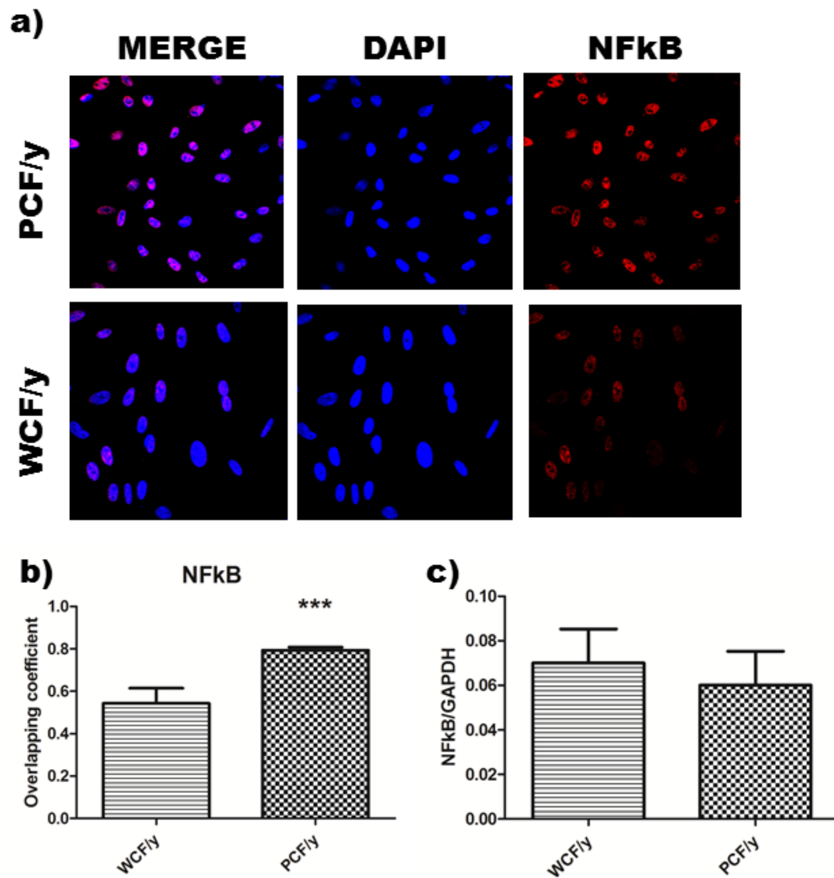

Fig. 25. NFkB nuclear translocation. ICF assay was performed on WCF/y and PCF/y between p1-6 to assess the nuclear translocation of NFkB. Panel a: representative images of NFkB ICF on both group of cells (40x magnification). Panel b: overlapping coefficient between NFkB signal and nuclear DAPI on 3 couples of matched WCF/y and PCF/y at different passages (mean ± SE, n=12 for WCF/y and n=15 for PCF/y, *** p <0.001 of PCF/y vs WCF/y). Panel c: western blotting analysis of total NFkB expression in one couple of WCF/y and PCF/y (mean ± SE of 3 independent experiments).

4.2.4 Inflammatory and CAF phenotype evaluation in WCF/y and PCF/y

To further investigate the pronounced inflammatory phenotype of PCF/y compared to WCF/y homologous, we measured COX-2 and NFkB expression in both cultures at different passages (0<p<6), both in control conditions and upon treatment for 24 h with LPS (10 µg/ml) or TNF-α (20 ng/ml). The release of IL-6 in cell media was also measured at the end of the treatments. Moreover, the expression of a known marker of CAFs, α-SMA, was assessed in both WCF/y and PCF/y in basal conditions and after a 24 h treatment with TGF-β (10 ng/ml). Concerning the inflammatory phenotype, PCF/y untreated (control, CTR) show significantly higher levels of COX-2 compared to the untreated WCF/y (fig. 26 b). Moreover, expression of COX-2 in PCF/y increases after 24 h stimuli with both LPS and TNF-α and this response is significantly different from the response observed in WCF/y as outlined by two-way ANOVA analysis (p <0.05).

Regarding the expression levels of total NFkB in response to the same stimuli (fig. 26 a) only a tendency towards increased expression were observed in both PCF/y and WCF/y.

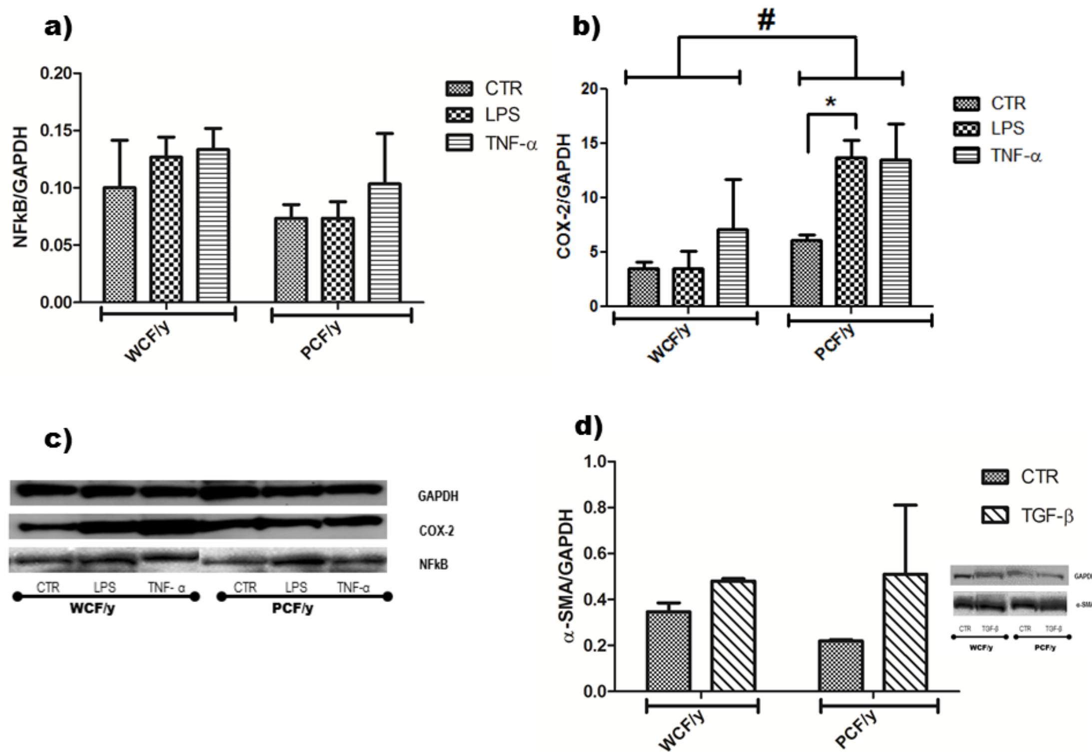

Fig. 26. PCF/y and WCFy response to different stimuli. Couples of matched WCF/y and PCF/y between p1-6 were treated for 24 h in low FBS medium (1%) with LPS 10 μg/ml, TNF-α 20 ng/ml or TGF-β 10 ng/ml: then proteins and media were collected. Panels a and b: NFkB and COX-2 expression respectively, in WCF/y and PCF/y (mean ± SE, n=3 independent experiments, * p <0.05 PCF/y vs WCF/y in CTR condition, # p <0.05 PCF/y vs WCF/y overall response). Panel c: representative images of western blotting membranes. Each band was normalized on its corresponding GAPDH (densitometry analysis was performed using Quantity One software, BioRad). Panel d: α-SMA protein levels measured by western blotting in one couple of WCF/y and PCF/y at p1-6 after 24 h treatment with TGF-β or in control conditions (CTR) (mean ± SE, n=2 independent experiments).

IL-6 ELISA assay in cell-conditioned media did not reveal any difference between PCF/y and WCF/y, although a trend towards higher levels released from PCF/y compared to WCF/y was observed. Again, the treatment with either LPS or TNF-α induced non-significant increases compared to controls, both in PCF/y and WCF/y (fig. 27).

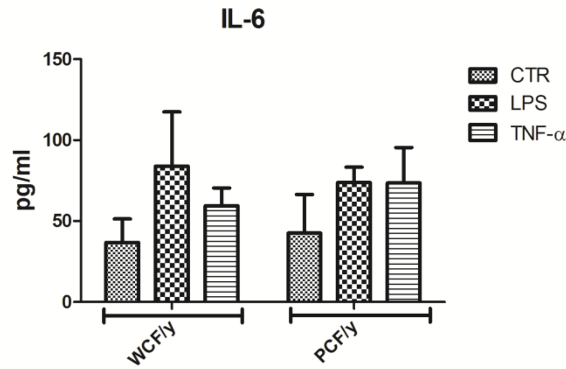

Fig. 27. IL-6 measured by ELISA assay in cell-conditioned media. After 24 h treatment with LPS 10 μg/ml or TNF-α 20 ng/ml or in control conditions (CTR), cell media from one couple of WCF/y and PCF/y were collected and used for performing an ELISA assay for IL-6 (means ± SE, n=3 independent experiments.

Finally, to verify whether the increased inflammatory phenotype showed above in PCF/y was associated to myofibroblast transition, we assessed the expression of a marker of fibroblast activation, alpha-SMA, by western blotting assay. No significant differences were observed between PCF/y and WCF/y basal alpha-SMA expression (fig. 26 d). TGF-β, known to favor fibroblast-myofibroblast transition, induced a tendency towards an increased expression, which appeared to be stronger in PCF/y than in WCF/y: however, all these differences were not significant.

4.3 DNA stability and epigenetic changes

The following experiments, in line with the aims of the *in vitro* ones, were aimed to assess the influence of *Apc* mutation on DNA stability at very early stages of colon carcinogenesis . Moreover, as outlined previously (par. Intro), global DNA methylation changes have a consistent impact on colon carcinogenesis: nevertheless, up to now the methods for assessing DNA methylation status are expensive and mostly time consuming. For this reason, the experiments described in the second part of this chapter were aimed at developing a new method for global DNA methylation assessment.

4.3.1 Apc mutation and DNA stability at very early stages of colon carcinogenesis

The possibility that the mutation in *Apc* could confer DNA instability to PCF, was investigated by COMET assay, both in the standard version and with the modification involving the use of the enzyme FPG enzyme, to detect both strand breaks and oxidized DNA bases, in primary fibroblast cultures. The data obtained on young PCF and WCF indicate that the amount of basal DNA breaks (SBs) is similar between the two groups of cells (fig. 28 a). Accordingly, the analysis of the phosphorylated histone 2Ax (γ-H2Ax), a marker of DNA breakage) performed on ICF images did not show differences between Pirc and wild type fibroblasts (fig. 28 b). Interestingly, DNA oxidative damage detected as FPG sites resulted to be significantly higher in WCF/y than in the PCF counterpart (fig. 28 a).

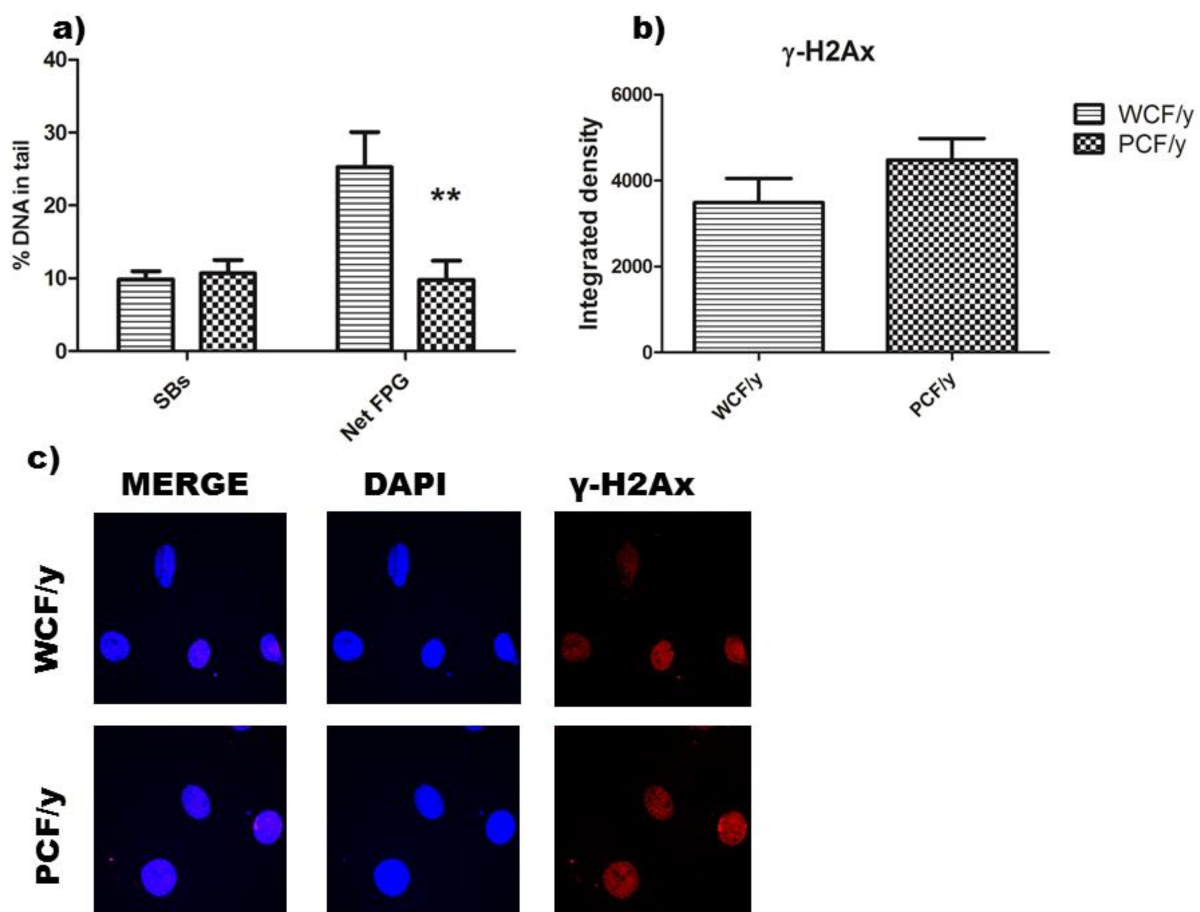

Fig.28. DNA breaks and oxidative damage. Panel a: DNA strand breaks (SBs) and FPG sites (oxidized bases, Net FPG) measured by COMET assay on three couples of matched WCF/y and PCF/y between p2-6 (mean ± SE n=2 independent experiments, ** $p < 0.01$ comparing PCF/y vs WCF/y for net FPG sites) . Panel b: integrated density calculated on ICF images of γ-H2Ax performed on three couples of matched WCF/y and PCF/y (mean ± SE, n=10 microscopic field for both group). Panel c: representative images of ICF for γ-H2Ax in PCF/y and WCF/y (100x magnification).

Interestingly, these data on colon fibroblasts are in line with results obtained performing the COMET assay on scraped NM samples from 4 couples of matched Wt and Pirc rats of 4 weeks age: indeed, in this case we again found the same levels of SBs between the two groups and higher levels of FPG sites in the Wt group compared to Pirc one, although in this last case a significance level was not reached, due to intra-group variability (fig. 29). Preliminary data obtained from one experiment performed on PCF/y and WCF/y cells at p8 showed that FPG sites were still higher in WCF than in PCF, whereas SBs resulted to be higher in PCF (data not shown).

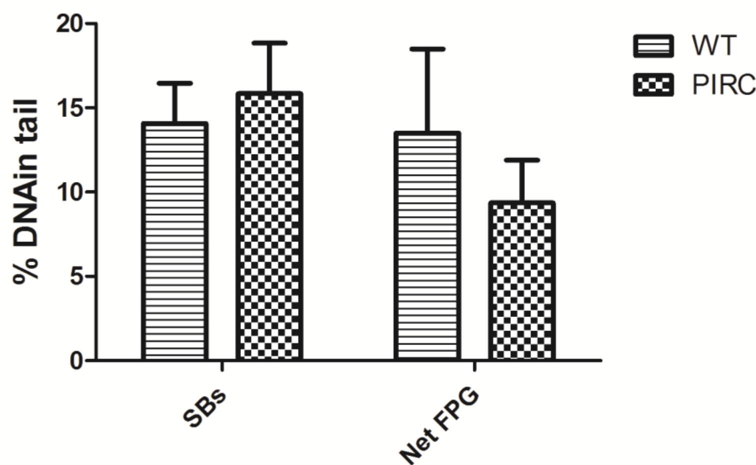

Fig. 29. COMET assay on NM samples of one month-old Wt and Pirc rats. Four couples of matched W (WT) and Pirc (PIRC) rats were euthanized at one month of age and samples of scraped NM were collected and subjected to COMET assay (data are means ± SE, n=4 for both groups).

4.3.2 Comet methylation assay development

A methyl-sensitive variation of the SCGE assay has been described previously (Lewies *et al*, 2014; Perotti *et al*, 2015; Kusari *et al*, 2017) but is not widely used yet. For this reason, in the laboratory of prof. Duthie (Robert Gordon University, Aberdeen - UK), we have tried to further develop this method, and we tested its ability to specifically detect perturbations in DNA methylation in normal human colon epithelial and fibroblasts cell lines (NCM460 and CCD-18Co respectively) subjected to folate depletion (hypomethylation) or exposed to nickel chloride which has been shown to induce DNA hypermethylation (Perotti *et al*, 2015). Preliminary experiments aiming at finding the best incubation conditions with enzymes HpaII and MspI were performed: ideally, the amount of breaks detected in the absence of the enzyme (slide incubation performed only with the fast digest buffer, FDB) should be low. The effect of the FDB alone is shown in fig. 30: it can be observed that in fibroblasts DNA damage began to increase over control (CTRL) levels at 30' incubation (145.5 ± 7.4 vs 107 ± 7.3); a further significant increase was found at 45' (173.3 ± 11.8) and 60' (236 ± 9.2). In the

epithelial cells, FDB alone significantly increased the total score at 60' (320.6 ± 31.2 vs 241 ± 22). For both enzyme activities, the chosen incubation time was15' in both cell lines, as already at 30' the increase in activity was associated with a slight increase in the FDB score.

DNA methylation was calculated as the ratio between the two separate enzyme digestions (HpaII/MspI) after subtracting the effect of buffer alone for each sample (Perotti *et al*, 2015). $NiCl_2$ induced DNA hypermethylation in both human colon cell lines (fig. 30 a), although the effect was statistically significant only in CCD-18Co fibroblasts (fig. 30 b). Short-term folate depletion (48 h) increased the HpaII/MspI ratio approximately 3-fold in the epithelial and 4-fold in colon fibroblasts (fig. 30 a and b respectively), indicating that HpaII digestion was increased, as it would be expected when DNA methylation is lower. A similar result was observed in response to longer-term folate deficiency (fig. 30 b). These data indicate that this modification of the comet assay can detect DNA methylation in cultured cells.

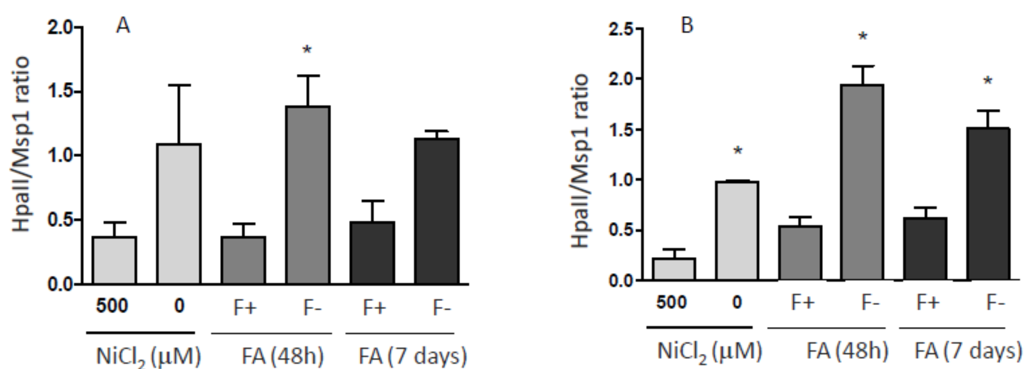

Fig. 30. Global DNA methylation in normal colon human cell lines. NCM460 (A) and CCD-18Co fibroblasts (B) were treated with $NiCl_2$ (24h) or made folate deficient (48 h or 7 days). Results are mean ± SE of 4 experiments. * $p < 0.05$, difference between cells grown in the presence or absence of folate, or incubated with or without $NiCl_2$.

Discussion and conclusions

This PhD project was conceived to address two important questions: firstly, to investigate the possibility to prevent the onset and/or to interfere with the progression of colon carcinogenesis with the use of natural compounds from different and non-canonical sources (fruit by-products), or targeting new molecular mechanisms (LMW-PTP oncoprotein). Secondly, we addressed the mechanisms of the effect of *Apc* mutation on colon carcinogenesis, by studying the changes in tissue microenvironment, particularly at the level of colon fibroblasts, and in DNA stability focusing on the very early stages of the carcinogenesis process (i.e. when no colonic lesions or only very few are present in the intestine).

The CRC genetic *in vivo* model Pirc rat maintained at the center for the housing of laboratory animals (CESAL) of the University of Florence, demonstrated to be suitable to pursue these two parallel research lines. Indeed, Pirc rat bears a germline heterozygote gene mutation in *Apc* as it can be found in FAP patients (an hereditary syndrome predisposing to early CRC onset), and in sporadic CRCs (Amos-Landgraf *et al*, 2007): in fact, mutations in this oncosuppressor gene allow enhanced nuclear translocation of β-catenin which in turn leads to hyper-activation of Wnt signaling pathway, a well-established key event in the onset of CRCs (Zhan *et al*, 2017). Pirc rat has been widely characterized by the group of prof. Caderni at University of Florence: differently from other genetic models of CRC, such as Min mice, this model develops polyps also in the colon, where they can be found since 4 months of age and whose number increases with the age of the animal. In addition, microscopic preneoplastic lesions MDFs can be detected in the colon of Pirc rats since one month of age and show nuclear translocation of β-catenin, suggesting that the colon carcinogenesis process is already started at this early phase (Femia *et al*, 2015a, 2016). Besides, MDFs number and multiplicity (number of crypts forming each lesion), increase with rats' age as also observed in other CRC animal models (DMH or AOM induced CRC models) (Femia *et al*, 2004, 2009, 2012): for this reason evaluation of these parameters is considered a reliable biomarker of colon carcinogenesis (Mori *et al*, 2004; Suzui *et al*, 2013).

5.1 New possible strategies for CRC chemoprevention

The possibility of inhibiting the carcinogenesis process is of particular relevance considering that colon carcinogenesis is a slow process compared to other types of cancer (it takes more than 10 years for a carcinoma development) both in

case of sporadic and hereditary types (Kuipers *et al*, 2015). As highlighted in the introduction, prevention can be made at two levels: primary prevention aims at completely avoiding cancer onset in the general population, promoting the implementation of healthy lifestyles. Secondary prevention is directed to high risk population, in which the risk is linked to unmodifiable factors such as age, genetic alterations or personal and/or familial history of neoplasia, which increase the risk of cancer. More recently, the concept of tertiary prevention (i.e. prevention of cancer recurrence either in the same body area or elsewhere) has gained attention, particularly in the case of CRC, in which CRC survivors represent the third largest group of long-term cancer survivors in Western countries (Labianca *et al*, 2010).

A plethora of data are present in the literature about the possibility of preventing CRC with the use of either products from "mother nature", or their principal active compound (Cruz-Correa *et al*, 2006; Citronberg *et al*, 2013). Polyphenols, terpens and flavonoids are the most studied classes of natural compounds which have been investigated for cancer chemopreventive purposes *in vitro*, *in vivo* and also in clinical trials, and extensively reviewed (Surh, 2003; Aggarwal *et al*, 2013). The interest on these molecules within the CRC prevention field is particularly due to their anti-inflammatory features: indeed, inflammation is a promoting factor of several cancers and also of CRC (Coussens & Werb, 2002). In addition, increasing interest is rising on the use of a combination of natural compounds instead of a single one. In fact, considering chemoprevention, the use of multiple agents could interfere simultaneously with multiple pathways involved in carcinogenesis; on the other hand, the combination of natural agents capable of working synergistically with drugs, could allow the use of lower doses of the last ones so reducing associated toxicities in the patients (Ulrich *et al*, 2006; Steward & Brown, 2013). Our *in vivo* studies with a flavonoid-enriched Bergamot juice extract (BJe) and with a pomegranate mesocarp decoction (PMD) stand in this scenario.

BJe has been characterized at the University of Messina by the research group of prof. Navarra and showed to be able to reduce the growth rate of different cancer cell lines (Delle Monache *et al*, 2013; Ferlazzo *et al*, 2015). In addition, an integrated network of case–control studies showed that *Citrus* fruit intake reduced the risk of several types of cancer, such as esophageal, stomach, colorectal and laryngeal (Foschi *et al*, 2010). In our study, BJe treatment was able to reduce colon tumors in Pirc rats in a dose-dependent manner (see fig.12 in "Results"): the 35 mg/kg dose reduced the number of colon tumors although the difference was not statistically significant, but a further and significant reduction was achieved with 70 mg/kg dose. Of note was the effect observed at the level of preneoplastic lesions: MDFs were significantly reduced with both 35 and 70 mg/kg, in a dose-dependent manner, so suggesting the ability of BJe to interfere with colon carcinogenesis (Navarra *et al*, 2018 submitte). Moreover, we showed that the administration of BJe

at a dose of 70 mg/kg in Pirc rats aged 4 weeks for 3 months, caused a marked down-regulation of inflammation-related genes in the colon tumors, along with up-regulation of *p53* (pro-apoptotic gene) and down-regulation of *survivin* and *p21* (anti-apoptotic genes), so suggesting that BJe at this dose is able to induce apoptosis in the tumors of treated rats. This hypothesis was further supported by the histological evaluation of tumors from both experimental groups: in fact, apoptosis, evaluated morphologically, was higher in tumors from the BJe treated group compared to controls (see fig. 13-14 in "Results").

Concerning PMD, this was prepared and characterized by prof. Mulinacci and colleagues (University of Florence) from pomegranate mesocarps (a by-product of pomegranate juice preparation) of Wonderful variety. This decoction, made as a powder, was rich in ellagitannins (about 15% w/w) among which punicalagin (peculiar of pomegranate fruit) was the most abundant. This was in line with data reported in literature showing that non-edible parts of pomegranate are richer in punicalagin compared to the edible part, namely arils, from which pomegranate juice is prepared: nevertheless, the majority of published data available on the chemopreventive properties of this fruit investigated the effects of the juice. In addition, PMD had a moderate content in soluble fibers which could act as a vehicle for polyphenols in the colon, improving their bioavailability. The study on PMD was divided in three parts: short-term chemopreventive *in vivo* study and mechanistic studies of the effects exerted by its main colon metabolites *in vitro* and *ex vivo*. Concerning the *in vivo* study, PMD administered for 6 weeks to Pirc rats at a dose corresponding to a daily intake of 50 mg/kg of polyphenols reduced significantly MDFs number and size (multiplicity index). In addition, an increase in apoptosis in MDFs from PMD-treated rats was observed compared to the controls, suggesting that PMD is able to reduce colon carcinogenesis possibly through the induction of apoptosis at the level of these pre-cancerous lesions, while it does not significantly affect proliferation and apoptosis in the apparently normal mucosa (NM) (see fig.17 in "Results").

The *in vitro* and *ex-vivo* experiments were conceived in order 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 hypothesized that the effects observed *in vivo* could be due to the action of PMD colon metabolites: considering PMD composition, we decided to investigate the effects of urolithin-A (u-A) and sodium butyrate (SB), i.e. the main metabolites of punicalagin and of soluble fibers respectively, as reported in literature (Cerdá *et al*, 2005; Bultman, 2014). We used to this aim commercially available compounds. The HT-29 human carcinoma cell line was used as it bears an *Apc* gene mutation like the *in vivo* model. u-A and SB alone demonstrated inhibitory effects on HT-29 cell viability with an IC_{50} of 43.9 μM and 3 mM respectively. Moreover, to better mimic the

administration of PMD *in vivo*, we evaluated the effect of the combination of these two metabolites: 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 (see fig. 19 in "Results"). 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. These three treatments (u-A 25 µM, SB 2.5 mM or their combination named USB) were tested on HT-29 cells for 24 and 72 h of incubation and followed by western blotting analyses of several protein markers involved in proliferative, inflammatory and apoptotic mechanisms. This analysis showed that USB treatment was capable of affecting these parameters in a more pronounced manner than single treatments (see fig. 19 in "Results"). In fact, USB treatment displayed the strongest reduction in proliferative activity (PCNA reduction) at 72 h and the strongest apoptotic induction (C-CASP-3 increase) at 24 h. 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 (Giménez-Bastida *et al*, 2012; Li *et al*, 2014), the anti-inflammatory effects of the combination was 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 tumors from 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 apparently normal mucosa (NMs) from eight-month-old PIRC rats were collected and maintained in culture for 24 h with or without USB, at the same concentrations tested in HT-29 cells.

First, to verify the reliability of this system, viability was assessed in the control samples at 24 h with the MTT method and resulted to be higher than 50% of the value recorded at time 0. Furthermore, proliferation and apoptosis were significantly higher in AD compared to NM, in agreement with studies documenting higher proliferation and apoptosis in AD compared to NM (Jaskiewicz *et al*, 1998): these results led us to consider this *ex-vivo* system as a reliable tool to test PMD as a chemopreventive treatment in more advanced stages of colon carcinogenesis. The treatment with USB increased apoptosis, as measured by BAK and C-CASP-3 protein levels in both AD and NM, compared to untreated controls and also determined a significant reduction in COX-2 expression; no significant effects were observed considering proliferation (PCNA) in both ADs and NMs (see fig. 20 in "Results"). As a whole, these data suggests for the first time that a preparation from a fruit by-product named PMD, is able to significantly reduce tumorigenesis *in vivo.* This effect could be due to the combined anti-inflammatory and pro-apoptotic action of its different components, ellagitannins and fibers, as *in vitro* and *ex vivo* studies indicate. Indeed, a combination of urolithin-A and sodium butyrate

was tested for the first time showing significant anti-inflammatory and pro-apoptotic effects both on human colon cancer cells and on adenomas of Pirc rats (Tortora et al. 2018).

As shown above, both these two treatments (BJe and PMD) appear to be able to prevent CRC carcinogenesis through anti-inflammatory and pro-apoptotic actions at the level of both microscopic preneoplastic lesions (MDF) and tumors. Additionally, in recent years the concept is emerging that natural compounds endowed with anti-inflammatory, anti-proliferative, pro-apoptotic and anti-oxidant activities could act synergistically or additively with drugs with the aim of enhancing their efficacy, reducing associated toxicity, or encompassing cell resistance (Rejhová *et al*, 2018). Cancer cells can develop resistance to therapies through different mechanisms such as, among others, enhanced DNA repair, epigenetic changes, microenvironment changes mediated by CAFs, and inhibition of apoptosis (Mansoori *et al*, 2017). Recently, Hoekstra et al. (Hoekstra *et al*, 2015) indicated the possible implication of the oncogenic low molecular weight (18 kDa) phospho-tyrosine phosphatase (LMW-PTP) in colon cancer cell drug resistance and its increase during CRC carcinogenesis. These data were confirmed by the group of prof. Raugei (University of Florence), who also demonstrated the ability of morin to lower LMW-PTP levels at micromolar concentrations and to restore 5-FU response in CRC resistant cell lines (Lori *et al*, 2018 under review). Morin is a polyphenolic compound (3,5,7,2',4'-pentahydroxyflavone), originally isolated from members of the *Moraceae* family, present in figs, mulberry, strawberries. In the present work, we evaluated the expression of this enzyme in the NM of Pirc rats, since it has been previously documented that the morphologically NM of Pirc rats is resistant to apoptosis (Femia *et al*, 2015a). Accordingly, over-expression of LMW-PTP was observed in the NM of Pirc rats aged 8 weeks, when no tumors and only few microscopic lesions are present in the colon mucosa. Moreover, we also found overexpression of LMW-PTP in Pirc colon tumors when compared to their corresponding NM (see fig. 15 in "Results"). Consequently, we tested whether morin could restore the apoptotic response of Pirc rats NM and lower LMW-PTP expression. Interestingly, we found that morin, at a dose of 50 mg/kg for 7 days before 5-FU treatment, restored the sensitivity to 5-FU-induced apoptosis in NM, along with a decrease in LMW-PTP expression. Finally, we assessed the chemopreventive effects of a short-term (6-weeks) treatment with morin at a dose of 50 mg/kg in one month old Pirc rats already showing higher expression of LMW-PTP compared to WTs. This treatment significantly decreased the number of MDFs in the colon (see fig.16 in "Results"). Thus, in this study we show for the first time that the preneoplastic mucosa of Pirc rats, prone to carcinogenesis and resistant to apoptosis, over-expresses LMW-PTP, a protein with oncogenic potential. We also show that morin is an

inhibitor of LMW-PTP *in vivo* in Pirc rats, restoring the sensitivity to apoptosis through this mechanism. Finally, this study suggests the possibility of using morin, a natural compound, to enhance the response to chemotherapy.

5.2 Effects of Apc mutation on colon tissue microenvironment at very early stages of CRC development

As outlined in par. 1.8, tissue microenvironment modifications occur during carcinogenesis and are mainly driven by the activation of normal tissue resident fibroblasts into cancer associated fibroblasts (CAFs). This activation is responsible for ECM remodeling, making the stroma a barrier to drug diffusion and immune cell infiltration. Moreover, CAFs promote cancer cell proliferation and migration, angiogenesis and invasion in various types of tumors, including CRCs, through the enhanced release of different growth factors, cytokines and chemokines (Kalluri, 2016). Given this wide range of modifications they promote, the prognosis of CRCs showing high CAFs infiltration is worse (Dienstmann *et al*, 2017). Data on colon fibroblasts isolated from CRC or NM specimens of CRC patients are available in literature (Mrazek *et al*, 2014; Hanley *et al*, 2017); on the contrary, to our knowledge few data on the characterization of colon fibroblasts mutated in *Apc* are available: Patel et al. (Patel *et al*, 2011) isolated fibroblasts from NMs of FAP patients and control individuals (undergoing colonoscopy within population based screening programs). Proteomic characterization was performed, but FAP and controls were not age-matched (39.3 ± 8.8 vs 60 ± 6.5 years in FAP and control group respectively) rising some doubts about the reliability of the differences found in protein expression. Moreover, adenomas can be detected in the colon of FAP patients since late adolescence (16 years), indicating that the analysis of these colon fibroblasts could show features associated to a middle/late stage of colon carcinogenesis. In fact, FAP patients have a 100% risk of CRC development unless preventive colectomy is performed: even with colectomy, the risk of CRCs is reduced but not completely eradicated, and is accompanied by a significant impairment of QoL. These considerations led us to characterize the phenotype of *Apc* mutated colon fibroblasts isolated from young Pirc rats (one month of age): at this age, there are no macroscopic lesions, but the carcinogenesis process is already ongoing due to the *Apc* mutation, as confirmed by the presence of some MDFs in the colon of Pirc rats at this age (Femia *et al*, 2015a). Thus, the isolation of colon fibroblasts from morphologically normal mucosa of matched age Pirc and Wt rats at one month of

age (referred to as PCF/y and WCF/y respectively) could provide a useful model to detect phenotypical differences between Wt and mutated cells at very early stages of CRC carcinogenesis.

Considering the altered Wnt pathway accounting for hyper-proliferation in *Apc* mutated cells, we expected to find higher proliferative activity in PCF/y compared to WCF/y: indeed, we found a surprising higher proliferative rate compared to WCF/y at initial passages in culture ($0<p<6$) indicating a nearly double proliferation rate of PCF/y population compared to WCF/y (see fig. 22 in "Results"). With increasing passages in culture, proliferative rates slowed down in both cell types; concomitantly, we observed increased senescence associated β-galactosidase activity (see fig. 23 in "Results"); these data indicate that replicative senescence was approached in both PCF/y and WCF/y at late passages in culture ($7<p<9$). However, it is interesting to note that β-galactosidase labelled cells could be found in WCF/y at intermediate passages in culture (p4-5) but were rare in PCF/y. Although *in vitro* cell senescence does not necessarily reflect what happens *in vivo*, these data suggest that *Apc* mutation could slow down replicative senescence, conferring to cells an advantage to live longer. A continuous cross-talk between epithelial cancer cells and CAFs supports the carcinogenesis process through modifications in both tissue components, and it is feasible that signals from tissue microenvironment *in vivo* could further contribute to slow down or block senescence in PCF/y. This hypothesis is in agreement with preliminary data obtained from colon fibroblasts isolated from old Pirc and Wt rats (PCF/o and WCF/o respectively) (see fig. 22 in "Results"): a significantly higher proliferative rate was still observed in cells from Pirc rats compared to Wt ones and, interestingly, PCF/o were not positive for β-galactosidase staining (see fig. 24 in "Results").

As a further step in characterizing the phenotype of PCF/y, we focused on the NFkB (p65) pathway, whose activation is associated with high Wnt signaling (Kaler *et al*, 2009; Myant *et al*, 2013). It is known that nuclear translocation of NFkB is associated with the activation of several processes which in turn promote carcinogenesis (Zhang & McCarty, 2017). We found significantly higher nuclear translocation of NFkB in PCF/y compared to WCF/y, which was not due to differences in protein expression, as shown by western blotting analysis (see fig. 25 in "Results"). Moreover, we found a significantly higher expression of the pro-inflammatory marker COX-2 in PCF/y compared to their Wt counterpart. Furthermore, PCF/y cells seemed to be more sensitive to pro-inflammatory stimuli as showed by COX-2 expression increase in response to LPS or TNF-α stimulation compared to WCF/y: indeed, two-way ANOVA analysis showed that the different response to the stimuli was significantly dependent from the genotype ($p < 0.05$) (see fig. 26 in "Results"). A trend towards an increase in the release of IL-6 in response to the same stimuli was also observed in PCF/y compared to WCF/y, although the effect was not statistically significant (see fig. 27 in "Results"). We also found no modification in

total NFkB levels measured by western blotting in response to the same stimuli. On the whole, these data strongly suggest for the first time that a pro-inflammatory phenotype is present in *Apc* mutated colon fibroblasts at very early stages of colon carcinogenesis, able to favor the establishment of a pro-tumorigenic environment for micro and macro preneoplastic lesion development. Future studies will have to clarify whether a similar scenario also occurs in FAP patients at very young age and/or in early phases of non-hereditary CRCs.

5.3 Apc and DNA stability

Finally, considering the involvement of *Apc* also in DNA repair mechanisms (BER systems) (Narayan & Sharma, 2015), we also brought about the assessment of DNA stability in both colon fibroblast cultures established from one month-old Pirc and Wt rats and in NM scraped samples from 4 couples of Pirc and Wt rats aged one month: in this last case, DNA stability was evaluated on all the tissue components, mainly epithelial cells and fibroblasts, whereas in the former the contribution of the colon fibroblast component was assessed separately. Interestingly, data obtained with the COMET assay performed to assess both DNA basal strand breaks (SBs) and oxidative damage (net FPG sites) on both types of samples (NMs from Wt and Pirc rats and PCF/y and WCF/y) showed a similar trend: considering NMs, SBs level were comparable in Wt and Pirc rats while oxidative damage was slightly higher in Wts (difference not statistically significant). Similarly, we found comparable amount of SBs in PCF/y and WCF/y, and these data were confirmed quantifying by ICF the levels of γ-H2Ax, a marker of DNA strand breaks (see fig. 28-29 in "Results"). Moreover, a significantly higher oxidative DNA damage level was observed in WCF/y compared to PCF/y. Previous analyses performed on NMs of Pirc and Wt rats of one month of age by our group (Femia et al, 2015) reported higher expression of *Sod2 (*superoxide dismutase 2*), Apex1* and *Ogg1* (both DNA repair enzymes) in Pirc rats. Thus, it can be hypothesized that increased levels of antioxidant and DNA repair enzymes in Pirc rats lead to lower levels of oxidative DNA damage, as we have shown with the comet assay. This could confer to *Apc*-mutated cells a selective advantage for carcinogenesis development: indeed, cancer cells produce high levels of ROS, so being exposed to constitutively high levels of endogenous oxidative stress which could in turn positively select anti-oxidant strategies to favor cancer cells survivorship, as reported in the literature (Ogasawara & Zhang, 2009; Trachootham *et al*, 2009). All considered, this study underlines a possible link between *Apc* mutation and resistance to oxidative stress as a mechanism which could favor cancer cells survival. Moreover, this mechanism might affect both epithelial and fibroblast colon cells.

DNA stability is strictly linked to epigenetic changes such as DNA methylation. DNA methylation and particularly changes in CpG islands methylation status, are involved in 18% of CRC cases (Gallois *et al*, 2016). In normal cells, CpG islands in the promoter region are not methylated while CpG islands occurring in genomic sequences are methylated: in cancer cells methylation induces both transcriptional silencing of tumor suppressor genes and an overall increased genome instability driving to overexpression of oncogenes (Rhee *et al*, 2017). Aberrant patterns of DNA methylation develop as a result of pathological processes such as chronic inflammation, and in response to various dietary factors, including imbalance in the supply of methyl donors, particularly folate, which can be obtained only from the diet; an imbalance in folate level leading to global DNA hypomethylation has been associated with an increased risk of CRC induction and progression (Duthie, 2011). Global DNA methylation status can be assessed with different types of assay, such as LC, MS/MS, microarray, whose limits to the use for research purposes are the costs and the required time. The development of a COMET assay modification able to measure in a reliable and fast manner global DNA methylation status can be of value and this was the aim of a short-mission at the Robert Gordon University in the research group of prof. Duthie. For setting up the method, taking into account previous attempts present in the literature (Lewies *et al*, 2014; Perotti *et al*, 2015) a fast digest approach was first developed using two human colon cell lines (NCM460 and CCD-18Co, epithelial cells and fibroblasts respectively) to develop and validate the method. The fast digest approach developed consists of the use of two restriction enzymes HpaII and MspI recognizing the same sequence within CpG islands, but only the latter can cut the sequence in the presence of a methylated group. Therefore, the level of HpaII digestion is inversely proportional to the level of global DNA methylation. Thus, global methylation changes can be evaluated as the ratio between the COMET scores of the two enzyme digestions after subtracting the score of the digest buffer alone. The incubation time for the best enzymes activity was found to be 15' in both cell lines: indeed, from 30' an increase in the enzyme activity was associated with an increased impact of the fast digest buffer on DNA damage. The validation of the method was performed on the same two cell lines treated with hypo- or hypermethylation stimuli ($NiCl_2$ and folic acid (FA) depletion, respectively) for 48 h and 7 days. $NiCl_2$ induced DNA hypermethylation in both human colon cell lines, although the effect was statistically significant only in CCD-18Co fibroblasts. In addition, 48 h folate depletion increased the HpaII/MspI ratio from approx. 3-fold in the epithelial to 4-fold in colon fibroblast cells, indicating that HpaII digestion was increased, as it would be expected when DNA methylation is lower (see fig. 30 in "Results"). Similar results were observed in response to 7 days FA depletion (Catala *et al*, 2018). These data indicate that this modification of the Comet

assay to detect DNA methylation in single cells is responsive to known modifiers of methylation status and it could thus represent an useful tool for assessing methylation in CRC models as well as in individuals at high risk for CRC.

5.4 Conclusions and future directions

The possibility to prevent or slow down the process of CRC carcinogenesis with the use of natural compounds is a promising strategy within secondary and tertiary prevention. The data we obtained with BJe, PMD and morin, endowed with pro-apoptotic and anti-inflammatory properties, suggest the possibility to modify CRC carcinogenesis in high risk individuals such as FAP patients or those with a previous history of CRC. As our data on PMD indicate, it is likely that the combination of different, synergistic compounds can provide more benefits than the use of a single compound. Indeed, considering the many pathways and players (epithelial cells, CAFs, stem cells) involved in CRC carcinogenesis, the combination of several natural compounds/drugs targeting different components can be anticipated to be more useful both in chemoprevention and in therapy. Moreover, the data on Morin suggest that natural compounds could act synergistically or in additive manner with therapeutics, enhancing drugs efficacy by inhibiting resistance mechanisms. In principle, this could also be associated with the need for lower drug doses and as a consequence lower associated toxicity. This last point is currently given much attention in recent research in cancer field, as it could improve the QoL of patients.

The different models developed within this PhD project have proven valuable for the study of metabolites and involved cellular and molecular mechanisms. They can be used to reduce the number of experimental animals, in agreement with the 3Rs principle, and can provide complementary findings compared to *in vivo* studies.

In the future, the methyl sensitive COMET assay developed here will be used to evaluate global methylation changes in the early stages of CRC carcinogenesis in Pirc rats, upon *in vivo* treatment with chemopreventive substances, and *in vitro* in colon fibroblast primary cultures from Pirc and wild type animals.

Finally, a further step in the development of an *in vitro* model for studying the effects of tumor microenvironment might be the setting up of a co-culture 3D model (epithelial cell spheroids plus fibroblasts).

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Acknowledgments

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List of in extenso publications

Reprint of the articles:

- Tortora K, Femia AP, Romagnoli A, Sineo I, Khatib M, Mulinacci N, Giovannelli L, Caderni G. Pomegranate By-Products in Colorectal Cancer Chemoprevention: Effects in Apc-Mutated Pirc Rats and Mechanistic Studies In Vitro and Ex Vivo. Mol Nutr Food Res. 2018 Jan;62(2). doi: 10.1002/mnfr.201700401. Epub 2017 Nov 22. PubMed PMID: 28948694. Attached.

- G.M. Catala, C.S. Bestwick, W.R. Russell, K. Tortora, L. Giovannelli, M.P. Moyer, E. Lendoiro, S.J.Duthie. Folate, genomic stability and colon cancer: the use of single cell gel electrophoresis in assessing the impact of folate *in vitro*, *in vivo* and in human biomonitoring. Mutation Research - Genetic Toxicology and Environmental Mutagenesis. Sept. 2018. https://doi.org/10.1016/j.mrgentox.2018.08.012. Attached.

In press:

G. Lori, P. Paoli, A.P. Femia, E. Pranzini, A. Caselli, K. Tortora, A. Romagnoli, G. Raugei, G. Caderni. Morin-dependent inhibition of low molecular weight protein tyrosine phosphatase (LMW-PTP) restores sensitivity to apoptosis during colon carcinogenesis: studies *in vitro* and *in vivo,* in an *Apc*-driven model of colon cancer. Molecular Carcinogenesis. Accepted Dec.2018. Attached.

Submitted:

M. Navarra, A.P. Femia, A. Romagnoli, K. Tortora, C. Luceri, S. Cirmi N. Ferlazzo, G. Caderni. Potential of a flavonoid-rich extract from Bergamot juice to prevent carcinogenesis in a genetic model of colorectal cancer, the Pirc rat (F344/NTac-Apc^{am1137}). European Journal of Nutrition.

Attachments

Molecular Nutrition & Food Research
www.mnf-journal.com

Pomegranate By-Products in Colorectal Cancer Chemoprevention: Effects in *Apc*-Mutated Pirc Rats and Mechanistic Studies In Vitro and Ex Vivo

Katia Tortora, Angelo Pietro Femia, Andrea Romagnoli, Irene Sineo, Mohamad Khatib, Nadia Mulinacci, Lisa Giovannelli, and Giovanna Caderni*

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) is administered for 6 weeks in the diet to Pirc rats, mutated in *Apc,* a key-gene in CRC. Mucin-depleted foci (MDFs), as CRC biomarkers, are reduced in PMD-fed rats compared to controls (MDF/colon: 34 ± 4 versus 47 ± 3, $p = 0.02$). There is an increase in apoptosis in MDFs from PMD-treated rats compared to controls (2.5 ± 0.2 versus 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), are tested alone or in combination in vitro (colon cancer cells), and ex vivo in adenoma (AD) and normal mucosa (NM) from Pirc rats. u-A 25 µm plus SB 2.5 mm (USB) causes a significant reduction in COX-2 protein expression compared to untreated controls (about –70% in cancer cell cultures, AD, and NM), and a strong increase in C-CASP-3 expression in cells (about ten times), in AD and NM (+74 and +69%).
Conclusion: These data indicate a chemopreventive 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), develops through a sequential multistep progression of epithelial cells initiated to a cancerous state with defined precancerous intermediaries. Numerous trials document the ability of nonsteroidal 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, and 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[4–6] (https://clinicaltrials.gov/). Among fruits, with a high polyphenolic content, pomegranate (*Punica granatum)* is endowed with antioxidative, anti-inflammatory, and anticancer activities.[7–9] The predominant phenolic component of pomegranate belongs to the ellagitannin family (ETs), among which punicalagin, showing pronounced antiproliferative and anti-inflammatory activities in addition to antioxidative effects, is peculiar of this fruit.[10–12] Several studies reported that total phenolic compounds (TPC) and punicalagin are more abundant in pomegranate mesocarp and peel (pericarp) than in arils and seeds, the latter being used for industrial juice production.[13] Beside polyphenols, the polysaccharide component, also present in mesocarp, has been suggested to exert anticancer activity, at least in in vitro systems.[14,15] Regarding the anticancer activity in vivo, two studies investigated in rats the effect of commercial pomegranate juice preparations on the induction of aberrant crypt foci (ACF), purported preneoplastic lesions and showed that the juice reduces the number of these lesions.[16,17] Besides the juice, other parts of pomegranate such as seed oil and peel have been reported to reduce chemically induced colon carcinogenesis in rats.[18–20] Similarly, Sadik and Shaker[21] reported that a commercial phytochemical extract of pomegranate might indeed have

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chemopreventive activity. Instead, the effect of mesocarp has not been investigated.

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), we thought it of interest 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 vivo in a genetic model of CRC, the Pirc rat. This strain bears an heterozygote mutation in the *Apc* gene, the key genetic event in colorectal carcinogenesis.[22] Due to this mutation, Pirc rats spontaneously develop colon polyps, thus mimicking both familial adenomatous polyposis (FAP) and sporadic colon cancer.[6,22,23] Pirc rats also present microscopic preneoplastic lesions in the colon, called mucin depleted foci (MDF), which represent the early step in the development of CRC and can thus be used as tumor biomarkers in chemoprevention studies.[23,24] In addition to the chemopreventive experiment in vivo, to investigate the mechanisms involved in the effects observed at the level of early carcinogenesis phases, and to assess the capacity of PMD to influence carcinogenesis at more advanced phases, we measured the activity of two main metabolites of PMD: urolithin-A (u-A) and sodium butyrate (SB), in vitro on HT-29 and HCT-116 colon cancer cells and ex vivo in biopsies of normal colon mucosa (NM) and colon adenomas (AD) from Pirc rats. u-A is the main punicalagin metabolite in the colon[25,26]; SB, one of the main short chain fatty acid produced by fermentation of the soluble fiber component in the colon, has anticancer activity in vitro and in vivo.[27,28]

2. Experimental Section

2.1 Chemical reagents

Gentamicin, Hank's Balanced Salt Solution (HBSS), DTT, thiazolyl blue tetrazolium bromide (MTT), RIPA buffer, and protease/phosphatase inhibitors were from Sigma–Aldrich (Milan, Italy). $\alpha + \beta$ punicalagins and ellagic acid were from Merck. AIN76 diet components were from Piccioni (Milan, Italy). NucleoSpin® TriPrep kit for RNA extraction was from Machery-Nagel GmbH & Co. KG (Duren, Germany). Cell culture materials were from GIBCO, BRL (Rodano-Milan, Italy). u-A was from Toronto Research Chemicals (Toronto, Canada). SB was from Merck-Millipore (Vimodrone-Milan, Italy). MTS solution was from Promega (Gessate, Italy). Protein assay was performed using DC Protein Assay kit from Bio-Rad (Segrate-Milan, Italy).

2.2. 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. Eighteen kilograms 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 1 h (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/100 g) = $N \times 6.25$ ($N =$ total nitrogen. Lastly, soluble and insoluble dietary fiber analysis was carried out 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 150 mm × 2 mm id, 4 μm, RP-18, column from Phenomenex (Bologna, Italy), with a flow rate of 0.2 mL min^{-1}. 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 reequilibration time of 10 min and a total time of analysis of 26 min. The ellagitannins determination was performed using as external standards $\alpha + \beta$-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.

2.3. 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 and bred as reported.[29] Male Pirc rats, aged 4 weeks, were randomly assigned to: controls ($n = 10$), fed with standard AIN-76 diet, or PMD-treated ($n = 11$) fed with the same AIN-76 diet supplemented with 10 000 ppm of PMD, corresponding, on the basis of the polyphenolic content of PMD, to a dose of 50 mg kg^{-1}/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 (Authorization number 323/2016-PR).

2.4. Processing of Colon, Sample Collection, and Determination of Mucin Depleted Foci

At sacrifice, the entire intestine was dissected, flushed with cold saline, and 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) as reported.[29] 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 fixed in formalin and stained with high-iron diamine Alcian blue (HID-AB). This technique highlights mucin production and allows, with a topographical observation of the unsectioned colon, the determination of the number of MDF (number of MDF/colon) and their multiplicity (number of crypts forming each MDF).[23] After the enumeration of MDFs, these were

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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) to determine apoptosis, see below.[29]

2.5. Cell Proliferation and Apoptosis in NM and MDF

Proliferative activity was assessed as previously reported[29] in the morphologically NM, determining proliferating cell nuclear antigen (PCNA) immunoreactivity with a mouse monoclonal antibody (PC-10, Santa Cruz, CA, USA) at 1:1000 dilution. Proliferative activity was expressed as labeling index (LI): number of cells positive to PCNA/cells scored × 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.[29] Apoptosis was also evaluated in histological sections of MDFs dissected as previously described.[30]

2.6. 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.[31] 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 **Table 1**).

2.7. Western Blotting

Protein expression was evaluated in samples from in vitro, in vivo, and ex vivo experiments. For cultured cell lines, 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 sec and centrifuged for 1 min at 14 000 rpm and 4 °C. For colon samples, scraped NM in the in vivo experiments and ex vivo samples of AD and NM were homogenized in RIPA-buffer in the proportion of 8 μL mg^{-1} tissue for no more than 2 min, sonicated, and centrifuged as above. Each supernatant was collected and the PC 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.[32] 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; NOS-2 (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.

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′

2.8. Cells Cultures and Treatments

HT-29 and HCT-116 cell lines (provided from ATCC) were grown in high glucose Dulbecco's Modified Eagle's Medium/RPMI1640 Medium respectively, both supplemented with 10% of fetal bovine serum, PenStrep (penicillin 100 U mL^{-1}, and streptomycin 0.1 mg mL^{-1}), maintained at 37 °C in a cell incubator (5% CO_2), and used during the linear phase of growth. For experimental treatments, cells were plated in 12 MW (130 000 cells/well) and treated starting on the following day with standard medium or: (a) u-A (3,8-diydroxybenzo(c)chromen-6-one, u-A) 0.1, 1, 10, 25, 50, and 100 μM; (b) SB 50, 100, 500 μM, 1, 5, and 10 mM; (c) u-A 25 or 50 μM + SB 500 μM, 1, and 5 mM (USB). Treatments lasted 24 or 72 h.

2.9. MTS Viability Assay in Cell Cultures

Cell proliferation assays were performed in 96 MW (8000 cells/well) after 72 h treatments. At the end of this time, media were removed, cell monolayers were washed two times with 1× PBS, and 100 μL/well of DMEM or RPMI 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-min incubation at 37 °C in 5% CO_2 humidified atmosphere.

2.10. 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 100 U mL^{-1} and streptomycin 0.1 mg mL^{-1}) and gentamycin (50 μg mL^{-1}), then opened to collect samples of AD and apparently 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^{-1} (HBSS/PSG), and dissected under microscope. ADs and NMs were dissected into approximately equal parts (weight range: 10–15 mg). Samples were then transferred in 48 MW containing DMEM (control) or DMEM containing u-A 25 μM + 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

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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^{-1} gentamycin and 1 mg mL^{-1} 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 + HCl 4 mM and 0.1% Nonidet-P40), weighted, and incubated at 37 °C with shaking for 3 h to extract MTT. Subsequently, they were centrifuged for 5 min 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^{-1}).[33] Finally, the viability at 24 h was expressed as percentage (%) of the corresponding T_0 counterpart.

2.11. Statistics

Differences in MDFs, immunological and morphological indexes and RT-PCR data between PMD group and controls were analyzed with the *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%), and fat was absent, as expected. The decoction contained also ellagitannins (151.47 ± 3.42 mg g^{-1}) with a prevalence of $\alpha + \beta$ punicalagins (67.5 ± 1.24 mg g^{-1}) and a minor content of ellagic acid and derivatives (21.7 ± 0.35 mg g^{-1}).

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 ($n = 10$) versus 217 ± 6 g in PMD treated ($n = 11$), means ± SE), with no apparent sign of toxicity of the treatment.

The number of the preneoplastic lesions MDFs was significantly reduced ($p = 0.02$) in PMD-treated rats compared to controls (**Figure 1**, panel A; Supporting Information, Figure S1). Moreover, PMD-treated rats showed MDF with a significantly lower multiplicity (i.e. the lesions were formed by a lower number of crypts) when compared to those in the control rats ($p = 0.03$) (Figure 1, panel B). Accordingly, measuring apoptosis in these lesions, we found a significant increase in apoptotic index in the MDFs dissected from PMD-treated rats (Figure 1, panel C).

3.3. Proliferation, Apoptosis, and Inflammation in the Normal Colon Mucosa of Pirc Rats

To understand the molecular mechanisms of action underlying this protective effect of PMD, we determined the gene expression of inflammatory proteins, apoptosis, and proliferation in the 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).

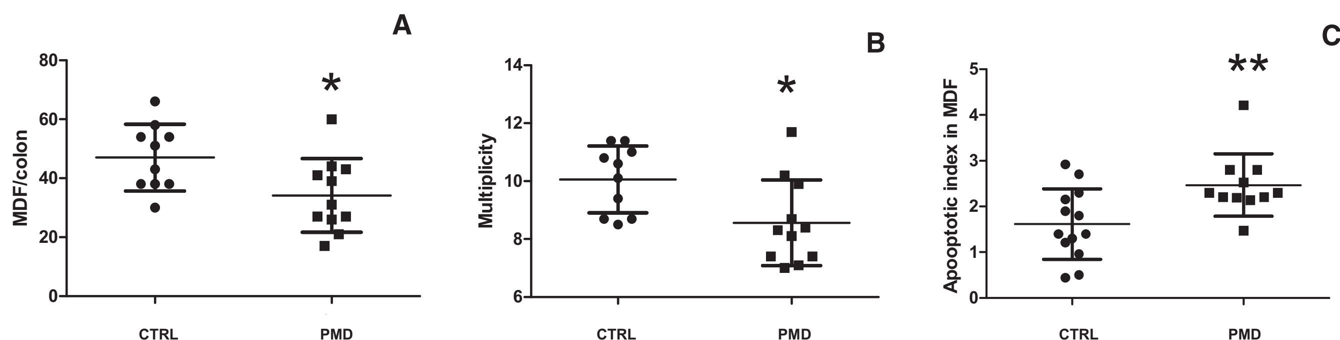

Figure 1. Pre-neoplastic lesions: MDF/colon (panel A), MDF multiplicity (number of crypts/MDF (panel B) and apoptotic index in MDF (panel C) in Pirc rats fed with AIN76 (CTRL) or AIN76 diet supplemented with 50mg kg^{-1}/die of PMD (means ± SD); controls: $n = 10$, PMD-treated: $n = 11$. Each point represents data from a single animal, means ± SD for each group are also shown. *, **: significantly different from controls (Student *t*-test), $p < 0.05$ and $p < 0.01$, respectively.

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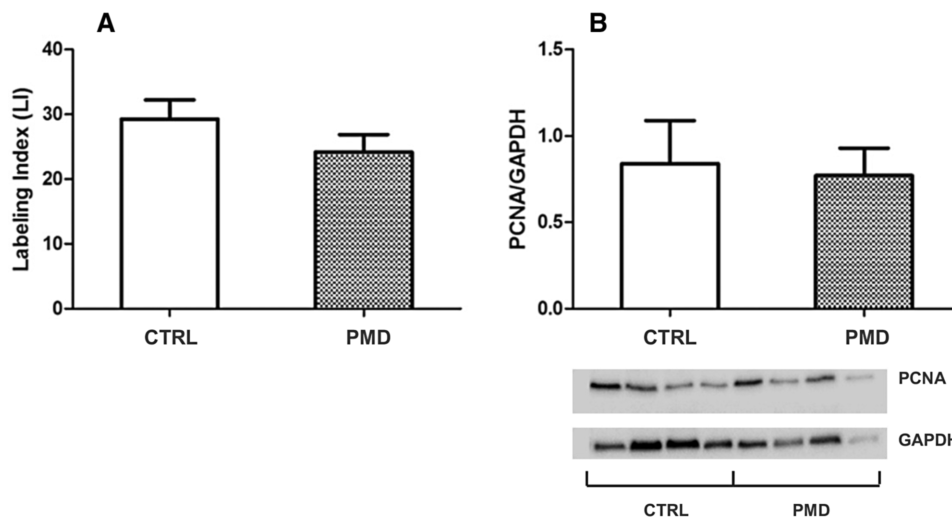

Figure 2. Effects of PMD in the Pirc normal colon mucosa: proliferation activity evaluated as LI in histological sections immunostained with a PCNA-antibody, and PCNA protein expression by Western blot (panels A and B respectively) in NM of Pirc rats treated with PMD. Bars are means + SE; controls: $n = 10$, PMD-treated: $n = 11$. Examples of Western blotting membranes showing bands relative to single animals are shown below the respective graph.

Concerning the impact on proliferation, the LI in colon sections immunostained with PCNA, as well as PCNA immunoblotting in NM samples, showed that proliferative activity was not affected by PMD (**Figure** 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 C-CASP-3 and BAK proteins showed no statistically significant difference between control and treated groups (CASP-3/GAPDH: 8.93 ± 1.9 versus 10.07 ± 2.02; BAK/GAPDH: 0.51 ± 0.21 versus 1.10 ± 0.29 in controls ($n = 10$) and in PMD treated ($n = 11$) groups respectively, means ± SE).

3.4. Effect of Pomegranate Metabolites on Colon Cancer Cells Viability

Based on the protective effects observed in vivo on microscopic preneoplastic lesions (MDFs), we also evaluated the effects of pomegranate products in more advanced steps of colon carcinogenesis, such as human colon cancer cell lines and macroscopic ADs from older Pirc rats. For these in vitro and ex vivo experiments, two main colon-relevant metabolites of the polyphenolic and fiber PMD components, u-A and 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 (**Figure 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 (Figure 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 copresence of u-A further reduced viability, (Figure 3 panel B, compare dotted and continue lines), suggesting an additive type interaction between the two metabolites. Similar results were found in HCT-116 human colon cancer cell line (IC_{50} for u-A 59.2 μM, for SB 0.7 mM). These data are shown in Supporting Information, Figure S2 (panels A–B).

3.5. Proliferation, Apoptosis, and Inflammation in Colon Cancer Cells Treated with Urolithin-A, Sodium Butyrate, or Both

The effect of u-A and SB 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 single compounds at concentrations below the respective IC_{50}s measured in the cell viability experiments, or with a combination of the two metabolites at concentrations giving additive effects on cell viability reduction: for HT-29, u-A 25 μM, SB 2.5 mM, and u-A 25 μM + SB 2.5 mM (USB); for HCT-116, u-A 50 μM, SB 0.5 mM, u-A 50 μM and SB 0.5 mM (USB). In HT-29 cells, 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, compared to the respective controls. 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 (Figure 3, panel C). As for apoptosis, activated Caspase-3 (C-CASP3) expression was strongly increased by the combination of u-A and SB by 24 h; during the following 48 h, the levels in treated cells were still higher compared to the respective controls, although the differences were no longer significant (Figure 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 (Figure 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

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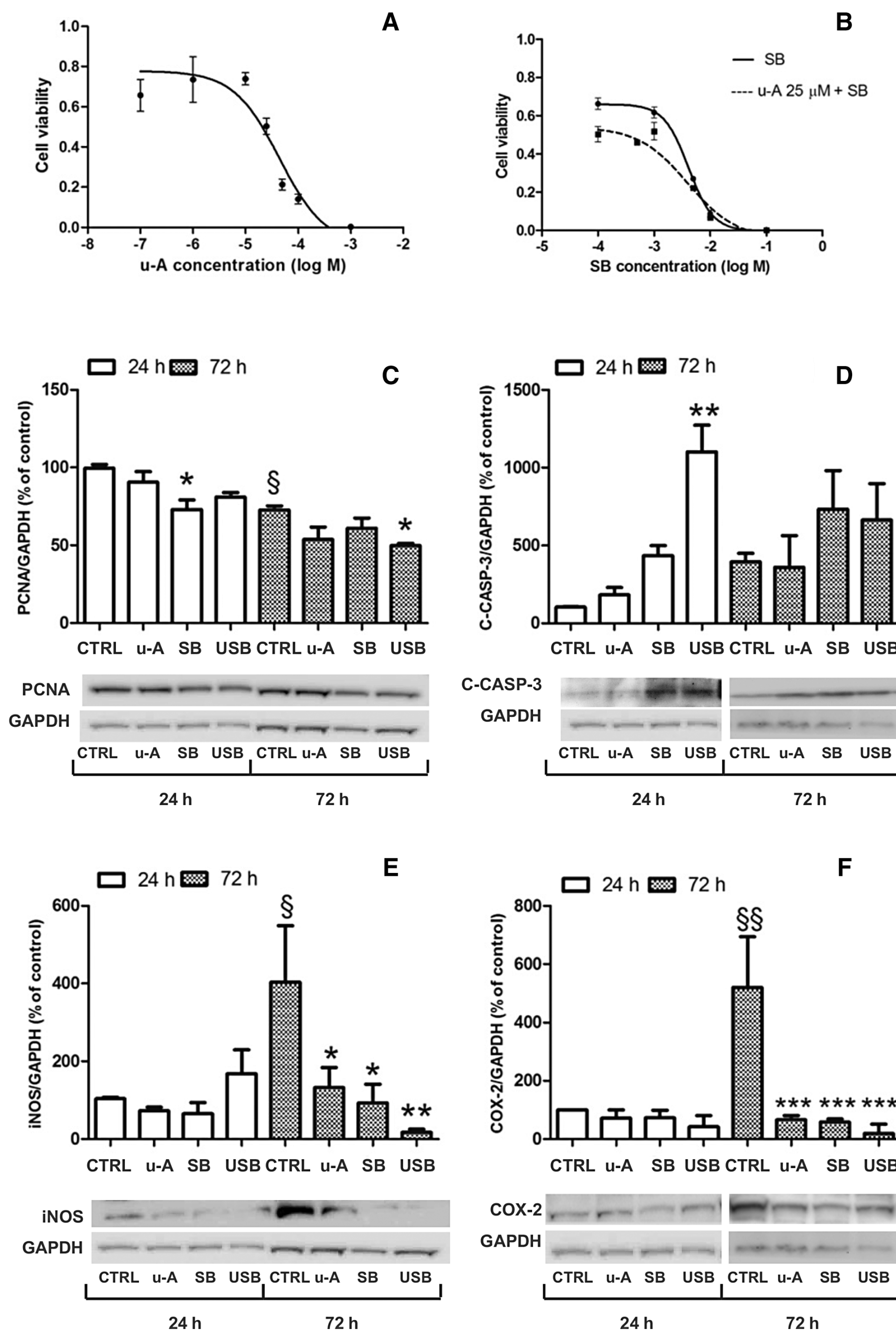

Figure 3. Effects of pomegranate metabolites on HT-29 cells: panel A: concentration-response curve of u-A (log IC_{50} = –4.358, R^2 = 0.8274); panel B: concentration-response curves of SB (logIC_{50} = –2.511, R^2 = 0.9224) and SB plus u-A 25 μM (dotted line, logIC_{50} = –2.405, R^2 = 0.9275); incubation time: 72 h. Panels C–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 (CTRL). Bars are means + SE of four experiments; *: $p < 0.05$, **: $p < 0.01$, and ***: $p < 0.001$ significantly different from respective controls. §: $p < 0.05$, §§: $p < 0.01$ significantly different from 24 h control (one-way ANOVA). Examples of Western blotting membranes are shown below the respective graph.

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. Similar results were obtained with HCT-116 cells (Supporting Information, Figure S1, panels C–F).

3.6. Effect of Pomegranate Metabolites on Proliferation, Apoptosis, and Inflammation in Biopsies from Pirc Adenomas and Normal Mucosa

Colon AD and NM samples from Pirc rats were used for short-term (24 h) ex vivo experiments, in which the combination of

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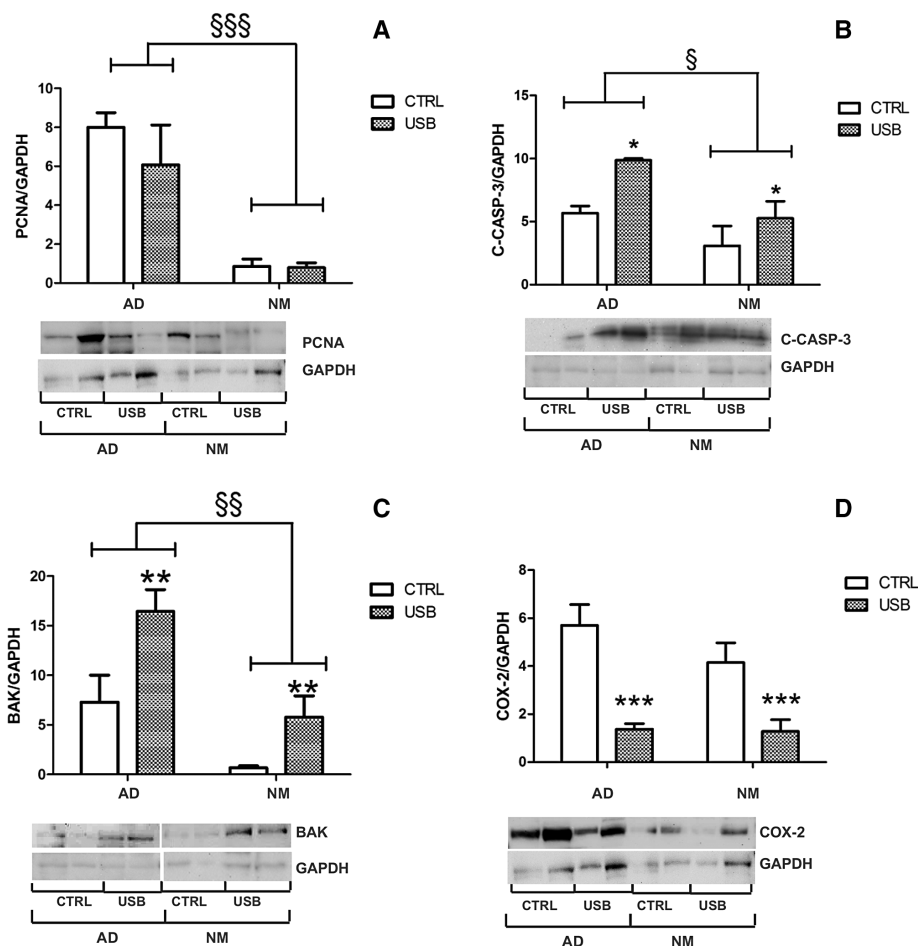

Figure 4. Effects of pomegranate metabolites in Pirc ADs and NM biopsies ex vivo: relative protein expression of PCNA, C-CASP-3, BAK, and COX-2 (panels A–D, respectively) in control (CTRL) and USB-treated AD and NM samples after 24 h in culture. Bars are means + SE of four experiments; §, §§, and §§§: $p < 0.05$, <0.01, and < 0.001, respectively, comparing AD versus NM (two way ANOVA). *, **, and ***: $p < 0.05$, < 0.01, and < 0.001, respectively, comparing treated versus untreated samples (two-way ANOVA). Examples of Western blotting membranes are shown below the respective graph: two different experimental points are present for each condition.

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 nonsignificant both in AD and in NM samples (**Figure 4**, panel A). On the contrary, USB showed a prominent proapoptotic effect in AD, as indicated by increased C-CASP-3 and BAK expression (Figure 4, panels B and C, respectively) compared to untreated controls; a significant increase in these two proapoptotic proteins was also observed in the NM. Finally, a marked anti-inflammatory effect on both AD and NM samples treated with USB was observed (Figure 4, panel D): COX-2 protein expression was decreased by 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 AD tissue compared to the NM.

4. Discussion

The majority of the published studies on chemopreventive effects of pomegranate focused on the juice obtained either from whole fruits or arils.[34] Despite the fact that peel and mesocarp also contain polyphenols, to which the beneficial effects of pomegranate are ascribed,[10,35] only few studies investigated the effects of peel[18,19] 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.[36] Interestingly, we recently demonstrated that the polysaccharide component of Wonderful pomegranate has prebiotic properties in vitro,[37] and our preliminary experiments

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on the advanced M-Shime® gastro-simulator also pointed out a beneficial effect of PMD, mainly in terms of short chain fatty acids production by the human microbiota (manuscript in preparation), suggesting prebiotic properties which have been associated with beneficial effects against colon carcinogenesis.[38] PMD was first tested in vivo, at a dose corresponding to a daily intake of 50 mg kg^{-1} of polyphenols, on Pirc rats. The Pirc rat, with a germ line mutation in the *Apc* gene, is a robust model for CRC chemopreventive studies,[6] as it spontaneously develops colon ADs and, at young age, microscopic preneoplastic lesions (MDFs) that can be used as an end-point in short-term chemopreventive studies.[39] Importantly, at variance with other genetic models such as Min mice, developing spontaneous tumors mostly in the small intestine and not in the colon, Pirc rats allow to study colon carcinogenesis and its chemoprevention in the very same environment, including the luminal content, in which the human disease develops. Our results clearly show that both the number of MDFs and their size in terms of crypts forming each MDF (multiplicity) were significantly reduced by PMD treatment, suggesting that indeed PMD is able to reduce colorectal tumorigenesis. Considering the number of MDF, the effect of PMD amounts to about 30% inhibition, a figure similar to that observed in rodents subjected to known chemopreventive treatments such as low fat diets, calcium, or aspirin.[40,41] We also observed that the level of apoptosis was significantly higher in MDFs from the PMD group than in MDFs from controls, suggesting that the observed protective effect may be due to increased apoptosis in these lesions, leading to elimination of precancerous cells. Increased apoptosis in tumors and preneoplastic lesions was previously observed in animals treated with compounds showing preventive activity.[42] In the NM of PMD-treated rats, only slight effects on proliferation, apoptosis, and inflammation were observed compared to controls.

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 cell lines and in macroscopic ADs), we tested the effect of two colon-relevant metabolites of the polyphenolic and fiber components of PMD: u-A and SB, respectively.[25,28] These were assessed in vitro on HT-29 human colon cancer cell line, bearing an *Apc* gene mutation, and on HCT-116 human colon cancer cell line, bearing a wild-type *Apc*. u-A and SB alone demonstrated inhibitory effects on cell viability with IC_{50} values in line with those reported previously.[8,43] We also evaluated the effect of the combination of these two metabolites, and found an additive effect on cell viability reduction of u-A and SB. We then focused on the combination of u-A and SB (USB), choosing concentrations of the two metabolites inducing per se less than 40% reduction in cell viability. 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 both cell lines. In fact, USB treatment was more effective on proliferative activity (PCNA reduction) and on apoptosis induction (C-CASP-3 increase). 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,[44,45] the anti-inflammatory effects of the combination were again more pronounced. The efficacy demonstrated by USB in cancer cell lines encouraged us to verify the efficacy of the combined treatment on macroscopic ADs of Pirc rats. For this purpose, and to reduce the number of experimental animals, we performed ex vivo experiments, in which small samples of ADs and NMs of 8-month-old Pirc rats were collected and maintained in culture for 24 h 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[29,46] documenting higher proliferation and apoptosis in AD compared to NM. Thus, 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 proapoptotic effect of PMD was also observed in vivo in MDFs 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. The concentrations of butyrate that we have used in the in vitro and ex vivo experiments are attainable in the colon in vivo.[26,28] For u-A, Nunez Sanchez et al.[4] demonstrated that in colon tissues of human subjects treated with a pomegranate peel extract for 15 days, u-A levels were around 850 ng g^{-1} of tissue, roughly corresponding to 4 μM concentration within the tissue. Thus, an extracellular concentration of 25 μM, as we have used in the present experiments, appears to be attainable.

5. Concluding Remarks

This is the first study to test the chemopreventive activity of pomegranate mesocarp in the Pirc rat, a relevant genetic model of colon carcinogenesis. Furthermore, we describe for the first time the combined effects of u-A and butyrate in vitro and the effect of this metabolite combination on ADs ex vivo.

As a whole, this work indicates that pomegranate mesocarp-based products have the potential to counteract the very initial, intermediate, and late stages of carcinogenesis, being potentially useful both in colon cancer primary and secondary prevention. The mechanism of action involves an increase in apoptosis in the preneoplastic lesions MDFs. Parallel in vitro and ex vivo experiments indicate that the chemopreventive effect of PMD may be due, at least in part, to the proapoptotic and anti-inflammatory effects of its colon metabolites u-A and SB, and especially to their combination.

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An added value to the use of by-products of juice production is the fact that PMD was obtained by a green simple process. Furthermore, 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. Thus, this study underlines the potential value of these by-products as a source of bioactive molecules that could be exploited for CRC prevention in high-risk subjects.

Abbreviations

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

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgments

KT carried out the in vitro and ex vivo experiments and 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 and carried out the relative chemical analyses together with NM. LG performed the in vitro experiments. GC determined apoptosis and proliferation with 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 conflict of interest.

Keywords

butyrate, colon carcinogenesis, mucin depleted foci, pomegranate mesocarp, urolithin

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Folate, genomic stability and colon cancer: The use of single cell gel electrophoresis in assessing the impact of folate *in vitro*, *in vivo* and in human biomonitoring

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A R T I C L E I N F O

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A B S T R A C T

Intake of folate (vitamin B_9) is strongly inversely linked with human cancer risk, particularly colon cancer. In general, people with the highest dietary intake of folate or with high blood folate levels are at a reduced risk (approx. 25%) of developing colon cancer.

Folate acts in normal cellular metabolism to maintain genomic stability through the provision of nucleotides for DNA replication and DNA repair and by regulating DNA methylation and gene expression. Folate deficiency can accelerate carcinogenesis by inducing misincorporation of uracil into DNA, by increasing DNA strand breakage, by inhibiting DNA base excision repair capacity and by inducing DNA hypomethylation and consequently aberrant gene and protein expression. Conversely, increasing folate intake may improve genomic stability.

This review describes key applications of single cell gel electrophoresis (the comet assay) in assessing genomic instability (misincorporated uracil, DNA single strand breakage and DNA repair capacity) in response to folate status (deficient or supplemented) in human cells *in vitro*, in rodent models and in human case-control and intervention studies. It highlights an adaptation of the SCGE comet assay for measuring genome-wide and gene-specific DNA methylation in human cells and colon tissue.

1. Introduction

Single cell gel electrophoresis (SCGE or the comet assay) is now established in human biomonitoring as a sensitive and specific assay capable of measuring low levels of DNA strand breakage, altered DNA bases and DNA repair capacity in human populations. However, the comet assay is also a particularly valuable investigative tool for exploring experimental mechanisms of genotoxicity linked to genomic instability, malignant transformation and carcinogenesis.

Intake of vitamin B9 (folate) is strongly linked with human cancer risk [1–4]. This review describes several important modifications of the comet assay for assessing DNA instability in response to folate status in human cells *in vitro*, in rats *in vivo* and in human studies, and highlights an emerging application of the SCGE assay in measuring aberrant DNA methylation.

2. Folate status and human cancers

Folates, a family of water-soluble B vitamins are found in a wide variety of foods including green leafy vegetables, cereals, beans, fruit and liver, and play a crucial role in preventing the development of human pathologies such as cancer and heart disease in adults, cognitive dysfunction and dementia in the elderly and congenital defects in babies. In the UK, suboptimal folate status is common, particularly in adolescents and the elderly. Overt folate deficiency has been implicated in the development of several human malignancies including cancer of the breast, ovary, lung and cervix [1,2]. Moreover, the evidence linking low folate status with an increased risk of colon cancer (CC) is particularly strong [1,2]. Globally, CC is the 3rd most commonly diagnosed cancer in the world (http://globocan.iarc.fr). Recent meta-analyses of case-control and prospective studies consistently report a reduced

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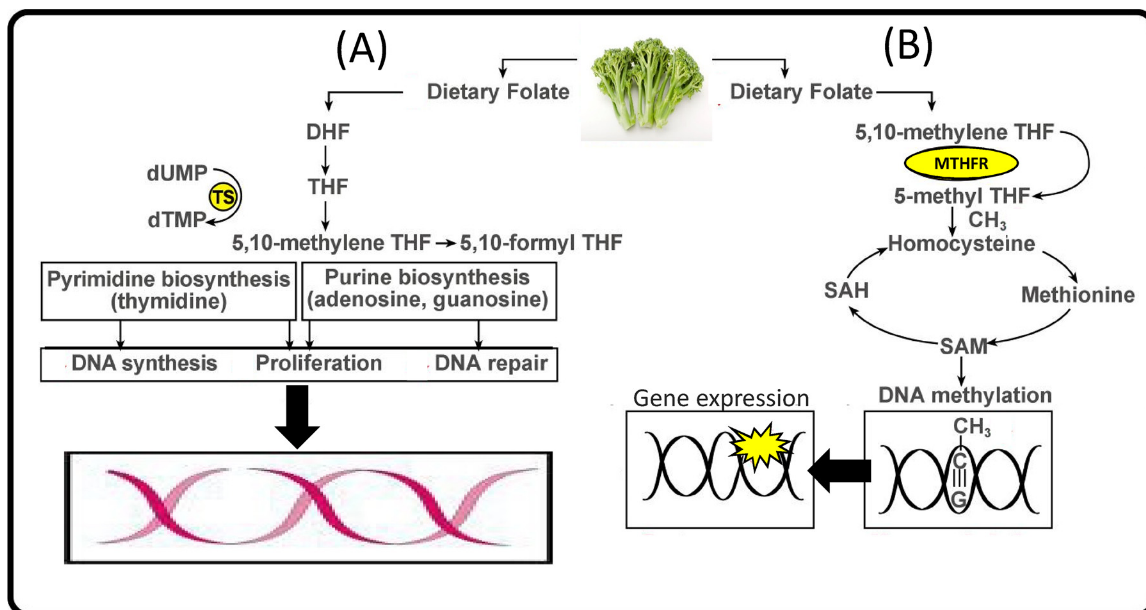

Fig. 1. Folate and one-carbon metabolism: regulation of DNA synthesis, DNA repair and DNA methylation.
A simplified scheme showing how dietary and cellular folates mediate (A) normal DNA synthesis, and repair and (B) DNA methylation and gene expression
Abbreviations: dihydrofolate: DHF; tetrahydrofolate: THF; 5,10-methylenetetrahydrofolate: 5,10-methyleneTHF; 5,10-formyltetrahydrofolate: 5,10-formylTHF; 5-methyltetrahydrofolate: 5-methylTHF; s-adenosylmethionine: SAM; s-adenosylhomocysteine: SAH; methylenetetrahydrofolate reductase: MTHFR; deoxyuridine monophosphate: dUMP; deoxythymidine monophosphate: dTMP; thymidylate synthase: TS. Adapted with kind permission from J. Inherited & Metabolic Disorders [1].

relative risk (RR) for CC in people with a high dietary folate intake compared with people with a low intake: RR 0.75; 95% CI = 0.64–0.89 [3]; RR 0.81; 95% CI = 0.66–0.99 [4].

3. Folate and mechanisms of carcinogenesis

Folate is critical in one-carbon metabolism and acts to maintain genomic stability by regulating DNA biosynthesis and DNA repair and by controlling DNA methylation and gene expression (Fig. 1). Within the methionine cycle, 5-methyltetrahydrofolate (5-methylTHF) remethylates homocysteine to methionine, which is further metabolised to S-adenosylmethionine (SAM). SAM controls gene transcription and ultimately protein expression through its ability to methylate cytosine in the DNA molecule. Similarly, folate is essential for the synthesis of both purines and the pyrimidine nucleoside thymidine. Deoxyuridine monophosphate (dUMP) is converted to deoxythymidine monophosphate (dTMP) by thymidylate synthase (TS), using 5,10-methylenetetrahydrofolate (5,10-methyleneTHF) as methyl donor. Subsequently, 5,10-formyltetrahydrofolate (5,10-formylTHF) is involved in the production of both adenosine and guanosine. Continual production of these DNA precursors is essential for normal DNA synthesis and for effective DNA repair. Folate insufficiency induces and accelerates carcinogenesis by perturbing each of these processes [1,2]. If dietary folate is limiting, the balance of purine and pyrimidine DNA precursors is altered and normal DNA synthesis and DNA repair are inhibited, leading to mutagenesis and malignant transformation. Moreover, folate depletion blocks the normal methylation of dUMP to dTMP, ultimately causing an accumulation of deoxyuridine triphosphate (dUTP). As dUTP and deoxythymidine triphosphate (dTTP) differ only by a single methyl group, the RNA base uracil, which is not normally present in DNA, is misincorporated into the DNA molecule in place of thymine (Fig. 2). This mismatch quickly triggers DNA base excision repair (BER) processes that remove the uracil from the DNA. However, if folate availability continually limits the synthesis of thymine, then uracil is misincorporated and cleaved from the DNA in what is described as “a catastrophic repair cycle”, resulting in DNA double strand breakage, chromosomal damage and ultimately malignant transformation [5]. Folate deficiency similarly perturbs the production of *S*-adenosylmethionine (SAM; Fig. 3), causing a reduction in the normal methylation of cytosine in the DNA molecule and leading to global DNA hypomethylation and inappropriate proto-oncogene activation [6,7]. This will be addressed in detail later in this review.

4. Using SCGE to understand the impact of folate status on genomic stability

4.1. Misincorporated uracil

As described above, folate deficiency induces misincorporation of uracil into DNA, subsequently inducing DNA single and double strand breakage and ultimately chromosomal damage and malignant transformation [1,5]. A valuable modification of the alkaline comet assay has allowed for uracil to be detected and measured specifically in human and animal cells and tissues. Here, DNA nucleoids are lysed and incubated with the bacterial DNA repair enzyme uracil DNA glycosylase (UDG; 1 unit/μg DNA for 30 min) which removes misincorporated uracil from the DNA molecule, leaving a single strand break which is detected by the comet assay in the standard manner [8]. This assay, specifically developed to measure uracil misincorporation, has been employed in the study of human peripheral blood mononuclear cells (PBMC), epithelial cells and colonocytes *in vitro* [8–10], in rodent studies [11,12] and in both human observational and intervention studies [13,14] to determine the influence of folate status on DNA stability.

Growing human cells in folate-free medium inhibits DNA synthesis and progressively increases uracil misincorporation (2–3-fold) in PBMC *ex vivo* [9], in human colonocytes [15], and in SV40-immortalised human colon epithelial cells (HCEC) with time in culture [10]. This detrimental effect of folate deficiency on DNA stability in HCEC was highly sensitive to folate, with uracil concentrations declining to baseline levels after repleting the cells with folate at concentrations normally observed in both human blood and colon tissue (10 ng/mL [10]).

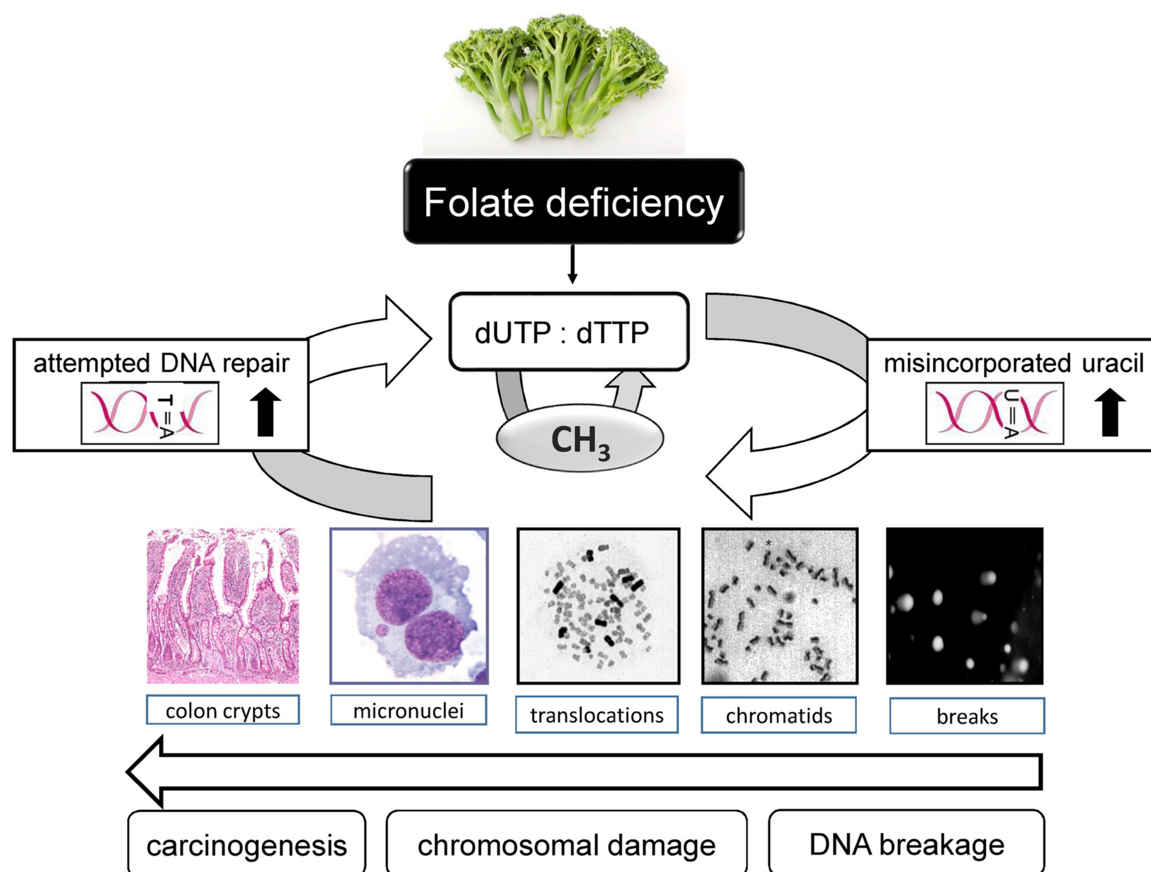

Fig. 2. Folate deficiency and uracil misincorporation.
The impact of dietary folate deficiency on uracil misincorporation and chromosomal instability. Abbreviations: dUTP: deoxyuridine triphosphate; dTTP: deoxythymidine triphosphate. Colon crypts by Nephron (Own work) [CC BY-SA 3.0 (http://creativecommons.org/licenses/by-sa/3.0) or GFDL (http://www.gnu.org/copyleft/fdl.html)] *via* Wikimedia Commons. Images of DNA damage are authors own.

Nutritional folate deficiency has also been shown to induce DNA instability in rodents *in vivo*. Here, DNA uracil misincorporation and DNA single strand breakage increased 2-fold ($P < 0.001$) in PBMC isolated from male Rowett Hooded Lister rats fed a folate-free diet either for 10 or 24 weeks [11,12]. However, the modified comet assay is probably most valuable when deployed in biomonitoring to understand better the relationship between folate and DNA stability in humans.

While elevated uracil misincorporation has been measured [by gas chromatography mass spectrometry (GC MS) or the highly invasive deoxyurydine assay] in PBMC and bone marrow from patients with severe folate deficiency and/or megaloblastic anaemia [5,16], little work has been carried out to establish whether there may be a beneficial effect on DNA stability by improving folate status in the general population. Supplementing apparently healthy men ($n = 15$) and women ($N = 15$) (mean age 41 years old with a red blood cell folate level of 250–650 nmol/L) with folic acid (1.2 mg/day) for 12 weeks substantially increased red blood cell folate (approx. 90%; $P < 0.01$), PBMC folate (approx. 85%; $P < 0.01$) and plasma 5-methylTHF (approx. 300%; $P < 0.01$), when compared with participants taking placebo [17]. Low levels of uracil were detected using SCGE in the PBMC of these healthy participants, and this strongly correlated with PBMC folate (Fig. 4; $r = -0.49$, $P < 0.01$), plasma 5-methylTHF ($r = -0.48$, $P < 0.01$) and intracellular red cell folate status ($r = -0.46$, $P < 0.01$) both pre- and post-intervention. Moreover, increasing blood folate concentration decreased significantly the level of uracil measured in PBMC isolated from subjects in the intervention group (approx. 20%, $P < 0.05$), highlighting that genomic stability can be improved by increasing folate levels even in folate-sufficient subjects [14,17]. However, the majority of human biomonitoring studies are (necessarily) limited in that they measure genomic stability in blood cells rather than in the target organ. Nonetheless, a few studies have determined the direct impact of folate status on genomic stability and specifically uracil misincorporation in the colon. In a study of patients with adenomatous ($n = 40$) or hyperplastic polyps ($n = 16$), folate levels in colon biopsy samples decreased (as much as 25%) with proximity to the polyp site when compared with control subjects ($n = 53$). Moreover, there was a corresponding increase in misincorporated uracil (measured using the UDG-modified comet assay) [18]. In a follow-up intervention study by the same research group [19], uracil misincorporation ratio (UDG-modified comet assay) declined by 0.5 ($P < 0.05$) at the site closest to the original polyp in polypectomised patients following treatment with folic acid (600 μg/day) for six months.

These findings show that uracil misincorporation is responsive to folate status both in surrogate and target tissue.

4.2. DNA strand breakage, DNA repair capacity and apoptosis

There are currently two ways in which the comet assay is used to measure DNA BER; the cellular repair assay, where whole cells are exposed in culture to a model damaging agent and complete repair of DNA strand breaks or DNA lesions is followed with time; and the *in vitro* cell-free repair assay where repair enzymes in a prepared cell extract (normally PBMC) incise model substrate DNA that contains an induced and specific lesion. Both of these assays have been used to assess how folate status affects DNA BER activity.

DNA BER capacity (cellular assay) is inhibited in folate-depleted human PBMC *ex vivo* and in SV40-immortalised HCEC in response to

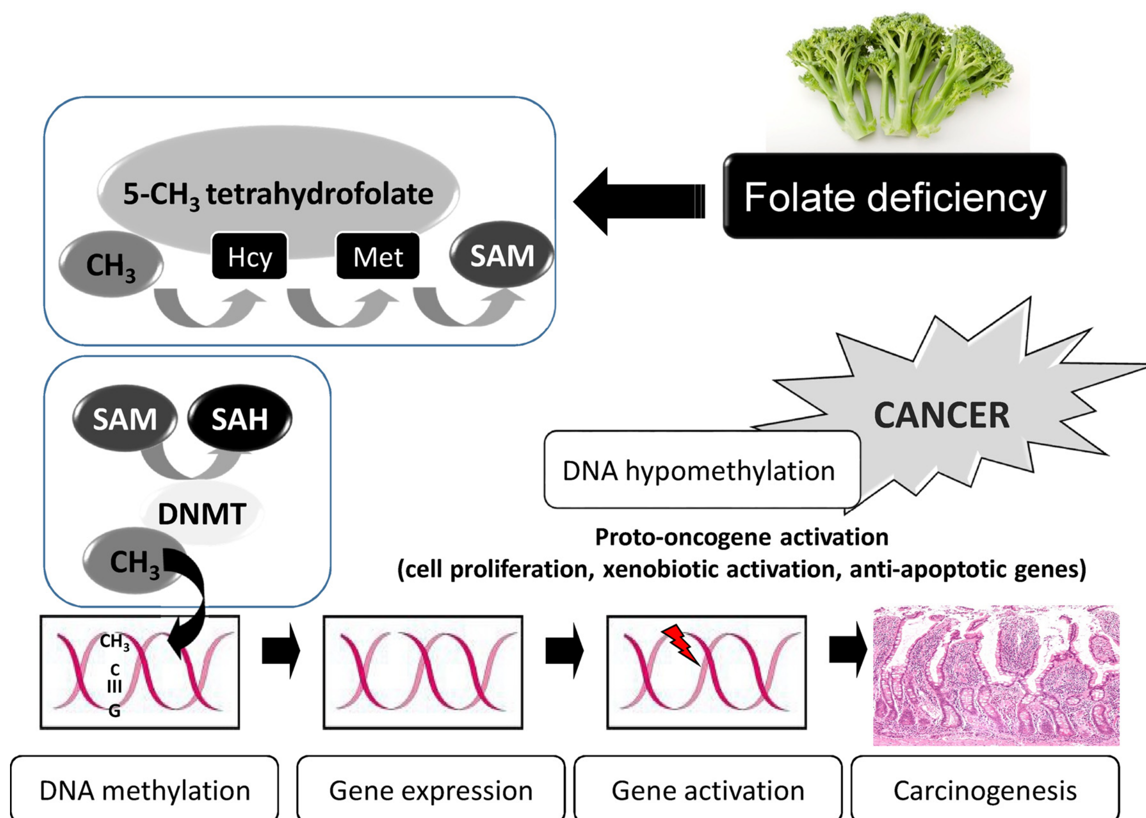

Fig. 3. Folate deficiency, aberrant DNA methylation and gene expression.
The effect of dietary folate deficiency on DNA hypomethylation and proto-oncogene expression. Abbreviations: 5-methyltetrahydrofolate: 5-methylTHF; s-adenosylmethionine: SAM; s-adenosylhomocysteine: SAH; DNAMT: DNA methyltransferase. Colon crypts by Nephron (Own work) [CC BY-SA 3.0 (http://creativecommons.org/licenses/by-sa/3.0) or GFDL (http://www.gnu.org/copyleft/fdl.html)] *via* Wikimedia Commons.

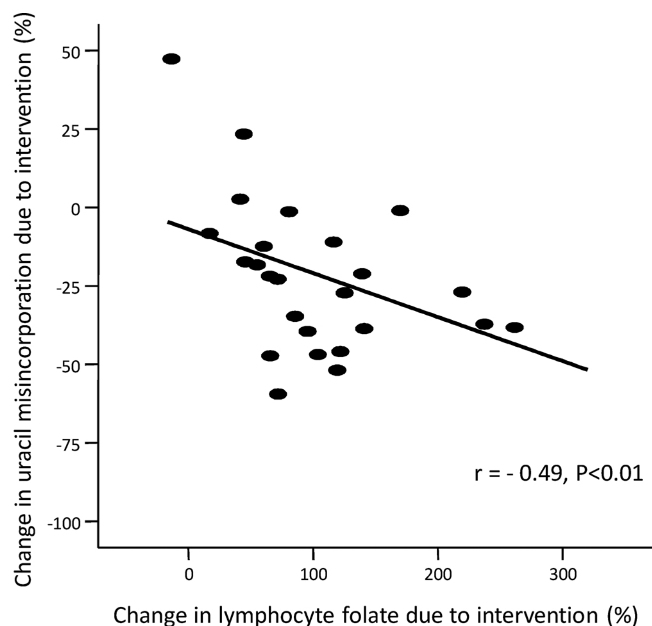

Fig. 4. Association between PBMC folate status and PBMC DNA uracil misincorporation in volunteers.
Change in uracil misincorporation with increasing PBMC folate status due to intervention with folic acid (1.2 mg/day for 12 weeks) in healthy human volunteers. Adapted with kind permission from the British Journal of Cancer [14].

both oxidative (hydrogen peroxide) and alkylation (methyl-methanesulfonate) damage [9,10].

A similar effect has been observed in non-transfected normal human colon epithelial cells. Originally isolated from a 68 year old Hispanic male, NCM460 cells (obtained under a material transfer agreement from INCELL, San Antonio USA) have been used to model B vitamin uptake and metabolism in the human intestine [20] and to assess the effect of folate deficiency on genomic stability [15]. The experiment described below demonstrates how folate depletion alters endogenous DNA strand breakage, DNA BER activity (measured using the comet assay), and ultimately apoptosis, in non-tumour derived human colon cells.

NCM460 cells were grown in A52 medium (BioConcept, Switzerland) in the absence (F-) or presence of folic acid (F+; 4 µg/mL). Intracellular total folate (measured by radioassay; [15]), cell proliferation (cell number), endogenous DNA strand breakage (comet assay), DNA BER capacity (comet assay) and apoptosis [polycaspase (1,3,4–9) activity using Vibrant FAM polycaspase assay kit (Invitrogen, UK) and by Flow cytometry (4 colour FACS Calibur, Becton Dickinson, Oxford, UK)] were measured up to 15 days in culture.

Intracellular total folate was depleted by more than 90% in NCM460 cells cultured in the absence of folic acid while cell growth was progressively inhibited to approx. 33% of the levels observed in folate supplemented cultures after 15 days (data not shown). Folate depletion induced genomic instability in normal human colonocytes, observed as a progressive increase in endogenous DNA strand breakage (Fig. 5) and inhibition of DNA BER capacity (Fig. 6) in response to oxidative challenge (20 µM H_2O_2). In this experiment, DNA strand breaks increased more than 2-fold [69.3 compared with 32.1 AU in F- *versus* F + NCM460 cells respectively ($P < 0.01$)] and BER rate was approx. 17% lower in folate depleted NCM460 after 14 days [3.67 compared

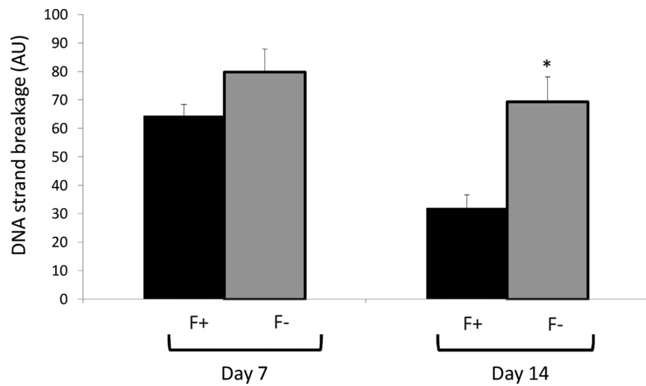

Fig. 5. Effect of folic acid deficiency on endogenous DNA damage.
Endogenous DNA strand breakage in NCM460 cells cultured under folic acid sufficient (F+; 4 µg/mL, black) or deficient (F-; < 1 ng/mL, grey) conditions. Results are mean ± SEM (n = 6). *p < 0.01, where P values refer to differences between cells grown in the presence or absence of folate by Students *T*-test.

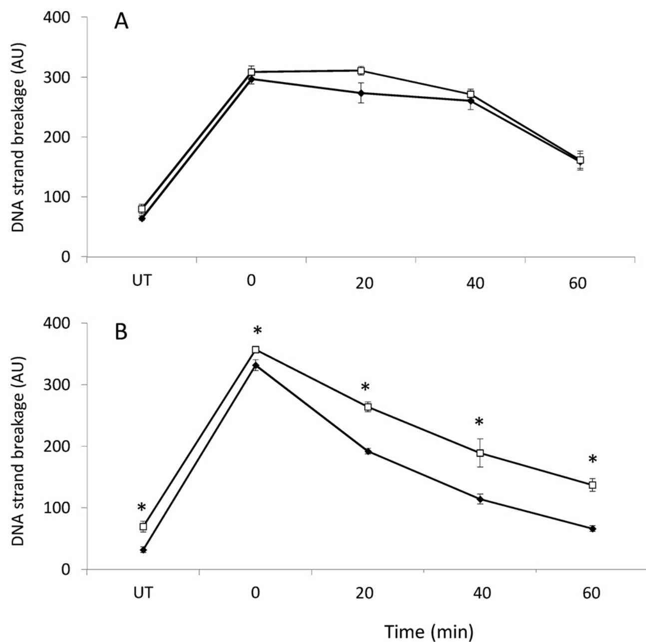

Fig. 6. Effect of folic acid deficiency on DNA base excision repair capacity (BER) with time.
NCM460 cells were grown in the presence or absence of folic acid for 7 (A) or 14 days (B) in either folic acid sufficient (4 µg/mL, ●) or deficient (< 1 ng/mL, □) conditions. Cells were exposed to hydrogen peroxide (200 µg) and BER capacity measured over 60 min. UT; untreated cells. Results are mean ± SEM (n = 6). *p < 0.05, where P values refer to differences between cells grown in the presence or absence of folate by Students *T*-test.

with 4.43 AU/min respectively (P < 0.05)]. Additionally, this time-dependent decrease in genomic stability as a consequence of folate depletion, translated into increased programmed cell death.

Caspase activation is used as an initial marker for the induction of programmed cell death such as results from folate depletion. Caspases are a family of cysteine proteases linked to programmed cell death events, and in particular to the development and execution of apoptosis [21]. Apoptosis is intimately linked to maintaining genomic stability by removal of damaged cells, thereby reducing the risk of malignant transformation leading to tumorigenesis.

While folate deficiency was initially associated with a small decrease in polycaspase activation as detected by flow cytometric analysis using the vibrant FAM polycaspase assay (which identifies the majority of caspase activies including caspase-1, 2, 4, 5, 7 and 9) in human colonocytes, this was followed by a substantial increase (approx. 50%) in enzyme activity after 14 days in culture (Fig. 7A). This elevation was observed without any concomitant change in colonocyte cell membrane integrity (as measured by propidium iodide uptake), indicating the likelihood of an apoptotic rather than necrotic mechanism of cell death (Fig. 7B). These data confirm that folate deficiency detrimentally impacts on genomic stability in normal human colon epithelial cells, by inducing DNA strand breakage and concurrently inhibiting effective DNA repair, resulting in an increased activity linked to elimination of critically damaged colonocytes from the population.

We have also shown that BER capacity is sensitive to folate status in humans. In the human intervention study described above, the BER repair capacity of participants receiving a folic acid supplement (1.2 mg daily for 12 weeks) or control participants was measured by employing the cell-free DNA BER comet assay. Extracts were prepared from PBMC sampled from the participants at the start and at the end of the study. Excision repair capacity was measured as the ability of PBMC extracts to excise 8-oxo-7,8-dihyroguanine (8-oxodG) from Chinese hamster Ovary (CHO) DNA over 20 min following exposure to the model sensitiser RO19-8022 [14]. Excision of 8-oxodG leaves a strand break, which is measured using the conventional alkaline comet assay. Increasing folate concentration in the majority of these normal healthy study participants had no significant effect on BER capacity. However, folate supplementation significantly reduced incision repair activity in those subjects with the lowest initial baseline red cell folate levels suggesting either that increased provision of DNA nucleotides or a reduction in DNA damage, may downregulate BER capacity only in relatively folate-depleted individuals [14].

5. The impact of nutrient-gene interactions on folate metabolism and genomic stability

An important but relatively neglected area of investigation pertaining to folate status and cancer risk is the impact of nutrient:gene interactions on genomic stability. The enzyme methylenetetrahydrofolate reductase (MTHFR; EC 1.5.1.20), which irreversibly converts 5,10-methyleneTHF to 5-methylTHF (Fig. 1), is a pivotal protein in folate metabolism which, when functional, directs methyl groups away from the production of thymidine towards homocysteine remethylation and the production of SAM. Two human polymorphisms in MTHFR (C677T and A1298C) are associated with a reduced enzyme activity, low circulating folate, low SAM and elevated homocysteine levels. Given that low SAM would result in an increased likelihood of DNA hypomethylation, and contrary to what might be expected, these variants are actually associated with a reduced risk of CC [22]. One hypothesis is that the homozygous recessive variant reduces genomic instability by ensuring the continual production of nucleotides for DNA synthesis and repair (Fig. 1). In a study of 199 individuals without cancer, homozygosity for the C677T polymorphism was associated with a reduced plasma and red cell folate (both approx. 25%) level, and decreased hyper-homocysteinemia (approx. 21%) when compared with wild type individuals. However, DNA strand breakage and uracil misincorporation (both measured by SCGE) were similar for all genotypes, indicating that in this study sequestration of 5,10-methyleneTHF for thymidine synthesis was not a major contributor to the apparent enhanced genomic stability in C677TT homozygous variants [13]. Nonetheless, other groups have found a lower level of plasma folate and of misincorporated uracil (measured in leukocytes using the uracil DNA glycosylase-modified comet assay) in individuals carrying the C677T polymorphism [23]. However, this study is limited by both the small sample size (n = 14) and the need to combine data for the C677CT and 677TT genotypes for the analyses.

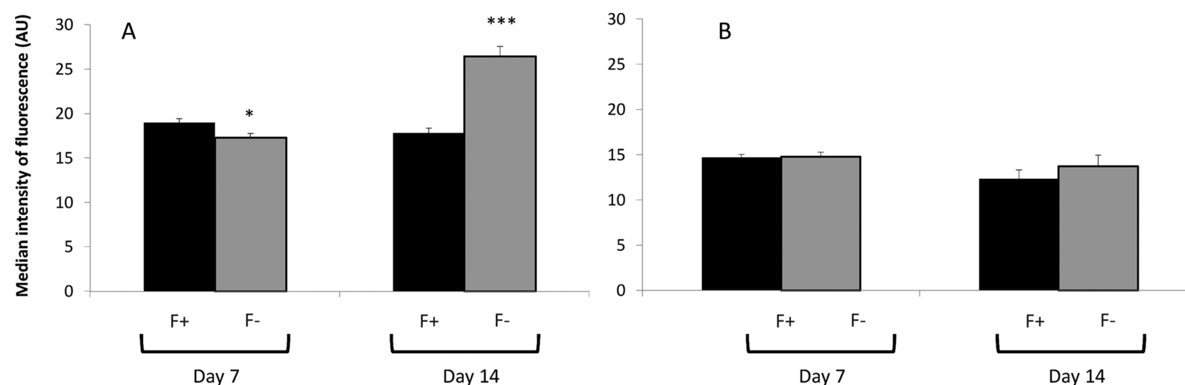

Fig. 7. Effect of folic acid deficiency on caspase (A) and cell membrane integrity (B).
Poly-caspase activation and cell membrane permeability was measured in NCM460 cells cultured under folic acid sufficient (F+; 4 μg/ml, black) or deficient (F-; < 1 ng/mL, grey) conditions for 7 or 14 days. Results are mean ± SEM (n > / = 5). *p < 0.05, **p < 0.001, where P values refer to differences between cells grown in the presence or absence of folate by Students *T*-test.

6. Innovations in the comet assay to detect DNA methylation

While the comet assay is now used widely to investigate the influence of folate on DNA repair capacity and genomic stability, new variations are being developed to improve further the specificity and effectiveness of the assay in establishing folate-modulated mechanisms of carcinogenesis.

It is now established that epigenetic changes, including aberrant DNA methylation, play a critical role in human carcinogenesis [24]. Moreover, it is estimated that dysregulated DNA methylation is involved in the aetiology of approx. one fifth of all CC cancer cases [25].

Folate (5-methylTHF) plays a critical role in the normal regulation of gene and protein expression by acting as methyl donor for the methylation of cytosines in the DNA molecule. Folate deficiency disrupts normal DNA methylation by attenuating the remethylation of SAH to SAM and inducing DNA hypomethylation in newly synthesised DNA. This effectively allows for the inappropriate expression (or over-expression) of genes, including proto-oncogenes, that increase the risk of malignant transformation and cancer [7; Fig. 3].

Methods for measuring genomic DNA methylation, which include Southern blotting, liquid chromatography tandem mass spectrometry (LCMS/MS) and a nucleotide extension assay, are expensive, require considerable biological material and can provide inconsistent data. As aberrant DNA methylation plays a role in both the early development of CC (global hypomethylation) and in advanced stages of tumours (gene-specific hypermethylation), the ability to detect and quantify aberrant methylation from blood samples or small tumour biopsies would offer a valuable predictive and prognostic biomarker. A methyl-sensitive variation of the SCGE assay has been described previously. This assay compared with other methods is simpler and more cost-effective [26–29]. For this modification, two isoschizomer endonucleases (HpaII and MspI) that share the same restriction site in CpG islands are deployed separately in the assay at the post-lysis step. As only HpaII activity is blocked by methylation in the restriction site, the level of HpaII digestion is inversely proportional to the level of global DNA methylation. Thus, global methylation changes can be evaluated as the ratio between the comet scores after the two separate enzymic digestions.

We have developed this method further and tested its ability to detect perturbations in DNA methylation in human colon cells as a consequence of folate depletion (hypomethylation) or exposure to nickel chloride which has been shown to induce DNA hypermethylation [28].

To induce a state of DNA hypomethylation, NCM460 human colon epithelial cells (cultured in A52 medium as described previously) or primary human colon fibroblasts [CCD-18Co obtained from the American Type Culture Collection and cultured in DMEM with low glucose (1.0 g/L)] were grown for 48 h, or 7 days, either in regular culture medium containing folic acid (4 μg/mL) or in the same medium without folic acid (BioConcept Inc, Switzerland). To induce DNA hypermethylation, colonocytes and fibroblasts in folate sufficient medium were exposed to $NiCl_2$ (500 μM) for 24 h. SCGE was carried out essentially as described previously with the subsequent addition of a fast digest endonuclease enzyme incubation step [27]. Post lysis, the slides were washed three times for 5 min each with PBS (4 °C) before incubation either with HpaII, MspI or Fast Digest Buffer [FDB; (ThermoFisher Scientific Inc, Massachusetts, USA] in a humidified chamber at 37 °C for 15 min. At the end of this incubation, the slides were subjected to alkaline unwinding (40 min), electrophoresis (40 min), staining (DAPI 1 μg/mL) and visual scoring as normal [30].

Genome-wide DNA methylation was calculated as the ratio between the two separate enzyme digestions after accounting for the effect of buffer alone [28], with a higher ratio reflecting a lower level of DNA methylation. $NiCl_2$ induced DNA hypermethylation in both human colon cell lines (Fig. 8A). However, the effect was statistically significant only in CCD-18Co fibroblasts. Short-term folate depletion (48 h) increased the HpaII/MspI ratio approx. 3-fold for the epithelial and 4-fold for the colon fibroblast cells (Fig. 8B), indicating that HpaII digestion was, as expected, more effective, when DNA methylation is lower. A similar result was observed after 7 days in response to longer-term folate deficiency (Fig. 8B).

These data indicate that this modification of the comet assay to detect DNA methylation in single cells is responsive to known modifiers of methylation status. Similar results have been reported using another methylation-sensitive variant of the comet assay [31]. Here DNA methylation status was measured by SCGE in folate deficient or replete human colonic adenocarcinoma SW620 cells (up to 14 days) using the methylation-sensitive restriction enzymes HpaII and HhaI (2.5 U/gel for 1 h). DNA global hypomethylation increased significantly (approx.6-fold) in folate depleted cells. Moreover, there was also significant DNA hypomethylation detected in the p53 gene region in these cells (approx. 2–3 fold), measured using a particularly elegant variant of the methylation-sensitive comet assay, which combines SCGE and FISH [31].

In view of the role of aberrant DNA methylation in cancer development, the ability to adapt the comet assay to specifically detect changes in DNA methylation patterns in human tissue will undoubtedly advance understanding of how epigenetics influences human cancer risk. An initial application of a methylation-sensitive variant of the comet assay [31] in the context of human carcinogenesis demonstrated that folate levels are significantly lower in colon tissue biopsies from patients with pre-cancerous polyps compared with non-diseased controls, and this is associated with genome-wide DNA hypomethylation [18]. Moreover, both colonocyte folate and DNA methylation declined with proximity to the original polyp site [18]. Global aberrant DNA methylation was found to be responsive to folate supplementation in a

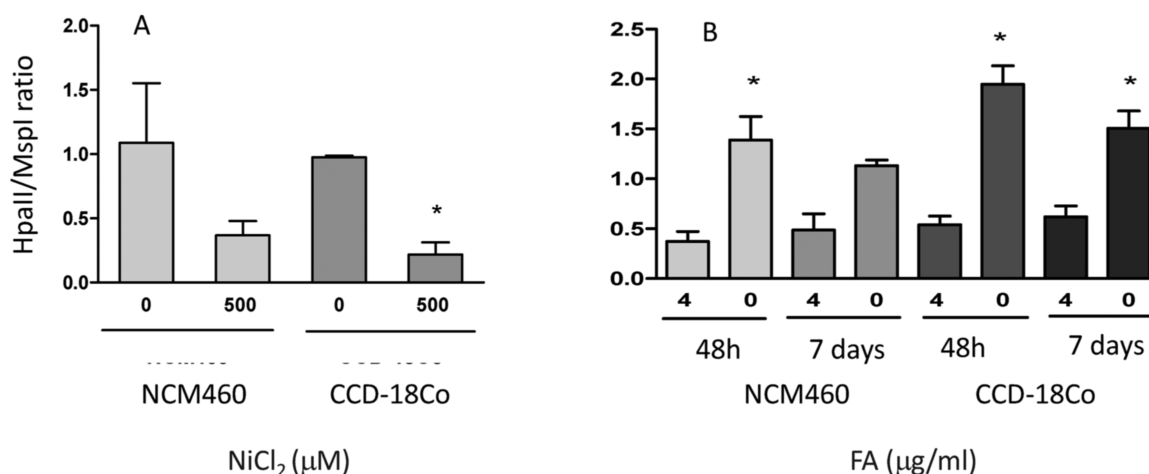

Fig. 8. Global DNA methylation measured in human colon cells and fibroblasts.
The methylation-sensitive variant of the comet assay was used in NCM460 colon epithelial cells and CCD-18Co fibroblasts. (A) Effect of a hypermethylating stimulus: 500 µM $NiCl_2$ treatment for 24 h. (B) Effect of folic acid (FA) depletion for 48 h or 7 days [F+ (4 µg/ml) or F- (0 ng/mL)]. Results show the mean ratio between HpaII and MspI DNA digestion ± SE (n = 4). *$p < 0.05$ where P values refer to differences between treated and untreated cells by one-way ANOVA.

subsequent intervention study, which demonstrated that taking folic acid (600 µg/day for 6 months) decreased genome-wide DNA hypomethylation in colonocytes isolated from polypectomised patients (n = 12) compared with non-supplemented individuals (n = 8) [19].

7. Summary

A considerable strength of SCGE is the genuine adaptability of the assay to measure multiple biomarkers of DNA stability. This review demonstrates that employing the comet assay to measure DNA strand breakage, misincorporated uracil, DNA BER capacity and now, DNA methylation in single cells, allows us to better understand in detail, how the B vitamin folate acts mechanistically on DNA stability and malignant transformation in human and rodent models. These studies serve to highlight the sensitivity of the assay to detect, and at least partially quantify, changes in DNA stability due to intervention. Here, we have been able to establish how folate deficiency works through increased uracil misincorporation, DNA strand breakage, defective BER and DNA hypomethylation to induce or accelerate genomic instability, while conversely, improved folate status reduces misincorporated uracil in patients with colon polyps and even in apparently healthy human volunteers.

Conflict of interest

All authors declare no conflict of interest.

Acknowledgments

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Molecular Carcinogenesis

Morin-dependent inhibition of Low Molecular Weight Protein Tyrosine Phosphatase (LMW-PTP) restores sensitivity to apoptosis during colon carcinogenesis: studies in vitro and in vivo, in an Apc-driven model of colon cancer

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Morin-dependent inhibition of low molecular weight protein tyrosine phosphatase (LMW-PTP) restores sensitivity to apoptosis during colon carcinogenesis: studies *in vitro* and *in vivo,* in an *Apc*-driven model of colon cancer.

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Running title

LMW-PTP inhibition enhances sensitivity to 5-FU in Pirc rats mutated in *Apc*

Keywords: Low Molecular Weight- Protein Tyrosine Phosphatase (LMW-PTP), colon carcinogenesis, Pirc rat, *Apc.*

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Abbreviations

Wt, wild type; Pirc, Polyposis in the rats colon; SDS-PAGE, Sodium Dodecyl Sulphate - PolyAcrylamide Gel Electrophoresis; NaF, Sodium Fluoride; EGTA, Ethylene Glycol Tetraacetic Acid; PVDF, Polyvinylidene fluoride; TPBS, Tween-PBS; DMEM, Dulbecco's Modified Eagle Medium; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide; DMSO, Dimethylsulfoxide; ANOVA, analysis of variance; Wnt, Wingless Type; STAT3, Signal transducer and activator of transcription 3; Bcl2, B-cell lymphoma 2; Bcl-XL, B-cell lymphoma extra-large; Min, multiple intestinal neoplasia; Myc, MYC proto-oncogene

ABSTRACT

LMW-PTP has been associated with the development of colorectal cancer (CRC) and with the resistance to chemotherapy in cancer cells. To clarify its role *in vivo,* we studied LMW-PTP expression in Pirc rats (F344/NTac$^{-Apc\ am1137}$), genetically prone to CRC and resistant to apoptosis. In the morphologically normal mucosa (NM) of Pirc rats, a dramatic over-expression of LMW-PTP was found compared to wt rats (about 60 times higher). Moreover, LMW-PTP levels further increase in spontaneously developed Pirc colon tumors. To understand if and how LMW-PTP affects resistance to apoptosis, we studied CRC cell lines, sensitive (HT29 and HCT-116) or resistant (HT29R, HCT116R) to 5-Fluorouracil (5-FU): resistant cells over-express LMW-PTP. When resistant cells were challenged with morin, a polyphenol inhibiting LMW-PTP, a fast and dose-related down-regulation of LMW-PTP was observed. 5-FU and morin co-treatment dramatically decreased cell viability, increased apoptosis, and significantly impaired self-renewal ability of all the cancer cell lines we have studied. Similarly, we observed that, in Pirc rats, one-week morin administration (50 mg/kg) down-regulated LMW-PTP and restored the apoptotic response to 5-FU in the NM. Finally, administration of morin for a longer period led to a significant reduction in colon precancerous lesions, together with a down-regulation of LMW-PTP. Taken together, these results document the involvement of LMW-PTP in the process of CRC *in vitro* and *in vivo*. Morin treatment may be envisaged as a system to increase the sensitivity to chemotherapy and to prevent carcinogenesis.

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INTRODUCTION

Colorectal cancer, one of the main forms of cancer in Western countries, develops through sequential steps each characterized by the acquisition of genetic and epigenetic alterations leading to activation or over-expression of proteins with oncogenic activity. [1] In the past, enzymes such as protein tyrosine phosphatases were considered merely onco-suppressors, being capable to counteract the oncogenic activity of kinases. [2] In the last decades, however, a possible role as oncogenes has been proposed for some phosphatases, such as LMW-PTP, an 18k Da protein encoded by the *ACP1* gene. [3-5] Accordingly, the increased expression level of this protein has been found in colorectal cancer, as well as in other tumors. [3,4] For colorectal cancer, it was also shown that LMW-PTP over-expression correlates with poor prognosis.[4] *In vitro*, LMW-PTP has been reported to interact with various targets such as Ephrin A2 receptor (EphA2), β-catenin, and platelet-derived growth factor receptors (PDGFR). [6-8] More recently, the involvement of LMW-PTP in controlling apoptosis, cell motility and resistance to chemotherapy has been reported in colorectal cancer cells, but the role of this protein in colorectal carcinogenesis *in vivo* remains to be elucidated.[3] Mutations in the Adenomatous Polyposis Coli (Apc) gene are early events in the multistep process of colorectal carcinogenesis.[1] Accordingly, germ-line mutations in this gene, as in the Familial Adenomatous Polyposis (FAP) syndrome, predispose the intestinal mucosa to the development of hundreds of adenomatous polyps, which progress to cancers if left untreated. *Apc* mutations are also very common in sporadic colon cancer.[1] Pirc rats (F344/NTac$^{-Apc\ am1137}$) carrying an *Apc* germ-line heterozygous mutation like FAP patients, spontaneously develop tumors in the colon and are thus a useful model to study carcinogenesis in the very organ in which colon cancer develops.[9,10-12] Interestingly, the morphologically normal colon mucosa (NM) of these rats, shows resistance to apoptosis when challenged with 1,2-dimethylhydrazine (DMH), a methylating agent inducing colon cancer in rodents.[10] It is not known whether Pirc rats are resistant to other cytotoxic

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chemicals such as chemotherapy drugs, like 5-fluorouracil (5-FU), currently used for the treatment of CRC. Nevertheless, these results point out that the resistance to apoptosis is not only a feature of cancerous cells but also a very early event in the process of carcinogenesis since it is already present in the Pirc NM prone to carcinogenesis.

Given the reported association between LMW-PTP and resistance to chemotherapy, together with the fact that the role of LMW-PTP in the early phases of colon carcinogenesis is not well known, we thought it of interest to study LMW-PTP expression in the normal mucosa of Pirc rats as well as in the colonic tumors, which spontaneously develop in this tumor-prone rat strain.[3,10] Moreover, to better understand if and how LMW-PTP over-expression confers resistance to apoptosis, experiments *in vitro* were set up to study the expression of LMW-PTP in colon cancer cell lines differing in their resistance to chemotherapy. These cells were also challenged with morin, a non-toxic polyphenolic compound that behaves as a non-competitive inhibitor of LMW-PTP and triggers transient degradation of LMW-PTP in cancer cells. [13] The *in vitro* results were then verified *in vivo,* using Pirc rats. Finally, since inhibition of LMW-PTP could be protective in the development of carcinogenesis, we administered morin to Pirc rats to determine whether this polyphenol could reduce colon carcinogenesis.

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MATERIALS AND METHODS

Animals and Treatments

Pirc (F344/NTac-Apc^{am1137}) and wild type (wt) Fisher F344/NTac rats were originally obtained from Taconic (Taconic Farms, Inc. USA) and bred at the University of Florence. The Pirc colony was maintained by mating heterozygous Pirc rats with wt rats and pups genotyped at one month of age. [9, 10].

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Pirc rats spontaneously develop colon carcinogenesis, accordingly, starting at an early age (4 weeks of age) microscopic precancerous lesions such as Mucin Depleted Foci (MDF) are already visible in the colon, while some weeks/months later, beside MDF, macroscopic tumours are also present. [9, 10] Rats were maintained in polyethylene cages under an experimental protocol approved by the Commission for Animal Experimentation of the Italian Ministry of Health. Unless otherwise specified, rats were fed AIN-76 diet ad libitum and euthanized by CO_2 asphyxia

To study resistance to apoptosis in Pirc and wt rats, we used 8-week-old Pirc and age-matched wt rats that were treated with 70 mg/kg (s.c.) of 5-Fluorouracyl (5-FU) and euthanized 24 hrs later as described below. The effect of morin (Sigma-Aldrich) treatment on the apoptotic response to 5-FU was studied in 8 Pirc rats (aged 9 months) who were randomly allocated to: Controls (4 rats), fed the standard AIN-76 diet or morin-treated (4 rats), fed for one week the same AIN-76 diet supplemented with morin to provide a dose of 50 mg/kg (body weight). The last day of the treatment week, rats (both Controls and morin-treated) were treated (s.c.) with 5-FU (50 mg/kg) and euthanized 24 hrs later to measure apoptosis and LMW-PTP expression as described below.

The chemiopreventive activity of morin was tested in 18 male Pirc rats that were randomly allocated at one month of age to the standard AIN-76 diet (Controls, n=9), or to the same diet supplemented with morin (n=9) to provide 50 mg/kg (body weight). This dose of morin was chosen based on previous carcinogenesis experiments with morin or with polyphenolic extracts from various sources. [14, 15] Similarly, the choice to administer morin mixed in the diet was based on previous experiments using this protocol to test potential chemopreventive agents. [16, 17] The diet containing morin was prepared weekly adjusting the quantity of morin to be added on the basis of the amount of diet eaten daily by rats and on their body weight determined each week. Animals were euthanized at 10 weeks of age, and the chemopreventive activity of morin determined

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comparing the number of preneoplastic lesions mucin depleted foci (MDF) in Controls and morin-treated animals. [10, 18,19]

The number of animals/groups depends on the size of the expected effects in each specific experiment. Regarding the experiment on the apoptotic response to 5-FU, the low number of animals is based on the expected large difference between wt rats and Pirc rats (as previously observed in Pirc rats and wt rats treated with DMH). [10] Therefore, we considered that 4 rats would be sufficient to observe a significant difference, this also in light of a reduction in the number of animals to be used (3R principle). Regarding the chemoprevention experiment with morin, we were unaware of the size of this effect and so we had to rely on a larger number of animals to obtain a statistically significant effect, and to avoid the risk to nullify the experiment and to waste animals. All the animals used in the study were male.

Determination of MDF

The entire colon was dissected, flushed with cold saline, longitudinally opened and fixed flat in formalin for at least 18 hrs. The colon was then stained with High-Iron Diamine Alcian blue (HID-AB) technique to determine MDF at the microscope.[10] As previously documented, staining the whole unsectioned colon with HID-AB, it allows to highlight mucin production at the opening of normal crypts; MDF which shows a defective mucous production, can be easily visualized as foci of crypts lacking HID-AB staining. [10, 18,19]

Determination of apoptosis in the colon

Apoptosis was evaluated in histological sections (4µm thick) of normal colon mucosa or tumors stained with hematoxylin eosin, determining cells with the following characteristics of apoptosis: cell shrinkage, loss of normal contact with the adjacent cells of the crypt, chromatin

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condensation or formation of round or oval nuclear fragments.[20] In the normal mucosa, apoptosis was quantified as apoptotic cells /crypt, observing at least 12 full longitudinal crypt sections. In tumors, apoptosis was quantified as the number of apoptotic cells/area measured using the ACT-2U software program (Nikon, Instruments Europe, Badhoevedorp, NL) connected via a camera to the microscope (Optiphot-2, Nikon, NL). The evaluation was performed at 1000x magnification.

Polyclonal LMW-PTPs antibodies production

Antisera were obtained by immunizing rabbits with the synthetic peptides [T S G Y E I G N P P D Y R G Q] and [V S D W N V G R S P D P R A V] respectively designed from the isoform-specific domain of IF1/LMW-PTP and IF2/ LMW-PTP (SIGMA-GENOSYS, Custom Peptide Antisera Services). Isoform specific antibodies were purified from the immunoglobulin fraction by affinity chromatography using Sepharose 4B coupled with the pure IF1/LMW-PTP protein or IF2/ LMW-PTP protein as immunoadsorbent. The purified antibodies were characterized with respect to their specificity and sensitivity by immunoblotting.

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Determination of LMW-PTP expression in the colon mucosa and tumors.

Morphologically normal mucosa from Pirc rats and age-matched wt rats (8 weeks of age) was stored in RNA-later™ (Qiagen) at -80 °C until analysis (n= 7 and 9 for wt and Pirc rats, respectively). The expression of LMW-PTP in colon tumors was evaluated in archival samples stored in RNA-later™ (Qiagen) at -80 °C from Pirc rats aged 9 months (n=7).

All samples were sonicated on ice in 300 µL of RIPA buffer (50mM TrisHCl pH 7,5, 150 mM NaCl, 100 mM NaF, 2 mM EGTA, 1% Triton X-100, 10µL/ml Protease and Phosphatase inhibitor, Sigma). Lysates were centrifuged at 4°C, 14000 rpm for 15 min: supernatants were collected. Cell extracts were resolved by SDS-PAGE and transferred to PVDF membranes (BioRad). Membranes were incubated overnight at 4°C with rabbit polyclonal anti-LMW-PTP produced as described above. After washing in TPBS-Tween 20 (0,1%) membranes were incubated with the appropriate horseradish peroxidase-

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conjugated secondary antibodies (Santa Cruz Biotechnology) for 1h. Proteins were detected using Clarity Western ECL (Biorad) by UVP.

Cell line and reagents

HT29, HCT-116, and Caco2 colorectal carcinoma cells were purchased from European Collection of Authenticated Cell Cultures (ECACC).

HT29 and HCT-116 resistant to 5-FU (HT29R and HCT-116R, respectively) were selected by exposing HT29 or HCT-116 sensitive cells to increasing doses of 5-FU during 6 months until the final concentration of 20 μM, as previously described. [21]

Cells were routinely grown in DMEM supplemented with 10% Fetal Bovine Serum, glutamine and penicillin-streptomycin (Sigma-Aldrich), in a humidified atmosphere with 5% CO_2 at 37°C.

5-FU was used at the concentration of 20 μM for HT29, HT29R cells and 5 μM for HCT-116, Caco2. Morin (Sigma Aldrich) was always used at the concentration of 1.5 μM for all cell lines.

Determination of cell viability

MTT assay (Sigma-Aldrich) was used to measure cell viability. Cells were incubated with 0.5mg/ml MTT solution for 1h at 37°C. After removing cell culture medium, DMSO was added to dissolve the insoluble MTT-formazane salt. Results were detected at 595 nm on a microplate reader (BioRad).

Annexin V and Propidium Iodid assay

Apoptosis was determined using Annexin-V-FLUOS Staining kit from Roche according to manufacturer's instructions. Briefly, $1x10^6$ cells were collected, washed and incubated with 100μL of Annexin-V-FLUOS labeling solution, for 10-15 min at room temperature. Cells were analyzed by flow cytometry BDFACS Canto. Q1: (PI labeling) necrotic cells; Q2: (PI/AnnV labeling) late apoptotic cells; Q3: (no labeling) living cells; Q4: (AnnV labeling) early apoptotic cells.

Colony formation assay

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After 24h treatment, 1000 cells were seeded into a six-well plate and cultured for 9-11 days. Subsequently, cells were fixed and stained with a solution containing 1% crystal violet (Sigma-Aldrich) and 10% methanol. Colonies were photographed and counted using ImageJ imaging system.

Statistical analysis

Data are presented as means ± SE. The data of the *in vitro* experiments are from at least three independent experiments; the number of animals for each *in vivo* experiment is specified in the legends to the figures. Statistical analysis of the data was performed by Student's t test or 2-way ANOVA (Bonferroni corrected). P values of ≤0.05 were considered statistically significant.

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RESULTS

Expression of LMW-PTP in the apparently normal mucosa of Pirc rats and in colon tumors

Pirc rats bearing *Apc* mutation spontaneously develop several colon tumors in their life. [9-12, 22] To confirm the involvement of LMW-PTP in colon carcinogenesis and to study its expression in the early phases of this process, we evaluated expression levels of this enzyme both in the morphologically NM and in spontaneously developed colon tumors in Pirc rats. Surprisingly, we find that expression levels of LMW-PTP in the NM of Pirc rats are dramatically higher when compared with those detected in the NM of wt rats. Moreover, considering Pirc rats, we observe that colon tumors express higher LMW-PTP levels with respect to their corresponding normal mucosa (Fig. 1 panels A, B). These results indicate that *Apc* mutated cells express high LMW-PTP levels, regardless they are in the apparently normal mucosa or in tumors.

To gain more insight into the role of LMW-PTP, we also evaluated the sensitivity of Pirc rats to apoptosis induced by 5-FU, a pyrimidine analogue, commonly used in the treatment of colon cancer. [23] To this aim, wt and Pirc rats were treated with a single 5-FU dose (70 mg/kg), measuring apoptosis in colon mucosa 24 hrs later. The results show that in Pirc rats the number of apoptotic

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3 cells is significantly lower (P = 0.02) than in wt rats treated with the same 5-FU dose (Fig. 1 panel C).
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6 This evidence, in agreement with our previous results with DMH, indicates that Pirc rats are also
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8 resistant to 5-FU and suggests that the overexpression of LMW-PTP may protect colon cells from
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11 cytotoxicity of 5-FU. [10]
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13 **LMW-PTP expression increases in colon cancer cells resistant to 5-FU**
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15 Data obtained with Pirc rats suggest a link between LMW-PTP and resistance to apoptosis.
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18 To confirm this hypothesis, we analyzed expression levels of LMW-PTP in two colon cancer cell lines
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20 resistant to 5-FU (HT29R and HCT-116R) (Fig. 2, panels A and B). We find that the expression level
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23 of the LMW-PTP is significantly higher in the cells resistant to 5-FU with respect to parental cells
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25 (Fig. 2, panels C, D, E, and F), Together, these findings indicate that overexpression of LMW-PTP
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28 correlates with the acquisition of resistance to anticancer drugs.
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30 **Inhibition of LMW-PTP enhances chemo-sensitivity of colon cancer cells**
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33 Recently, Hoekstra et al. demonstrated that pharmacological inhibition of LMW-PTP
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35 improves the sensitivity of cancer cells to anticancer drugs. [3] Based on this report, we decided to
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38 study morin, a polyphenolic compound that we have recently shown to be a non-competitive
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40 inhibitor of LMW-PTP in melanoma cell lines. [13] Accordingly, here we find that morin leads to a fast
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43 down-regulation of LMW-PTP expression in both HT29 and HT29R cells (Fig. 3, panels A and B). The
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45 same phenomenon is also observed in Caco2 and in HCT-116R colon cancer cells treated with 1.5
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47 μM of morin (Fig. 3, panels C and D).
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50 To clarify the effect of LMW-PTP inhibition in these cell lines, we evaluated whether
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52 treatment with morin enhances their sensitivity to 5-FU. The results of this experiment (Fig. 4) show
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55 that 5-FU or morin alone do not significantly impair the viability of these cell lines. However, when
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57 cells were treated with a combination of 5-FU and morin, cell viability was dramatically reduced (Fig.
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60 4). In keeping with this evidence, we also demonstrate that combined treatment (5-FU + morin)

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strongly increases the fraction of apoptotic cells in HCT-116, Caco2 and HT29R cell lines (Fig. 5). Similarly, co-treatment with morin and 5-FU significantly impairs self-renewal ability in all the three cell lines we have studied (Fig. 6). These results indicate that morin acts as a potent sensitizing agent with respect to the toxic effects of 5-FU.

***In vivo* pre-treatment with morin enhances apoptotic response in Pirc normal mucosa**

To evaluate the effectiveness of morin in enhancing apoptotic response also *in vivo*, we studied its effects in Pirc rats. Morin (50 mg/kg) was administrated to rats mixed in the diet for one week before treatment with a single dose of 5-FU (50 mg/kg). Twenty-four hours after 5-FU treatment, the animals were euthanized to evaluate the number of apoptotic cells in the colon (Fig. 7, panel A). We observe that the number of apoptotic cells is higher in the rats pre-treated with morin compared with control rats not treated with morin (Fig. 7, panel B, and Supplementary Figure 1). At the same time, testing LMW-PTP expression in the NM of Pirc rats pre-treated with morin, we find a statistically significant lower expression of LMW-PTP compared with Pirc rats not treated with morin (Fig. 7, panels C and D), confirming that morin down-regulates LMW-PTP also *in vivo*. It is also interesting to note that the same morin treatment does not affect 5-FU induced apoptosis in wt rats. In facts, apoptotic cells/crypt were 1.6 ± 0.2 in wt control rats (not treated with morin) and 1.2 ± 0.3 in wt morin-treated rats (means ± SE, n=5).

Morin treatment reduces colon precancerous lesions in Pirc rats

Finally, we evaluated whether morin is able to inhibit colon carcinogenesis. Starting at one month of age, Pirc rats were fed for 6 weeks with a standard diet or with a diet supplemented with morin (50 mg/kg) and then euthanized to evaluate the number of MDF in the colon (Fig. 8, panel A, and Supplementary Figure 2). The results of this experiment show a significantly lower number of MDF in morin-treated rats compared to control rats (Fig. 8 panel B). As expected, LMW-PTP expression in the NM is significantly lower in the rats treated with morin (Fig. 8, panels C and D).

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Few macroscopic tumors were also present in these rats (2.3±0.8 and 1.3±0.5 in controls and morin treated rats, respectively) (Supplementary Figure 3). Interestingly, measuring apoptosis in the tumors dissected from rats treated with morin, we show a significantly higher apoptosis in comparison with the tumors dissected from control rats (Fig. 9). On the contrary, basal levels of apoptosis in the normal mucosa are not affected by the morin treatment. In fact, apoptotic cells/crypt are 0.25 ± 0.04 and 0.24 ± 0.03 in controls and morin-treated rats, respectively (means ± SE; n=8 in both groups).

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Discussion

The ability to resist to death stimuli, a hallmark of the cancerous cell, may cause resistance to chemotherapy agents in CRC, leading to a consequent failure of cancer therapy. [24, 25] Resistance to apoptotic stimuli is also important in the early phases of cancer development since it may allow the growth of defective cells that would be otherwise eliminated by apoptosis. [20, 26, 27] We have previously documented that the morphologically normal colon mucosa of Pirc rats, (rats that are prone to carcinogenesis via a heterozygous mutation in *Apc* gene) is resistant to apoptosis. In fact, Pirc NM, when challenged with a toxic dose of DMH, shows low levels of apoptosis and fails to stop proliferation.[10]

In the present paper, we show that the NM of Pirc rats over-expresses LMW-PTP when compared to the NM of wt rats.

LMW-PTP over-expression has been reported so far in several types of cancer, including CRC, as we showed in clinical colon cancer samples and in rodent DMH-induced colon cancers. [4, 28] Although Hoekstra and colleagues reported a step-wise increase in LMW-PTP expression in different stages of colon dysplasia, there were no data on the over-expression of LMW-PTP at such an early stage of carcinogenesis, as the NM of Pirc rats can be considered. [3, 10, 27, 29] Accordingly, in the

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present paper, the over-expression of LMW-PTP was observed in the NM of rats aged 8 weeks, when no tumors and only a few microscopic lesions are present in the colon mucosa. [10] We also show that Pirc colon tumors over-express LMW-PTP when compared to their corresponding NM, in agreement with previous data with colon tumors in rodents. [28] Taken together these data suggest that *Apc* mutated cells express high LMW-PTP levels and that LMW-PTP levels increase in the process of colon carcinogenesis. *Apc* mutations have been linked to constitutive activation of the Wnt-signaling pathway causing up/down regulation of several genes connected with cell proliferation and differentiation. [30] So far, LMW-PTP has not been described as a possible target of the Wnt-signaling, but it is possible that, at least indirectly, this protein may be linked to the activation of the Wnt-pathway, a hypothesis that should be verified in the future. Noteworthy, recent data by our group obtained in melanoma cell lines document that LMW-PTP regulates the phosphorylation status of several targets[31], but how this phenomenon correlates with tumorigenesis is not known yet.

To better understand the role of LMW-PTP in the process of colorectal carcinogenesis, we used an inhibitor of LMW-PTP, morin, a polyphenolic compound (3,5,7,2',4'-pentahydroxyflavone), originally isolated from members of the Moraceae family, present in figs, mulberry, strawberries. [31] [32] In a first set of *in vitro* experiments, we show that CRC cells resistant to 5-FU over-express LMW-PTP. We also show that LMW-PTP can be lowered by the use of morin in a dose-dependent manner acting at micromolar concentrations. These results are in agreement with our recent report on melanoma cell lines, documenting that morin inhibits LMW-PTP action mainly because it markedly induces degradation of this enzyme through a proteosomal-dependent mechanism. Furthermore, in the same paper, we demonstrated that LMW-PTP silencing strongly improves the sensitivity of different types of cancer cells to anti-cancer drugs (dacarbazine, 5-FU and docetaxel), thereby confirming the key role of LMW-PTP in modulating the resistance to these drugs [13]. The exact mechanism by which morin increases the sensitivity to 5-FU in colon cancer cells has not been fully

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clarified yet. A study conducted by Gupta and colleagues on human myeloma, breast cancer, and head and neck cancer cells, demonstrated that morin acts as a sensitizing agent. In fact, the authors observed that treatment with morin suppresses STAT3 pathway, leading to down-regulation of several anti-apoptotic proteins including Bcl2, Bcl-XL, and survivin. [33] We recently documented similar effects of morin on anti-apoptotic proteins in melanoma cell lines. [13]

We then tested the effect of morin in cancerous cells resistant to apoptosis, finding that, upon treatment with morin, apoptosis induced by 5-FU is restored. These data are in agreement with previous studies reporting anticancer effects of morin in various cancer cell lines (melanoma, hepatoma, prostate cancer, HeLa cells) with mechanisms involving an increase of apoptosis. [34-38] Based on these strong effects of morin in CRC cells *in vitro*, we then tested whether morin could restore the apoptotic response in Pirc rats NM and lower LMW-PTP expression *in vivo*. We first show that the defective apoptotic response observed in Pirc rats challenged with DMH was also observed with 5-FU, a drug currently used in the therapy of colorectal cancer. [10, 23] Interestingly, we find that morin, administered to a dose of 50 mg/kg for 10 days before 5-FU treatment, restores the sensitivity to 5-FU induced apoptosis in the NM, together with a decrease in LMW-PTP expression, in agreement with our observations in cancer cell lines. In a similar way, previous studies in Min mice (carrying a germ-line mutation in *Apc* gene, but developing tumors mostly in the small intestine), showed that Sulindac, a non-steroidal anti-inflammatory drug with chemopreventive properties, may restore the defective apoptotic response to a genotoxic carcinogen. [26]

We also show that morin, administered for 6 weeks to Pirc rats, decreases the number of preneoplastic MDF in the colon. This result is in agreement with reports showing that morin, (administered at the same dosage of the present study) may decrease colon carcinogenesis when given chronically (up to 30 weeks of treatment) to carcinogen-initiated rats. [15, 39] Since morin is present in some fruits [40, 41], one could wonder how our study relates to a modulation of cancer risk

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just with the diet. Dietary consumption of vegetable and fruits (both rich in polyphenolic compounds) has been associated with a lower risk of CRC, but it is thought that this beneficial effect is due to the presence in these foods of several compounds (e.g. fibers, for one thing) and certainly not just to a single polyphenol like morin, which is present in small quantity in foods. [42, 43] In the present study morin was used since it is a potent inhibitor of LMW-PTP *in vitro*, and, as we show, it also acts *in vivo* to lower LMW-PTP and to decrease carcinogenesis. Although the dose we used is higher than the presumed dietary intake of morin in humans, since morin is not toxic, these studies suggest that morin treatment (as supplement) could be envisaged as a strategy to prevent CRC in subjects at high risk such as those operated for CRC or FAP patients. Furthermore, the results of the experiments with 5-FU and morin co-treatment suggest the possibility of a therapeutic use of morin as a sensitizing agent to potentiate anticancer drug effectiveness in humans.

Various mechanisms have been implicated in this protective effect of morin (i.e. variation in fecal and mucosal enzymes, in tissue and serum lipids, or in *Myc* expression), none of these studies investigated the expression of LMW-PTP and apoptosis. [15, 42, 43] Here we show that morin is able to lower LMW-PTP expression in the colon mucosa in agreement with our *in vitro* findings. In addition, we find that in the tumors from animals treated with morin, apoptosis is higher than in those from control animals, suggesting that this phenomenon might selectively favor the elimination of defective cells during carcinogenesis. An increase in apoptosis in tumors, has been described as a mechanism through which chemopreventive agents act in the colon. [30,44,45] Interestingly, basal levels of apoptosis in the normal mucosa are not affected by morin.

In conclusion, we show for the first time that the preneoplastic mucosa of Pirc rats, prone to carcinogenesis and resistant to apoptosis, over-expresses LMW-PTP, a protein with oncogenic potential. We also show that morin is an inhibitor of LMW-PTP in colon cancer cells and that this

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inhibitor restores the sensitivity to apoptosis and it decreases other characteristics of malignancy in these cells.

Importantly, we document that the same effects observed *in vitro* can be reproduced *in vivo* in Pirc rats where morin restores the sensitivity to apoptosis and lowers LMW-PTP.

Finally, we show that morin decreases the spontaneous formation of colonic preneoplastic lesions when given chronically to Pirc rats; the mechanism involves a selective increase in apoptosis in tumor cells and a down-regulation of LMW-PTP.

Conclusions

Taken together these results document the involvement of LMW-PTP in the process of colon carcinogenesis both in the early and in the late phases of this process. Morin treatment may be envisaged as a system to increase the sensitivity to chemotherapy and to prevent carcinogenesis in patients at risk to develop CRC.

Author Contributions

G.C. and G.R. supervised the project. P.P. and A.C. designed the experiments. G.L. performed most experiments. E.P., K.T., AP.F., A.R., and A.C. helped with all the experiments. P.P., G.C. and G.R. discussed the results. P.P., G.R., and G.C. wrote the manuscript. All authors read and approved the final manuscript

Legends to the Figures

Figure 1: (A), Protein expression levels of LMW-PTP in normal mucosa of wt and Pirc rats and in colon tumors from Pirc rats. Data are means ± SE, n = 9, and 7, for the normal mucosa of wt and Pirc rats, respectively and n = 7 for colon tumors. All data were normalized with respect to the values in

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the wt normal mucosa. *: P < 0.01; #: P < 0.05. (B), Representative western blot showing the expression levels of LMW-PTP in: colon mucosa of wild type rats (left), normal colon mucosa (NM) of Pirc rats (middle), and spontaneous colon tumor of Pirc rats (right). (C), apoptotic cells/crypt in wt and Pirc rats treated with 5-FU (70 mg/Kg). Apoptosis was measured in histological sections obtained from normal mucosa stained with hematoxylin-eosin. Data represent means ± SE (n = 4 rats in both groups). * P : < 0.05, compared with wt rats.

Figure 2: Panels A and B: MTT test on parental (HT29, HCT-116) and 5-FU resistant cells (HT29R, and HCT-116R). Panels C and D: LMW-PTP expression level in parental and resistant cells. Panels E and F: densitometric analysis of the data in panels C and D, carried out by Kodak MI software. Data reported represent mean value ± SE. * P < 0.05.

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Figure 3: Morin down-regulates LMW-PTP expression in colon cancer cells. Panel A: colon cancer cells, HT29 and HT29R, resistant to 5-FU, were treated with increasing morin concentration for 4 hours. After treatment, expression levels of LMW-PTP was analysed by western blot. Panel B: quantification of western blot in panel A was obtained using Kodak MI software. Panels C and D: Caco2 and HCT-116R cells were incubated in the presence of 1.5 μM morin. After 4 hours, expression levels of LMW-PTP were evaluated by western blot.

Figure 4: MTT viability assay. Cells were starved for 20 hours, and then incubated with 1.5 μM Morin, 5-FU, or with a combination of both for further 24 hours. After this time, cell viability was evaluated using MTT assay. Panel A: HT29; Panel B: HT29R; Panel C: HCT-116; Panel D: HCT-116R; Panel E: Caco2. For HT29, HT29R, and HCT-116R cells concentration of 5-FU used was 20 μM; for HCT-116 and Caco2 cells, 5 μM of 5-FU was used. # P < 0.01; * P < 0.05 when compared with cells treated with 5-FU alone.

Figure 5: Evaluation of apoptosis in different cells lines. Panel A: HCT-116; Panel B: Caco2; Panel C: HT29R. Cancer cells were starved for 20 hours, and then incubated in the presence of 5-FU (5 μM),

morin (1.5 μM), or with a combination of both. After 24 hours, the fraction of apoptotic cells was evaluated using Annexin V/Pi assay. For each assay, 10000 events were acquired. Data reported represent mean values ± SE (n= 4). Q1, necrosis; Q2, late apoptosis; Q3 viable cells; Q4, early apoptosis. * P < 0.05 when compared with cells treated with 5-FU alone.

Figure 6: Colony assay. Panel A: HCT-116; Panel B: Caco2; Panel C: HT29R. Cells were starved for 20 hours and then treated with 5-FU, morin or with a combination of both. After 24 hours, cells were detached, counted, plated in new dishes and incubated at 37°C. After 10 days, colonies were stained with crystal violet, and counted to determine the surviving fraction. * P < 0.05 when compared with cells treated with 5-FU alone.

Figure 7: Morin treatment restores apoptotic response to 5-FU and downregulates LMW-PTP in normal mucosa of Pirc rats. The scheme of animal treatment is presented (panel A). Pirc rats were treated with morin for one week (50 mg/kg in the diet) and the last day of the week injected with a single dose of 5-FU (50 mg/Kg). Twenty-four hours after injection, rats were euthanized to evaluate the number of apoptotic cells in the intestinal crypts (Panel B). (n = 4) * P < 0.01

Expression levels of LMW-PTP in the same NM samples as in panel A was analysed by western blot (panel C). Quantification of western blot in panel C was obtained using Kodak MI software (panel D). Bars represent mean values ± S.E. (n = 3) * P < 0.05.

Figure 8: Morin treatment decreases colon precancerous lesions in Pirc rats. The scheme of animal treatment is presented (panel A). Preneoplastic lesions (MDF) in the colon of Pirc rats fed for six weeks with the AIN-76 standard diet (Controls, white bar) or the same AIN-76 diet supplemented with morin (50 mg/kg). Each bar represents a mean ± S.E. (n = 9 in both groups) (panel B). * P<0.05 with t-test.

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Expression levels of LMW-PTP in specimens of normal mucosa obtained from the same rats of panel (B) were evaluated by western blot (panel C). Quantification of western blot was carried out using Kodak MI software (panel D). Bars are mean values ± SE. * P<0.05 with t-test.

Figure 9: Morin increases apoptosis in colon tumors. Panel A: apoptotsis in the tumors of Pirc rats treated for 6 weeks with morin or with a standard rodent diet. Bars are mean values ± SE. (Control group, n= 6; morin group n = 5). * P < 0.05.

Supplementary data

Daily diet consumption by rats

Supplementary Figures

Suppl. Figure 1: histological sections of two colon crypts (panels A and B) showing apoptotic cells (arrows). Sections were stained with haematoxylin and eosin. Original magnification: 1000x.

Suppl. Figure 2: topographical view of Pirc colon stained with HID-AB showing two MDF (arrows, panel A) and one MDF (arrow, panel B). Original magnification: 40x.

Suppl. Figure 3: histological sections of Pirc colon tumours (panels A and B) stained with haematoxylin and eosin. Original magnification: 40X

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45. Swamy MV, Patlolla JM, Steele VE, Kopelovich L, Reddy BS, Rao CV. Chemoprevention of familial adenomatous polyposis by low doses of atorvastatin and celecoxib given individually and in combination to APC Min mice. *Cancer Res*; 2006; 66: 7370-7.

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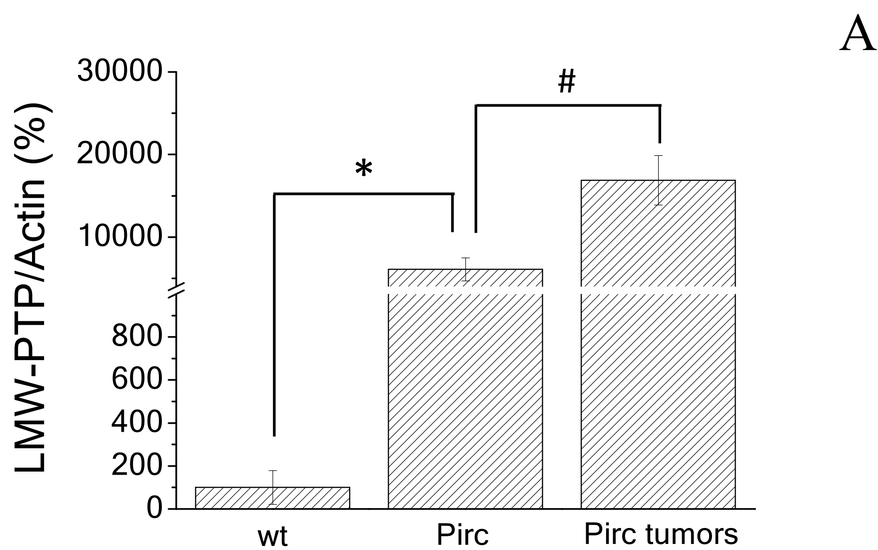

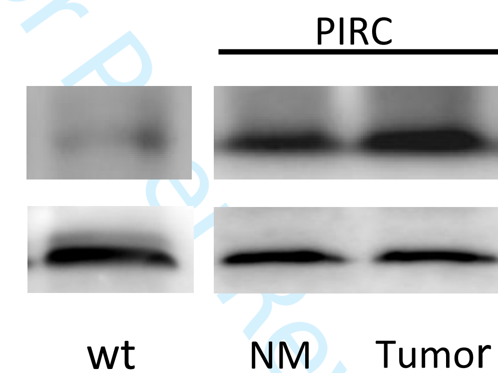

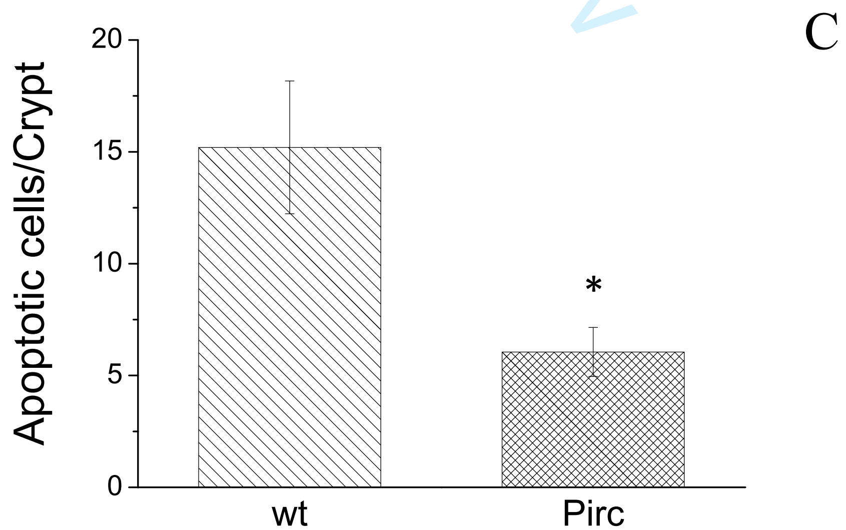

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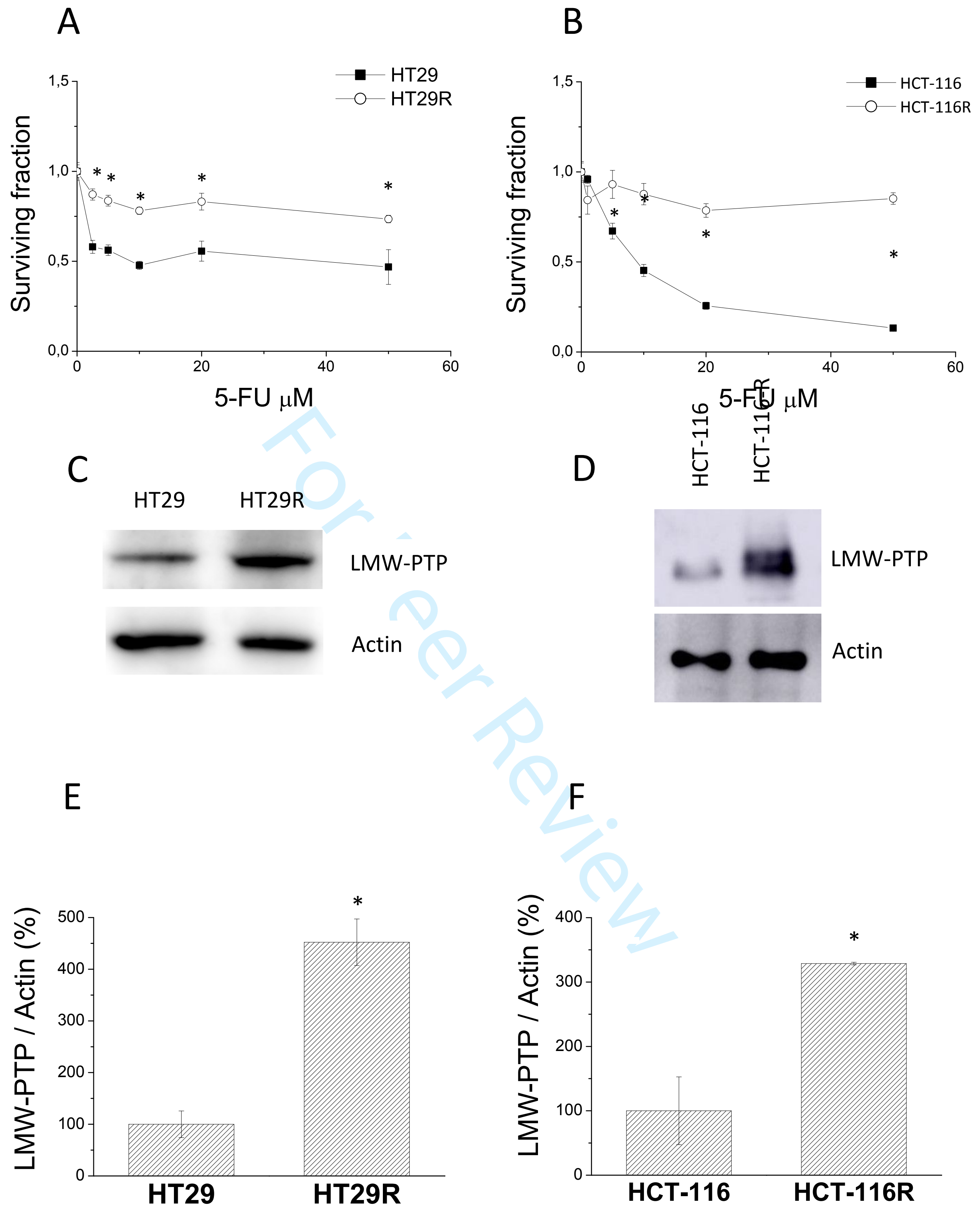

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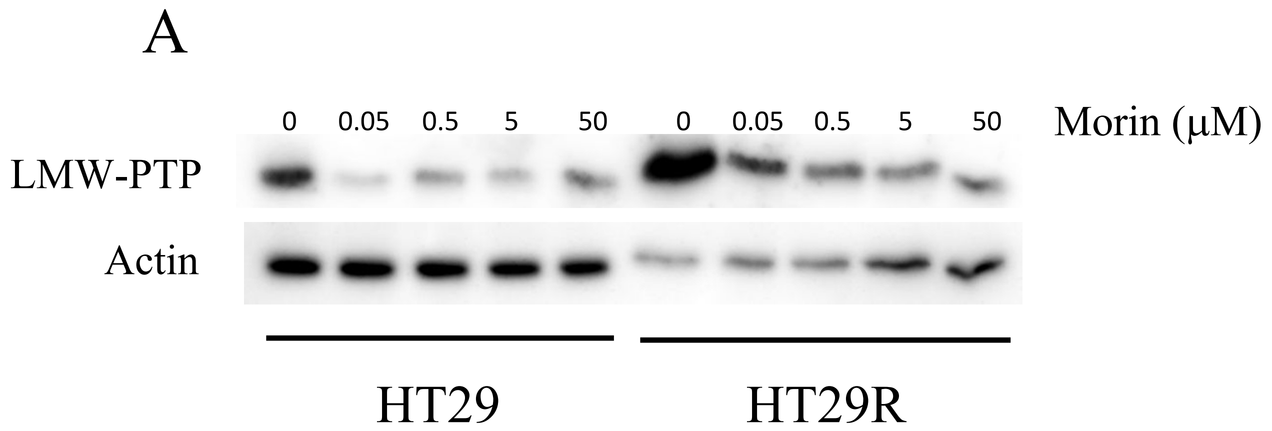

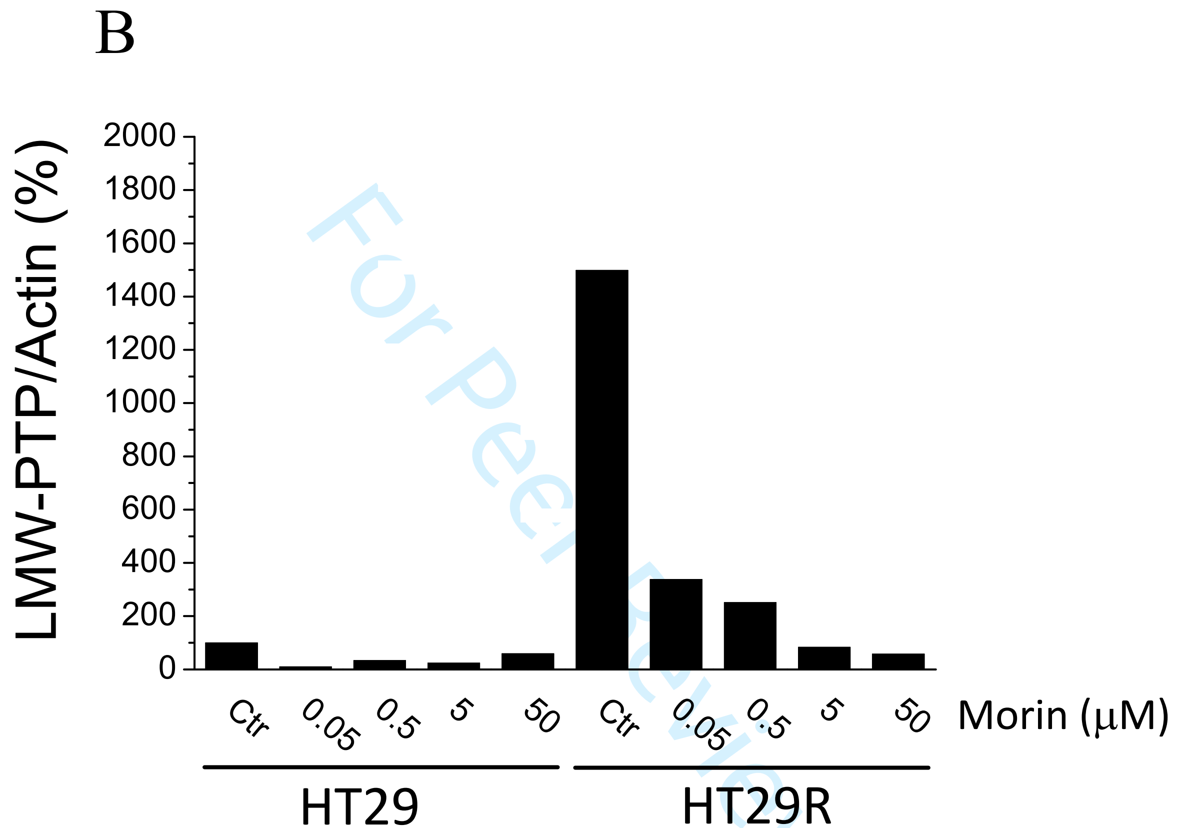

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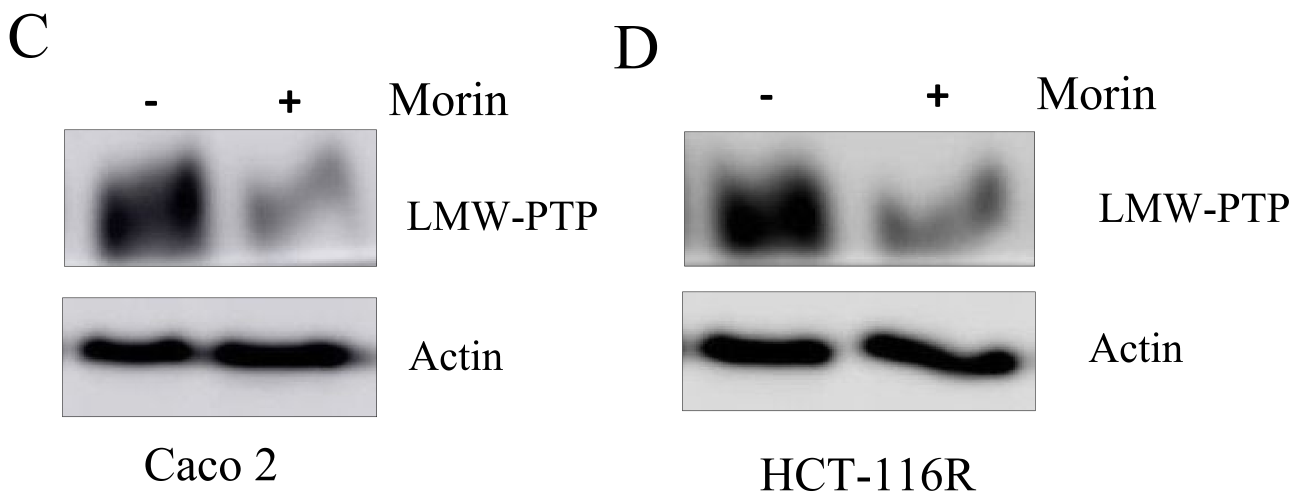

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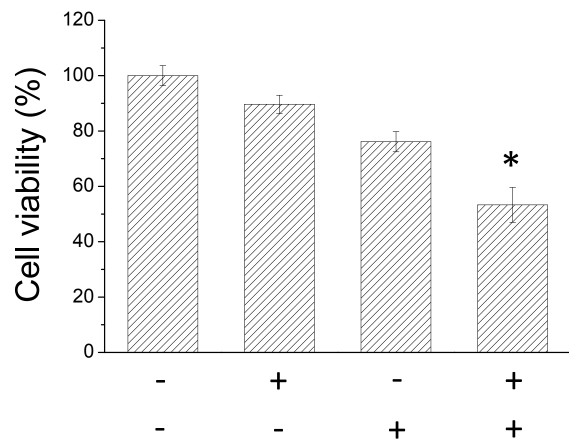

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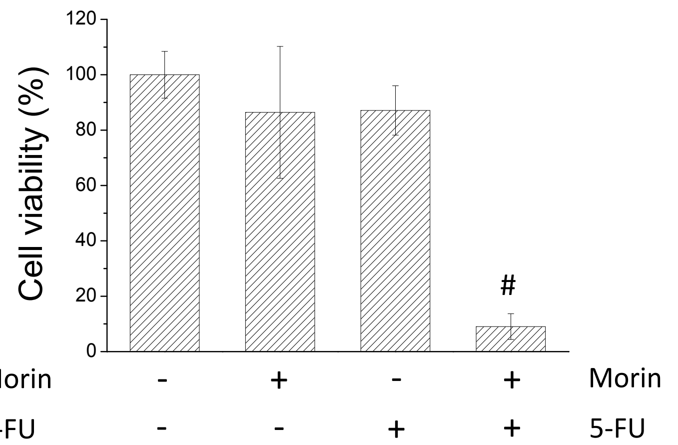

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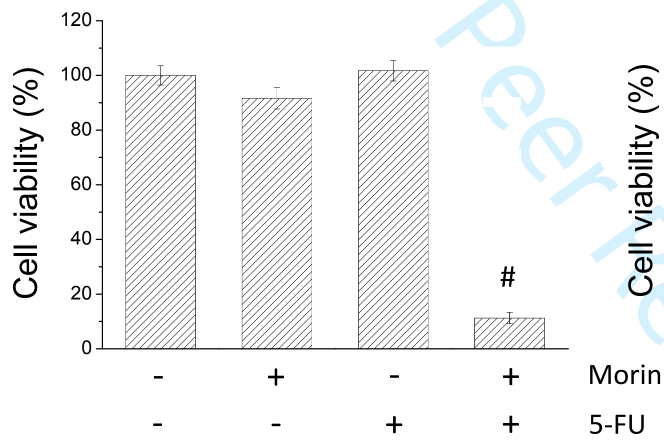

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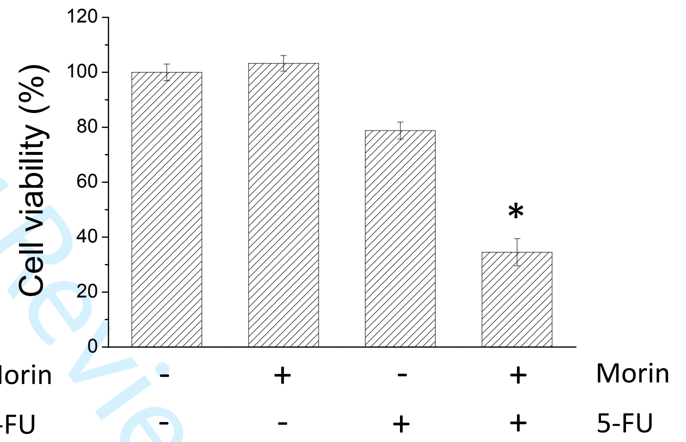

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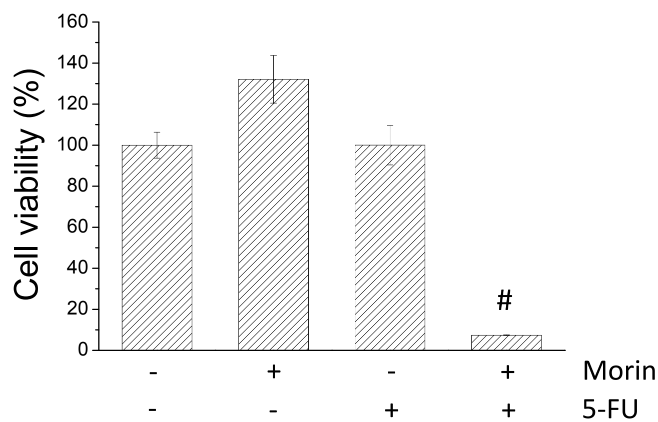

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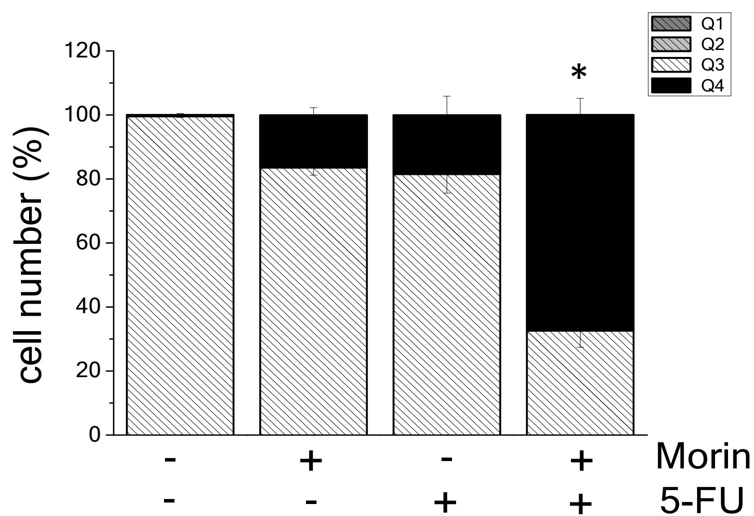

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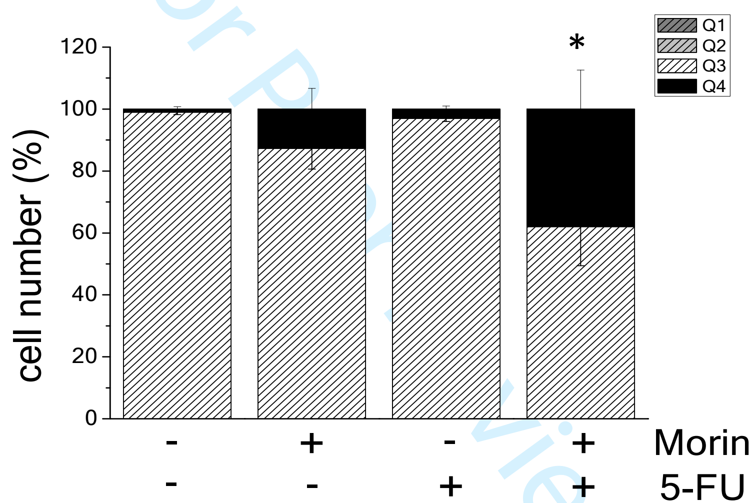

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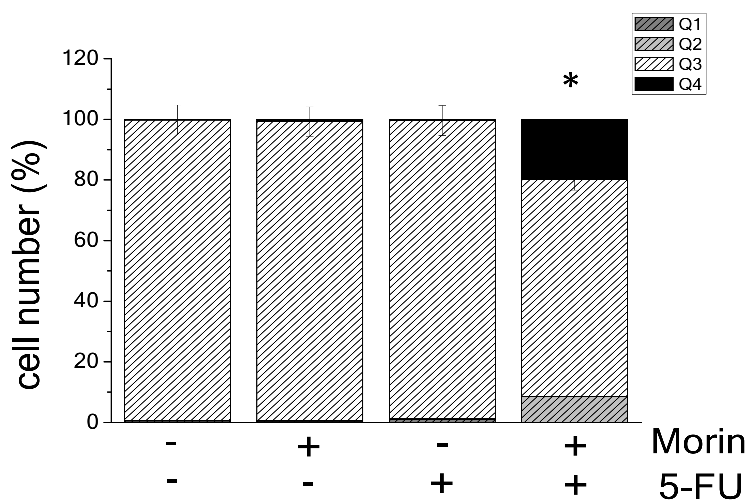

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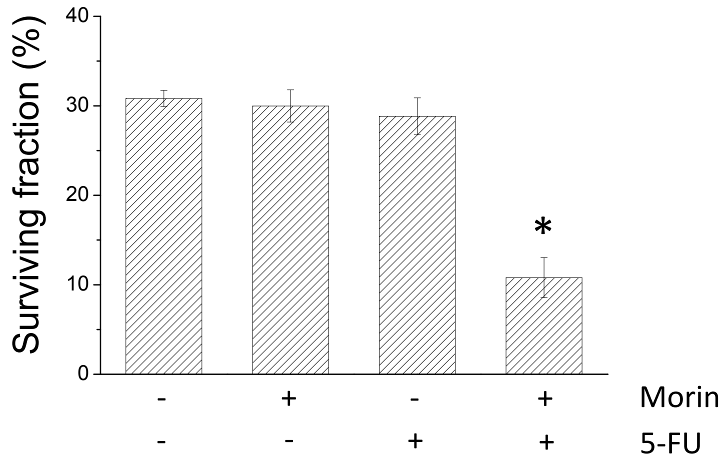

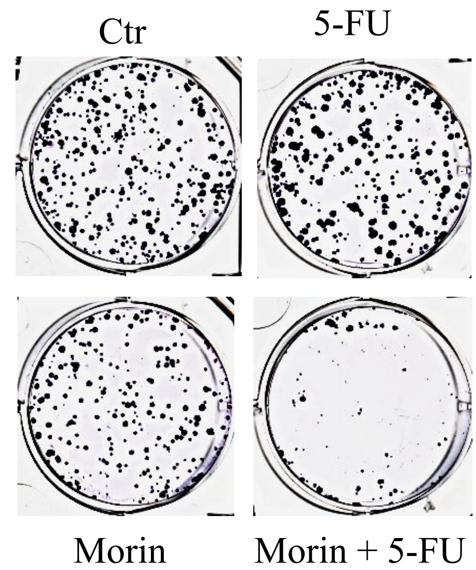

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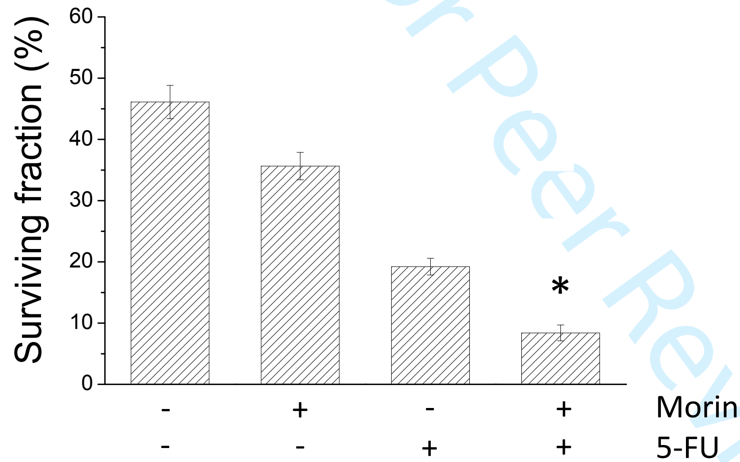

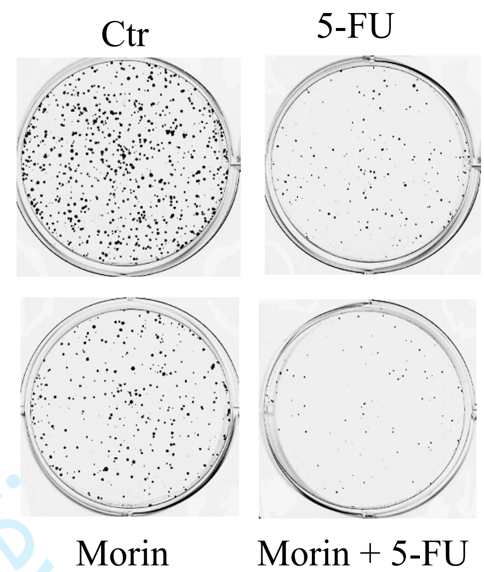

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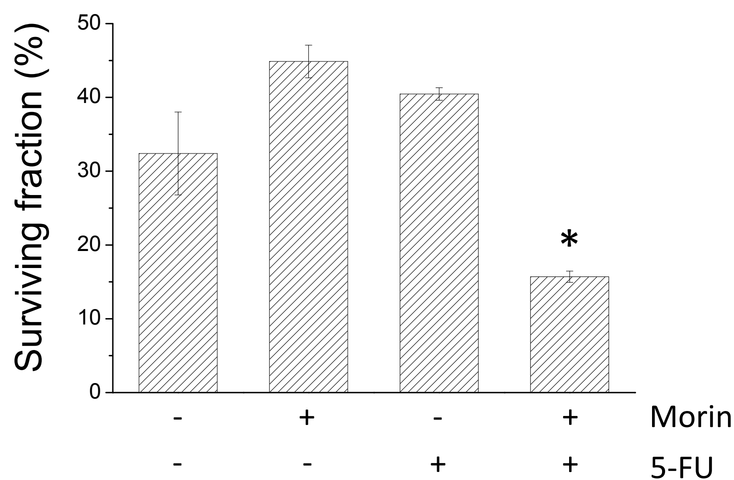

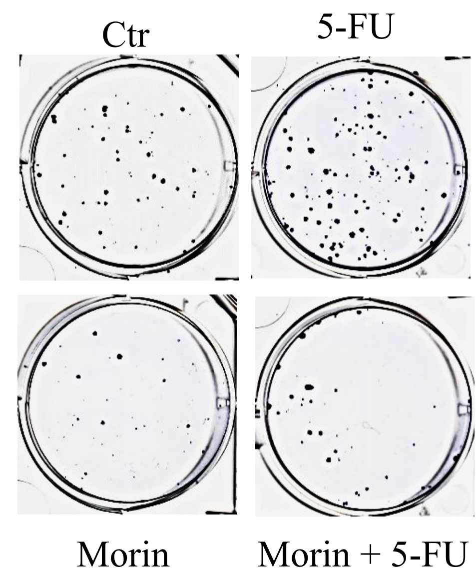

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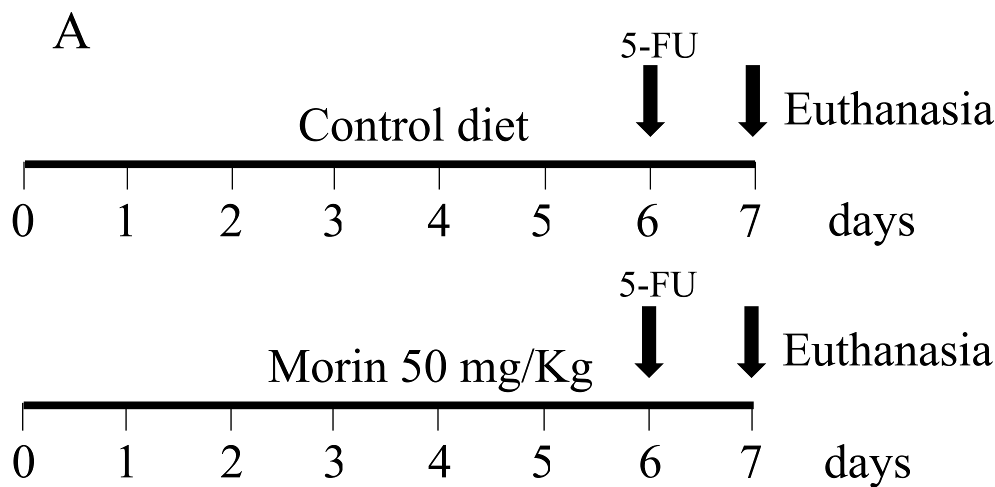

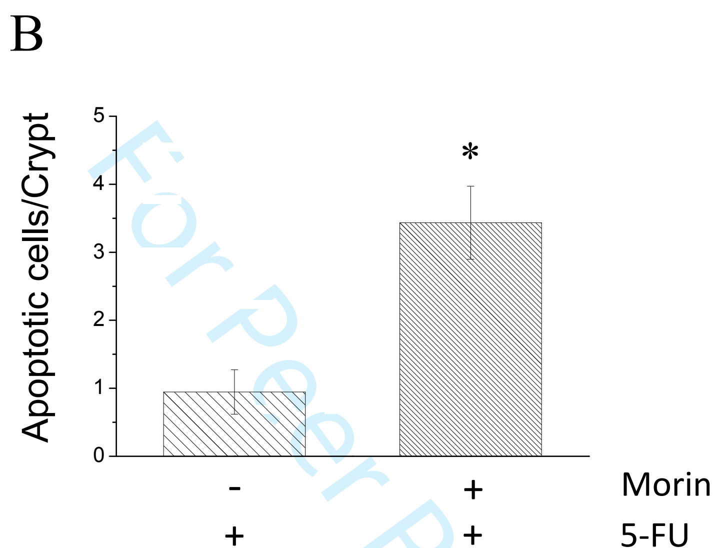

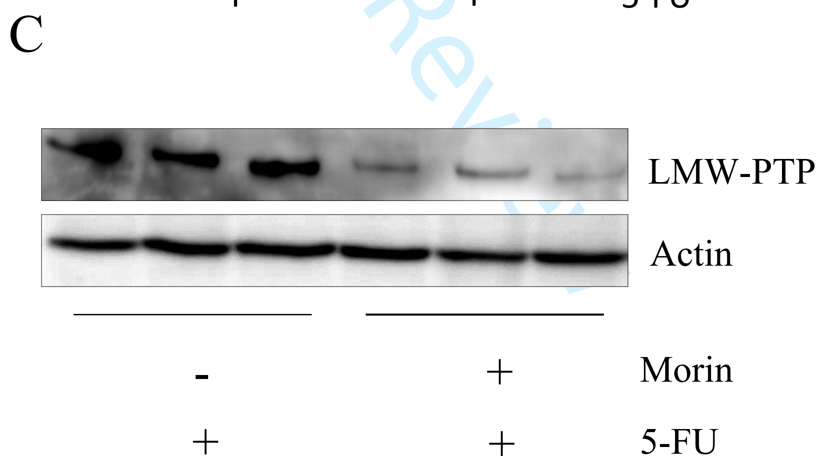

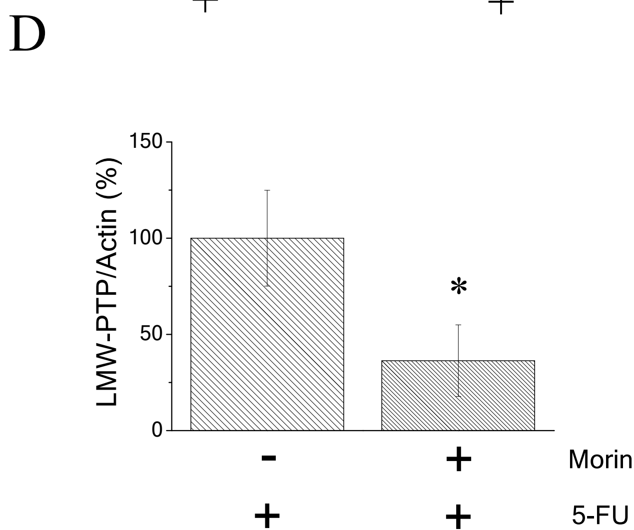

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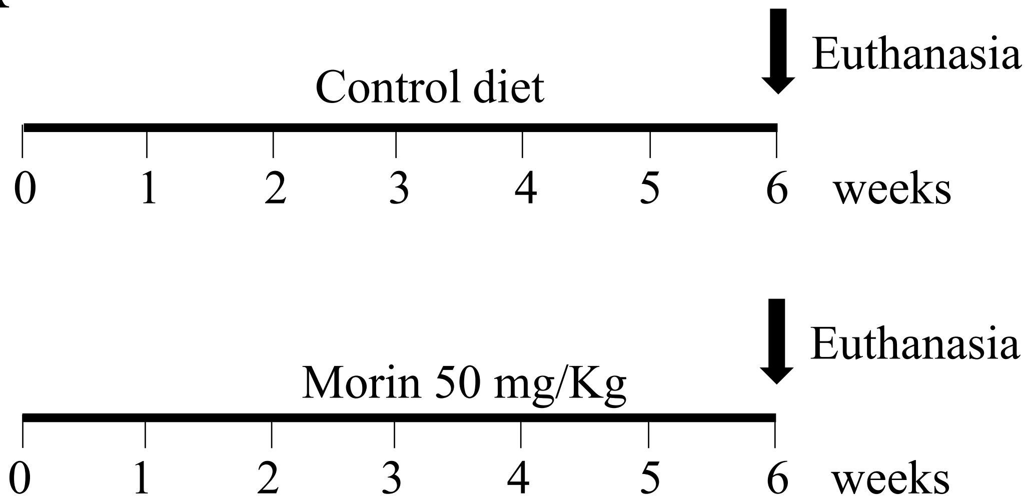

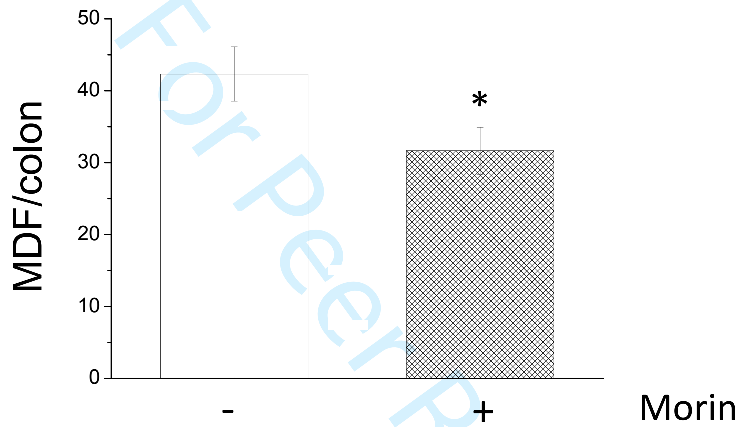

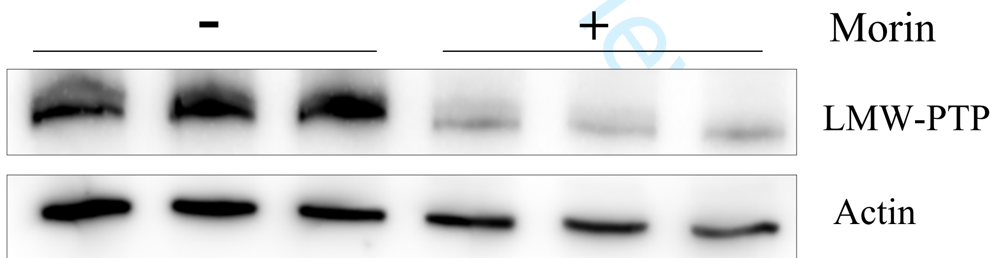

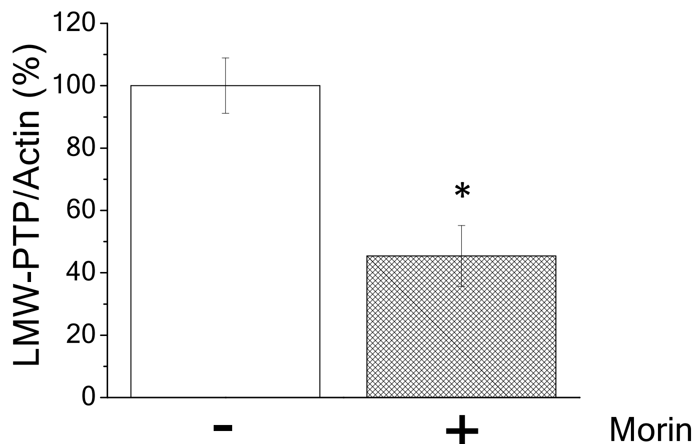

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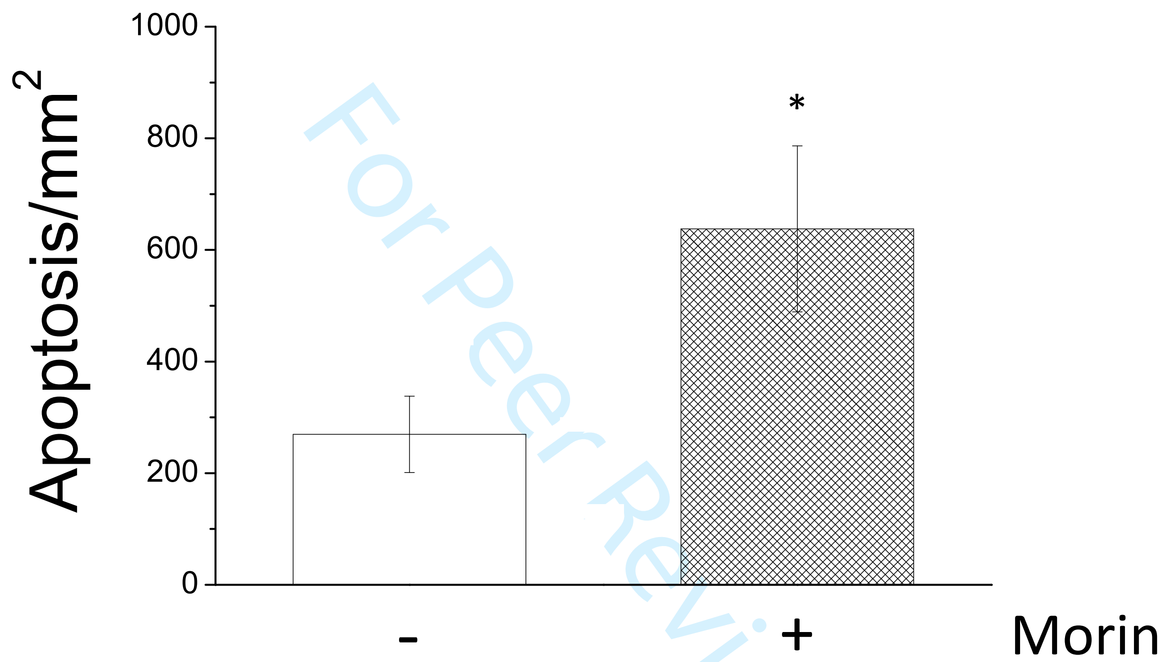
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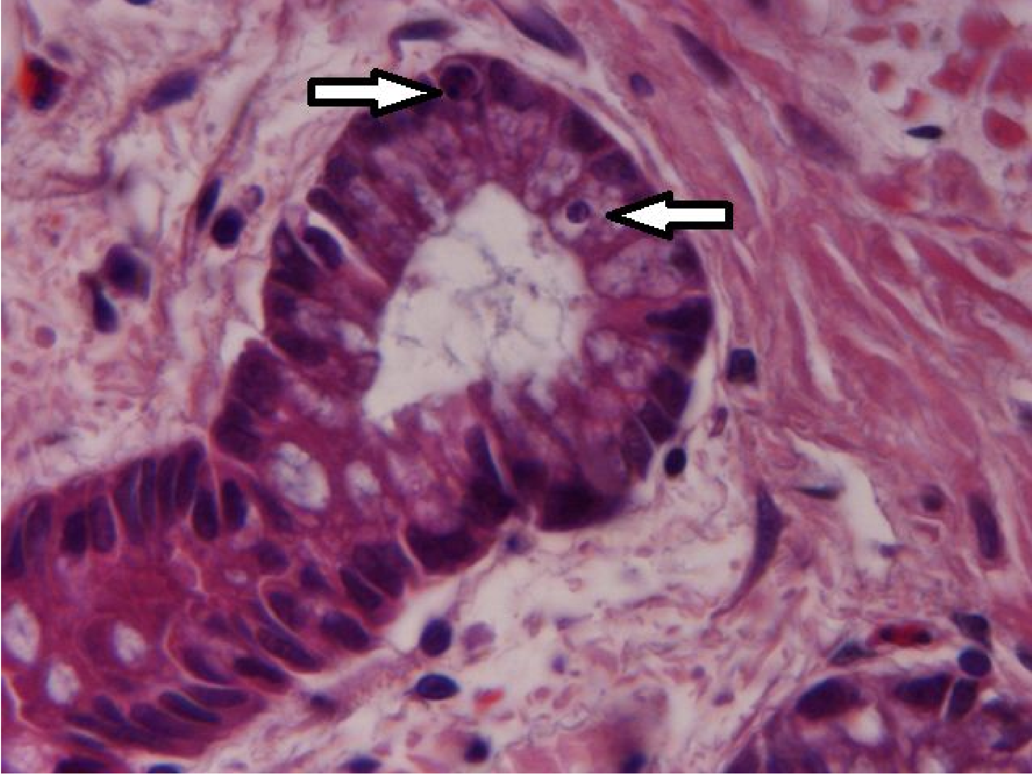

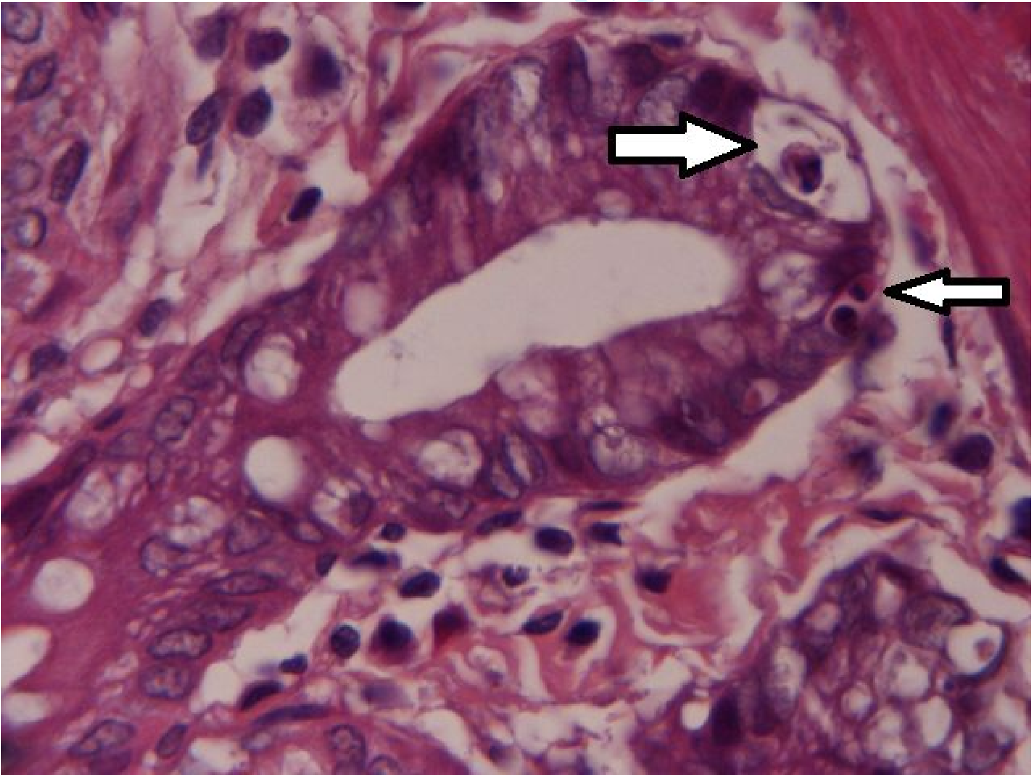

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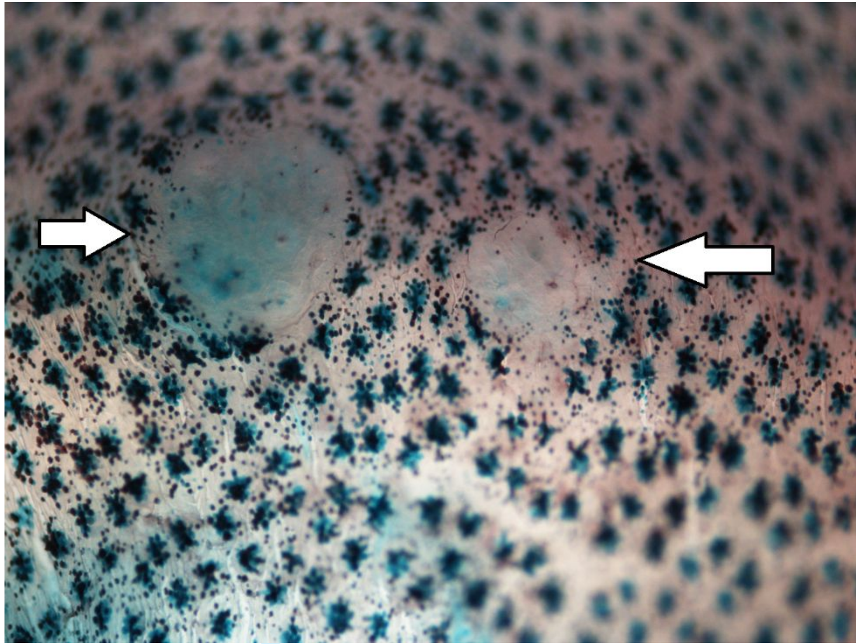

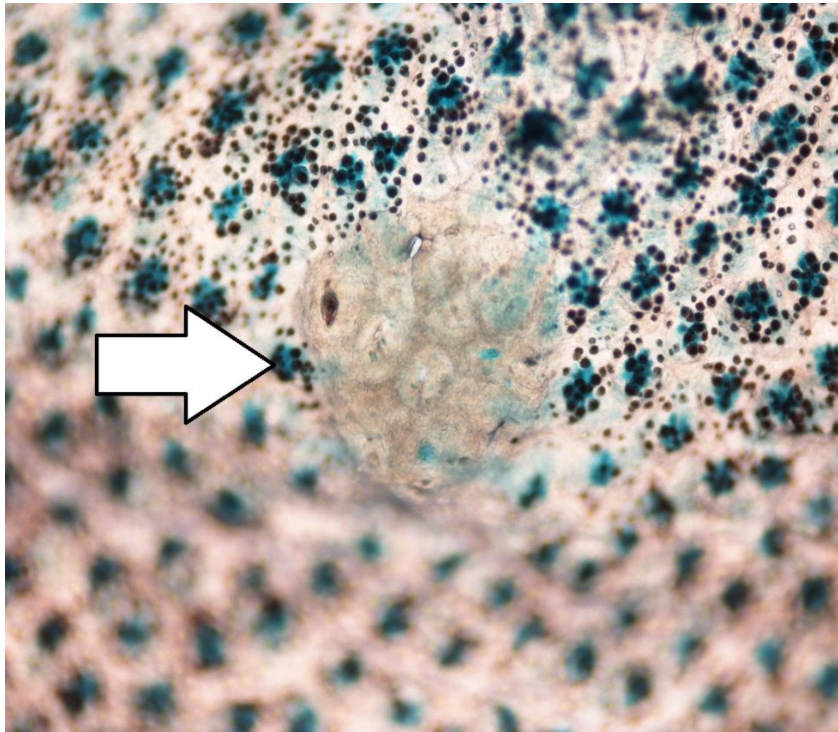

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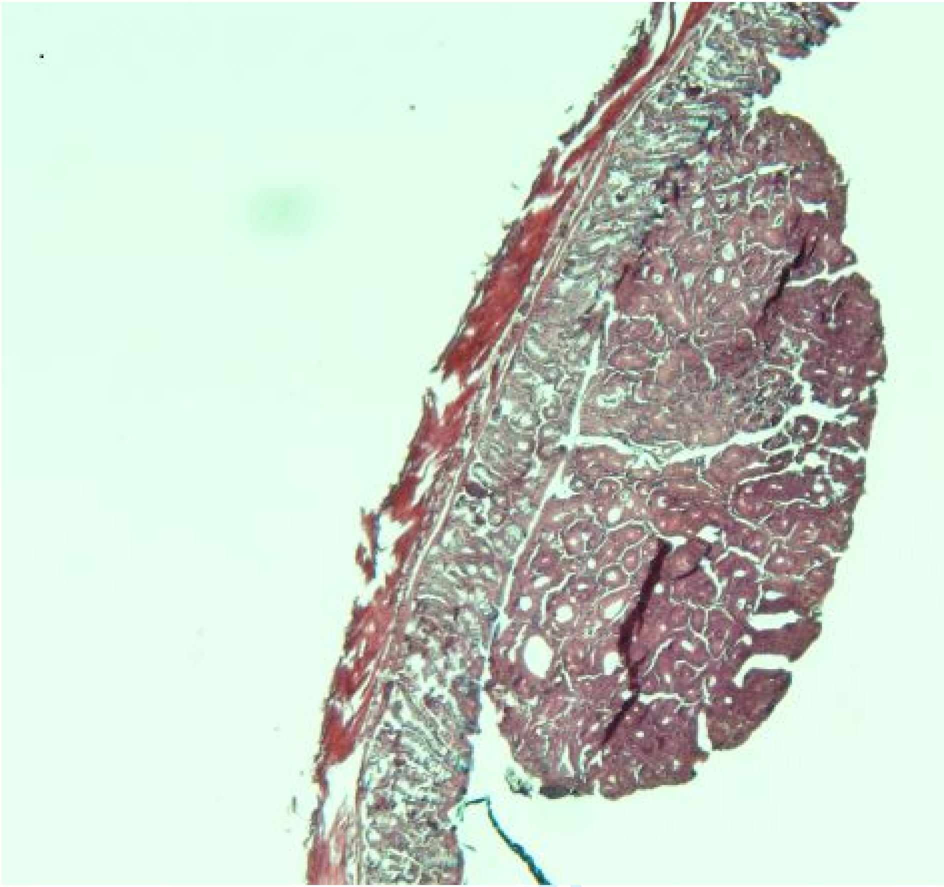

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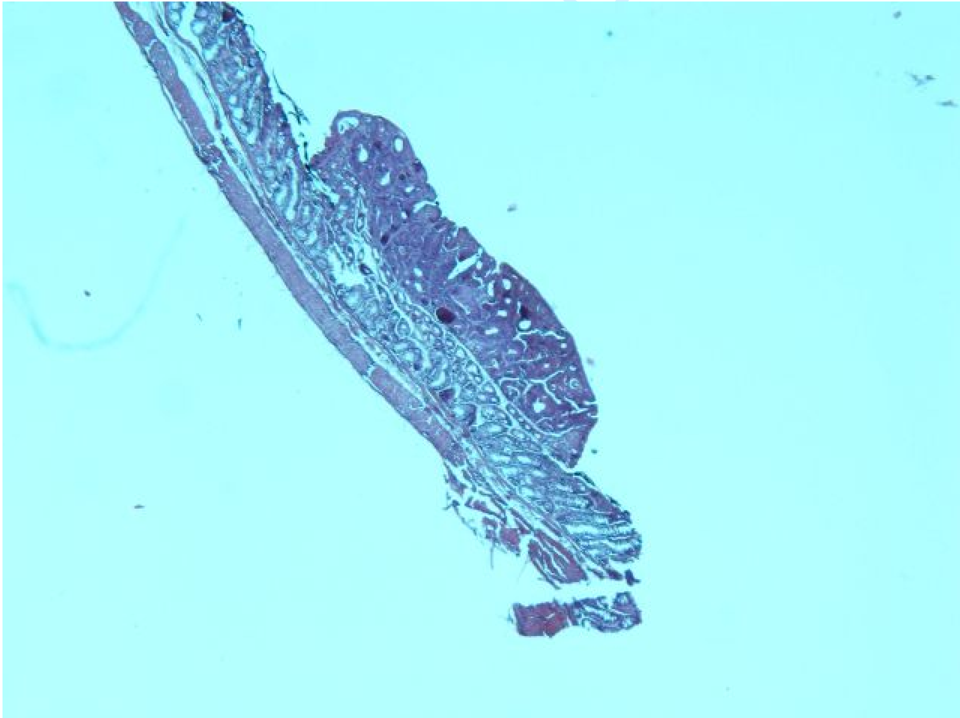

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