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HNE AND CHOLESTEROL OXIDATION PRODUCTS IN COLORECTAL INFLAMMATION AND CARCINOGENESIS

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Abstract

Consistent experimental data suggest the importance of inflammation-associated oxidative stress in colorectal cancer (CRC) pathogenesis. Inflammatory bowel disease with chronic intestinal inflammation is now considered a precancerous condition. Oxidative stress is an essential feature of inflammation. Activation of redox-sensitive pro-inflammatory cell signals and inflammatory mediators concur to establish a pro-tumoral environment. In this frame, lipid oxidation products, namely 4-hydroxynonenal and oxysterols, can be produced in big quantity so as to be able to exert their function as inducers of cell signaling pathways of proliferation and survival. Notably, an important source of these two compounds is represented by a high fat diet, which is undoubtedly a risk factor for inflammation and CRC development. Current evidence for the emerging implication of these two oxidized lipids in inflammation and CRC development is discussed in this review.

Keywords: 4-hydroxynonenal; oxysterols; IBD; CRC; colon; intestinal; antioxidant response; survival.

Abbreviations: **7K**, 7-ketocholesterol; **7 β OH**, 7 β -hydroxycholesterol; **22OH**, 22-hydroxycholesterol; **24OH**, 24-hydroxycholesterol; **25OH**, 25-hydroxycholesterol; **27OH**, 27-hydroxycholesterol; **8-OHdG**, 8-hydroxy-2'-deoxyguanosine; **Akt**, AKT serine/threonine kinase 1; **AP-1**, activator protein-1; **CAC**, colitis-associated cancer; **CCR7**, chemokine receptor-7; **CD**, Crohn's disease; **COX**, cyclooxygenase; **CRC**, colorectal cancer; **DSS**, dextran sulfate sodium; **ERK**, extracellular signal-regulated kinase; **FOXO**, forkhead box protein O1; **GSH**, glutathione; **GSSG**, glutathione disulfide; **GSTA4**, glutathione S-transferase alpha 4; **HNE**, 4-hydroxynonenal; **HNE-dG**, HNE-deoxyguanosine; **HO-1**, heme oxygenase-1; **IBD**, inflammatory bowel disease; **I κ B**, inhibitor of κ B; **IKK**, I κ B kinase; **IL**, interleukin; **JAK**, Janus kinase; **JNK**, c-Jun N-terminal kinase; **LOX**, lipoxygenase; **LT**, leukotriene; **LXR**, liver X receptor; **MAPK**, mitogen activated protein kinase; **MCP-1**, Monocyte Chemoattractant Protein-1; **MMP**, matrix metalloproteinase; **NADPH**, nicotinamide adenine dinucleotide phosphate; **NF- κ B**, nuclear factor- κ B; **NLRP3**, NLR family pyrin domain containing 3; **NOX**, NADPH oxidase; **NRF2**, nuclear factor-erythroid 2-related factor 2; **p38**, protein 38; **PG**, prostaglandin; **PI3K**, phosphoinositide 3-kinase; **PUFA**, polyunsaturated fatty acid; **ROS**, reactive oxygen species; **RNS**, reactive nitrogen species; **STAT**, signal transducer and activator of transcription; **TGF β 1**, tumor growth factor β 1; **TNBS**, 2,4,6-trinitrobenzene sulfonic acid; **TNF**, tumor necrosis factor; **TRL**, toll like receptor; **TrxR1**, thioredoxin reductase 1; **UC**, ulcerative colitis.

1. Introduction

Chronic inflammation contributes to the pathogenesis of the majority of diseases considered as leading causes of mortality in Western countries. Its influence in the development and progression of different types of cancer has been widely accepted, especially in those tissues that are easily exposed to injury by environmental agents such as intestinal mucosa. Inflammation can be triggered by cellular stress and dysfunction caused by excessive calorie consumption, with high production and storage of lipids, and elevated blood glucose levels involved in oxidative metabolic pathways [1].

On the basis of epidemiologic studies, colorectal cancer (CRC) risk is strongly associated with red and processed meat intake resulting in high quantity of fats [2]. World red meat consumption has increased from 1990 to 2010 especially in emerging economies in relation with rising income and rapid urbanization [3]. Diet with high animal fat and low in fruits and vegetables is the most common pattern associated with an increased risk of developing Inflammatory Bowel Disease (IBD), a group of intestinal diseases characterized by chronic intestinal inflammation that includes Ulcerative colitis (UC) and Crohn's Disease (CD) [4]. Therefore, policy efforts should be especially focused on limiting the consumption of red meat and avoiding the consumption of processed meat as recommended by worldwide nutritional organizations [5].

Cholesterol and fatty acids, which are the major constituents of cellular membranes, are crucial for the maintenance of their structure and normal functioning, and represent an important part of diet. Unfortunately, excess dietary fats of animal origin can induce the formation of large quantity of oxidized molecules that initiate lipid oxidation processes able to generate different end-products that could contribute to the loss of intestinal epithelial barrier function and the production of pro-inflammatory molecules. Vice versa, during inflammation activated leukocytes can contribute to maintain an oxidative microenvironment which, in all likelihood, leads to functional impairment and dysplasia of the enteric mucosa.

Among various secondary non-enzymatic lipid oxidation compounds, 4-hydroxynonenal (HNE), derived by peroxidative breakdown of ω -6 polyunsaturated fatty acids (PUFAs) in biological membranes, and oxysterols, which are cholesterol oxidation products, have received particular attention for their potential involvement in the pathogenesis of different human diseases, including cancer.

In this review, we summarize the recent progress on understanding the role of HNE and oxysterols in CRC pathogenesis, and focus on their involvement in inflammatory signaling pathways activated during carcinogenesis.

2. Inflammation and oxidative tissue reactions in colorectal cancer

The mechanism underlying CRC pathogenesis continues to require extensive investigation in the field of cancer research. In order to understand what the role of specific lipid oxidation compounds is in colorectal carcinogenesis, it is important to point out what the possible events triggering the formation of such compounds are in the tumor microenvironment.

The opinion that CRC is a consequence of specific sequences of mutated genes (*apc/β-catenin* pathway, *ras/raf*, *p53*), and epigenetic modifications has been widely accepted [6]. Of all CRCs, 5-6% accounts for hereditary types, 20-25% has a positive family history or genetic predisposition, while the majority of cases occurs sporadically as the result of somatic mutations in response to environmental factors [7].

A large number of environmental factors is strictly involved in the induction of inflammation, which, in turn, is well recognized as a major driving force in CRC initiation and promotion, as well as progression. It is, in fact, a key event in the induction of cancer genetic instability [8].

Colitis-Associated Cancer (CAC) is a subtype of CRC, which is associated with IBD. This association has been found to be responsible of deaths in up to 15% of IBD patients [9]. IBD patients with active disease show hyper-responsiveness of the host mucosa against intestinal flora with exaggerate immune and inflammatory responses. This can be caused by altered functions of pathogen recognition receptors and can lead to mucosal barrier defect with corresponding uncontrolled induction of proliferative and survival cell signals of neoplastic transformation [10, 11]. The increased risk in CAC development in IBD depends on the persistence of inflammation, actually linked to longer colitis duration and the extent of inflamed colonic mucosa [12]. The hallmark of IBD is massive infiltration into lamina propria of innate and adaptive immune cells, which generate many inflammatory cytokines included IL-1 β , IL-6 and TNF α , which play a crucial role in colorectal carcinogenesis [13].

The promoting role of inflammation in CAC has been strongly proved in different animal models in which colitis-associated neoplasia is induced chemically [14]. The most widely used mice model consists of the administration of inflammatory promoting agent dextran sulfate sodium (DSS) in association with a single initiating dose of carcinogen azoxymethane [15]. This experimental model shows IBD similar intestinal inflammatory infiltrate with multiple colonic tumors [14].

Sporadic CRC and CAC share inflammation as pathogenetic process involved in different phases of colorectal carcinogenesis. While inflammation in CAC has been considered to be involved in tumor initiation and promotion, inflammatory microenvironment in sporadic CRC can contribute to tumor progression by producing specific cytokines and chemokines, which increase aggressiveness of tumor by promoting survival and angiogenesis.

A major link between oxidized lipids production and CRC development is represented by activated inflammatory cells, which are critical actors to start and maintain the oxidative environment through repeated generation of inflammatory mediators and high levels of reactive oxygen (ROS) and nitrogen species (RNS) produced during oxidative burst. Increased levels of ROS and RNS have been found in inflamed tissues from patients with active IBD [16]. All these species participate to the different phases of colorectal carcinogenesis, being the cause of initial and additional cell mutations that can initiate CAC or favor CRC progression. Reactive radicals such as $O_2^{\cdot-}$ and HO^{\cdot} can act indirectly by non-enzymatic breakdown of membranes PUFAs leading to reactive aldehyde-end products such as HNE involved in CRC. On the other hand, H_2O_2 , $O_2^{\cdot-}$ and HO^{\cdot} can function as signaling molecules of proliferation and survival [17] or can cause massive oxidative DNA lesions such as 8-hydroxy-2'-deoxyguanosine (8-OHdG); the concentration of 8-OHdG, which is considered a marker of oxidative DNA damage relevant for mutagenesis, increases in IBD patients [18].

Cell signals involved in inflammation and CRC are jointed by the induction of the common redox sensitive nuclear factor κB (NF- κB), which is frequently activated in the carcinogenic process [19, 20]. NF- κB activation represents the rate limiting event that concurs to the induction of survival and the inhibition of apoptosis in dysplastic cells turning into tumor phenotype. Chronic activation of NF- κB in IBD intestinal tissue has been associated with oxidative stress. Increased H_2O_2 levels counteract the activity of thioredoxin1, an antioxidant small protein that can inhibit NF- κB activation by interacting with I $\kappa B\alpha$.

inhibitor and thereby preventing its phosphorylation by I κ B kinase (IKK); O $_2^{\cdot-}$ production, and consequently NF- κ B activation, has been also ascribed to the TNF α -mediated induction of NADPH oxidase enzyme, which is actively involved in phagocytic function [21].

The activity of NF- κ B itself is regulated by other transcription factors, in particular signal transducer and activator of transcription (STAT) 3, whose activity has been strongly associated to oncogenesis [22]. NF- κ B, IL-6 and STAT3 have been shown to represent the signaling axis, which can regulate proliferation and survival of tumor initiating intestinal epithelial cells [23]. Persistent activation of STAT3 in UC colonic tissue involves cytokine production such as IL-22, IL-6 and TNF α enabling progression towards CRC [24]. TNF α activity is also strongly involved in IBD pathogenesis for its pro-inflammatory and pro-apoptotic properties; in fact, TNF α blockers have become a mainstay in the therapy of IBD [25]. NF- κ B and STAT3 have been shown to improve β -catenin transcription, whose hyperactivation is present in the majority of sporadic and familial CRCs [26]. Targeting NF- κ B signaling pathway by using antioxidants such as polyphenols has been now considered as an emerging approach to prevent CRC development [27, 28]. The discovery of redox-sensitive Nuclear Factor-Erythroid 2-Related Factor 2 (NRF2) has been very interesting. This transcription factor plays a key role in the maintenance of redox balance in intestinal mucosa, being activated by increased oxidative cell conditions to transcribe several genes coding for antioxidant enzymes [29]. NRF-2 has been suggested protecting against CRC-associated inflammation in colitis animal model [30]. However, NRF-2 plays a contradictory role in carcinogenesis depending on different phases of cancer. It suppresses tumor development at the earliest stages, but its over-expression has been found in later stages of malignancy, during which the already transformed cancer cells need to maintain intracellular antioxidant status by inducing cellular resistance against anticancer therapies [31]. Principal events implicated in the induction of mucosal barrier damage during inflammatory reactions that can lead to tumor growth are shown in Figure 1.

Dietary lipids, in particular ω 6-PUFAs, provide the main substrates for cyclooxygenases (COXs) and lipoxygenases (LOXs) enzymes, and for lipid peroxidation. Phagocytes in the colonic lamina propria generate high levels of eicosanoids produced through COX-2 induction in presence of ω 6-arachidonic acid as substrate. Eicosanoids prostaglandins (PGs) and leukotrienes (LTs) may in turn activate lymphocytes to synthesize either pro- or anti-inflammatory mediators in response to dietary antigens [32].

Different studies suggest COX-PG pathway as an emerging signal axis in the regulation of tumorigenesis [33, 34]. The well known inflammatory mediator PGE2 has been demonstrated to be responsible for the activation of phosphoinositide 3-kinase (PI3K) p85 α , extracellular signal-regulated kinase 1 (ERK1), and NF- κ B pathways in epithelial tumor cells isolated from human CRC specimens and cecal tumor tissues from *Apc^{min}* mice [35]. The activation of these cell signals by PGE2 can be responsible for increased β -catenin signaling in CRC [36]. PGE2 and LTD4 were found to favor tumor microenvironment in inducing cancer stem cells and their survival in a nude mouse xenograft model [37, 38].

High levels of the inducible COX-2 have been experimentally detected in association with poor prognosis in CRC patients [39]. COX-2 and PGE2 synthase mPGES1 have been shown to be co-expressed in both colorectal cancer lines and CRC [40]. Blocking the COX pathway with acetyl salicylic acid has been considered as chemopreventive strategy for colon cancer [41]. The administration of non-steroidal anti-inflammatory 5-aminosalicylates can reduce the risk of CRC development in IBD patients [42, 43], probably having anti-cancer effect by blocking DNA strand break induced by radicals [44]. Therefore, cancer chemoprevention through COX-2 inhibition by therapeutic agents has been considered for their ability to prevent oxidative damage during inflammatory process in intestinal diseases.

3. Role of 4-hydroxynonenal in colorectal carcinogenesis

The implication of 4-hydroxynonenal (HNE) in the pathogenesis of a wide variety of human diseases characterized by strong activation of inflammatory processes has been established unequivocally.

HNE has been identified as a breakdown product of cell membrane ω 6-PUFAs as linoleic acid and arachidonic acid. HNE is an amphiphilic molecule, being water-soluble with stronger lipophilic properties normally occurring in a number of different tissues and body fluids. Due to its high chemical reactivity, HNE is considered a crucial molecule in cell signaling activation. The main biological effects of HNE are due to covalent modifications of important biomolecules including proteins, DNA, and phospholipids containing amino and thiol groups. Low levels of this aldehyde are generated during physiological turnover of cell membranes through lipid peroxidation. However, an elevated status of oxidative stress critically enhances HNE production. Therefore, HNE activity depends on its concentration in cellular systems: its low

concentration can be involved in enzyme modulation, cell signal transduction and gene expression, whereas high HNE levels can exert deleterious events such as inflammatory and cytotoxic effects [45, 46].

In fact, HNE is believed to be one of the major player contributing to the mutagenic and carcinogenic effects of lipid peroxidation [47].

Most of the biochemical effects of HNE are due to its structure containing three functional groups, which consist of a C=C double bond at carbon 2, a C=O carbonyl group at carbon 1, and a hydroxyl group at carbon 4. Altogether they provide high positive charge to HNE and frequently act synergistically to form covalent adducts with macromolecules by a) modifying lipid membrane properties, i.e. alteration of membrane fluidity or loss of phospholipid asymmetry, b) inducing both conformational changes of functional proteins, especially those involved in cell signaling pathways determining antioxidant response, and c) reacting with all four DNA bases, preferably with deoxyguanosine to form stereoisomeric HNE-deoxyguanosine (HNE-dG) adducts. An increase in etheno-modified DNA bases generated by DNA reaction with HNE produced during oxidative inflammatory processes has been found in the inflamed pancreatic tissue of patients with chronic pancreatitis and in the colon mucosa of IBD patients [48].

Proteins are the most important group of biomolecules targeted by HNE (about 1-8 % of produced HNE is able to modify proteins). HNE forms adducts with three different side chains in proteins, namely Cys, His, and Lys. HNE-targeted proteins are more susceptible to degradation by the proteasomal pathway, which is responsible for most intracellular proteolysis. Cys residues are always the preferential targets of HNE in proteins; rapid conjugation of HNE with glutathione (GSH) markedly depletes this antioxidant in different cell types [45].

HNE exerts a plethora of effects of pathophysiological significance, often in a dose-dependent manner and with opposite carcinogenic or anti-carcinogenic activity, depending on the type of proteins targeted by HNE and their functional relevance in biological systems [49].

Bioactive low concentrations of HNE (ranging from 1 to 10 μ M) have been found to induce apoptosis and inhibit cancer cell growth in CaCo-2 and HT-29 cell lines by modulating cell cycle progression: it inhibits c-myc and p21 gene expression [50], and telomerase activity [51], reinforces TGF β 1 pro-apoptotic effect by activating c-Jun N-terminal kinase (JNK) that synergistically cooperates in increasing cytokine transduction signaling molecule Smad4 [52].

Thanks to the applied technique based on the detection of antibodies directed against HNE-protein adducts in biological systems, high levels of HNE have been consistently detected in CRC. Formation of HNE-protein adducts in colon cancer tissues associated to the decrease of non-enzymatic level of antioxidants, such as vitamins C and E as well as GSH have been related to the growth and progression of CRC [53].

On the other hand, HNE has been suggested to be more cytotoxic in normal cells than in neoplastic cells at same concentration, suggesting its involvement in colon carcinogenesis in the selection of mutated cell clones. Apc-mutated cells show high capability in HNE biotransformation, being able to upregulate enzymes involved in HNE metabolism, such as aldehyde dehydrogenase, glutathione S-transferase alpha 4 (GSTA4) or cystine transporter, thus allowing survival advantage for the mutated cells exposed to a peroxidative environment [54]. Increased production of HNE-protein adducts in human colon adenoma and CRC has been found associated with strong expression of phase II detoxifying enzyme GSTA4, which is involved in the metabolism of both carcinogens and HNE. GSTA4 has been found activated during the early stages of inflammation in macrophages and the epithelial tissue deriving from an animal model of colitis/CRC consisting of interleukin-10 knockout (IL-10^{-/-}) mice colonized by *E. faecalis*. [55]. Notably, the expression of GSTA4 depends on HNE ability in inducing NRF2 and c-Jun in colon cancer cells, two components of AP-1 oncogenic transcription factor [55]. HNE can function as alarm signal for oxidative changes, which activate NRF2 in vascular cells [56]. NRF2 controls the transcription of important antioxidant cytoprotective enzymes including heme oxygenase-1 (HO-1), and thioredoxin reductase 1 (TrxR1) that may have oncogenic potential in fully transformed cancer cells especially in resistance to anticancer treatments [13]. This evidence underlines that transformed tumor cells undergo metabolic changes by inducing inactivation of oxidative mediators favoring tumor growth. This behavior is consistent with previously observed results in CRC patients highlighting a decreased content of HNE-protein adducts in T2 CRC tissue compared to normal tissue [57]. Notably, TGFβ1 that could promote selective growth of neoplastic cells have been shown to be up-regulated by HNE and cholesterol oxidation products in colon adenocarcinoma CaCo-2 cell line [58].

Intestinal inflammation related to CRC induction plays a major role in the alteration of intestinal barrier structure and function. HNE can be involved in the mucosal layer derangement due to increased

generation of intracellular H₂O₂, thus causing reduction in intestinal permeability and defense function against luminal agents [59]. Matrix metalloproteinases (MMPs) have been clearly implicated in tumor invasiveness because of their properties to regulate the integrity of physical barriers and transmigration of leukocytes from vasculature to tissue. MMP-9 induction affects intestinal epithelial membrane permeability contributing to the severity of experimental DSS colitis mice model [60]. These findings are consistent with previous observations showing a net increase of serum levels of MMP-9 and IL-8 in advanced stages of CRC [61]. Furthermore, HNE has been found to enhance cell release of IL-8, IL-1 β , and TNF α and to up-regulate MMP-9 via TLR4/NF- κ B-dependent pathway in inflammatory cells, possibly implicating p38 MAPK and ERK activation [62, 63] (Figure 2).

Diet may influence tissue levels of HNE being its precursors arachidonic and linoleic acids often abundant in Western diets [64]. HNE can be produced upon peroxidation of dietary lipids from food transformation and digestion, reaching significant high toxic concentrations at intestinal level. Different concentrations of malonaldehyde, HNE, and 4-hydroxyhexenal were evaluated in gastric and intestinal lumen during digestion of meals containing fish or isolated herring oils [65]. A meta-analysis of prospective cohort studies on relative risk of colon cancer in association with heme iron intake with red meat supports evidence that consumption of highly processed meats induces the formation and activity of cyto- and genotoxic oxidation products including HNE [66].

Increased concentration of mercapturic acid of dihydroxynonane, which is one of the major HNE urinary metabolite, has been found highly dependent on the dietary factors in rats fed a diet rich in heme iron, ferric citrate, ω -6 or ω -3-rich oils [67, 68].

However, the amount of HNE also depends on antioxidant intake as well as by intestinal microbiota. HNE and its urinary metabolites have been found to be reduced in humans in response to increased plasma antioxidant concentration [69]. Antioxidant dietary supplementation with α -lipoic acid reduces HNE plasma levels and prevents diabetes mellitus complications in rats, by inhibiting oxidative DNA damage in peripheral lymphocytes [70]. An *in vitro* model of dietary fiber fermentation has shown a reduction of HNE-induced DNA damage and increased GST in intestinal colon cancer cells [71]. In male Sprague-Dawley rats, in which colitis was induced by lipopolysaccharide injection, a transient increase of HNE has shown to reduce bactericidal activity by promoting IgA immunoglobulins polymerization in intestinal mucosa [72]. In

experimental models of colitis-associated dysplasia induced in mice by DSS or 2,4,6-trinitrobenzene sulfonic acid (TNBS) the observed increased proliferation of colon *Enterobacteriaceae* able to produce high levels of HNE and malonaldehyde, which in turn may activate TLR-4/NF- κ B inflammatory pathway has been suggested as possible mechanism of colitis induction by the two chemicals [73].

The consistent evidence of HNE production during inflammatory processes, which shows strong reactivity with important functional biomolecules, stresses the role of this aldehyde as an effector of oxidative intestinal damage in IBD, which can lead to CRC development.

4. Role of oxysterols in colorectal carcinogenesis

Besides aldehydes produced by lipid peroxidation of cell membranes, at least as for high dietary consumption of animal fats, other oxidation products may be involved in activation of inflammatory processes that can lead to CRC development in the intestine. Among these, oxysterols produced during cholesterol oxidation have recently been considered for their potential impact on different phases of colorectal carcinogenesis.

Cholesterol is a multifunctional molecule, since it is an essential component of eukaryotic cell membranes and hydrophobic core of lipoproteins, it is a precursor for biosynthesis of bile acids, steroid hormones, and vitamin D. Like PUFAs, cholesterol is highly susceptible to oxidative reactions, and oxysterols are the most important intermediates of cholesterol biotransformation playing a main role in the regulation of cholesterol homeostasis. Oxysterols can originate endogenously both by enzymatic activity of cytochrome P450-dependent/independent hydroxylases and by non enzymatic cholesterol auto-oxidation. The exogenous source of oxysterols is food with high content of cholesterol (dairy products, milk, eggs, dried egg powder, clarified butter, meat products, and dried or stored fish), which can undergo non enzymatic auto-oxidation during their processing, mainly during the exposure to heat treatments and long-term storage.

For many years oxysterols have been mainly considered for their physiological role associated to cholesterol function. Over the last two decades much emphasis has been given to oxysterols for their potential action as signaling molecules. In particular, oxysterols-derived enzymatically such as 22-, 24-, 25- and 27-hydroxycholesterol (22OH, 24OH, 25OH and 27OH) have been recognized as very good ligands of

Liver X Receptors (LXRs) [74], which act as master transcription factors in cell metabolism and proliferation, inflammation and immunity [75, 76].

However, altered cholesterol metabolism with production of high content of oxysterols in biological systems are unanimously recognized to be of interest in human inflammatory degenerative pathophysiology. Today, substantial evidence shows that certain oxysterols contribute to the development and progression of atherosclerosis, neurodegenerative Alzheimer's disease, age-related macular degeneration and more recently IBD [77]. High cholesterol diet in azoxymethane-treated mice has been shown to promote CRC by increasing inflammatory IL-1 β and up-regulating NLRP3 inflammasome activity, which is particularly involved in colitis development [78].

The interest on oxysterols' impact is recently increased because of their potential role in controlling immune cell functions of the innate and adaptive responses through LXR modulation [for a comprehensive review on the role of oxysterols in the control of immune system see 79].

LXR signaling activation negatively affects proliferation; therefore, those oxysterols that are agonist of LXR have been generally considered to act as negative modulators of tumor growth. LXR activation by 22OH and 24OH has been found to inhibit proliferation of Colo205 and HCT116 colon cancer cell lines. Oxysterols delay colon cancer growth by blocking cell cycle progression in G1 phase, and inducing apoptosis via caspase activation; experiments performed in xenograft models of mice where HT29 cells injected subcutaneously into the flanks confirmed these findings [80, 81].

On the other hand, this signaling pathway might concur to create an immunosuppressive tissue microenvironment, which could favor tumor growth by facilitating immune escape process. Different tumor cells of human and murine origin including colon cancer can release oxysterols that block the expression of CC chemokine receptor-7 (CCR7) on dendritic cells (DCs) maturing by LXR activation thus allowing tumor escape from immune surveillance by inhibiting DC migration to tumor-draining lymph nodes [82, 83].

The potential involvement of oxysterols in inducing cancer through an LXR-independent mechanism that modulate immune and inflammatory cells has also been suggested [76]. Oxysterols 22OH, 24OH and 27OH have been observed to accumulate in the microenvironment of different tumor grafts in mice, where they are potentially able to chemoattract pro-tumorigenic inflammatory cells [84]. Consistent evidence of oxysterols involvement in cell pro-survival response and metastatization has been provided by studies

performed on U937 promonocytes: cell treatment with 27OH induced ERK and Akt phosphorylation, O₂^{·-} and H₂O₂ increased levels, NRF2 up-regulation, and MMP-9 activation via TLR4 and NF-κB induction [85, 86, 62]. Notably, ten years ago Bai and colleagues already suggested the pro-inflammatory properties of oxysterols. They found a strong increase of IL-8 mRNA expression and synthesis in Caco-2 cells stimulated with IL-1β in presence of 25OH, which exerted its effect in concentration-dependent manner [87].

Oxysterols produced by non-enzymatic reactions are undoubtedly strongly involved in inducing pro-inflammatory, pro-oxidant and cytotoxic effects that concur to the functional impairment of enteric mucosa. Particular attention must be paid to the oxysterols originated from diet, as they are totally absorbed from the gut, which is strongly exposed to their effects.

Processed cholesterol-rich food undergoes rapid oxidation by generating a mixture of oxysterols mainly consisting of 7-ketocholesterol (7K), 7α-hydroxycholesterol, 7β-hydroxycholesterol (7βOH), 5α,6α-epoxycholesterol, and 5β,6β-epoxycholesterol [88, 89].

The activity of 7K and 7βOH has been deeply investigated because they are the major non-enzymatic oxysterols present in most tissues, being produced both exogenously and endogenously. They have been widely considered in the pathogenesis of different human diseases for their cytotoxic and pro-apoptotic effects in different cytotypes [90, 91, 92]. Cytotoxicity of 7K in intestinal cells has been considered for its ability to induce metabolic alterations of mitochondrial functionality with caspase 3/7 activation, as well as decrease in RNA proportion in the G1 population [91, 93]. 7βOH was also shown to have apoptotic effect with loss of mitochondrial membrane potential and cytochrome c release [94], and in the meantime to have much stronger cytotoxic capacity than other oxysterols in colorectal cancer cells suggesting this molecule as a potential anticancer agent [95].

First evidence that 7K may influence survival and death signals has been provided by Lizard's group, who observed in human monocytic cells the activation of ERK signal before inducing apoptotic death through transient and net inhibition of Bad pro-apoptotic protein [96].

In the same period, 7βOH was shown to be pro-inflammatory by increasing IL-8 mRNA expression in Caco-2 cells [87]. Increased production of IL-8 by 7βOH has been confirmed by other studies, which underlined pro-inflammatory effect of other oxysterols, mainly 7K, in the same types of colon cells [95, 97].

More recently, 7K has been demonstrated to decrease barrier integrity of vascular endothelium and intestinal epithelial monolayer [98]. Authors have demonstrated in CaCo-2 cells co-cultured with DCs that 7K affects intestinal epithelial barrier functions by altering IL-10 expression in DCs [99]. These results suggest the importance of oxysterols in the induction of inappropriate inflammatory response of the intestinal epithelium versus food compounds, which might have CRC promoting significance.

Anyway, based on the evidence that oxysterols are present in food always in mixture, *in vitro* studies on intestinal cells treated with a mixture of dietary oxysterols have been performed as experimental model which better mimics intestinal pathophysiological conditions. Experiments in differentiated CaCo-2 cells with normal enterocyte-like phenotype demonstrated that mixed oxysterols can interfere with the homeostasis of the human digestive tract by inducing apoptosis and inflammation. Oxysterol-dependent activation of the intestinal form of NADPH oxidase NOX1 and consequent $O_2^{\cdot-}$ overproduction have been demonstrated to be central in the induction of these events [97, 100]. Dietary oxysterols have been found to increase a large pattern of pro-inflammatory mediators, such as IL-1 β , IL-6, IL-8, IL-23 and MCP-1, as well as of recognition receptors TLR2 and TLR9 [97]. Temporally activation of NOX1, p38 MAPK and NF- κ B in differentiated intestinal cells has been suggested as a specific signaling pathway axis involved in the induction of pro-inflammatory effects mediated by excessive dietary intake of oxysterols [101]. Finally, Swan and colleagues [102] have recently shown that individual oxysterol metabolizing enzymes are overexpressed in colorectal cancer associated with poor prognosis in CRC patients and in mismatch repair proficient CRC cohorts, thus confirming the role of oxysterols in metabolic pathways involved in CRC.

Collectively, these studies suggest oxysterols as important oxidized products able to interfere in different steps of colon carcinogenesis (see Figure 3). Oxysterols, either generated endogenously or absorbed with the diet, exert remarkable properties in modulating inflammatory and apoptotic cell signaling cascades, which can concur to sustain tumor growth and survival. In particular, two new important molecular mechanisms occur: a) the ability to disrupt intestinal barrier integrity with consequent development of an aberrant inflammatory response against gut luminal antigens; b) tumor immune escape strategy by both inhibiting DC activation and recruiting pro-tumoral inflammatory cells into the tumor microenvironment that favor invasiveness, through the generation of MMPs and pro-angiogenic factors.

5. Conclusions and perspectives

All the studies described above provide emerging molecular evidence of the importance of lipid oxidation products, namely HNE and oxysterols, in colorectal carcinogenesis where inflammation represents the fundamental link.

In a setting of chronic intestinal inflammatory diseases active proliferation of immune cells, induction of several inflammatory mediators and local enrichment of reactive oxygen species, which decrease cell and tissue antioxidant defenses, contribute to develop a cancer-prone microenvironment.

High dietary intake is believed to be a major contributory factor to the aggravation of gut inflammation and CRC development, due to the formation of high reactive metabolized compounds. HNE and oxysterols represent principal molecules able to modulate different steps of this process. Both these molecules exert common role in activating signaling processes of proliferation and survival, by influencing the continuous cross talking between the enterocytes and inflammatory cells present in the mucosal layer stroma. On one hand HNE, which is mainly produced by ROS-induced lipid peroxidation, can exert its action essentially intracellularly, by altering redox balance of normal cells, and activating antioxidant response signals, which drive cells towards tumor resistance. On the other hand, oxysterols can exert their pro-inflammatory and pro-tumorigenic action by producing ROS, which behave as signaling molecules stimulating cell growth during early phases of cancer development. Oxysterols can also play an important role in cancer progression for their new reported mechanisms to stimulate pro-tumoral activity of inflammatory/immune cells in the tumor-surrounding environment. In this context, it is clear the significant role of reactive oxygen species in CRC carcinogenesis and progression, in particular when cellular antioxidant defenses are not sufficient. Numerous therapies have been considered in order to reverse or prevent oxidative status and, therefore, the formation lipid oxidation compounds in tumor microenvironment.

Dietary polyphenols present in fruits and vegetables are considered therapeutic candidates in counteracting cell growth in preneoplastic and neoplastic cells. They have been shown to modulate different cell signals involved in cytochrome c and caspase activation, NF- κ B and JAK/STAT signaling pathways regulation [103, 104]. Other dietary compounds such as ω -3PUFAs and their metabolites resolvins, involved in the resolution phase of inflammation, have been suggested to exert a potential anti-cancer role [105]. Notably, resolvins have been found to reduce cytokines production and leukocytes infiltration in murine

peritonitis induced by intraperitoneal injection of glutathionyl-HNE adduct [106]. Recently, different studies have pointed out the dual role of NRF2 in tumorigenesis, whose activation can be essential in cancer progression, but detrimental during cancer promotion. Its exogenous induction during the early stages of carcinogenesis has been suggested as a new approach targeting CRC development [31, 107]. In this context, the capability of low levels of HNE in inducing NRF2 [55] and recent suggestions of antitumoral properties of HNE encapsulated in β -cyclodextrin nanoparticles polymers (108) could represent a new attractive strategy in cancer therapy. In addition, specific oxysterols such as 7β OH and epoxycholesterols have been also found to increase sensitivity of tumor cells to chemotherapy drugs or gamma radiation [109, 110].

In conclusion, oxidation compounds, especially those originated in high fat diets, such as HNE and oxysterols are actively involved in the events that link inflammation to CRC. However, the picture of molecular mechanisms controlled by these compounds in the different phases of colorectal carcinogenesis is still incomplete: future studies will help draw a more definite one.

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Figure Legends

Figure 1. Mucosal barrier defect with altered inflammatory response induces intestinal epithelial cells to uncontrolled proliferative and survival cell signals. Excess of inflammatory reactions and alteration of mucosa layer function, often present in IBD, can lead to exaggerate production of H_2O_2 , $O_2^{\cdot-}$, HO^{\cdot} , $HOCl$ and inflammatory mediators by inflammatory cells, thus favoring colon carcinogenesis. Hyper-responsiveness of the host mucosa against intestinal microbiota induced by altered functions of pathogen recognition receptors (TLRs) can increase mucosal barrier defect. Consequently, the amplification of oxidative status occurs both directly by $O_2^{\cdot-}$ generation NADPH oxidase-dependent, and indirectly by the synthesis of pro-tumorigenic chemokines such as $TNF\alpha$ and IL-6; mitochondria also represent a source of $O_2^{\cdot-}$ generation. Furthermore, inflammatory and oxidative reactions trigger the activation of specific transduction and transcription factors mainly implicated in inflammation-associated tumor development (STAT3, NF- κ B), and induce mutations and genomic alterations. All these events result in the induction of survival and the inhibition of apoptosis in dysplastic cells turning into tumor phenotype.

M: Macrophage; Th: T helper lymphocytes; MMPs: Matrix Metalloproteinases; NIEC: Normal Intestinal Epithelial Cell; NC: Necrotic Cell; AC: Apoptotic Cell; TJs: Tight Junctions.

Figure 2. HNE effects on colonic tumor epithelial cells. The presence of $O_2^{\cdot-}$ and HO^{\cdot} in the tumor environment can favor PUFAs peroxidation leading to HNE generation. By its amphiphilic properties, HNE can easily cross cell membranes and covalently modify macromolecules, which can either induce a direct DNA damage or interfere with the activity of important proteins involved in the activation of pro-inflammatory survival and proliferation signals. On the other hand, HNE is able to strongly affect redox balance and create an oxidative status that activate antioxidant cell response, thus providing survival advantage for neoplastic cells.

Figure 3. Oxysterols effects on colorectal cancer growth. Oxysterols are able to induce an oxidative status in intestinal cells by increased $O_2^{\cdot-}$ production mediated by NOX activity up-regulation. This activation triggers pro-inflammatory and proliferative signaling pathways involving p38MAPK and NF- κ B activation, with the induction of different interleukins favoring tumor promotion. Furthermore, oxysterols are able to

gather in the tumor surrounding environment; here they exert chemotactic activity by recruiting activated pro-tumoral immune/inflammatory cells *in situ*. Oxysterols could also escape immune surveillance by altering dendritic cells in their antigen presentation function.

M: Macrophage; PMN: Polymorphonuclear leukocyte; DC: Dendritic Cell; TJs: Tight Junctions; NOX: NADPH oxidase.

Figure 1

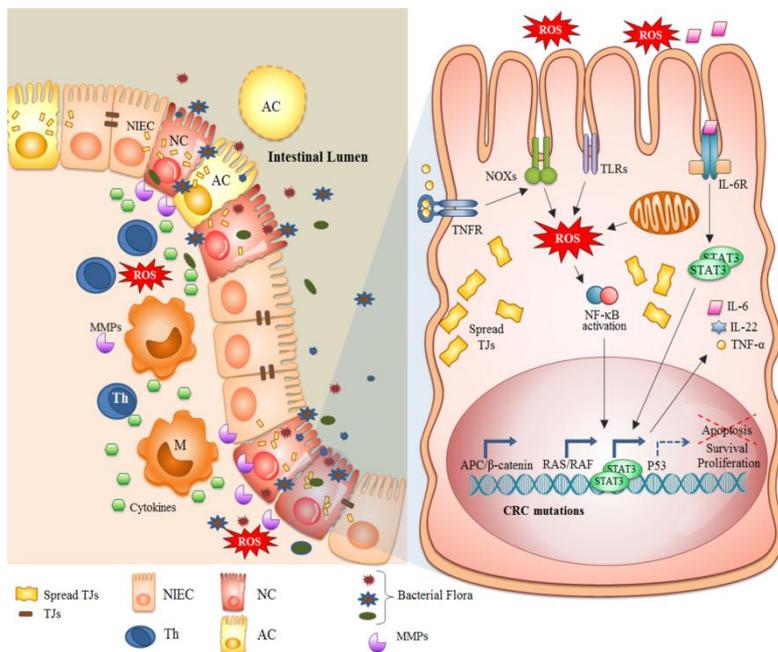


Figure 2

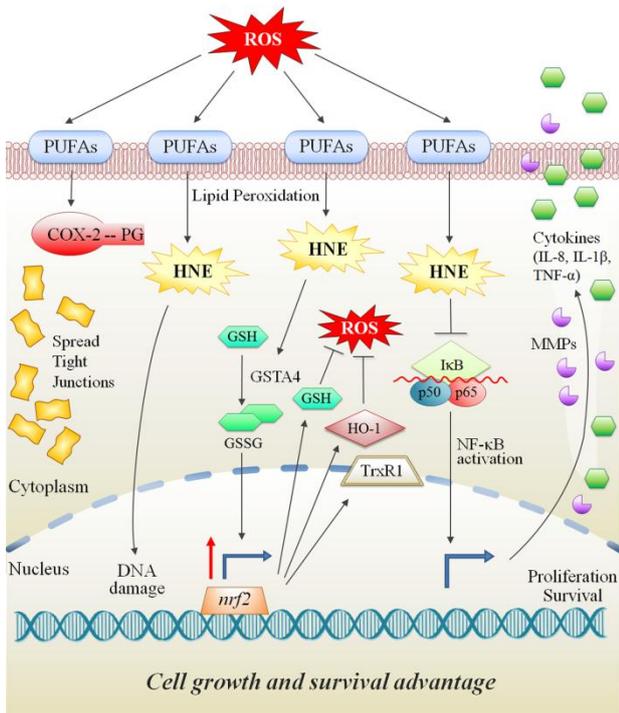


Figure 3

