**Universidade do Minho**
Escola de Ciências

Dalila Fernanda Neto Pedro

# Diet in the promotion or prevention of colorectal cancer

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Tese de Doutoramento em Biologia Molecular e Ambiental

Trabalho realizado sob a orientação da
**Professora Doutora Cristina Pereira Wilson**

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DECLARAÇÃO

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"If something is wrong, fix it if you can. But train yourself not to worry. Worry never fixes anything." - Ernest Hemingway

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**Abstract**

Colorectal cancer incidence is highly associated with one's lifestyle, such as lack of physical activity, which leads to obesity, smoking habits, and most importantly, diet. Diet is an important risk factor for colon carcinogenesis and several studies have shown that high red meat and saturated fat intake increases the incidence of this disease significantly. On the other hand, a healthy balanced diet with intake of fruits and vegetables can decrease the risk of this disease. Dietary strategies for colon cancer chemoprevention, and even during treatment, are needed to help reduce the incidence of colorectal cancer. The aim of this work was to investigate dietary compounds that can be used in these dietary strategies for colon cancer prevention. Also, we initiated the development of new models to be used for compound screening processes.

Initially, we focused on the effects of two bile acids, deoxycholic acid (DCA), with colon cancer promoting capacity, and ursodeoxycholic acid (UDCA), a chemopreventive compound, induced in Caco-2 cell line. We found that DCA in fact increase DNA damage and apoptosis in Caco-2 cell line. Also, activation of MAPK/ERK and PI3K/AKT pathways was also observed. UDCA did not induce DNA damage, but did induce the same activation in the signaling pathways. So, it seems that DCA increase cell turnover by increasing apoptosis and also cell proliferation in the remaining cells. UDCA only increase cell proliferation. When UDCA was administered as a pretreatment before DCA treatment, apoptosis was increased and this increase was accompanied by a constant activation of the JNK signaling pathway. Also, pretreatment with UDCA significantly decreased expression of the repair proteins MGMT and MLH1.

One of the aims of this work was to develop an *in vitro* model of the *in vivo* azoxymethane (AOM)-induced colon cancer model. With the conditions tested we were able to induce a slight increase in cell proliferation in Caco-2 cells. This increase of cell proliferation could possibly be explained by the activation of the MAPK/ERK pathway, which was also activated with AOM treatment. Although we observed this increase in cell proliferation, we found no induction of $O^6$-methylguanine lesions by our CoMeth assay or DNA damage observed by the comet assay.

In the *in vivo* assays, the potential of an herbal tea, sage, and two isolated compounds found in foods from our diet, ursolic acid (UA) and EGCG, were evaluated for their chemopreventive effects against colorectal cancer. In the first study, sage tea was given to Fischer 344 rats before or after AOM treatment. Sage tea was able to reduce the number of pre-

neoplastic lesions when given before AOM treatment, demonstrating chemopreventive potential. This reduction of pre-neoplastic lesions was accompanied by a reduction of the number of proliferating cells in colon crypts, as seen by Ki67 marker. Also, it conferred protection against DNA damage induced by AOM and by $H_2O_2$ *ex vivo* in colonocytes and lymphocytes. In the second study, UA and EGCG were added to the diet of healthy Fischer 344 rats. The potential of these compounds to protect against DNA damage was assessed. We found that both compounds protected against endogenous DNA damage in colonocytes and lymphocytes. The effects of the two compounds on protection against alkylating DNA damage induced *ex vivo* was also evaluated. UA and EGCG conferred protection against this type of damage in colonocytes, but not in lymphocytes.

Finally, it has been shown that epigenetics has an important role in colon carcinogenesis, so we tried to develop a new, simple method to evaluate demethylating agents. We used the CoMeth assay developed in our group in a MMR-deficient cell line, in which one of the intervenients, *MLH1*, is epigenetically silenced by hypermethylation. Using 5-azacytidine, we were able to revert the hypermethylation and induce DNA damage in these cells. We further characterized the model, showing increase in apoptosis, and effects on reexpression of MLH1 protein levels by western blot. We also tested a few natural compounds with the model and found that EGCG, which is well-known that it has demethylating ability, induced similar levels of DNA damage as 5-azacitidine, suggesting that the model is functional.

In conclusion, this work demonstrated the potential of sage tea as a chemopreventive agent and UA and EGCG as compounds with interest for chemopreventive strategies. The AOM *in vitro* model needs to be improved, but the adapted CoMeth assay for demethylating compounds is functional. Altogether, but with some additional studies, these natural compounds could be considered as chemopreventive agents and have possible interest in dietary strategies for cancer prevention.

**Resumo**

A incidência do cancro colorectal está altamente relacionada com o estilo de vida das pessoas. A falta de exercício, hábitos de tabagismo, obesidade e a dieta são alguns fatores de risco. A dieta assume um papel importante na carcinogénese do cólon. Vários estudos demonstraram que dietas ricas em carnes vermelhas e gorduras saturadas aumentam significativamente a incidência desta doença. No entanto, o seu risco de aparecimento pode ser reduzido com uma dieta saudável, rica em frutas e vegetais. Estratégias de quimioprevenção do cancro do cólon baseadas na dieta, assim como no decorrer do seu tratamento, são necessárias para ajudar na redução da sua incidência. Com este trabalho, pretendeu-se avaliar a capacidade preventiva de compostos presentes na dieta no aparecimento do cancro do cólon. Foi, também, desenvolvido trabalho no sentido de desenvolver novos modelos que possam ser usados no *screening* de outros compostos.

Inicialmente, o trabalho focou-se no estudo da resposta da linha celular Caco-2 à presença de dois ácidos biliares, o ácido deoxicólico (DCA), um promotor do cancro do cólon, e o ácido ursodesoxicólico (UDCA), um composto quimiopreventivo. Descobriu-se que o DCA induz o aumento de danos no DNA, assim como promove a apoptose na linha celular. Verificou-se, também, a ativação das vias de sinalização MAPK/ERK e PI3K/AKT. No caso do UDCA, observou-se uma ativação das mesmas vias de sinalização, no entanto, não houve um aumento nos danos do DNA. Desta forma, o DCA aparenta ser capaz de aumentar a renovação celular, visto que promove a apoptose e também a proliferação celular. O UDCA é apenas capaz de aumentar a proliferação celular. Quando o UDCA foi administrado como pré-tratamento ao tratamento com DCA, verificou-se um aumento da apoptose, o qual foi acompanhado de uma ativação constante da via de sinalização JNK. O pré-tratamento com UDCA foi, também, responsável por uma redução significativa da expressão das enzimas de reparação MGMT e MLH1.

Com este trabalho, também se pretendeu desenvolver um modelo *in vitro* do modelo *in vivo* de indução do cancro do cólon com azoximetano (AOM). As condições usadas permitiram induzir um ligeiro aumento da proliferação celular. Este aumento da proliferação pode ser explicado pela ativação da via de sinalização MAPK/ERK, a qual também foi ativada pelo tratamento com AOM. Apesar do aumento observado da proliferação celular, não se identificaram danos do tipo $O^6$-metilguanina, recorrendo ao método CoMeth, nem danos no DNA, observados por *comet assay*.

Nos estudos *in vivo*, avaliou-se o potencial quimiopreventivo do chá de Sálvia e de dois compostos isolados presentes na dieta, o ácido ursólico (UA) e o (-)-epigalocatequina-3-galato (EGCG), contra o cancro colorectal,. No primeiro estudo, ratos Fischer 344 consumiram chá de Sálvia antes ou após o tratamento com AOM. O chá foi capaz de reduzir o número de lesões pré-neoplásicas quando administrado antes do tratamento com AOM, demonstrando o seu potencial quimiopreventivo. Essa redução de lesões pré-neoplásicas foi acompanhada duma redução do número de células proliferativas nas criptas do cólon, observado com o marcador Ki67. Foi, também, observada proteção do DNA contra danos induzidos por AOM e por $H_2O_2$ em ensaios *ex vivo* com colonócitos e linfócitos. No segundo estudo, incluiu-se UA e EGCG na dieta de ratos Fischer 344 saudáveis, avaliando-se o seu potencial protetor contra danos de DNA. Os resultados mostraram que, em colonócitos e linfócitos, ambos os compostos conferem proteção contra danos endógenos do DNA. O seu potencial contra danos alquilantes induzidos no DNA foi, também, avaliado em ensaios *ex vivo*. Nos colonócitos, foi possível observar proteção conferida pelo UA e pelo EGCG contra este tipo de danos, ao contrário do observado no caso dos linfócitos.

Por fim, uma vez que a epigenética assume um papel importante na carcinogénese do cólon, criou-se um método novo, e simples, para avaliação de agentes desmetilantes. O método CoMeth, desenvolvido do nosso grupo de investigação, foi usado numa linha celular deficiente no sistema de *Mismatch repair* (MMR), na qual o gene *MLH1* se encontra epigeneticamente silenciado por hipermetilação. Usado a 5-azacitidina, foi possível reverter a hipermetilação e induzir danos no DNA nestas células. Foi, também, observado um aumento da apoptose e, pela técnica de *western blot*, percebeu-se que existe reexpressão da proteína MLH1. Vários compostos naturais foram usados para validar o método e, entre eles, o EGCG, que é conhecido pela sua capacidade de desmetilação, induziu níveis de danos no DNA semelhantes àqueles observados com a 5-azacitidina, validando a funcionalidade do método.

Assim, este trabalho comprovou não só o potencial quimiopreventivo do chá de Sálvia mas também o potencial do UA e do EGCG como parte integrante duma estratégia quimiopreventiva. O método CoMeth provou ser funcional na avaliação de potenciais agentes desmetilantes, contudo, o modelo *in vitro* do AOM precisa de ser melhorado. No seu conjunto, apesar de ser necessário completar os estudos já realizados, os compostos naturais estudados podem ser considerados agentes quimiopreventivos, conferindo-lhes o potencial para aplicação em estratégicas de prevenção de cancro baseadas na dieta.

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**Abbreviation list**

| | |
|---|---|
| 5-aza | 5-Azacytidine |
| ACF | Aberrant crypt foci |
| AOM | Azoxymethane |
| AP | Apurinic/apyrimidinic |
| *APC* | *Adenomatous polyposis coli* |
| APE1 | Human AP endonuclease |
| ASK1 | Apoptosis signal-regulating kinase-1 |
| BCAC | β-catenin accumulated crypts |
| BER | Base excision repair |
| BG | $O^6$-benzylguanine |
| BSA | Bovine serum albumin |
| CA | Cholic acid |
| CDCA | Chenodeoxycholic acid |
| CDNB | 1-Chloro-2,4-dinitrobenzene |
| CIMP | CpG island methylator phenotype |
| CIN | Chromosomal instability |
| CK1 | Casein kinase 1 |
| CRC | Colorectal Cancer |
| DAB | 3,3′-Diamino-benzidine |
| DAPK | Death-associated protein kinase |
| DCA | Deoxycholic acid |
| DCF-DA | 2′,7′-Dichlorofluorescein diacetate |
| DCFH | Dichlorofluorescin |
| DMEM | Dulbecco's modified Eagle medium |
| DMH | 1,2-dimethylhydrazine |
| DMSO | Dimethyl sulfoxide |
| DNMT | DNA methyltransferases |
| DTT | Dithiothreitol |
| DVL | Disheveled |
| EDTA | Ethylenediaminetetraacetic acid |
| EGCG | (-)-Epigallocatechin-3-gallate |

| | |
|---|---|
| EGF | Epidermal growth factor |
| EGFR | EGF receptor |
| ERK | Extracellular signal-regulated kinase |
| FAP | Familial adenomatous polyposis |
| FBS | Fetal bovine serum |
| FPG | Formamidopyrimidine DNA glycosylase |
| Fzd | Frizzled |
| GAP | GTPase activating proteins |
| GDP | Guanosine diphosphate |
| GEF | Guanine exchange factor |
| GRB2 | Growth factor receptor-bound protein 2 |
| GSH | Glutathione |
| GSK3β | Glycogen synthase kinase 3 β |
| GST | Glutathione-S-transferase |
| GTP | Guanosine triphosphate |
| HAT | Histone acetyltransferases |
| HATs | Histone acetyltransferases |
| HBSS | Hank's buffered salt solution |
| HDAC | Histone deacetylases |
| HNPCC | Hereditary nonpolyposis colorectal cancer |
| IQ | 2-Amino-3-methylimidazol[4,5-f]quinoline |
| JNK | c-Jun amino N-terminal kinase |
| LCA | Lithocholic acid |
| LRP | Low-density lipoprotein receptor-related protein |
| LS | Lynch syndrome |
| MAM | Methylazoxymethanol |
| MAPK | Mitogen-activated protein kinase |
| MBD | $m^5$CpG-binding domain proteins |
| MDF | Mucin-depleted foci |
| MeCPs | $m^5$CpG-binding proteins |
| MGMT | $O^6$-methylguanine-DNA methyltransferase |
| Min | Multiple intestinal neoplasms |
| MLK2 | Mixed lineage kinase 2 |

| | |
|---|---|
| MMR | Mismatch repair |
| MMS | Methyl methanesulfonate |
| MNU | *N*-Methyl-*N*-nitrosourea |
| MPG | Methylpurine-DNA glycosylase |
| MSI | Microsatellite instability |
| MSS | Microsatellite stable |
| MTT | Thiazolyl blue tetrazolium bromide |
| $O^6$-MeG | $O^6$-methylguanine adduct |
| OGG1 | 8-Oxoguanine glycosylase |
| PCNA | Proliferating cell nuclear antigen |
| PDK1 | Protein serine/threonine kinase-3'-phophoinositide-dependent kinase 1 |
| PH | Pleckstrin homology |
| PhIP | 2-Amino-1-methyl-6-phenylimidazol[4,5-b]pyridine |
| PI3K | Phosphatidylinositol-3-kinase |
| PIP2 | Phosphatidylinositol-4,5-bisphosphate |
| PIP3 | Phosphatidylinositol-3,4,5-triphosphate |
| *Pirc* | Polyposis in the rat colon |
| PKB | Protein kinase B |
| PNP | *p*-nitrophenol |
| *RB1* | Retinoblastoma tumor suppressor gene |
| RNS | Reactive nitrogen species |
| ROS | Reactive oxygen specie |
| RT | Room temperature |
| RTKs | Receptor tyrosine kinases |
| SAPK | Stress-activated protein kinase |
| SOS | Son of sevenless homologue |
| STS | Staurosporin |
| TAK1 | Transforming growth factor-β-activated kinase 1 |
| TCF/LEF | T-cell factor/lymphoid enhancer factor |
| UA | Ursolic acid |
| UDCA | Ursodeoxycholic acid |

# GENERAL INTRODUCTION

Overview of the past years

*Chapter 1*

# 1 – Colorectal Cancer

## 1.1 – Incidence and general concepts

Colorectal cancer (CRC) is a common malignancy and significant cause of mortality in Western societies. Worldwide, CRC is the third most common cancer in men and second in women (Figure 1a), while being the fourth most common cause of death from cancer. In Portugal, it is the second most common cancer in men and in women, behind prostate and breast cancer, respectively (Figure 1b) [Ferlay, J., *et al.*, 2010].

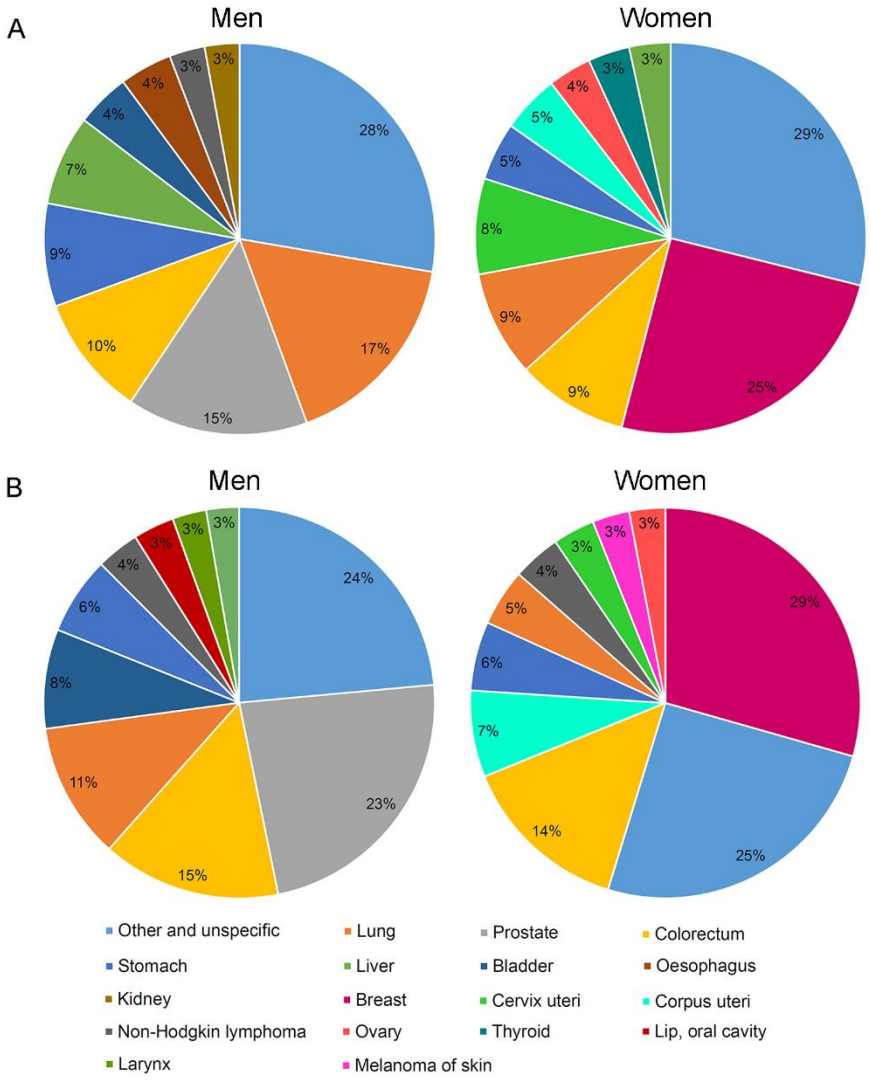


**Figure 1 –** Representation of the incidence of different cancers in men and in women, in the world (A) and specifically in Portugal (B). Adapted from [Ferlay, J., *et al.*, 2010].

The carcinogenic process is multi-step and usually several years are needed for a tumor to reach an invasive stage. This process can be divided into three distinct phases: initiation, phase where genetic alterations occur and the irreversible changes confer an intrinsic capacity to proliferate uncontrollably; promotion, phase when the initiated cells continue to divide giving rise to a detectable neoplasm; and progression, where the neoplasm acquires increased invasiveness and metastatic potential [Frank, S.A., 2007; Grady, W.M., *et al.*, 2008].

CRC usually develops through the inheritance of a genetic defect or induction of DNA damage. Afterwards, the accumulation of further genetic and epigenetic alterations transforms normal colon cells and gives them growth advantage over their neighboring cells. Other factors that have been implicated in the onset of the disease are diet, obesity, lack of physical activity, smoking habits and exposure to chemical or biological carcinogens. The evolution of normal tissue to adenocarcinoma usually follows a known progression of histological changes and simultaneous genetic and epigenetic modifications [Frank, S.A., 2007].

The first alterations that appear during the carcinogenic process usually involve gatekeeper or caretaker genes. A gatekeeper gene is a gene that controls the initiation of a neoplasm, such as the *adenomatous polyposis coli* (*APC*) tumor suppressor gene, while a caretaker gene may also be one controlling the rate of accumulation of genetic alterations, such as the DNA mismatch repair (MMR) genes [Fearon, E.R., 2011; Frank, S.A., 2007; Redston, M., 2001]. With these alterations, progressive defects in important cellular pathways are activated, inducing cell proliferation, preventing apoptosis and senescence, and predisposing to failure to recognize and repair DNA damage [Walker, J., *et al.*, 2001]. Some altered pathways in colorectal cancer are the mitogen-activated protein kinase pathway and WNT signaling pathway, which will be described in more detail in the following segment.

## 1.2 – Signaling pathways

### 1.2.1 – WNT signaling

The WNT signaling pathway is important for embryonic development, but also regulates homeostasis in self-renewing tissues, such as the colonic epithelial. At the center of this pathway is the highly regulated β-catenin protein, encoded by the *CTNNB1* gene. β-catenin is found in three different sites in the cell: first, at cellular adherent junctions,

interacting with E-cadherin; in the cytosol; and finally in the nucleus. In normal cells, the absence of WNT signals favors the proteasomal degradation of cytoplasmic β-catenin and this degradation is initiated by a multi-protein "destruction" complex (Figure 2). This complex is formed by adenomatous polyposis coli (APC) and AXIN, which are important for binding of β-catenin to the complex, and glycogen synthase kinase 3 β (GSK3β) and casein kinase 1 (CK1), which are responsible for the phosphorylation of the protein. The "destruction" complex phosphorylates the N-terminus of the protein, targeting it for proteasomal degradation. This regulation maintains the cytosolic levels of the protein relatively low. Binding of Wnt signals to specific receptors in the cell membrane, frizzled (Fzd) and low-density lipoprotein receptor-related protein (LRP), inhibit β-catenin phosphorylation, enabling this protein to accumulate in the cytosol and translocate to the nucleus. In this cellular compartment, β-catenin interacts with a family of transcription factors, the T-cell factor/lymphoid enhancer factor (TCF/LEF), to activate target genes, mostly genes involved in differentiation, proliferation, migration, and adhesion [Burgess, A.W., *et al.*, 2011; White, B.D., *et al.*, 2012].

Aberrant WNT signaling is widely implicated in diseases, such as cancer. In CRC, a high number of tumors have an alteration in a protein of the WNT pathway, the most common being *APC* or *CTNNB1*, in which most of these tumors show β-catenin accumulation in the nucleus. Although mutations in these genes result in activation of the pathway and accumulation of β-catenin in the nucleus, it seems that they are not functionally equivalent. It seems that *APC* and *CTNNB1* mutations are mutually exclusive, so tumors either have one or the other. *CTNNB1* mutations have been associated with small colorectal adenomas and also hereditary nonpolyposis colorectal cancer (HNPCC), while *APC* mutations are associated with the familial adenomatous polyposis (FAP). But even between cells within the same tumor, there are differences in WNT signaling. CRC tumors with activating WNT signaling mutations show inconsistent levels of signaling, suggesting other mechanisms of pathway regulation, such as epigenetic silencing or pathway crosstalk. Also, *APC* mutations have been found to cause significant alterations at the cell membrane, influencing cell adhesion and migration. These mutations activate the pathway at different levels, promoting tumor progression by different mechanisms, ultimately prompting different types of tumors [Burgess, A.W., *et al.*, 2011; White, B.D., *et al.*, 2012; Zeller, E., *et al.*, 2013].

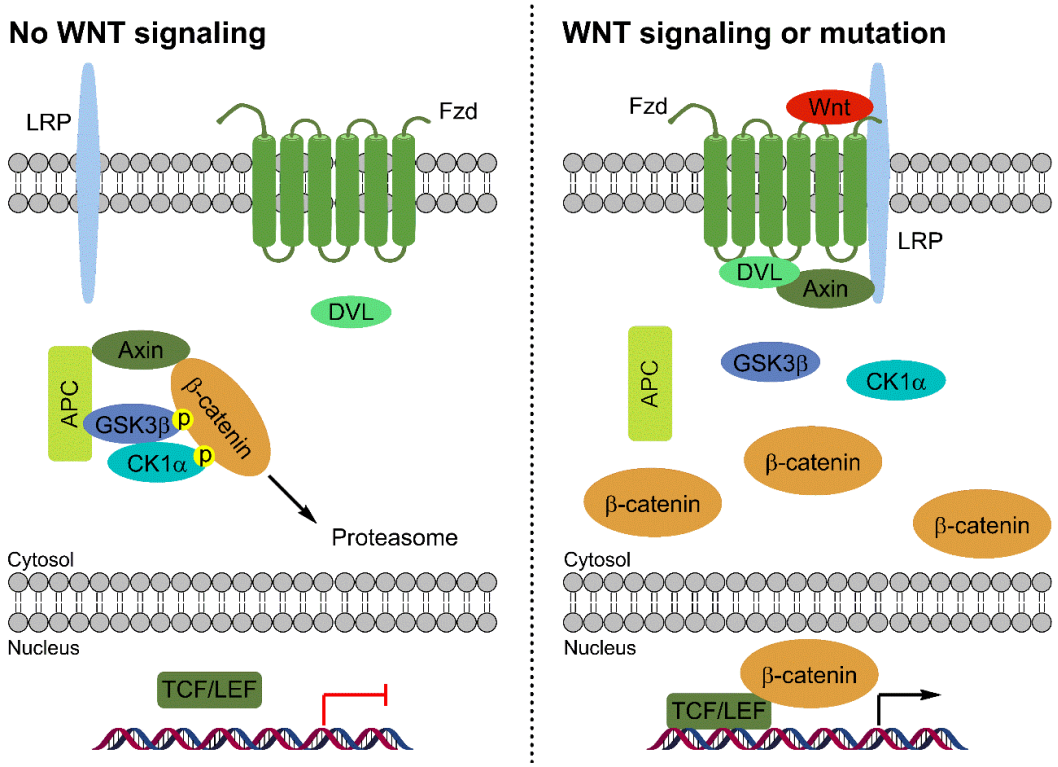


**Figure 2 –** Schematic figure depicting the Wnt/β-catenin pathway. (A) In the absence of Wnt signal, β-catenin is phosphorylated by a destruction complex containing AXIN, APC, GSK3, and CK1 proteins. N-terminal phosphorylated β-catenin is targeted proteasomal degradation. (B) Wnt ligands initiate signaling through FZD and LRP receptor, activating and recruiting DVL (Disheveled) and Axin to the membrane, thereby disrupting the destruction complex. Higher cytosolic levels of β-catenin result in its translocation into the nucleus, where it binds TCF/LEF transcription factors, activating WNT target genes. Adapted from [White, B.D., *et al.*, 2012].

### 1.2.2 – Extracellular signal-regulated kinase (MAPK/ERK) pathway

The mitogen-activated protein kinase (MAPK) pathway is a highly conserved signaling pathway that controls important cellular processes that include cell proliferation and differentiation. Three distinct groups of regulated MAPK cascades are known in humans that lead to altered gene expression. One of them is the extracellular signal-regulated kinase (MAPK/ERK) pathway. Activation of this pathway starts with an extracellular stimulus, such as a ligand binding to its respective receptor, for example epidermal growth factor (EGF) and EGF receptor (EGFR), which becomes dimerised and phosphorylated (Figure 3). A complex of proteins, that include growth factor receptor-bound protein 2 (GRB2) and son of sevenless homologue (SOS), can now bind to the phosphorylated domain of the receptor. Simultaneously, a protein with guanine exchange factor (GEF) activity, which is also in the protein complex, binds to RAS protein. This GEF protein displaces guanosine diphosphate (GDP) molecules from RAS and allows

guanosine triphosphate (GTP) molecules to bind, thus activating RAS. Active RAS binds to RAF, recruiting it to the membrane and activating it. After activation, RAF proteins phosphorylate and activate MEK, which in turn, phosphorylate and activate ERK1/2. ERK1/2 protein then either phosphorylates target substrates in the cytosol or is translocated to the nucleus where it activates a range of transcription factors, for example, Jun and Fos, and these, in turn, bind to the AP-1 DNA domain promoting transcription of genes involved in regulation of cell proliferation. In normal cells, regulation of the pathway is done by RAS-GTPase activating proteins (GAP), which hydrolyze GTP to GDP, inactivating RAS protein, and blocking the pathway [Meister, M., *et al.*, 2013; Zenonos, K., *et al.*, 2013].

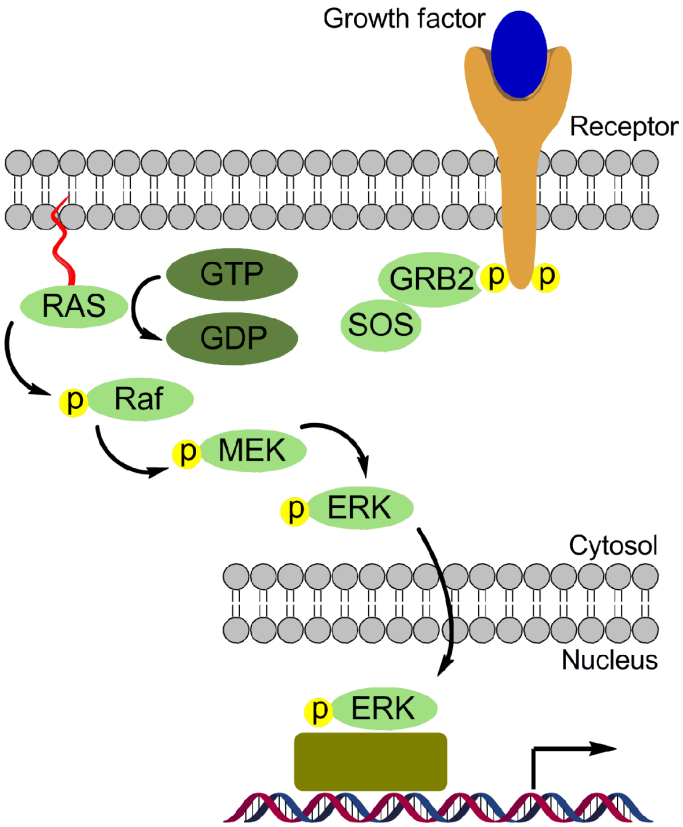


**Figure 3 –** Schematic representation of the MAPK/ERK cascade. Activation of the pathway by binding of a growth factor to a receptor results in recruitment of GRB2 and SOS, which then interacts with and activates RAS. This results in phosphorylation of Raf and, thereby, the initiation of sequential phosphorylation steps of MEK and ERK. Activated ERK can phosphorylate either cytosolic or nuclear substrates. Adapted from [Meister, M., *et al.*, 2013].

Deregulation of the MAPK/ERK pathway is common in carcinogenesis. The RAS family of proteins was the first oncogene discovered. There are several subtypes of RAS

protein, such as HRAS, NRAS, ERAS, MRAS and KRAS, but the most common gene mutated in colorectal cancer is *KRAS*, followed by *NRAS*. Among the most frequent alterations, we find *RAS* and *RAF* mutations. About half of all CRC cases present a *KRAS* mutation, while *NRAS* mutation is found in only fewer cases of CRC. Mutations in the *KRAS* gene are usually found in specific positions at codons 12, 13, and 61, stabilizing the protein into a constant active state. These mutations usually lead to conformational changes in the protein, interfering with RAS-GAP activity and maintaining RAS constantly active, promoting tumorigenic properties by constant MAPK pathway activation. *BRAF* mutations appear in about 5% to 10% of all CRC cases. There are three isoforms of the RAF protein, C-RAF, also RAF-1, A-RAF and B-RAF. Mutations in *BRAF* lead to increased kinase activity, also promoting MAPK pathway activation. The most common mutation of the *BRAF* gene is the V600E mutation, a transversion of a thymine by an adenine, causing a substitution of amino acids. Both types of mutations lead to deregulation of the MAPK/ERK pathway and increased cell proliferation and tumor growth [Arends, M.J., 2013; Nandan, M.O., *et al.*, 2011; Zenonos, K., *et al.*, 2013].

### 1.2.3 – c-Jun amino N-terminal kinase (JNK) pathway

Another pathway of the MAPK is the c-Jun amino N-terminal kinase (JNK) pathway. The JNK pathway, also known as the stress-activated protein kinase (SAPK), is mainly activated in response to stress signals. In humans, three JNK genes can be found, JNK1 and JNK2, which are ubiquitously expressed, and JNK3, which is mainly expressed in the brain, cardiac smooth muscle and testes. Being part of the MAPK cascade, it is also a cascade with triple kinase (Figure 4). The signaling cascade starts with a stress stimulus. Various stresses strongly activate JNK pathway, such as cytokines, irradiation, cytotoxic drugs, DNA damaging agents, and reactive oxygen species (ROS). These stress stimuli activate various MAP3 kinases, such as MEKK1, mixed lineage kinase 2 (MLK2), MLK3, transforming growth factor-β-activated kinase 1 (TAK1) and apoptosis signal-regulating kinase-1 (ASK1). These, in turn, activate by phosphorylation MKK-3, MKK-4, MKK-6 and MKK-7, and these ultimately activate JNK1 and JNK2. JNK can stay in the cytosol or translocate to the nucleus, so not only does JNK regulate transcription factors in the nucleus, such as c-Fos, c-Jun, ATF-2, AP-1, p53, and ELK1, but also modulates cytoskeletal and mitochondrial proteins, example of the latter being Bcl-2 and Bcl-xl [Sehgal, V., *et al.*, 2013; Sui, X., *et al.*, 2014].

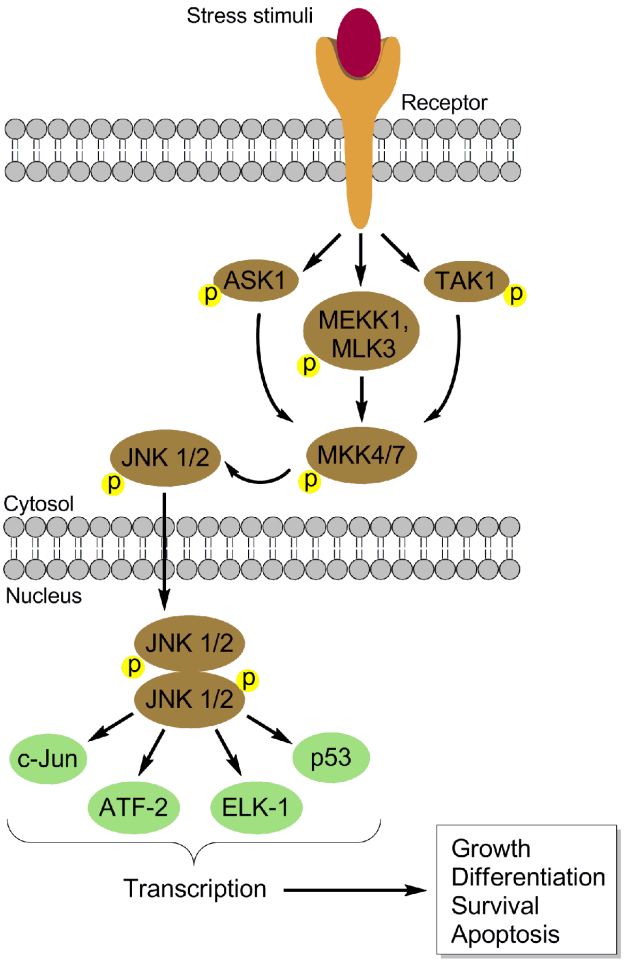


**Figure 4 –** Schematic representation of the JNK pathway. Activation of the pathway by stress stimulus. These stress stimuli activate various MAP3 kinases, such as MEKK1, MLK3, TAK1, and ASK1. These, in turn, phosphorylate MKK4 or MKK7. JNK1 and JNK2 are phosphorylated by these last kinases, and activate various proteins in the cytosol or in the nucleus.

JNK pathway has been associated with a plethora of cellular processes, such as apoptosis, autophagy, metabolism, cell proliferation, and DNA repair. Whether JNK activation leads to cell proliferation or apoptosis depends on the type of cell involved and the stimuli received. Apoptosis is regulated by JNK through two different mechanisms. First, promotion of c-Jun and ATF-2 phosphorylation results in AP-1 activation and expression of Fas/FasL signaling pathway-related proteins. This Fas/FasL binding can mediate caspase 8 and caspase 3 activation, which will lead to apoptosis. Second, in the cytosol, JNK phosphorylates Bcl-2/Bcl-xL, which are anti-apoptotic proteins, changing the mitochondrial membrane potential and inducing caspase 9 and caspase 3 activation, also leading to apoptosis. So, looking at the classification of signaling pathways that initiate apoptosis as extrinsic or intrinsic, it is safe to say that JNK activation has an important role in both pathways. [Dhanasekaran, D.N., *et al.*, 2008; Sui, X., *et al.*, 2014].

### 1.2.4 – Phosphatidylinositol-3-kinase (PI3K)/AKT pathway

The phosphatidylinositol-3-kinase (PI3K)/AKT pathway has an important role in regulating cell survival in response to cellular stress. It is activated by growth factors that bind to receptor tyrosine kinases (RTKs), as in the MAPK pathways (Figure 5). After binding to the receptor, PI3K protein is recruited to the receptor, stimulating the conversion of phosphatidylinositol-4,5-bisphosphate (PIP2) to phosphatidylinositol-3,4,5-triphosphate (PIP3). This conversion can be reversely regulated by the protein PTEN, converting PIP3 into PIP2. PI3K protein has two subunits: p110, which has catalytic activity, and p85, which is the regulatory subunit. PIP3 provides a docking area for proteins with pleckstrin homology (PH) domains, such as protein serine/threonine kinase-3'-phophoinositide-dependent kinase 1 (PDK1) and AKT/protein kinase B (PKB). AKT/PKB regulates several cell processes after activation [Porta, C., *et al.*, 2014; Zhang, L., *et al.*, 2013].

In normal cells and after appropriate stimuli, AKT/PKB protein can inhibit apoptosis by inactivating pro-apoptotic factors, such as BAD and caspase-9. It also phosphorylates GSK3β at Serine 9, which blocks the protein activity. As referred before, GSK3β is involved in β-catenin regulation and degradation. With GSK3β inhibited, there is an accumulation of β-catenin in the nucleus, inducing cell proliferation. AKT may also promote survival by phosphorylating HDM2, and this, in turn, promotes translocation to the nucleus, where it triggers p53 degradation [Manning, B.D., *et al.*, 2007; Zhang, L., *et al.*, 2013]. Cancer cells may present mutations in components of this pathway, leading to increased proliferation and decreased apoptosis. *PI3KCA* mutations have been found in some colorectal tumors. These mutations are mostly found in the catalytic subunit of the protein, affecting PIP2 to PIP3 conversion. It has been shown that, unlike other mutations that are mutually exclusive, PI3KCA mutations are significantly associated with KRAS mutations and loss of expression of a DNA repair enzyme $O^6$-methylguanine-DNA methyltransferase (MGMT). PTEN is a phosphatase that acts as a tumor suppressor by suppressing cell proliferation by inhibiting AKT activation. *PTEN* mutations are frequently found in advanced stages of carcinogenesis. Mutations in this gene prevent the negative regulatory control of this protein, allowing for constant cell growth [Cathomas, G., 2014; Porta, C., *et al.*, 2014].

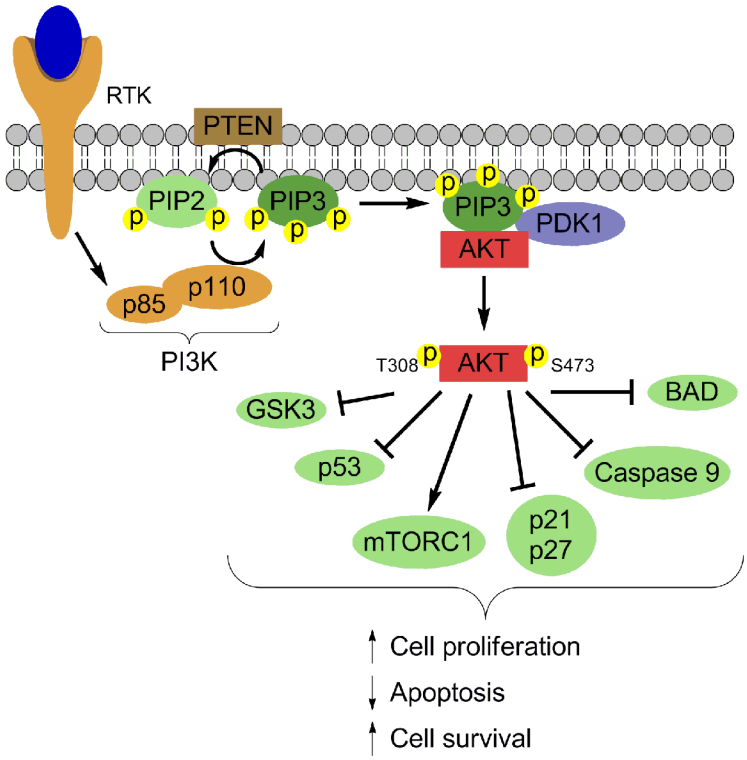


**Figure 5 –** Schematic representation of the PI3K/AKT pathways. Upon RTK activation, PI3K catalyzes the conversion of PIP2 to PIP3. PIP3 allows recruitment to membrane of AKT, where it gets phosphorylated and induces proliferation and inhibits apoptosis. Adapted from [Zhang, L., *et al.*, 2013].

### 1.3 – DNA repair systems

Genomic instability has been recognized as a trait of the majority of solid tumors. There are many forms of genomic instability. One of the most common forms found in cancer is chromosomal instability (CIN). CIN is characterized by a high rate of changes in chromosome structure and number over time. It seems that cancer cells acquire new chromosomal abnormalities over time. Another type of genomic instability found in cancer cells is microsatellite instability (MSI). This type of abnormality is characterized by the increase or decrease of the number of oligonucleotide repeats present in a microsatellite sequence. Increased frequencies of base-pair mutations can also contribute to genomic instability in cancer cells.

These genomic alterations and mutations can alter the cell's behavior, in the initiation and/or progression process, and how the tumor will respond to therapy in cancer treatment. Due to the destructive effects of genomic instability, and the fact that the integrity of DNA is constantly being challenged, cells have DNA repair systems to detect and correct the lesions before being passed to daughter cells by division [Lord, C.J., *et*

*al.*, 2012; Negrini, S., *et al.*, 2010]. These repair systems recognize and repair specific and different types of damage [Ramos, A.A., *et al.*, 2011]. In the next section, we will describe in more detail a few of these repair systems.

### 1.3.1 – The DNA Mismatch Repair system

The mismatch repair (MMR) system comprises a number of proteins that recognize and directly repair nucleotide mismatches, mainly base-base mismatches and insertion/deletion loops. The target DNA sequences of this repair system are residual errors that have escaped the normal proofreading function of the replication system. These mispairs, if not corrected, may cause nucleotide transitions or transversions, and consequently cause point mutations. These point mutations can appear in genes that regulate cell growth and accumulation of these in cells may promote neoplastic growth [Lord, C.J., *et al.*, 2012; Marra, G., *et al.*, 2005; Ramos, A.A., *et al.*, 2011].

In human cells, at least six MMR proteins are needed for correct repair (Figure 6). MSH2 protein forms a heterodimer with MSH6 or MSH3 for mismatch recognition. The binding depends on the type of mispair found, where MSH2-MSH6 (hMutSα complex) detects base-base mispairs and small insertion-deletion loops while MSH2-MSH3 (hMutSβ complex) only detects larger loops. After recognition by the first complex, another heterodimer, hMutLα, composed of MLH1 and PMS2, coordinates the interaction between the recognition complexes and the other proteins necessary for repair, such as PCNA, DNA polymerases and single-stranded DNA–binding protein. All these interactions lead to the excision of the mispair or loop and resynthesis of DNA by DNA polymerase [Lord, C.J., *et al.*, 2012; Marra, G., *et al.*, 2005; Ramos, A.A., *et al.*, 2011]. DNA repair pathways are important for the normal cell correction, but also for cancer treatment. Defects in the mismatch repair system, specifically mutation or epigenetic modifications of *MLH1* or *MSH2*, have been observed in some cases of CRC. This is the case of hereditary non-polyposis colorectal cancer, which is associated with loss-of-function mutations of these mismatch repair genes.

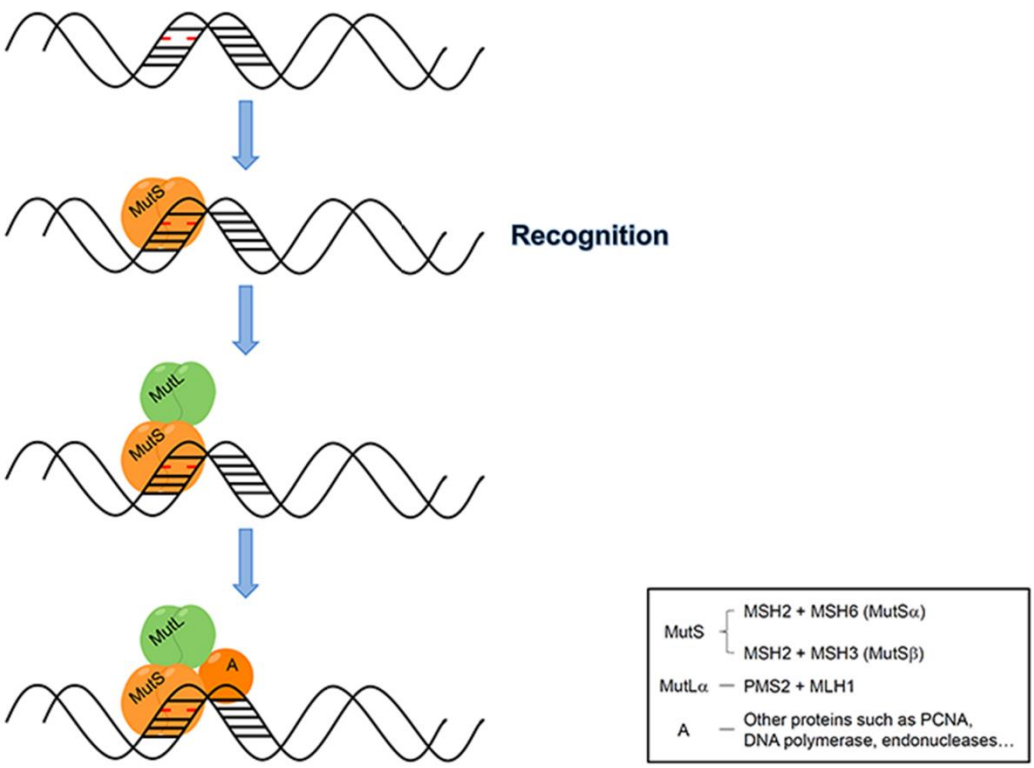


**Figure 6 –** Schematic representation of the mismatch repair system recognition. A hMutS complex identifies the lesion and recruits the MutL complex to the location. This complex coordinates the interaction between the recognition complexes and the other proteins necessary for repair, such as PCNA, DNA polymerases and single-stranded DNA–binding protein.

### 1.3.2 – Direct damage reversal repair

We are constantly in contact with mutagens from our environment, as well as endogenous metabolic products, both generating reactive electrophilic species which alkylate DNA. The $O^6$-methylguanine adduct ($O^6$-MeG) is a crucial lesion due to its mutagenic and toxic potential. It allows DNA replication to continue but causing a mispair with thymine instead of cytosine and, during the replication process, causing GC→AT transitions. This lesion is very stable if not repaired by MGMT. MGMT repairs $O^6$-alkylation adducts, by direct damage reversal, in a one-step alkyl transfer reaction, transferring the alkyl group from the oxygen in the guanine nucleotide in the DNA to a cysteine residue of the protein, a process that inactivates MGMT [Kaina, B., *et al.*, 2007; Ramos, A.A., *et al.*, 2011]. If MGMT fails to remove the alkyl group, the mispair is recognized by the MMR pathway, removing the thymine and inserting a gap. In the next round of replication, $O^6$-MeG mispairs again, and the thymine is removed once more. This loop continues, if $O^6$-MeG remains in one of the templates, creating a "futile repair loop", eventually resulting in toxic double-strand breaks, leading to apoptosis or

chromosomal aberrations (Figure 7). It is known that $O^6$-MeG is a common cause of mutations responsible for carcinogenesis initiation, especially activation of oncogenes and inactivation of tumor suppressor genes. MGMT is also frequently silenced in sporadic colon tumors by promoter hypermethylation, demonstrating the important role of this protein in cancer prevention [Kaina, B., *et al.*, 2007; Ramos, A.A., *et al.*, 2011].

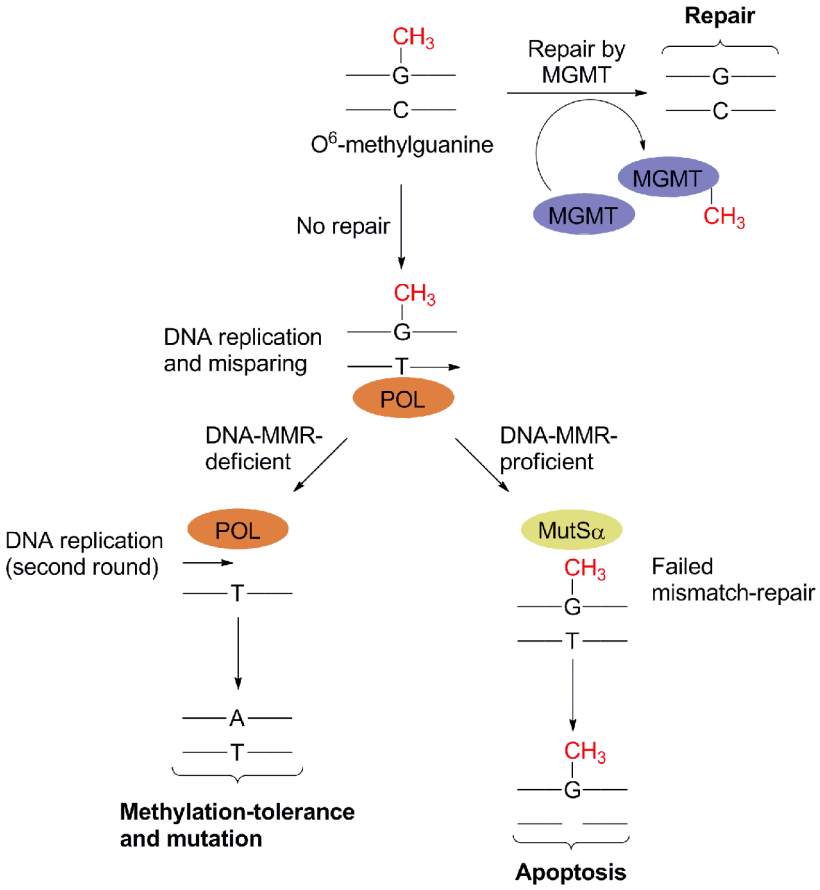


**Figure 7** – MGMT repair of $O^6$-MeG lesion. MGMT repairs the lesion by direct damage reversal inactivating the protein. If the lesion is not repaired, the mispair is recognized by the MMR pathway, removing it and inserting a gap. MMR keeps inserting a gap in every replication cycle, creating a "futile repair loop", which ultimately leads to apoptosis. If MMR is not functional, then the mispair continues in the strand and, in the next replication, thymine is paired with an adenine, resulting in a point mutation. Adapted from [Allan, J.M., *et al.*, 2005].

## 1.4 – Epigenetics and cancer

In the past decade, it has been found that carcinogenesis does not arise by just genetic alterations in the DNA sequence, but also by alterations in gene expression that do not involve changes in the DNA sequence. This regulation is termed epigenetic

regulation and they are reversible, being transmitted during mitosis or meiosis. Epigenetic DNA modifications are an alternative pathway for colorectal carcinogenesis, affecting gene function even in the absence of mutations. Most colorectal tumors have epigenetic modifications that coexist with classical genetic alterations, such as *P53*, *β-catenin* and *KRAS* mutations. Epigenetic alterations found to contribute to malignant phenotypes are 5' cytosine methylation of CpG island and histone modifications [Goel, A., *et al.*, 2012].

### 1.4.1 – Epigenetic silencing by DNA methylation

Gene expression is regulated by interactions between transcription factors and the start codon (ATG) and the DNA sequences immediately before or after the start sequence. Cytosine methylation is the most well-known epigenetic change. The promoter regions are rich in C-G sequences, termed CpG islands, around 60% of all the human promoters, and in normal cells, methylation is usually found in CpG-poor regions and CpG-rich regions are normally protected from this type of modification [Kulis, M., *et al.*, 2010]. DNA methylation, controlled by DNA methyltransferases (DNMT), is necessary during development, but aberrant DNA methylation is known to be an important event in cancer development. Occasionally, the first exon and promoter regions of "housekeeping" genes or tissue-specific genes have unmethylated CpG islands. Fully methylated CpG islands are important in promoter regions of silenced alleles for selected imprinted autosomal genes and multiple silenced genes on the inactivated X-chromosome of females [Baylin, S.B., *et al.*, 2000; Goel, A., *et al.*, 2012; Kondo, Y., *et al.*, 2004].

As referred previously, the methylation of cytosines is mediated by a class of enzymes termed DNMT. Five members of this family have been identified until now in mammals: DNMT1, DNMT3a, DNMT3b, DNMT2 and DNMT3L, although only the first three have been found to participate in the assembly of the methylation pattern. These enzymes catalyze the addition of a methyl group to the 5' carbon of a cytosine, forming 5-methyl-cytosine. DNMT1 is the most abundant of the DNMT in somatic cells and interacts with the proliferating cell nuclear antigen (PCNA). DNMT1 is thought to be responsible for the copying of the methylation pattern after DNA replication, being referred as the "maintenance" methyltransferase. Both DNMT3a and DNMT3b are required for the *de novo* methylation that occurs in the genome following embryonic implantation, being referred as the "*de novo*" methyltransferases [Baylin, S.B., *et al.*, 2000].

DNMT1 is the major enzyme for maintenance of the methylation status of DNA during replication, but is also important for appropriate histone H3 modification, crucial for chromatin domain organization. DNMT1 is located at the replication fork and methylates the newly synthesized, hemimethylated DNA strand to maintain precisely the original DNA methylation pattern. DNMT1 recruits chromatin-modifying enzymes, such as HDAC1, HDAC2 and histone methyltransferase, and is important for the initiation of chromatin remodeling and, consequently, regulation of gene expression. The N-terminal domain of DNMT1 is essential to discriminate between hemimethylated and unmethylated DNA strands, having preference for the first one, decreasing the "*de novo*" methylation activity of this enzyme [Turek-Plewa, J., *et al.*, 2005]. Loss of DNMT1 function results in embryonic lethality in mice, demonstrating the importance of this protein for normal cell function [Kulis, M., *et al.*, 2010].

DNMT3a and DNMT3b methylate CpG dinucleotides and are responsible for the "*de novo*" methylation of DNA, especially during embryogenesis and their levels remain low in adult somatic tissues. Unlike DNMT1, these methyltransferases methylate without preference for hemimethylated DNA. DNMT3a expression is ubiquitous, but DNMT3b is expressed at low levels in most tissues and is intensely increased in tumor cell lines. These two enzymes can interact with DNMT1 and activate HDAC1, repressing gene transcription which indicates that DNMT3a and 3b may be involved in chromatin remodeling associated with gene transcription regulation. This may indicate that DNMT3b is important for tumorigenesis [Turek-Plewa, J., *et al.*, 2005]. Inactivation of these proteins leads to embryonic lethality, DNMT3b, or death shortly after birth, DNMT3a, in knockout mice. This could indicate that DNMT3b is important in the early stages of development, while DNMT3a is relevant in the later stages or after birth [Kulis, M., *et al.*, 2010].

The methylation status of regulatory DNA sequences is correlated with the transcriptional activity of genes. The presence of methylated CpG dinucleotides in the promoter region or first exon of the gene may have a direct or indirect effect on transcription. Methylation affects the promoter region and the start site of the gene, altering the configuration of the DNA strand, interfering with binding of transcription factors, silencing gene expression. This way, transcription factors do not bind correctly to the promoter region, preventing transcription. Indirectly, specific DNA-binding proteins will bind to the methylated CpG dinucleotides, which then block the interaction of the transcription factors with the DNA. It is considered that DNA methylation is the

initial step for establishing the inactive chromatin state. These proteins include $m^5$CpG-binding domain proteins (MBD) and $m^5$CpG-binding proteins (MeCPs) which are able to form complexes with histone deacetylases (HDAC) and ATP-dependent chromatin remodeling proteins. These proteins, in turn, are involved in the stabilization of the heterochromatin (transcriptionally inactive chromatin) structure, and gene silencing is achieved. These modifications are stably passed to the daughter cells, maintaining the methylation pattern in the next generation [Baylin, S.B., *et al.*, 2000; Goel, A., *et al.*, 2012; Kulis, M., *et al.*, 2010].

### 1.4.2 – Epigenetic silencing by histone modifications

Along with DNA methylation-induced transcriptional gene expression control, posttranslational covalent modifications of histones constitute another epigenetic mechanism. Histone modifications in the N-terminal tail control chromatin structure, and therefore gene expression. In the nucleus of eukaryotic cells, DNA is tightly condensed into chromatin, functioning as a scaffold in the regulation nuclear processes, such as transcription, DNA replication and repair, apoptosis and mitosis. The status of chromatin structures affects the ability of transcriptional regulatory proteins and RNA polymerase to access specific promoter regions, and consequently activate gene transcription. So, unmethylated chromatin is typically active while methylated chromatin is inactive [Baylin, S.B., *et al.*, 2000; Zheng, Y.G., *et al.*, 2008].

As referred before, histone modifications is another mechanism of epigenetic silencing of genes. Genomic DNA is folded by both histone, that are organized in cylindrical structures and constitute the histone core, and non-histone proteins, forming nucleosomes and a more structured form, referred to as chromatin. This dynamic packaging of the DNA is important to switch between on and off states of transcription, controlling gene activation and silencing. It is known that histone tail domains can be modified by a variety of processes, such as acetylation, phosphorylation and methylation. Histones modifications are governed by two factors: the type of modification and the amino acid involved in the modification. Histone methylation and acetylation/deacetylation are epigenetic modifications that are reversible and responsible for the activation or silencing of transcription, respectively. Histone modifications usually occur at 4 histones, H2A, H2B, H3, and H4. Histone acetyltransferases (HAT) and HDAC maintain the level of acetylation of the chromatin histones, balancing the euchromatin/heterochromatin structure. It is known that hypoacetylation of histones is

typically involved in gene silencing, while hyperacetylation activates gene expression. HATs add acetyl groups to lysine residues in histone tails, neutralizing their positive charges and relaxing the chromatin structure. HDACs interact with transcriptional activators and co-activators, reversing the action of HAT, condensing chromatin and not allowing transcription to occur. Methylation/demethylation is also controlled by enzymes termed histone methyltransferases and histone demethylases, respectively [Fang, J.Y., *et al.*, 2004; Goel, A., *et al.*, 2012].

### 1.4.3 – Epigenetic silencing and cancer

It has been found that tumors are overall hypomethylated, with the exception of specific hypermethylated genes. This hypomethylation contributes to genomic instability and activation of silenced oncogenes. Promoter hypermethylation is accepted as a mechanism for gene silencing, especially of tumor suppressor genes. This was first demonstrated in studies of retinoblastoma patients in which the promoter region of retinoblastoma tumor suppressor gene (*RB1*) was hypermethylated. Gene inactivation resulting from DNA promoter hypermethylation and histone deacetylation is a frequent epigenetic event in malignant transformation. This phenotype in which tumor suppressor genes are methylated and carcinogenesis occurs through silencing is termed "CpG island methylator phenotype" or CIMP. Many genes have been found to be hypermethylated in CRC tissue, such as DNA repair genes, *hMLH1* and *MGMT*, and cell cycle regulators, *p16* and *p15*, and substantial degrees of hypermethylation have been found already in early colon cancer lesions, aberrant crypt foci (ACF) [Goel, A., *et al.*, 2012; Kulis, M., *et al.*, 2010]. It is possible to classify tumors as non-CIMP, CIMP-low and CIMP-high according to the proportion of promoters that surpass a certain degree of DNA methylation. CIMP-high appears in around 15% of CRC cases and is almost always present in tumors with *MLH1* silencing [Goel, A., *et al.*, 2012]. The death-associated protein kinase or DAPK is a protein involved in apoptotic and autophagic cell death, tumor and metastasis suppression. This protein induces cell death when overexpressed in cells. It has been found to possess a hypermethylated promoter in more than 20 types of human cancers including lymphomas, lung, breast, colon, prostate and brain. This fact further implies the tumor suppressor activity of this protein [Gozuacik, D., *et al.*, 2006].

As referred previously, hyperacetylation of histones opens chromatin, a process necessary for transcriptional activation. It has been shown that transcriptionally inactive gene promoters are characterized by trimethylation of histone H3 lysine 9 and 27,

H3K9me3 and H3K27me3, respectively. Transcriptionally active modifications found are dimethylation and trimethylation of histone H3 lysine (H3K4me2/me3) and acetylation of H3/H4 (H3K9Ac and H4K9Ac). So, HDAC inhibitors, such as sodium butyrate (a short chain fatty acid), have been investigated as possible candidates for cancer treatment because of the effects on genes regulating proliferation [Fang, J.Y., *et al.*, 2004; Spurling, C.C., *et al.*, 2008; Zheng, Y.G., *et al.*, 2008].

### 1.5 – Molecular pathways to CRC

Colorectal cancer is a major health problem, as it is one of the most common types of cancer worldwide. With the scientific advances, CRC has been shown to be a very heterogeneous disease. Nowadays, the molecular and genetic features of the tumors have an important role in the prognosis and treatment of the disease. The majority of CRC cases arise as sporadic carcinomas and develop through the classical adenoma-carcinoma sequence (Figure 8). Common *APC* changes which lead to a truncated protein, affecting β-catenin degradation by the proteasome, are considered to be initiating events. As mentioned above, destabilization of the complex allows β-catenin to be translocated to the nucleus and activate transcription of key players in the regulation of proliferation and apoptosis [Arends, M.J., 2013; Fearon, E.R., 2011]. In a study conducted with human samples of CRC, WNT signalling pathway was altered in 93% of all the cases studied, either by inactivation of APC, activating mutation of β-catenin, and alterations in other pathway intervenients [Network, T.C.G.A., 2012]. APC has also been found to be involved in exporting β-catenin from the nucleus, reducing transcription of β-catenin target genes. *KRAS* mutations are thought to be important for adenoma progression. The same study with human samples found that 55% of non-hypermutated tumors had activating alterations affecting *KRAS*, *NRAS* or *BRAF* [Network, T.C.G.A., 2012]. The tumor suppressor p53 protein is the “guardian of the genome” due to its pivotal role in responding to DNA damage-induced stress by interrupting the cell cycle for DNA repair or induction of apoptosis. Mutations in this gene are found in more than 60% of CRC, usually appearing at later stages of the adenoma-carcinoma transformation [Arends, M.J., 2013].

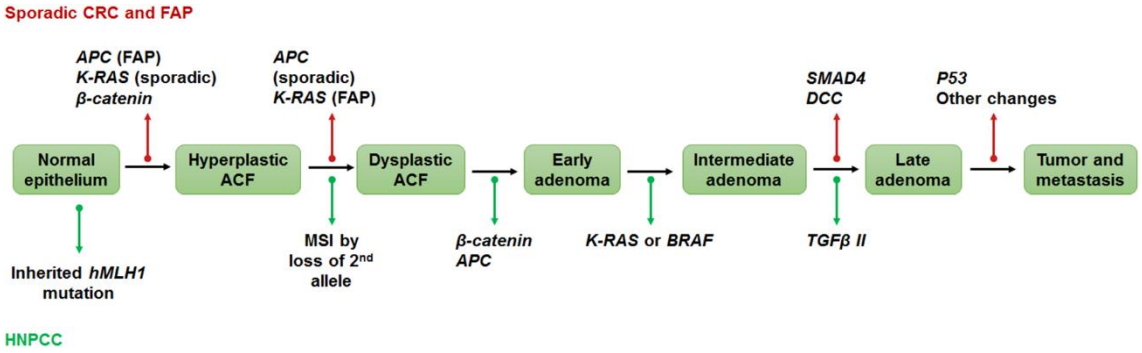


**Figure 8** – The colorectal adenoma-carcinoma sequence. The adenoma-carcinoma sequence shows the transitions from normal through the earliest stage of aberrant crypt foci to adenomas and ultimately carcinomas. Key molecular alterations are highlighted to distinguish sporadic, FAP and HNPCC. Adapted from [Fearon, E.R., 2011; Frank, S.A., 2007].

Less than half of CRC cases can be classified as hereditary colon cancer. Familial adenomatous polyposis coli syndrome (FAP) is a type of hereditary colon cancer that starts with an inherited mutated *APC* gene. This alteration causes the development of hundreds of adenomatous polyps in the colon and increases the risk of CRC to nearly 100%. FAP patients inherit an *APC* mutation in one allele and are wild-type in the other allele, acquiring the second *APC* alteration, which can be a mutation or promoter methylation. All the cells of a FAP patient have the initial *APC* mutation and the second alteration happens in a number of colorectal cells, leading to hundreds of adenomas [Arends, M.J., 2013; Fearon, E.R., 2011; van Wezel, T., *et al.*, 2012]. In hereditary nonpolyposis colorectal cancer (HNPCC) syndrome, also known as Lynch syndrome (LS), there is an inherited alteration, mutation or epigenetic silencing, in a MMR gene in one allele, in which the most commonly inherited mutant genes are *MLH1* and *MSH2*. After the inactivation of the same gene in the second allele, the cell's MMR system becomes faulty, increasing the rate of mutation due to the inability to repair DNA damage. When the mutations occur at nucleotide repeats, termed microsatellites, microsatellite instability arises, and this is also a common characteristic of LS. These patients develop a smaller number of polyps, but it is believed that these progress at a faster rate to carcinoma [Arends, M.J., 2013; Banno, K., *et al.*, 2012; Fearon, E.R., 2011].

Apart from the conventional adenoma-carcinoma, the serrated neoplasia pathway can be responsible for around 30% of CRC cases. This pathway involves the transition of hyperplastic polyps, which are found in patients with hyperplastic polyposis syndrome, to serrated adenomas, and then further to invasive adenocarcinomas. In this pathway, two molecular features, MAPK pathway activation and CIMP, in which the MAPK pathway

activation can appear due to *BRAF* or *KRAS* mutation. There are three major categories of serrated polyps: hyperplastic polyps, sessile serrated adenomas, and traditional serrated adenomas. The first are common large polyps, with *BRAF* or *KRAS* mutation and are usually not considered potential malignant precursors. In sessile serrated adenomas, the initiating event is thought to be a *BRAF* mutation, while promoter silencing of *MLH1* is found in adenomas with high-grade dysplasia. Other alterations are found in subsets of these types of adenomas, including activation of WNT signalling and methylation of *MGMT*. These lesions are more common on the right side of the colon, more specifically in the cecum, ascending colon and transverse colon. The traditional serrated adenomas are less frequently found, around 1% of colorectal polyps and are more common in the distal side of the colon, sigmoid colon and rectum. These lesions are still poorly understood as they are confused with the other types of polyps. *KRAS* mutation and *MGMT* silencing are common in advanced lesions and CIMP-H has been reported in many cases, but *BRAF* mutations can also be found [Arends, M.J., 2013; Bettington, M., *et al.*, 2013].

Considering the pathological, molecular, and clinical characteristics of CRC tumors, Jass *et al.* developed a 5 subtype classification for CRC [Jass, J.R., 2007]:

1) CIMP-H/MSI-H/*BRAF* mutation;
2) CIMP-H/MSI-L or microsatellite stable (MSS)/*BRAF* mutation;
3) CIMP-L/MSS or MSI-L/*KRAS* mutation;
4) CIMP-negative/MSS;
5) HNPCC/CIMP-negative/MSI-H.

It is clear now that colorectal cancer is not just one type of disease, it comprises a heterogeneous group of diseases. Knowing the characteristics of the tumors can help provide better prognostic and treatment options [Bettington, M., *et al.*, 2013; Bogaert, J., *et al.*, 2014].

## 1.6 – Animal models of colorectal cancer

As referred previously, CRC is an accumulation of genetic and epigenetic modifications, giving cells advantage to divide uncontrollably. This process is stepwise, with diverse intermediates before reaching the carcinoma stage. The various stages of the colorectal carcinogenic process can be studied using adequate animal models, investigating effects in the different pathways that can be altered. There are several animal

models for CRC, and these can be divided into induced and transgenic models, excluding the rare cases where CRC develops spontaneously in aged rodents [Johnson, R.L., *et al.*, 2013; Perse, M., *et al.*, 2011]. Due to the diversity of human CRC, it is difficult to find an adequate model for all types of CRC in a single animal model. Nevertheless, animal models can be used to study specific alterations important to colon carcinogenesis, as long as the model maintains three important characteristics [Johnson, R.L., *et al.*, 2013]:

1 – The cancer should be limited to organ of interest, in this case the colon, so there is no interference with diseases in other organs;

2 – The lesions studied should be similar to the lesions found in the human form of the cancer;

3 – The models should seize the complex cellular interactions that are important for carcinogenesis.

### 1.6.1 – Chemically induced models - 1,2-dimethylhydrazine/azoxymethane

Among the induced CRC models, 1,2-dimethylhydrazine (DMH) and azoxymethane (AOM) are the chemicals mostly used. Identification of these carcinogens was found when large quantities of cycad flour were fed to rats and colon adenocarcinomas were found [Rosenberg, D.W., *et al.*, 2009]. Usually, the endpoint of studies with these carcinogens are intermediate, pre-neoplastic lesions termed aberrant crypt foci (ACF), in short-term studies, or colonic tumors in long-term studies. The DMH/AOM model shares many similarities to human sporadic colon cancer, including responses to some preventive and promotional agents. Due to this fact, they are widely used today to study chemopreventive, dietary and environmental agents. DMH is metabolically activated, in the liver, through a cascade of intermediates, including AOM and methylazoxymethanol (MAM), until the highly reactive carcinogenic metabolite, methyldiazonium ion. Once converted, MAM can enter the blood stream and travel to epithelial cells in the colon. In the colon, MAM is metabolized into methyldiazonium ion, which, in turn, induces methylation of DNA, increasing loss of epithelial cells by apoptosis, increasing proliferation and the rate of mutation. The most important lesion this carcinogen generates is $O^6$-methylaguanine, which, can mispair with thymidine, resulting in a point mutation, if not repaired correctly [Perse, M., *et al.*, 2011; Rosenberg, D.W., *et al.*, 2009; Washington, M.K., *et al.*, 2013].

ACF were first visualized by Bird in 1987 [Bird, R.P., 1987] and since then these lesions have been used as biomarkers for CRC studies. In the last few years, there have

been additional lesions identified as biomarkers, such as dark ACF, flat ACF, mucin-depleted foci (MDF), and β-catenin accumulated crypts (BCAC). ACF can be seen with a simple methylene blue staining and have been found in human colon as well as carcinogen treated rodents. These lesions are a heterogeneous group, as it is fairly easy to find ACF with hyperplasia but it is also possible to find lesions with dysplasia. These lesions with dysplasia are accepted as indicators of increased risk for cancer progression and, after identification, can be classified as flat ACF or dark ACF, depending on surface morphology. When using high-iron diamine alcian blue, one can visualize crypts with mucous production. Aberrant crypts with little or no production of mucous are termed mucin-depleted foci, and these are dysplastic crypts which share frequent genetic and epigenetic alterations with colon cancer. After carcinogen administration, MDF appear over a period of around 7 weeks and increase in number and multiplicity with time. BCAC are only found with immunohistochemical protocols, searching for β-catenin accumulation in the nucleus of colon cells. This accumulation is often due to a mutation in the *β-catenin* gene, giving these lesions a higher relevance as biomarkers for CRC [Perse, M., *et al.*, 2011].

In the DMH/AOM model, *Apc* mutations are found to a lesser extent than β-catenin mutations, although these alterations are only found in neoplastic lesions and have not been found in hyperplastic ACF. *Apc* alterations have been found to be missense or truncated point mutations, while β-catenin mutations are G to A point transitions in the GSK3β phosphorylation motif due to $O^6$-MeG damage. Either mutation gives the same result as in human carcinogenesis, inability to phosphorylate β-catenin and, therefore, β-catenin accumulation in the nucleus and activation of transcription factors. *Kras* point mutations in codon 12 have been found in hyperplastic lesions, in adenomas and in adenocarcinomas of the DMH/AOM animal model, while it is very rare to find *p53* mutations. In this model, *Kras* mutations have the same effect as in human CRC, increased MAPK activation leading to increased proliferation. Also, the frequency of this mutation in small adenomas and large adenoncarcinomas is similar to the frequencies found in human cancer. This type of alteration has also been found frequently in hyperplastic ACF, more than in dysplastic ACF. In human CRC, *TP53* mutations are frequently found in later stages of the adenoma to carcinoma transition, but in the DMH/AOM animal model, this alteration is very rarely found [Perse, M., *et al.*, 2011; Takahashi, M., *et al.*, 2004].

### 1.6.2 – Transgenic models

One of the most used genetically modified animal model is the *APC*$^{min/+}$ mice model, a model extensively used to study the prevention, development or treatment of CRC with somatic *APC* alterations. This model was reported in 1990 after a germline truncating mutation of *Apc* was induced with ethylnitrosourea in C57B1/6J mice. These mice develop multiple intestinal neoplasms or "Min", where most tumors appear in the small intestine rather than in the large intestine in heterozygotic mice. Although this difference in the model compared to human FAP, the phenotypic and histopathological features are similar to those found in colon tumors in FAP patients, so this model is widely used in studies to understand the role of *APC* mutation in cancer. As in FAP, loss of the functional protein in the second allele is needed for adenoma formation [Johnson, R.L., *et al.*, 2013; Suman, S., *et al.*, 2012].

Another model for the study of hereditary CRC, FAP, is the *Pirc* (polyposis in the rat colon) rat, which was also induced by ethylnitrosourea in F344 rats. This model harbors a heterozygous point mutation at the *Apc* gene that results in a truncated protein. These rats develop adenomas throughout the intestine and colon and they mimic the morphology of human adenomas and the progression to invasive adenocarcinoma sequence. In homozygotes, this mutation is embryonic lethal, while in the heterozygous, animals become mortally ill in less than a year [Johnson, R.L., *et al.*, 2013; Washington, M.K., *et al.*, 2013].

Heterozygous or homozygous mice for DNA MMR genes are also used, mostly to study Lynch syndrome. Lynch syndrome, as referred before, is an inherited condition where cells present a deficient MMR system, usually caused by mutation or epigenetic silencing of one of the MMR genes, mainly *MLH1* and *MSH2*. Mouse models lacking *Mlh1*, *Msh2* and *Msh6* have been developed and tumors are found in the organs of the intestinal tract, including colon [Suman, S., *et al.*, 2012]. *Mlh1-/-* mice develop cancer in the intestine and lymphoid tissue, but usually die by the age of 9 months due to this phenotype. *Msh2* knockout mice develop intestinal tumors after 6 months of age, but often die due to lymphomas. Crossing mice that are *Mlh1* or *Msh2* knockout with heterozygous mutated *Apc* mice increase intestinal carcinogenesis, the same occurring with crossing *Msh2* and inducible *Kras* mutation, although in this last case there are more tumors in the small intestine than the colon [Johnson, R.L., *et al.*, 2013].

As most of these models only show the effects of one type of alteration, and as carcinogenesis is an accumulation of genetic modifications, investigators are crossing

different rodent strains, generating models representing key molecular events involved in CRC, for example, the MMR deficiency (*Msh2*$^{-/-}$) in combination with APC$^{Min/+}$. Due to the complexity of CRC, there seems to be no one ideal animal model to study all the genetic and epigenetic alterations that can lead to CRC. With this in mind, the selection of the appropriate model to use in compound chemopreventive/therapeutic studies is important to correctly understand the mechanistic, preventive or therapeutic action of the compound [Johnson, R.L., *et al.*, 2013; Suman, S., *et al.*, 2012].

## 2 – Diet and cancer

### 2.1 – General concepts

A large percentage of the etiology of colorectal cancer has been attributed to factors associated with lifestyle, such as obesity, lack of physical activity, smoking habits, and most importantly, diet. Modification of these factors may, therefore, be beneficial for prevention of the disease [Andersen, V., et al., 2013]. Diet seems to have a major role not only on the onset of the disease, but also in the possible chemoprevention. Chemoprevention can be considered the use of natural or synthetic compounds to prevent or delay the development of a disease and the fact that one of the risk factors of CRC is diet makes this disease an excellent case for chemoprevention studies. Animal studies give us the opportunity to test the role of environmental factors, such as dietary constituents, in the prevention or progression of colon cancer. The concept of colon cancer prevention involves inhibition, regression or elimination of precancerous lesions, reducing the incidence of cancer. It has been seen that bioactive dietary compounds may alter the expression of certain genes involved in a variety of cellular events and regulatory processes [Andersen, V., *et al.*, 2013].

Diet may also have adverse effects on colon cancer, being one of the main risk factors of this disease [Andersen, V., *et al.*, 2013; Durko, L., *et al.*, 2014; Yusof, A.S., *et al.*, 2012]. Red and processed meat has been associated with increased risk of CRC. Several hypotheses have been suggested for the reason behind this association, such as high heme intake, which induces ROS and these induce mutations and expression of cytokines, the production of malondialdehyde, a lipid peroxidation product, or the production of heterocyclic amines and *N*-nitroso compounds [Durko, L., *et al.*, 2014;

Oostindjer, M., *et al.*, 2014]. Also, diets with high intake of saturated fats are associated with CRC incidence. A proposed mechanism for this risk factor is the stimulation of bile acids discharge. Although bile acids are needed for proper digestion, some have been shown to be CRC promoters. The next section will explain bile acids and the mechanism for tumor promotion [Barrasa, J.I., *et al.*, 2013].

## 2.2 – Bile acids

It is known that diet is one of the risk factors of colon cancer development. Research has shown that high saturated fat intake increases the risk of CRC. This effect seems to be related with the increase of endogenous cholesterol biosynthesis that, when combined with exogenous cholesterol intake, can lead to increased bile acid biosynthesis. Fatty acids have the ability to irritate the colon and induce a local inflammatory response, increasing the production of ROS or reactive nitrogen species (RNS) in colon cells. High ingestion of fats also stimulate the release of bile acids, which induce an increase in cell proliferation, acting as promoters of CRC. It has been shown that there is an increase of fecal bile acid concentrations in individuals with increased risk for CRC [Bernstein, H., *et al.*, 2009].

Bile acids are natural detergents that facilitate the uptake of lipids and fat-soluble vitamins. The primary bile acids cholic (CA) and chenodeoxycholic acid (CDCA) are synthesized in the liver from cholesterol and high intake of fat induces the release of these bile acids into the small intestine, after conjugation with glycine or taurine. The bile salts are then actively absorbed in the ileum and return to the liver, by the enterohepatic circulation, although some bile salts are able to escape this process and enter the colon. In the colon, these bile salts suffer various enzymatic reactions, such as deconjugation and dehydroxylation, by bacteria in the colonic lumen, forming secondary bile acids, such as deoxycholic (DCA) and lithocholic acid (LCA). Ursodeoxycholic acid (UDCA) is also formed by the gut microbiota by epimerization of CDCA [De Preter, V., *et al.*, 2011; Payne, C.M., *et al.*, 2008]. A representation of the chemical structures of the bile acids are shown in Figure 9.

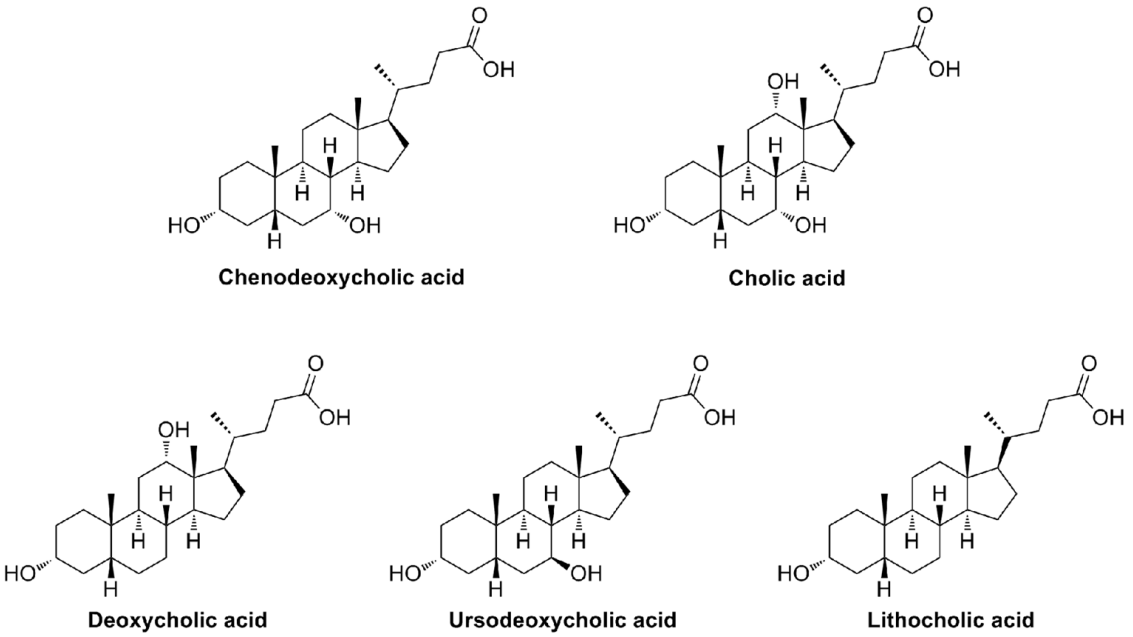


**Figure 9** – Representation of the chemical structures of the different bile acids referred in the text.

It has been found that it is possible that the bacteria in the colonic lumen can influence multiple processes involved in colon carcinogenesis. There has been a link between bacteria and production of toxic and genotoxic metabolites that affect colon cell signal transduction. Also, carcinogenic agents can be formed during bacterial transformation in the gut. There is a higher bacterial density in the colon than in the small intestine. In 1975, the first study showing the link between gut microbiota and colon carcinogenesis was by Reddy *et al*. [Reddy, B.S., *et al.*, 1975]. In this study, Reddy demonstrated that only 20% of germ-free rats developed colon cancer after treatment with a chemical inducer, while in conventionally maintained rats 93% developed colon tumors. There are several strains of bacteria found in the human intestine, and some of these strains have been implicated in the development of cancer, such as *Streptococcus bovis*, *Bacteriodes* spp, *Clostridia* spp, and *H. pylori*, while other strains have been shown to have the opposite effect, such as *Lactobacillus acidophilus* and *Bifidobacetrium longum*. For example, *S. bovis* has been shown to induce increase of proliferation in colonic crypts and increase proliferation markers in carcinogen-treated rats [Ellmerich, S., *et al.*, 2000]. Also, it seems that diet has a high impact on the type of strains present in the microbiota. Gut microbiota appears to increase colon cancer risk by inducing inflammation, generating reactive metabolites, which can induce damage, and converting primary bile acids to tumor promoting secondary bile acids [Del Chierico, F., *et al.*, 2014; Zhu, Q., *et al.*, 2013].

Secondary bile acids have been shown to be promoters of colon carcinogenesis. Although concentration of bile acids in the colon are relatively low, compared to concentrations in the gall bladder or small intestine, some secondary bile acids, such as DCA, can achieve concentrations of 700 to 800 μM in the cecum with high-fat diets or pathological anomalies [Barrasa, J.I., *et al.*, 2013]. *In vivo*, deoxycholic acid promotes growth of ACF and increases translocation of β-catenin to the nucleus, which provides an increase of proliferation [Flynn, C., *et al.*, 2007]. Nevertheless, the effects of secondary bile acids to the promotion of carcinogenesis are vast. One of the first immediate effects of bile acids to colon cells is the induction of ROS and RNS. This increase can be caused by damage to mitochondria or by release of arachidonic acid from the cell membrane and, consequently, ROS production by partial reduction of $O_2$. Also, DCA has been found to activate NF-κB in colon cells. This activation may induce and increase nitric oxide synthase 2, which generates NO. The production of ROS/RNS increases DNA damage and, consequently, favors the appearance of mutations. With the increase of ROS/RNS, it is possible to conclude that bile acids can induce DNA damage and cause mutation while decreasing DNA repair enzymes, which expression decreases with the increase of oxidative stress [Bernstein, H., *et al.*, 2009; Payne, C.M., *et al.*, 2008].

DCA has been shown to produce more damage to the cell membrane than any other bile acids and their conjugates. It was found that DCA alters membrane composition, through redistribution of cholesterol, and microdomains, dysregulates membrane-bond receptors, and activates surface enzymes, such as epidermal growth factor receptor and protein kinase C. The activation of these surface proteins can be the cause for DCA modulation of signaling pathways [Jean-Louis, S., *et al.*, 2006]. Other effects that have been found to be due to bile acid exposure are micronuclei formation, induction of endoplasmic-reticulum stress, and consequently apoptosis, autophagy or necrosis [Barrasa, J.I., *et al.*, 2013; Payne, C.M., *et al.*, 2008].

On the other hand, UDCA, a less hydrophobic bile acid, has been shown in several publications that it has chemopreventive potential. This compound was initially used for treatment of gallstones and primary biliary cirrhosis. UDCA not only has different biological effects than DCA, but can also inhibit DCA's deleterious effects. In one study, it was found that UDCA is able to inhibit cell proliferation in a colon cancer cell line, suppressing c-Myc expression [Peiro-Jordan, R., *et al.*, 2012]. In other studies, UDCA was found to inhibit DCA-induced apoptosis in HCT116 colon cancer cell line [Im, E., *et al.*, 2004; Powell, A.A., *et al.*, 2006]. This same group also demonstrated that UDCA can

induce differentiation in colon cancer cells by inducing histone hypoacetylation [Akare, S., *et al.*, 2006]. In high-risk populations, UDCA was also found to decrease the risk of colorectal cancer development. In animal models, UDCA was found to inhibit *Ras* mutations and cyclooxygenase-2 expression in AOM-induced carcinogenesis. As UDCA is a hydrophilic bile acid and DCA has a hydrophobic character, the differences in mode of action could be due to the hydrophilicity of the compounds [Barrasa, J.I., *et al.*, 2013].

### 2.3 – Natural compounds

Natural products have been shown to be promising agents that may play a role in cancer prevention as well as in cancer therapy, by modulating common signaling pathways in cancer development, but also in DNA damage prevention or repair. So, chemoprevention can be targeted to specific molecular processes. Many epidemiological studies have found a correlation between consumption of fruits and vegetables and the decrease risk of CRC.

Folic acid (vitamin B9) is not synthesized by the human organism, so its source is fruits and dark green vegetables. It has been found that deficiency of this vitamin is linked to increased colorectal lesions, while supplementation correlates with decreased risk in patients with ulcerative colitis. The assumed molecular mechanisms for folic acid CRC prevention include DNA synthesis and repair, but also the methylation state of genes. Epidemiological studies have shown also an association of folic acid and decreased colon carcinogenesis [Link, A., *et al.*, 2010].

Polyphenols have been shown to have a wide variety of beneficial effects for overall human health. Curcumin has antioxidant, anti-inflammatory and anticarcinogenic properties and has been found to inhibit cell invasion and decrease DNMT activity, reducing gene silencing [Link, A., *et al.*, 2010; Vanden Berghe, W., 2012]. Quercetin, a flavonol, has been found to interact with β-catenin, decreasing cell proliferation, but also to inhibit HAT activity, reducing gene expression [Pericleous, M., *et al.*, 2013; Vanden Berghe, W., 2012]. Quercetin has also been found to suppress ACF formation in a colon cancer induced model [Bordonaro, M., *et al.*, 2014; Warren, C.A., *et al.*, 2009]. It was found that (-)-epigallocatechin-3-gallate (EGCG), a major polyphenolic compound from green tea, inhibits DNMT activity and reactivates methylation-silenced genes present in cancer cells, such as *MGMT* and *hMLH* [Fang, M.Z., *et al.*, 2003]. The chemical structures of some natural compounds are shown in Figure 10.

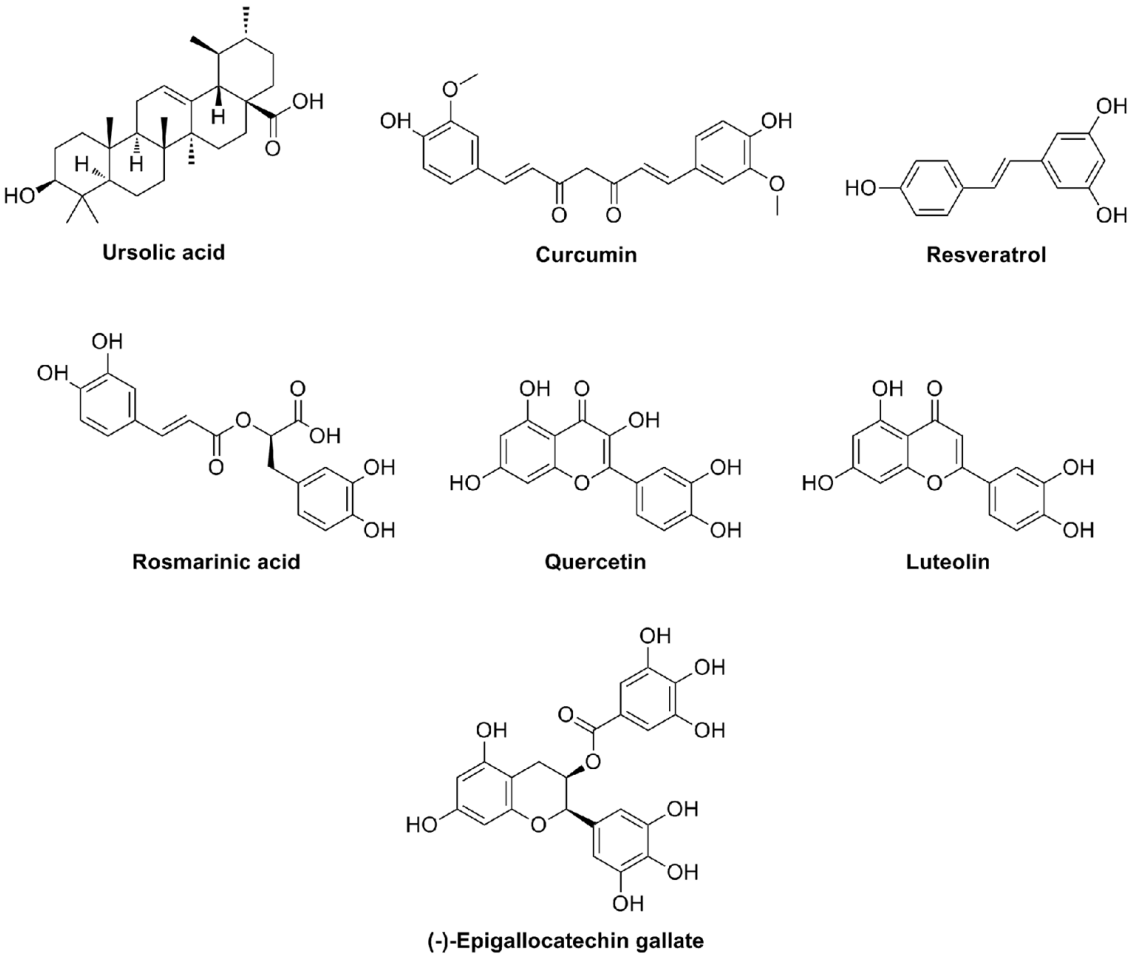


**Figure 10** – Representation of the chemical structures of the different natural compounds referred in the text.

Studies in our lab have demonstrated other effects of diet on CRC prevention: at the level of DNA protection and/or induction of repair and of molecular targets of cell proliferation and death. These studies demonstrated that ursolic acid and luteolin protect DNA from oxidative damage, increase DNA repair activity [Ramos, A.A., *et al.*, 2010b], but also have antiproliferative and proapoptotic effects [Xavier, C.P., *et al.*, 2009b]. Also, extracts from the plant genius *Salvia* had chemopreventive potential by stimulating DNA repair and protecting cells against oxidative DNA damage in Caco-2 [Ramos, A.A., *et al.*, 2010a] and in CO115 and HCT15 [Ramos, A.A., *et al.*, 2012]. In another study, these extracts induced apoptosis and inhibited cell proliferation by interfering in the MAPK signaling pathway [Xavier, C.P., *et al.*, 2009a].

Alterations in lifestyle and diet can help lower colorectal incidence and/or progression. The introduction of foods with compounds that have chemopreventive activity can also aid in the prevention of CRC. The introduction of a healthy dietary pattern and good lifestyle habits would not only bring benefits for colorectal cancer prevention, but also the overall health of the individual.

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# THE EFFECTS OF BILE ACIDS ON COLON CARCINOGENESIS

*Chapter 2*

# 1 - Effects of deoxycholic acid and ursodeoxycholic acid in Caco-2 cells

## 1.1- Introduction

Bile acids are amphiphilic acidic steroids which are synthesized from cholesterol in the liver, originating cholic and chenodeoxycholic acid as primary bile acids. After synthesis, these bile acids are conjugated with taurine or glycine, generating taurocholic or glycocholic acid and taurochenodeoxcholic or glycochenodeoxycholic acid, respectively, and released into the bile. In the intestine, bile acids aid in fat digestion and absorption and protect lipolytic enzymes from proteolytic degradation. Intestinal microflora metabolize bile acids converting them into secondary bile acids, for example, deoxycholic acid (DCA) [Bernstein, H., *et al.*, 2009; Zhu, Q., *et al.*, 2013]. Deoxycholic acid has been associated with increased risk of colon cancer development, whereas ursodeoxycholic acid (UDCA), also produced in the gut by the microflora, has been associated as a chemopreventive compound [Barrasa, J.I., *et al.*, 2013; Bernstein, H., *et al.*, 2009].

It is known that the content of the intestinal lumen is dependent on diet and a diet with high saturated fat intake stimulates bile acid discharge. The constant exposure to high levels of bile acids in the large intestine can have two main consequences that may explain bile acid promotion of colorectal cancer: promotion of DNA damage by oxidative stress and selective growth of apoptotic-resistant cells. The apoptotic effect of bile acids on hepatocytes has been extensively studied, but there is also an interest on the cellular effects of these compounds on colonocytes and colon cancer. There are already several studies that have shown the effect of bile acids, especially DCA, in the promotion of colon carcinogenesis, but also the potential of being carcinogens themselves [Bernstein, C., *et al.*, 2011; Flynn, C., *et al.*, 2007; Payne, C.M., *et al.*, 2010]. In colon cancer cell lines, various studies have shown some mechanisms on how DCA or UDCA can affect cell proliferation and apoptosis [Glinghammar, B., *et al.*, 2002; Im, E., *et al.*, 2004; Jean-Louis, S., *et al.*, 2006; Powell, A.A., *et al.*, 2006; Qiao, D., *et al.*, 2001].

In this study, we used Caco-2 cells, a human colonic adenocarcinoma cell line, to study the effects of DCA and UDCA in cell proliferation, apoptosis and DNA damage. Most studies use HCT116 or HT29 cell lines to study the impact of bile acids on colon cells, however these cell lines harbor various genetic or epigenetic alterations in the

MAPK signaling pathways, giving us information on the effect of bile acids in tumor cells. Caco-2 cells are microsatellite stable, mismatch repair proficient, and *KRAS* and *BRAF* wild-type, having characteristics closer to normal colonocytes.

## 1.2 – Material and methods

### 1.2.1 – Reagents and antibodies

*N*-Methyl-*N*-nitrosourea (MNU), $O^6$-benzylguanine (BG), thiazolyl blue tetrazolium bromide (MTT), DCA, UDCA, Dulbecco's modified Eagle medium (DMEM), penicillin/streptomycin, and trypsin solution were purchased from Sigma–Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS) was purchased from Biochrom KG (Berlin, Germany). SYBR Gold (nucleic acid gel stain) was from Invitrogen Molecular Probes (Eugene, OR, USA). The protein quantification DC protein assay was purchased from Bio-Rad Laboratories (Hercules, CA, USA). Primary antibodies were purchased from the following sources: anti-actin was purchased from Sigma–Aldrich; anti-JNK, anti-phospho-JNK, anti-phosphoERK1/2, anti-K-Ras, anti-B-Raf, anti-MGMT, anti-MLH1 Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA); and anti-p44/42 MAPK (ERK1/2), anti-phospho-Akt (ser473), and anti-Akt from Cell Signaling (Danvers, MA, USA). Peroxidase-conjugated goat anti-mouse antibody and Immobilon Western blotting detection reagents were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and Millipore (Billerica, MA, USA), respectively. All other reagents and chemicals used were of analytical grade.

### 1.2.2 – Cell line and culture conditions

Caco-2 cells, derived from human colon carcinoma, were maintained as monolayer cultures in DMEM supplemented with 10% FBS and antibiotics (100 U/mL penicillin and 100 mg/mL streptomycin), under an atmosphere of 5% $CO_2$ at 37 ºC. Cells were seeded onto 6- (2 mL) or 12- (1 mL) well plates at a density of $0.1\times10^6$ cells/mL. Test compounds were added to culture medium to the desired concentration ensuring that the DMSO concentration did not exceed 0.5% (v/v); controls received vehicle only.

### 1.2.3 – Assessment of cell viability/proliferation by MTT reduction test

A MTT reduction assay was performed in order to evaluate the toxicity of DCA and UDCA in this colon cancer cell line. Cells were treated with test compounds for 6,

24 and 48 h, including 2 h incubation with MTT (final concentration 0.5 mg/mL). The formazan crystals were then dissolved in a solution of DMSO/ethanol (1:1). The number of viable cells in each well was estimated by the cell capacity to reduce MTT. The results were expressed as percentage relative to the control (cells without any test compound).

### 1.2.4 – Nuclear condensation assay

The effects of DCA (500 µM) or UDCA (500 µM) treatment on induction of apoptosis in Caco-2 cells was assessed by nuclear condensation assay. The number of apoptotic cells was counted after 48 and 72 h as previously described [Xavier, C.P., *et al.*, 2009]. Staurosporin (STS) at 0.5 µM was used as positive control. At least 500 cells were counted and the number of apoptotic cells was divided by the total number cells counted to give the percentage of cell death.

### 1.2.5 – Genotoxicity assay

The alkaline version of the single cell gel electrophoresis (comet) assay was used to evaluate DNA damage [Collins, A.R., 2004]. Caco-2 cells were incubated for different time points (15 min, 30 min, 1 h, 6 h, 24 h and 48 h) at 37 ºC with DCA or UDCA at different concentrations (100, 500 and 750 µM). Cells were collected by trypsinization and around 50,000 cells were centrifuged for 1 min at 5,000 rpm, ressuspended in low melting point agarose and spread onto agarose-coated slide using a cover slip. After 10 min at 4 ºC, the coverslips were removed and slides were placed in lysis solution (2.5 M NaCl, 100 mM $Na_2EDTA$, 10 mM Tris base, pH 10, plus 1% Triton X-100) for 1 h at 4 ºC. Slides were then placed in a horizontal electrophoresis chamber with electrophoresis solution (0.3 M NaOH, 1 mM $Na_2EDTA$, pH > 13) for 40 min at 4 ºC for the DNA to unwind before electrophoresis for 20 min at 0.8 V/cm and ~300 mA. After electrophoresis, slides were washed twice with PBS and dried at room temperature (RT). For analysis of the comet images, slides were stained with SYBR Gold solution for 30 min at 4 ºC; after drying, the slides were analyzed using a fluorescence microscope and the Comet IV analysis system (Perceptive Instruments Ltd, Haverhill, UK) was used to calculate the parameter percentage of DNA in the tail. About 100 randomly selected cells were analyzed per sample.

### 1.2.6 – DCFH oxidative stress assay

The capacity of DCA to induce reactive oxygen species was evaluated using the dichlorofluorescin (DCFH) oxidative stress assay. Briefly, culture medium was removed and cells were washed with PBS. Afterwards, cells were incubated with 2′,7′-dichlorofluorescein diacetate (DCF-DA) (100 µM) or DMSO (vehicle) for 30 min at 37 ºC. This solution was removed and cells were washed with PBS. Cells were then incubated with DCA (500 µM) or *tert*-butyl hydroperoxide (200 µM), diluted in serum-free medium, for 30 min at 37 ºC. The compounds were removed and cells were lysed with a solution of 90% DMSO/10% PBS for 10 min at RT in the dark. Fluorescence was read at 520 nm upon excitation at 485 nm.

### 1.2.7 – Western blot analysis

Cells were incubated with DCA or UDCA (500 µM) for 6, 24 and 48 h. Total cell lysates were prepared to measure expression of different proteins. The cells were washed with PBS 1x and lysed for 5 min at 4 °C with ice-cold RIPA buffer (1% NP-40 in 150 mM NaCl, 50 mM Tris (pH 7.5), 2 mM EDTA) supplemented with 20 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 20 mM $Na_3VO_4$ and protease inhibitor cocktail (Roche, Mannheim, Germany). Protein concentration was quantified using the DC protein assay following the manufacturer's instructions, and 20 µg/well was separated by 12% SDS–PAGE and transferred to polyvinylidene difluoride membranes. Membranes were blocked and incubated with primary antibody overnight. After washing, membranes were incubated with secondary antibody conjugated with IgG horseradish peroxidase for 1 h, and immunoreactive bands were detected using the Immobilon solutions (Millipore, Billerica, MA, USA) under a chemiluminescence detection system (Chemi Doc XRS; Bio-Rad Laboratories, Inc.).

Band area intensity was quantified using the Quantity One software from Bio-Rad. β-Actin was used as loading control.

### 1.2.8 – Statistical analysis

Statistical analyses were done using t test, one-way or two-way analysis of variance, using GraphPad Prism 4.0 software, when appropriate (San Diego, CA, USA). P values $\leq 0.05$ were considered statistically significant. All results are presented as mean

± SEM of at least three independent experiments. Images are representative of three independent experiments.

### 1.3 – Results and discussion

In this study, we evaluated the effect of two bile acids, deoxycholic acid and ursodeoxycholic acid, on proliferation and DNA damage in Caco-2 cell line. One of the most used cell lines is HCT116, but as this cell line has many alterations in signaling pathways and DNA damage repair pathways, it gives us evidence of the effects of bile acids in tumor cells. Caco-2 cell line has fewer alterations in key pathways and has functional $O^6$-methylguanine-DNA methyltransferase (MGMT) and mismatch repair systems, giving more information on possible mechanisms in normal colonocytes. Few studies have used Caco-2 for the evaluation of bile acids effects. In this study, high concentrations (500 and 750 µM) of DCA induced cell death at 24 and 48 h of treatment (Figure 1). This data is in accordance with a study by Milovic, V *et al*., where they found that apoptosis was induced with higher concentrations of the same bile acid, in Caco-2 cell line [Milovic, V., *et al.*, 2002]. We also tested a hydrophilic bile acid, UDCA, which is considered to be protective against colon carcinogenesis. With this bile acid, we found no significant effects on cell proliferation at any of the incubation times or the concentrations tested (Figure 1). Krishna-Subramanian *et al*., have shown that, in IEC-6 intestinal cells, a 72 h incubation of UDCA induced growth arrest of these cells [Krishna-Subramanian, S., *et al.*, 2012]. They used the IEC-6 cell line due to the fact that this cell line mimicked the effects of UDCA in epithelial colonic cells from mice, while HCT116 and HCT8 did not. As seen in Figure 1, for 750 µM of UDCA we also see a slight tendency (22%) of decreased proliferation with 48 h incubation. This tendency could suggest an effect of inhibition of proliferation of UDCA in this cell line, similar to the effects seen with the IEC6 cell line.

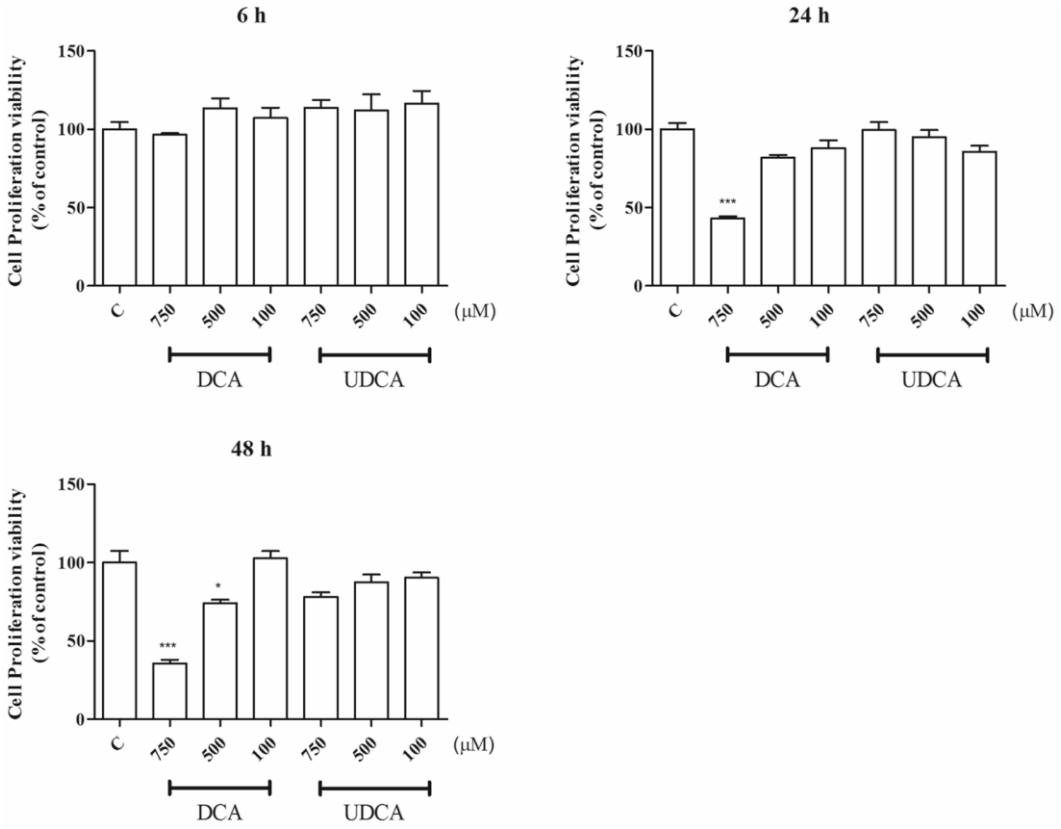


**Figure 11 –** The effect of different concentrations (750, 500 and 100 μM) of DCA and UDCA on cell viability at 6, 24 and 48 h, in Caco-2 cells. Cell viability was measured by the MTT assay and results are the mean ± SEM of at least 3 independent experiments. *P≤ 0.05 and ***P≤ 0.001, when compared with control were determined by one-way ANOVA followed by Newman-Keuls multiple comparison test.

As we found that 500 μM of DCA decreased cell proliferation, and this concentration is widely used in other studies, we tested this concentration for induction of cell death and for comparative reasons, the same concentration of UDCA was used. After 72 h incubation of DCA, there was a significant increase of cell death, while UDCA treatment showed no effect at this concentration (Figure 2). It has been reported that DCA is a stress inducer, causing membrane alterations, DNA damage, mitotic stress, and other perturbations, ultimately leading to apoptosis in various cancer cell lines [Payne, C.M., *et al.*, 2008]. In cancer cells, as survival pathways are usually activated, apoptosis is evaded, so with this cell line we have a result possibly similar to what happens in normal cells.

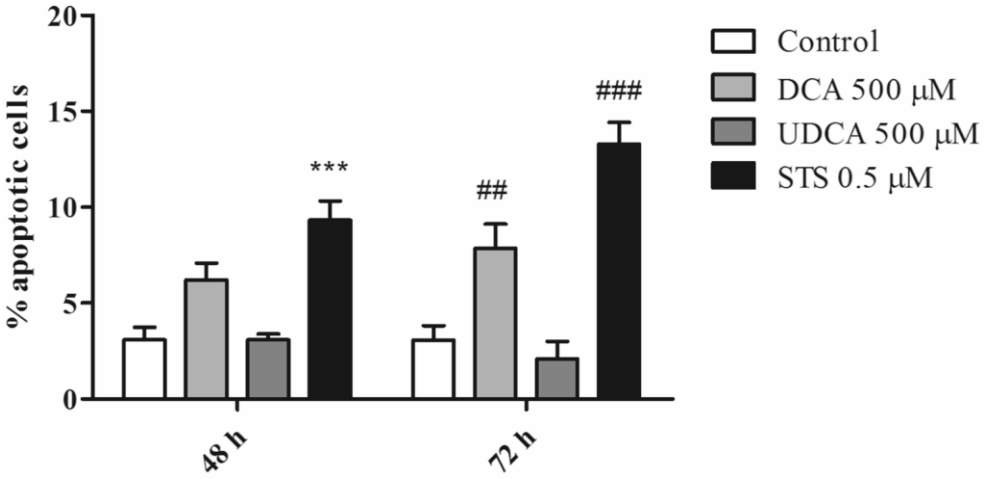


**Figure 12 –** Effects of DCA, 500 µM, and UDCA, 500 µM, on apoptosis induction in Caco-2 cells. Apoptosis was measured by the TUNEL assay after 48 and 72 h incubation with the compounds. Staurosporin (STS) at 0.5 µM was used as a positive control. Results are expressed as mean ± SEM of at least 3 independent experiments. *** P≤ 0.001, ## P≤ 0.01 and ### P≤ 0.001, when compared with respective control, were determined by two-way ANOVA followed by Bonferroni post-tests.

As it was found that DCA induces apoptosis, then it could be speculated that some kind of stress is being induced. Cells are under constant attack from reactive oxygen species (ROS) and alkylating species generated from endogenous and exogenous sources. It is known that high concentrations of bile acids, caused by high intake of fat, in the colon lumen provides a stressful environment, disrupting the normal cellular redox homeostasis, favoring an oxidant state. This redox state may also help the selection of mutated cells that survive this type of environment, propagating the mutator phenotype and increasing the chances of colon cancer development [Payne, C.M., *et al.*, 2008; Ramos, A.A., *et al.*, 2011]. ROS can damage molecules in the cell, such as proteins, lipids, and DNA, resulting in metabolic deregulation. DNA damage is a significant consequence of ROS and alkylating agents attack and is considered to be involved in the development of mutations and cancer [Payne, C.M., *et al.*, 2008]. The ability of DCA and UDCA to induce DNA damage was measured using the Comet assay (Figure 3). UDCA did not induce DNA damage at any of the time points or concentrations used. DCA induced DNA damage after 1, 6 and 24 h incubation at the highest concentration used, 750 µM. At 48 h incubation, it seems that either the cells are repairing the damage or are redirecting to cell death, as the level of DNA damage begins to decrease.

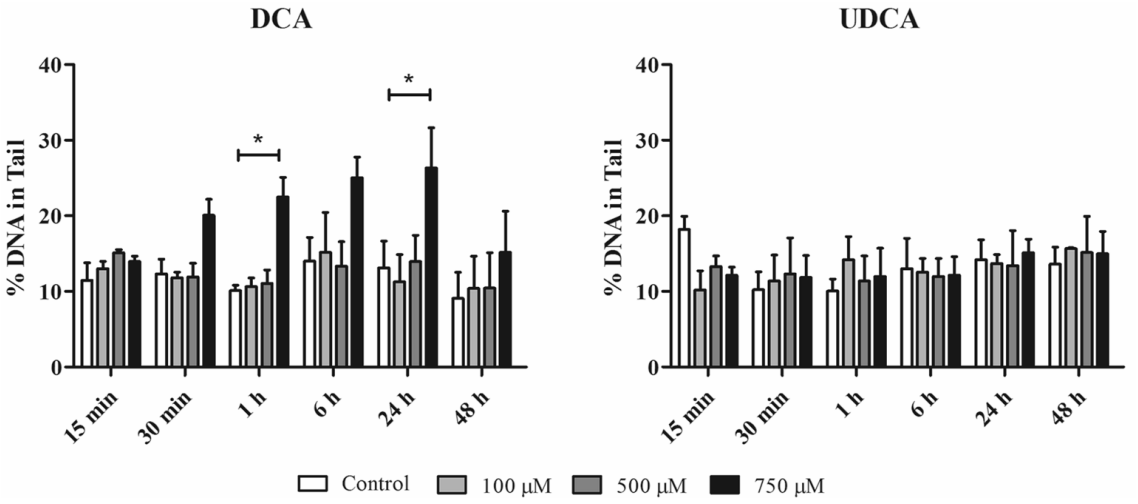


**Figure 3 –** Effects of different concentrations (100, 500, and 750 µM) of DCA and UDCA on DNA damage induction in Caco-2 cells. DNA damage was measured by the comet assay at different time points (15 min, 30 min, 1, 6, 24, and 48 h). Results are expressed as mean ± SEM of at least 3 independent experiments. *P≤ 0.05 when compared with respective control was determined by one-way ANOVA followed by Newman-Keuls multiple comparison test.

We also evaluated the potential of DCA to induce ROS to find a possible cause for the DNA damage seen by the comet assay. As UDCA did not induce any kind of damage, we did not use this compound in this assay. Treatment with 500 µM of DCA for 30 min produced significantly more ROS when compared with control, and comparable to the positive control used (Figure 4). It has been shown that DCA induces ROS production in other cell lines, such as Barrett's epithelial cells [Huo, X., *et al.*, 2011] and HCT-116 [Smith, A.F., *et al.*, 2012]. So, in our model, DCA is inducing DNA damage, at least in part, by ROS production.

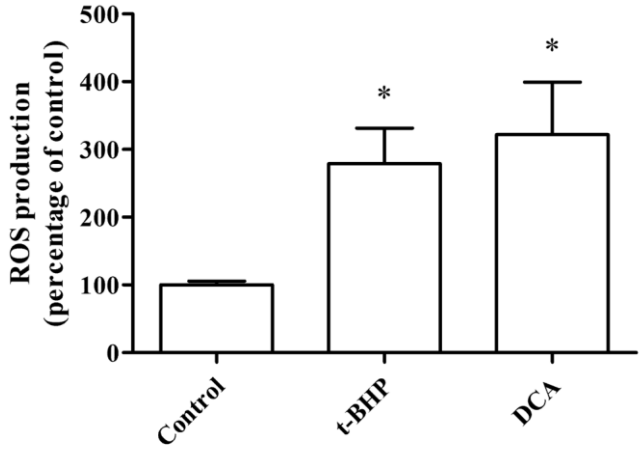


**Figure 4 –** Effects of DCA (500 μM) on the production of ROS. Results are expressed as mean ± SEM of at least 3 independent experiments. ROS production was measured by DCFH oxidative stress assay and *tert*-butyl hydroperoxide (200 μM) was used as postive control. *P≤ 0.05 when compared with control was determined by one-way ANOVA followed by Newman-Keuls multiple comparison test.

Increased DNA damage by bile acids may be due, in part, to the decrease in DNA repair enzymes. $O^6$MeG, a highly mutagenic lesion, is corrected by the MGMT protein. If there is a problem with the MGMT protein, then the lesion will cause a mispair in the DNA and then the mismatch repair system (MMR) will recognize it and excise the mispair. MGMT and MLH1, a protein from MMR system, are commonly altered in colon carcinogenesis. So, the effect of the two bile acids on the protein expression of these two repair enzymes was assessed (Figure 5). We found a significant effect of DCA on MLH1 expression at 48 h incubation, a slight decrease of MLH1 expression, while UDCA showed a tendency (26.13%) to decease the expression of this protein. There was a significant decrease of MGMT expression by both bile acids after 48 h incubation. It has been shown that bile acids can modulate the expression of some DNA repair enzyme, either directly or by oxidative stress [Payne, C.M., *et al.*, 2008]. With our results, we found that deoxycholic acid decreases the expression of MGMT and MLH1 over time. The decrease of the expression of these proteins can be the source of deoxycholic acid cancer promotion effect, not causing DNA damage itself, but modulating the expression of a key enzyme in DNA repair, which are important for repair of highly mutagenic lesions, such as $O^6$methylguanine [Nyskohus, L.S., *et al.*, 2013]. We also found that UDCA decreases significantly MGMT expression.

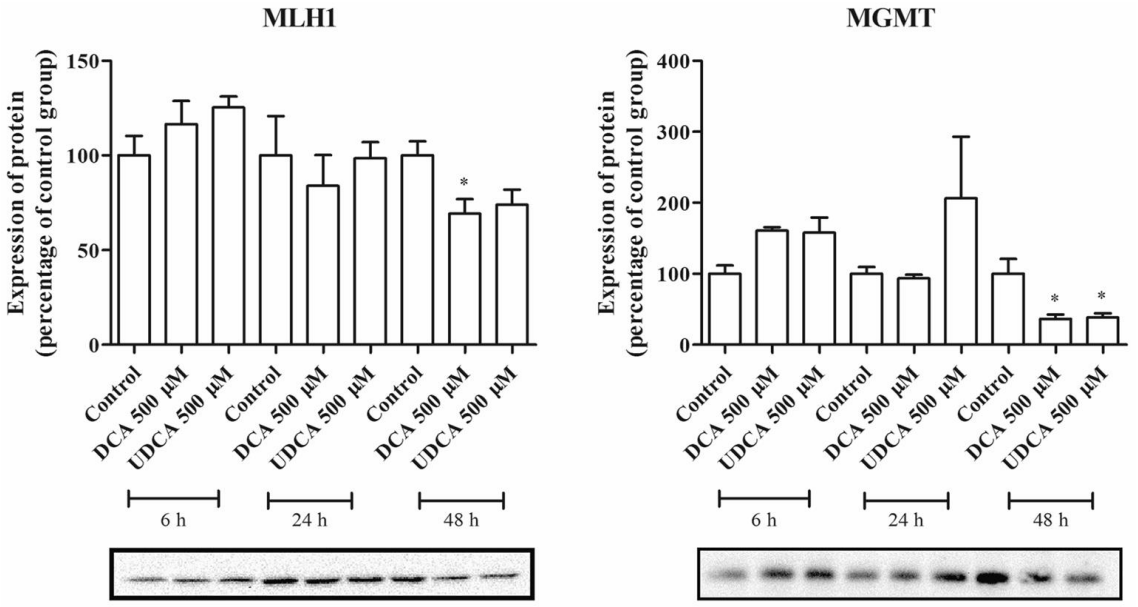


**Figure 5 –** Effects of treatment with DCA and UDCA (500 µM) for 6, 24, and 48 h on MLH1 and MGMT protein expression in Caco-2 cells, using western blot. A representative blot is shown under the quantification. β-actin was used as loading control. Values are mean ± SEM of at least three independent experiments. *P ≤ 0.05 when compared to control was determined by one-way ANOVA followed by Newman-Keuls multiple comparison test.

As bile acids can modulate signaling pathways involved in cell proliferation and apoptosis, we investigated the expression of some key players in the MAP kinase signaling pathway. We found no significant effects of DCA or UDCA on BRAF and KRAS expression (Figure 6). The expression of phospho-ERK and phospho-JNK was also evaluated and an increase of phospho-ERK at 6 and 24 h incubation was observed, while no effects were seen in phospho-JNK (Figure 8). It has been demonstrated that in HCT116, DCA activates phospho-ERK, but not phospho-JNK [Qiao, D., *et al.*, 2001], while in a more recent study by Zeng, H. *et al.*, in the same cell line, they found activation of phospho-ERK and phospho-JNK [Zeng, H., *et al.*, 2010]. In the cell viability assay, we found no significant effects on increase or decrease of proliferation at 6 h incubation, but it seems that the MAPK/ERK pathway is activated. As KRAS is a membrane anchored protein and bile acids have been found to perturb the cell membrane [Jean-Louis, S., *et al.*, 2006], there could be alterations in the membrane composition and this effects KRAS expression. This would possible decrease the activation of the MAPK/ERK pathway, opposite of the effects in this study. One hypothesis to explain our results is that DCA has been shown to activate PKC pathway, and this pathway can activate MAPK/ERK via BRAF, without KRAS involvement [Wu, J., *et al.*, 2008]. Also, the effect of the bile acids

on phospho-AKT was investigated and there was also an increase by the bile acids of the expression of this protein (Figure 7). So, although DCA induces cell death to some cells, as seen by the cell viability assay, it activates proliferation and cell survival in other cells, seen by induction of MAPK/ERK and PI3K/AKT signaling pathways, respectively.

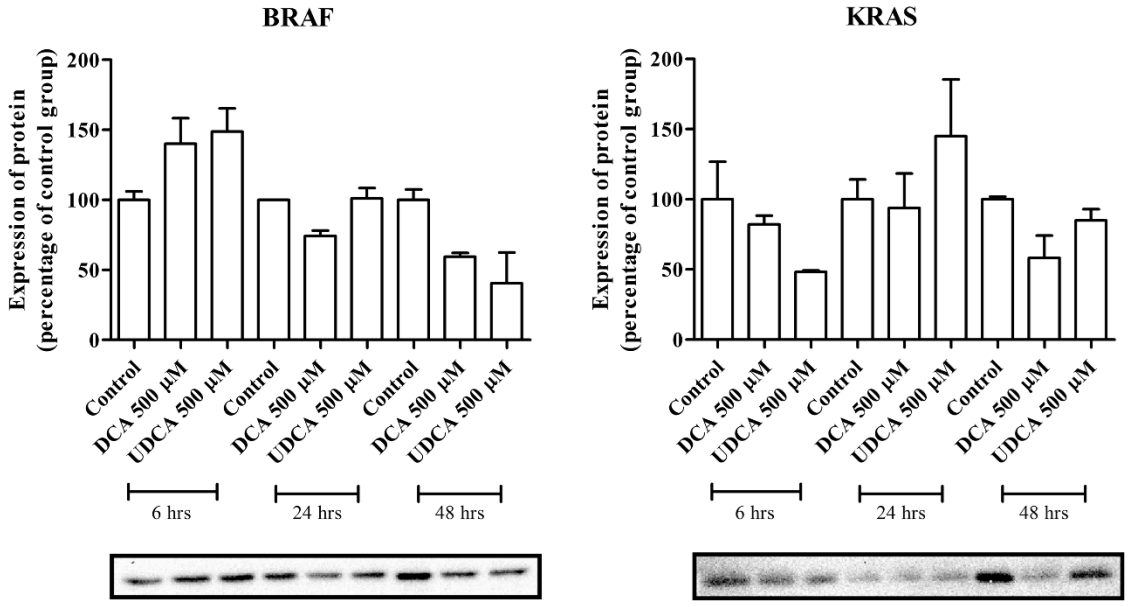


**Figure 6 –** Effects of treatment with DCA and UDCA (500 µM) for 6, 24, and 48 h on BRAF and KRAS protein expression in Caco-2 cells, using western blot. A representative blot is shown under the quantification. β-actin was used as loading control. Values are mean ± SEM of at least three independent experiments.

In conclusion, our study showed that, in Caco-2 cells, DCA induces cell death at high concentrations. This cell death can be, in part, due to DNA damage and decrease of DNA repair. Also, although DCA induces cell death, it also induces cell growth and survival in the remaining cells by activation of the MAPK/ERK and PI3K/AKT signaling pathways. In our study, UDCA did not induce apoptosis or DNA damage in this cell line, but did decrease DNA repair enzymes. This decrease is a characteristic of carcinogenesis and not chemoprevention. Also, UDCA activated the same signaling pathways that DCA did, this suggesting increased proliferative potential. These effects are characteristic of carcinogenesis and not chemoprevention. High levels of DCA appear to induce rapid cell turnover in the colon and this can be beneficial for propagation of a mutator phenotype.

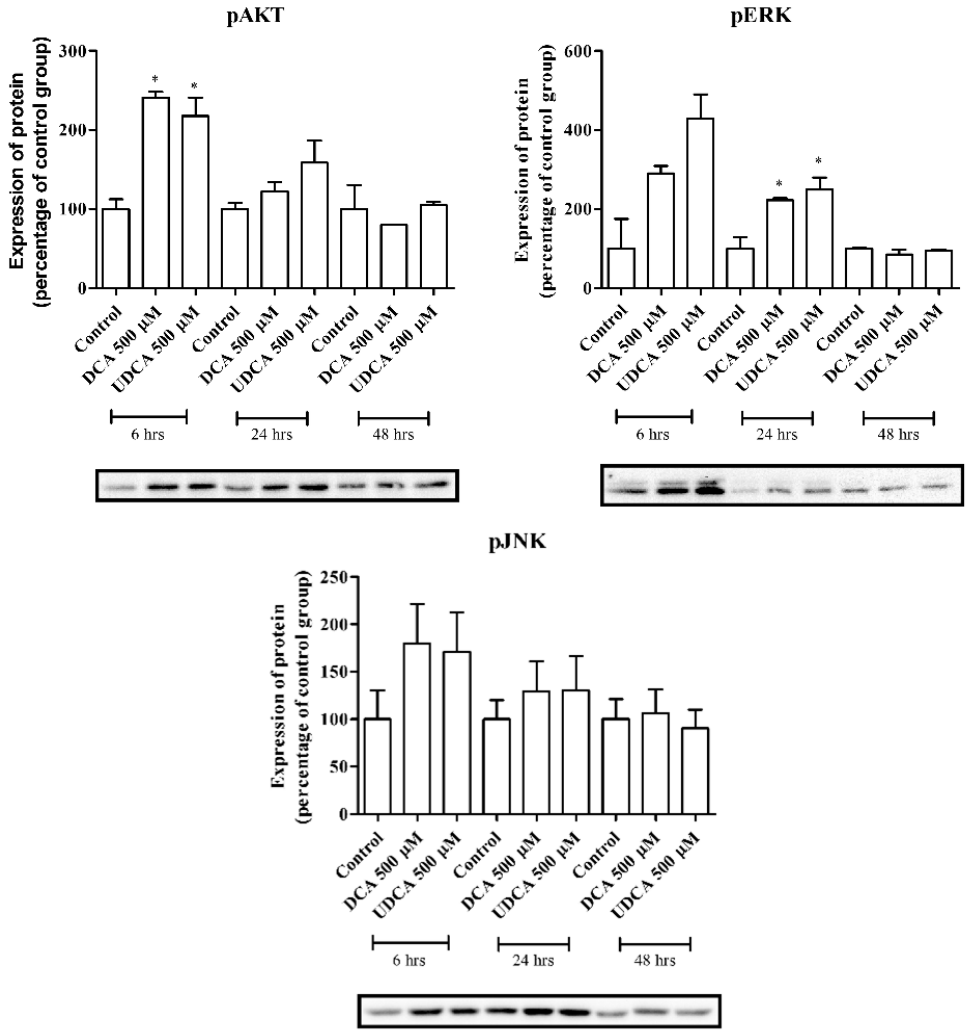


**Figure 7 –** Effects of treatment with DCA and UDCA (500 µM) for 6, 24, and 48 h on phospho-ERK, phospho-JNK and phospho-AKT protein expression in Caco-2 cells, using western blot. A representative blot is shown under the quantification. The total protein was used to normalize the values. Values are mean ± SEM of at least three independent experiments. *$P \leq 0.05$ when compared to control.

# 2 – Effects of UDCA pretreatment on DCA induced alterations

## 2.1 – Introduction

Hydrophobic bile acids, such as deoxycholic acid (DCA), has been shown to be linked to colon carcinogenesis as a promotor and also as an inducer. But more hydrophilic bile acids, such as ursodeoxycholic acid (UDCA), are considered cytoprotective and is used in the treatment of some diseases [Barrasa, J.I., *et al.*, 2013]. It is known that the cytoprotective effect of UDCA may be due to the reduction of the apoptotic threshold through the classical mitochondrial pathways. In HCT116 cell line, it has been found that

UDCA induces differentiation and senescence, inhibits DCA-induced activation of EGFR/Raf-1/ERK signaling pathway and suppresses the apoptosis induced by high concentrations of DCA [Amaral, J.D., *et al.*, 2009; Barrasa, J.I., *et al.*, 2013]. Also, UDCA can protect HCT116 cells against DCA-induced apoptosis by stimulating AKT pathway [Im, E., *et al.*, 2005]. But, HCT116 has an activating mutation in the *KRAS* gene [Ahmed, D., *et al.*, 2013], so these effects are tumor-related. Caco-2 cell line has no mutation in the EGFR/Raf-1/ERK nor the PI3K/AKT pathways.

In this study, we evaluated the pretreatment for 48 h of UDCA on cell death and activation of signaling pathways induced by DCA. We used Caco-2 cell line, which does not harbor *KRAS* or *BRAF* mutations, to give us some evidence on how UDCA affects DCA alterations in a "normal" colon cell.

## 2.2 – Material and methods

### 2.2.1 – Reagents and antibodies

DCA, UDCA, Dulbecco's modified Eagle medium (DMEM), penicillin/streptomycin, and trypsin solution were purchased from Sigma–Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS) was purchased from Biochrom KG (Berlin, Germany). The protein quantification DC protein assay was purchased from Bio-Rad Laboratories (Hercules, CA, USA). Primary antibodies were purchased from the following sources: anti-actin was purchased from Sigma–Aldrich; anti-JNK, anti-phospho-JNK, anti-phosphoERK1/2, anti-K-Ras, anti-B-Raf, anti-MGMT, anti-MLH1 Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA); and anti-p44/42 MAPK (ERK1/2), anti-phospho-Akt (ser473), and anti-Akt from Cell Signaling (Danvers, MA, USA. Peroxidase-conjugated goat anti-mouse antibody and Immobilon Western blotting detection reagents were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and Millipore (Billerica, MA, USA), respectively. All other reagents and chemicals used were of analytical grade.

### 2.2.2 – Cell line and culture conditions

Caco-2 cells, derived from human colon carcinoma, cells were maintained as monolayer cultures in DMEM supplemented with 10% FBS and antibiotics (100 U/mL penicillin and 100 mg/mL streptomycin), under an atmosphere of 5% $CO_2$ at 37 ºC. Cells were seeded onto 6- (2 mL) well plates at a density of $0.1\times10^6$ cells/mL. Test compounds

were added to culture medium to the desired concentration ensuring that the DMSO concentration did not exceed 0.5% (v/v); controls received vehicle only.

### 2.2.3 – Nuclear condensation assay

The effects of 48 h pretreatment with UDCA (500 µM) before incubation with DCA (500 µM) for 72 h on induction of apoptosis in Caco-2 cells was assessed by nuclear condensation assay. The number of apoptotic cells was counted as previously described [Xavier, C.P., *et al.*, 2009]. At least 500 cells were counted and the number of apoptotic cells was divided by the total number cells counted to give the percentage of cell death.

### 2.2.4 – Western blot analysis

Cells were incubated with UDCA (500 µM) or medium for 48 h. Then, medium was removed and DCA (500 µM) was added for 6, 24 and 48 h. Total cell lysates were prepared to measure expression of different proteins. The cells were washed with PBS 1x and lysed for 5 min at 4 °C with ice-cold RIPA buffer (1% NP-40 in 150 mM NaCl, 50 mM Tris (pH 7.5), 2 mM EDTA) supplemented with 20 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 20 mM $Na_3VO_4$ and protease inhibitor cocktail (Roche, Mannheim, Germany). Protein concentration was quantified using the DC protein assay following the manufacturer's instructions, and 20 µg/well was separated by 12% SDS–PAGE and transferred to polyvinylidene difluoride membranes. Membranes were blocked and incubated with primary antibody overnight. After washing, membranes were incubated with secondary antibody conjugated with IgG horseradish peroxidase for 1 h, and immunoreactive bands were detected using the Immobilon solutions (Millipore, Billerica, MA, USA) under a chemiluminescence detection system (Chemi Doc XRS; Bio-Rad Laboratories, Inc.).

Band area intensity was quantified using the Quantity One software from Bio-Rad. β-Actin was used as loading control.

### 2.2.5 – Statistical analysis

Statistical analyses were done using *t*-test, one-way or two-way analysis of variance using GraphPad Prism 4.0 software, when appropriate (San Diego, CA, USA). P values $\leq 0.05$ were considered statistically significant. All results are presented as mean ± SEM of at least three independent experiments. Images are representative of three independent experiments.

## 2.3 – Results and discussion

UDCA has been shown to inhibit DCA-induced alterations in HCT116 cell line. We evaluated the effect of 48 h pretreatment with UDCA (500 µM) on DCA-induced cell death in Caco-2 cell line (Figure 8). DCA induced an increase of cell death at 72 h incubation. UDCA alone did not induce any kind of cell death, being comparable to the control group. When UDCA was given before DCA treatment, there was an increase, although not significant when compared with DCA alone, of cell death with the two bile acids, which is significant when compared with UDCA alone. In studies with HCT116 cell line, it was found that UDCA decreased DCA-induced apoptosis in an AKT-dependent manner [Im, E., *et al.*, 2005; Im, E., *et al.*, 2004]. The difference in the effects may be due to the genetic alterations present in the HCT116 cell line that are not present in the Caco-2 cell line. This could indicate that there are differences in the effects of UDCA in cells with genetic alterations and normal colonocytes.

The same study showed that UDCA pretreatment reduced the expression of phospho-ERK when compared with DCA treatment alone [Im, E., *et al.*, 2004]. The effect of pretreatment with UDCA before DCA on the expression of phospho-ERK, phospho-JNK, and phospho-AKT was also evaluated (Figure 9). In our study, we found no effect of pretreatment with UDCA in phospho-ERK expression at 6 h incubation, but at 24 h incubation we found a significant decrease in expression. As Caco-2 cells grow slower than HCT116, this could account for differences between the time points of these two studies. We also evaluated the effect on phospho-JNK and phospho-AKT, to compare with our previous results and to understand the increase of apoptosis seen with pretreatment with UDCA. In our previous study, we found no effect of either bile acid on phosphor-JNK expression, but with pretreatment with UDCA before DCA incubation, we found an increase in this protein expression at all time points (Figure 9). Upon phosphorylation of JNK, this protein enters the nucleus where it activates c-Jun, and this activation leads to the formation of AP-1, which, in turn, regulates the expression of several proteins, some of them involved in pro-apoptotic signaling [Dhanasekaran, D.N., *et al.*, 2008]. As we found an increase in in the number of apoptotic cells with pretreatment of UDCA, and we find an increase in phospho-JNK expression, we can speculate that possibly, in Caco-2 cells, UDCA treatment before DCA incubation induces an increase in apoptosis *via* JNK pathway and this activation is prolonged until at least 48 h incubation. This indicates, with the addition of the previous results on the decrease

of DNA repair proteins, that in this cell line, UDCA does not have a chemopreventive effect. Further studies are needed to support this hypothesis.

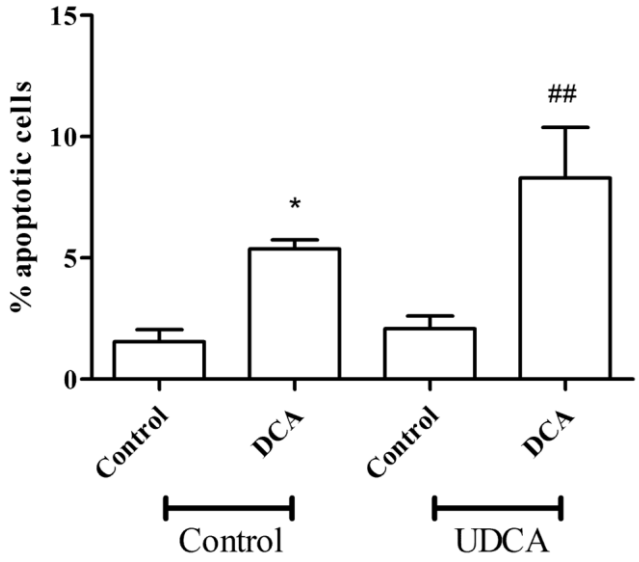


**Figure 8 –** The effects of 48 h pretreatment with UDCA (500 µM) before incubation with DCA (500 µM) for 72 h on induction of apoptosis in Caco-2 cells. Apoptosis was measured by the nuclear condensation assay. Results are expressed as mean ± SEM of at least 3 independent experiments. * P≤ 0.05 when compared with control and ## P≤ 0.01 when compared with UDCA alone, were determined by Student's *t*-test.

In our previous study, we observed an increase of phospho-AKT with both bile acid treatments at 6 h. In this study, we observed an increase of the same protein at 6 h with DCA alone and pretreatment with UDCA significantly increased when compared with DCA (Figure 9). The PI3K/AKT pathway regulates different metabolic pathways, but also inhibits apoptosis [Testa, J.R., *et al.*, 2005]. In this case, the effects on JNK signaling may be stronger than the effects of AKT signaling, hence having an increase in apoptosis.

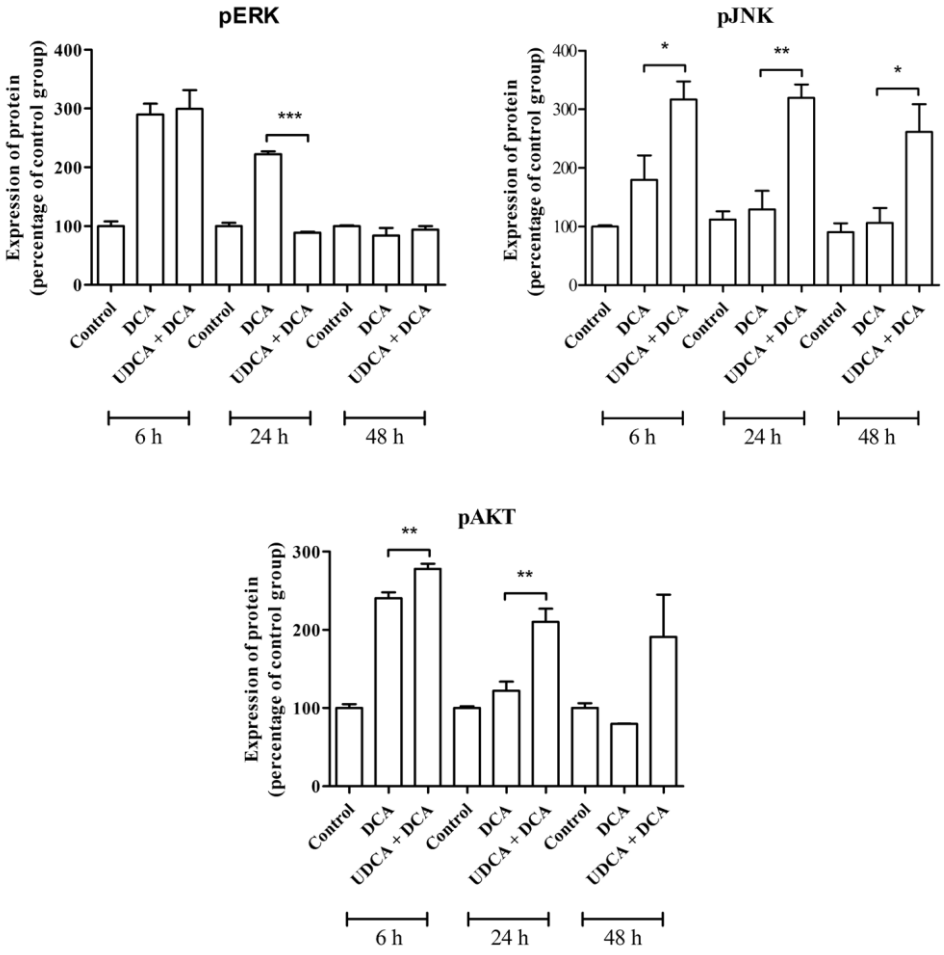


**Figure 9 –** Cells were incubated with UDCA (500 µM) or medium for 48 h. Then, medium was removed and DCA (500 µM) was added for 6, 24 and 48 h. Effects on phospho-ERK, phospho-JNK and phospho-AKT protein expression in Caco-2 cells were evaluated using western blot. The total protein was used to normalize the values. Values are mean ± SEM of at least three independent experiments. *P≤ 0.05, ** P≤ 0.01 and *** P≤ 0.001 were determined by one-way ANOVA followed by Newman-Keuls multiple comparison test.

We also evaluated the effects of UDCA pretreatment on BRAF, KRAS, MGMT, and MLH1 protein expression. No significant effects were found on BRAF and KRAS expression (Figure 10) when comparing with DCA treatment alone. With the DNA repair enzymes, there was a decrease of expression of both proteins with UDCA pretreatment when compared with DCA treatment alone (Figure 11). This could be due to a decrease in DNA damage associated to UDCA cytoprotective effects as seen in a study with male rats [Rodrigues, C.M., *et al.*, 1998]. In our previous study, we found that DCA and UDCA alone decreased MLH1 and MGMT expression. This effect could be an accumulation of the effects with each bile acid alone. Studies on UDCA pretreatment on ROS production

and DNA damage would clarify this hypothesis. On the other hand, with the increase of apoptosis, the cell may be decreasing the production of these proteins, as it directs transcription to pro-apoptotic proteins. Further studies are needed to answer these questions.

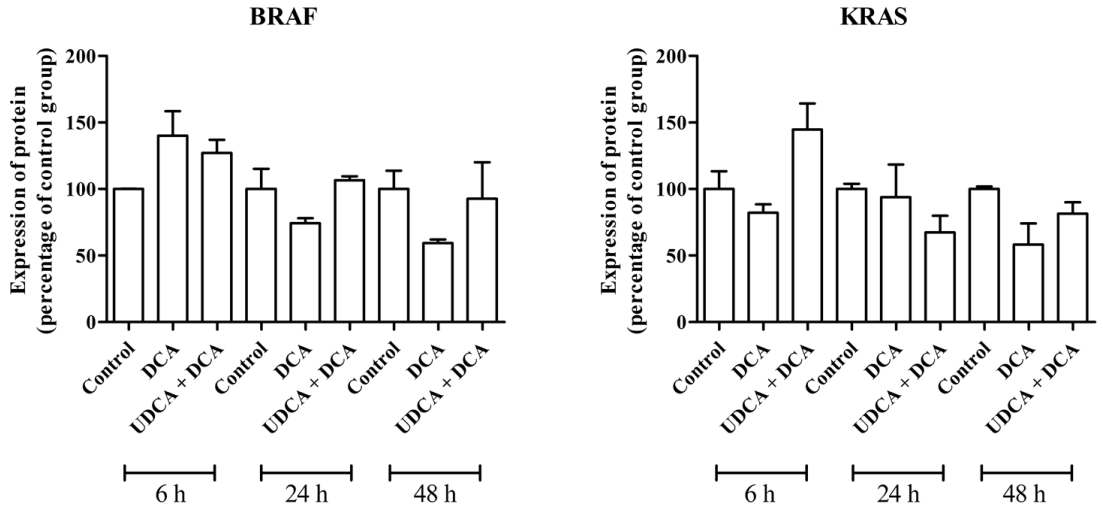


**Figure 10 –** Cells were incubated with UDCA (500 µM) or medium for 48 h. Then, medium was removed and DCA (500 µM) was added for 6, 24 and 48 h. Effects on KRAS and BRAF protein expression in Caco-2 cells were evaluated using western blot. The total protein was used to normalize the values. Values are mean ± SEM of at least three independent experiments.

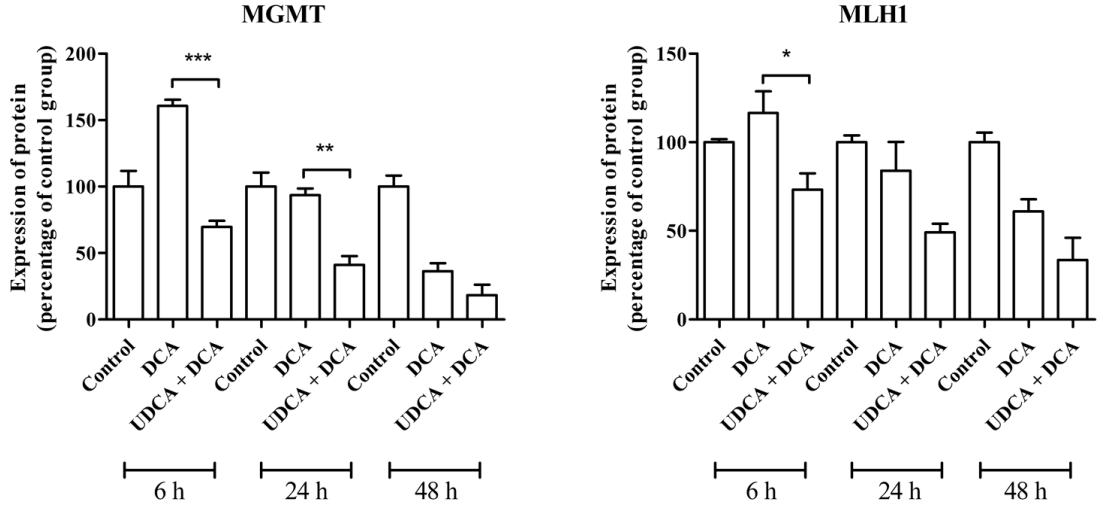


**Figure 11 –** Cells were incubated with UDCA (500 µM) or medium for 48 h. Then, medium was removed and DCA (500 µM) was added for 6, 24 and 48 h. Effects on MGMT and MLH1 protein expression in Caco-2 cells were evaluated using western blot. The total protein was used to normalize the values. Values are mean ± SEM of at least three independent experiments. *P≤ 0.05, ** P≤ 0.01 and *** P≤ 0.001 were determined by one-way ANOVA followed by Newman-Keuls multiple comparison test.

In conclusion, in Caco-2 cell line, UDCA enhances apoptosis when given before DCA treatment. This increase is accompanied by a prolonged increase in JNK phosphorylation. These results contradict some studies that were done in HCT116 cell line, but perhaps the genetic background of the two cell lines may account for the discrepancy. We found a decrease in phospho-ERK expression, which is in accordance with the HCT116 cell line study. UDCA pretreatment also induced a decrease in MGMT and MLH1 DNA repair proteins, which needs further studies to explain this effect. UDCA has shown potential cytoprotective effects in many studies, but in this study, UDCA showed characteristics of promoting potential.

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*Chapter 3*

# *IN VITRO* MODEL OF AZOXYMETHANE-INDUCED DAMAGE

# 1 – *In vitro* azoxymethane model for colon carcinogenesis

## 1.1 – Introduction

Colon cancer is one of the most common types of cancer in humans, so there is a need to improve colon cancer research and find ways to diagnose, prevent, and treat the disease. But the use of animal models always has its drawbacks, especially when it is used to evaluate potential dietary chemopreventive compounds. In chemoprevention and mechanistic studies, in addition to animal models, there has to be a complete cell culture study to screen for the compound or compounds that have higher potential for the effect that is desired. But, as cell culture is great for large scale screening of compounds, very often the effects do not hold up when used in animal models. In animal models, the compounds 1,2-dimethylhydrazine (DMH) and its derivative, azoxymethane (AOM), are the two most commonly used colon cancer chemical inducers. This model shares many similarities with sporadic colon cancer and is widely used to evaluate chemopreventive compounds [Suman, S., *et al.*, 2012].

In the animal model, AOM is metabolized in the liver by CYP2E1 enzyme to methylazoxymethanol (MAM). This compound then circulates in the blood stream and reaches the colon where it is metabolized to the ultimate carcinogenic metabolite, the methyldiazonium ion. This metabolite is highly reactive and induces DNA damage in the cells, mainly $O^6$-methylguanine ($O^6$meG), giving rise to mutations in colon cells. $O^6$-methylguanine methyltransferase (MGMT) is the key enzyme for DNA repair and apoptotic removal in this model. $O^6$meG is rapidly formed within 2 h of AOM administration in the rat colon. MGMT quickly repairs the lesion, being possible to see a quick depletion of the expression of this protein in a dose and time dependent manner. Apoptosis is only triggered when MGMT is no longer able to correct the lesion. But when even apoptotic removal is insufficient, $O^6$meG quickly transforms in mispairs, leading to point mutations [Nyskohus, L.S., *et al.*, 2013]. It has been shown that genes that are mutated in sporadic cases of colon carcinogenesis are also found mutated in the AOM-induced colon carcinogenesis model. Some of these mutations alter key pathways involved in cell proliferation and apoptosis. These pathways include the WNT and MAPK pathways. APC is a protein involved in the WNT signaling pathway and it is involved in the regulation of free β-catenin. *APC* mutation is believed to be one of the first alterations

in colon carcinogenesis. With the AOM model, *Apc* mutations are rare (around 33% of tumors have this alteration), but a *β-catenin* mutation that has the same effect as the *APC* mutation in humans is induced in around 80% of AOM-induced tumors [Perse, M., *et al.*, 2011; Takahashi, M., *et al.*, 2004]. Another mutation that is found in the AOM animal model is the *Kras* mutation. In this model, mutations in the *Kras* gene are point mutations most commonly observed in codon 12, promoting a G to A transition, which constitutively activates the MAPK signaling pathway. *Kras* mutations seem to be as frequent in the AOM model as in human carcinogenesis. This alteration has been observed more frequently, in humans and in the AOM model, in hyperplastic lesions, so it seems that this mutation is involved in early events of carcinogenesis [Perse, M., *et al.*, 2011; Takahashi, M., *et al.*, 2004].

Animal models have been useful to explore the different features of colon carcinogenesis. But there are limitations in the use of animal models. Different models can give different results in the study of the effects of the same compound in colon carcinogenesis. So, in this study, we initiated a possible development of an *in vitro* model with the characteristics of the AOM-induced animal model for effective screening processes. We used the Caco-2 cell line, a colon cancer cell line with no alterations in the MAPK and WNT signaling pathways. Using AOM, we characterized the alterations induced by the carcinogen in our *in vitro* model and compared them to the AOM *in vivo* model, to ensure that the same modifications were being induced.

## 1.2 – Material and methods

### 1.2.1 – Reagents and antibodies

*N*-Methyl-*N*-nitrosourea (MNU), $O^6$-benzylguanine (BG), thiazolyl blue tetrazolium bromide (MTT), AOM, S9 liver fraction, Dulbecco's modified Eagle medium (DMEM), penicillin/streptomycin, and trypsin solution were purchased from Sigma–Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS) was purchased from Biochrom KG (Berlin, Germany). SYBR Gold (nucleic acid gel stain) was from Invitrogen Molecular Probes (Eugene, OR, USA). The protein quantification DC protein assay was purchased from Bio-Rad Laboratories (Hercules, CA, USA). Primary antibodies were purchased from the following sources: anti-actin was purchased from Sigma–Aldrich; anti-JNK, anti-phospho-JNK, anti-phosphoERK1/2, anti-K-Ras, anti-B-Raf, anti-MGMT, anti-MLH1, anti-β-catenin, and anti-histone H1 from Santa Cruz Biotechnology, Inc.

(Santa Cruz, CA, USA); and anti-p44/42 MAPK (ERK1/2), anti-phospho-Akt (ser473), and anti-Akt from Cell Signaling (Danvers, MA, USA. Peroxidase-conjugated goat anti-mouse antibody and Immobilon Western blotting detection reagents were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and Millipore (Billerica, MA, USA), respectively. All other reagents and chemicals used were of analytical grade.

### 1.2.2 – Cell line and culture conditions

Caco-2 cells, derived from human colon carcinoma, cells were maintained as monolayer cultures in DMEM supplemented with 10% FBS and antibiotics (100 U/mL penicillin and 100 mg/mL streptomycin), under an atmosphere of 5% $CO_2$ at 37 ºC. Cells were seeded onto 6- (2 mL) or 12- (1 mL) well plates at a density of $0.1\times10^6$ cells/mL. Test compounds were added to culture medium to the desired concentration ensuring that the DMSO concentration did not exceed 0.5% (v/v); controls received vehicle only.

To enhance the enzymatic biotransformation of AOM, a mixture of S9 from human liver and an exogenous NADPH-regenerating system was added to the medium at the same time of the procarcinogen (0.5% v/v S9 liver fraction, 1 mM $NADP^+$, 5 mM glucose-6-phosphate, 0.4 U/mL glucose-6-phosphate dehydrogenase, and 3.3 mM magnesium chloride).

### 1.2.3 – Assessment of cell viability/proliferation by MTT reduction test

A MTT reduction assay was performed in order to evaluate the toxicity/increase proliferation of AOM in this colon cancer cell line. Cells were treated with different concentrations of AOM for 6, 24, 48 and 72 h, including 2 h incubation with MTT (final concentration 0.5 mg/mL). The formazan crystals were then dissolved in a solution of DMSO/ethanol (1:1). The number of viable cells in each well was estimated by the cell capacity to reduce MTT. The results were expressed as percentage relative to the control (cells without any test compound).

### 1.2.4 – Nuclear condensation assay

The effects of AOM (15 µM) treatment, with and without S9 liver fraction mixture, on induction of apoptosis in Caco-2 cells was assessed by nuclear condensation assay. The number of apoptotic cells was counted after 48 and 72 h as previously described [Xavier, C.P., *et al.*, 2009]. Staurosporin (STS) at 0.5 µM was used as positive control. At

least 500 cells were counted and the number of apoptotic cells was divided by the total number cells counted to give the percentage of cell death.

### 1.2.5 – Genotoxicity assay

The alkaline version of the single cell gel electrophoresis (comet) assay was used to evaluate DNA damage [Collins, A.R., 2004]. Caco-2 cells were incubated for different time points (1, 2, 4, and 6 h) at 37 ºC with AOM (5 and 15 µM), with and without S9 liver fraction mixture. Cells were collected by trypsinization and around 50,000 cells were centrifuged for 1 min at 5,000 rpm, ressuspended in low melting point agarose and spread onto agarose-coated slide using a cover slip. After 10 min at 4 ºC, the coverslips were removed and slides were placed in lysis solution (2.5 M NaCl, 100 mM $Na_2EDTA$, 10 mM Tris base, pH 10, plus 1% Triton X-100) for 1 h at 4 ºC. Slides were then placed in a horizontal electrophoresis chamber with electrophoresis solution (0.3 M NaOH, 1 mM $Na_2EDTA$, pH > 13) for 40 min at 4 ºC for the DNA to unwind before electrophoresis for 20 min at 0.8 V/cm and ∼300 mA. After electrophoresis, slides were washed twice with PBS and dried at room temperature (RT). For analysis of the comet images, slides were stained with SYBR Gold solution for 30 min at 4 ºC; after drying, the slides were analyzed using a fluorescence microscope and the Comet IV analysis system (Perceptive Instruments Ltd, Haverhill, UK) was used to calculate the parameter percentage of DNA in the tail. About 100 randomly selected cells were analyzed per sample.

### 1.2.6 – CoMeth assay

The adapted version of the comet assay (CoMeth) was used to measure the potential of AOM to induce specifically the $O^6$-methylguanine ($O^6$meG) lesion [Ramos, A.A., et al., 2013]. Briefly, cells were pretreated with or without BG, 100 µM, for 2 h before treatment for 1, 24, 48 or 72 h of AOM (15 µM), with or without S9 liver fraction mixture, or MNU (used as positive control). Afterwards, the comet assay was continued as referred previously.

### 1.2.7 – DCFH oxidative stress assay

The capacity of DCA to induce reactive oxygen species was evaluated using the dichlorofluorescin (DCFH) oxidative stress assay. Briefly, culture medium was removed and cells were washed with PBS. Afterwards, cells were incubated with 2′,7′-Dichlorofluorescein diacetate (DCF-DA) (100 µM) or DMSO (vehicle) for 30 min at 37

ºC. This solution was removed and cells were washed with PBS. Cells were then incubated with AOM (15 µM), with and without S9 liver fraction mixture, or *tert*-butyl hydroperoxide (200 µM), diluted in serum-free medium, for 30 min at 37 ºC. The compounds were removed and cells were lysed with a solution of 90% DMSO/10% PBS for 10 min at RT in the dark. Fluorescence was read at 520 nm upon excitation at 485 nm.

### 1.2.8 – Western blot analysis

Cells were incubated with AOM (15 µM) for 6, 24 and 48 h, with and without S9 liver fraction mixture. For the pretreatment with the bile acids, bile acid treatment was done for 6 h prior to a 24 h incubation with AOM, with and without S9 liver fraction mixture. Total cell lysates were prepared to measure expression of different proteins. The cells were washed with PBS 1x and lysed for 5 min at 4 °C with ice-cold RIPA buffer (1% NP-40 in 150 mM NaCl, 50 mM Tris (pH 7.5), 2 mM EDTA) supplemented with 20 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 20 mM $Na_3VO_4$ and protease inhibitor cocktail (Roche, Mannheim, Germany). Protein concentration was quantified using the DC protein assay following the manufacturer's instructions, and 20 µg/well was separated by 12% SDS–PAGE and transferred to polyvinylidene difluoride membranes. Membranes were blocked and incubated with primary antibody overnight. After washing, membranes were incubated with secondary antibody conjugated with IgG horseradish peroxidase for 1 h, and immunoreactive bands were detected using the Immobilon solutions (Millipore, Billerica, MA, USA) under a chemiluminescence detection system (Chemi Doc XRS; Bio-Rad Laboratories, Inc.).

Band area intensity was quantified using the Quantity One software from Bio-Rad. β-Actin was used as loading control.

### 1.2.9 – Statistical analysis

Statistical analyses were done using *t*-test, one-way or two-way analysis of variance using GraphPad Prism 4.0 software, when appropriate (San Diego, CA, USA). P values $\leq 0.05$ were considered statistically significant. All results are presented as mean ± SEM of at least three independent experiments. Images are representative of three independent experiments.

## 1.3 – Results and discussion

In this study, we searched for an *in vitro* model of the AOM-induced carcinogenesis, for easier screening purposes of chemopreventive compounds. To our knowledge, very few studies have used azoxymethane in *in vitro* models. IEC-6 intestinal cells are from rat small intestine epithelium and have been used in *in vitro* studies with AOM [Sasaki, T., *et al.*, 2005; Sasaki, T., *et al.*, 2006]. The authors used these cells to evaluate the potential of deoxycholic acid and linoleic acid to promote or inhibit AOM-induced carcinogenic transformation, respectively. In these two studies, the authors found that, in both cases, the compounds inhibit IEC-6 transformation. For the effects of AOM, the authors used a 40-week incubation period, but then did not characterize fully the effect of the carcinogenic transformation and compare it to the *in vivo* model, specifically the signaling pathways that could be altered and induce this transformation.

Contrarily to the studies mentioned above, in this study, a human colon cancer cell line was used, Caco-2 cells, and the characterization of the effects of AOM in this cell line was assessed. The time points used were up to 72 h, and not 40 weeks as in the study by Sasaki, T., *et al.*, to see if there are immediate effects of AOM in the cell line, as it has been shown that $O^6$meG is rapidly formed within 2 h of AOM administration in the rat colon. The effect on cell proliferation was evaluated by the MTT assay (Figure 1). No differences were found with the different concentrations (from 0.05 to 5 µM) of the carcinogen at any of our time points (6, 24, 48 and 72 h). It is known that AOM has to be metabolized to methylazoxymethanol, and this is done through the CYP2E1 enzyme. It has been shown that Caco-2 cells express this enzyme and the enzyme is active [Lampen, A., *et al.*, 1998], but, as in studies in cell lines that do not have active metabolizing enzymes, we used a liver S9 fraction to enhance the metabolization of AOM. Using 5 and 15 µM of AOM for 24 and 48 h incubations, we detected a significant increase of proliferation with 15 µM of AOM at 48 h, with S9 mixture (Figure 2). So with this result it seems that the S9 liver fraction enhanced AOM metabolization and alterations occurred that increased slightly cell proliferation. This increase of proliferation could suggest an alteration in signaling pathways that regulate this process.

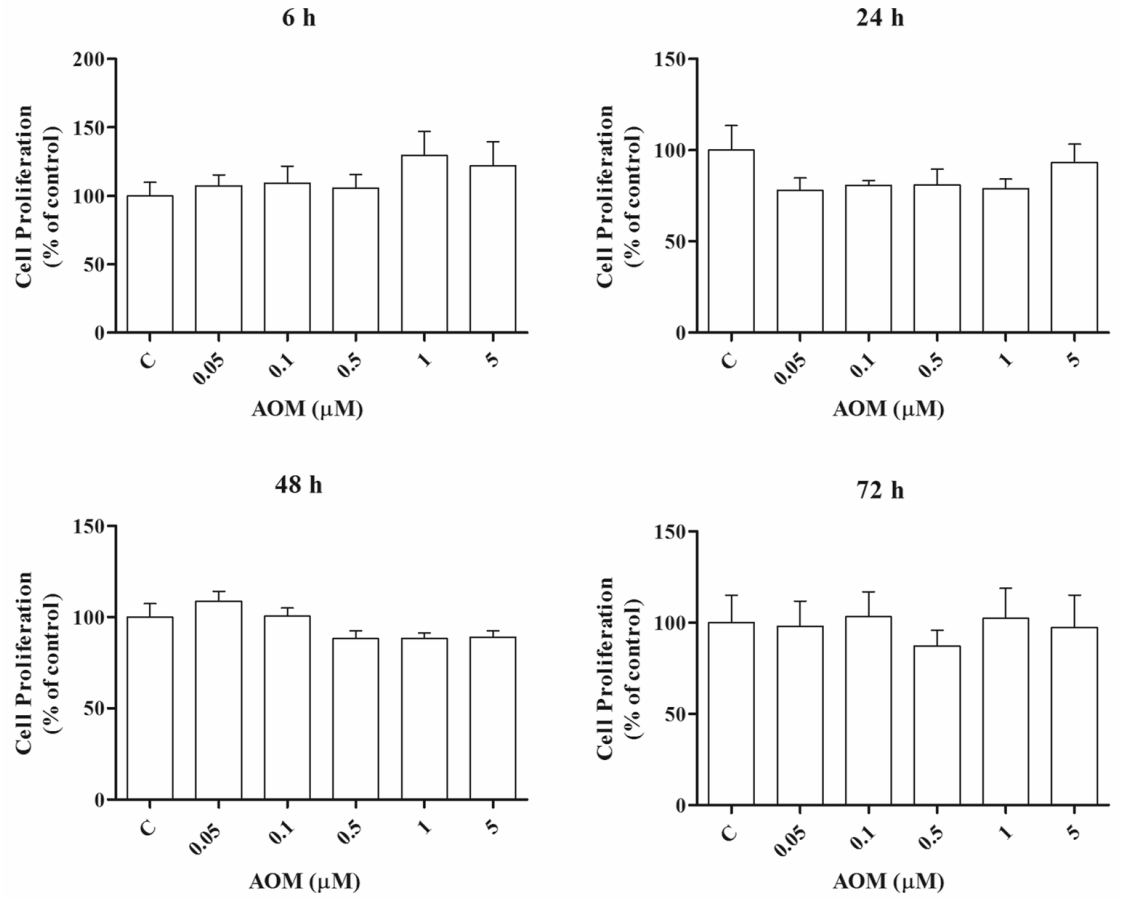


**Figure 1 –** The effect of different concentrations (0.05, 0.1, 0.5, 1 and 5 μM) of AOM on cell viability at 6, 24, 48 and 72 h, in Caco-2 cells. Cell viability was measured by the MTT assay and results are the mean ± SEM of at least 3 independent experiments.

We found no significant decrease in proliferation, but to evaluate if the concentration of AOM used would be too cytotoxic, we evaluated the induction of cell death through nuclear condensation assay. AOM, at 15 μM, did not induce cell death in any of the conditions tested, when compared with respective control group (Figure 3). So, this far in the characterization of our model, AOM induces a slight, but significant, increase in cell proliferation after 48 h incubation and does not induce cell death in Caco-2 cells.

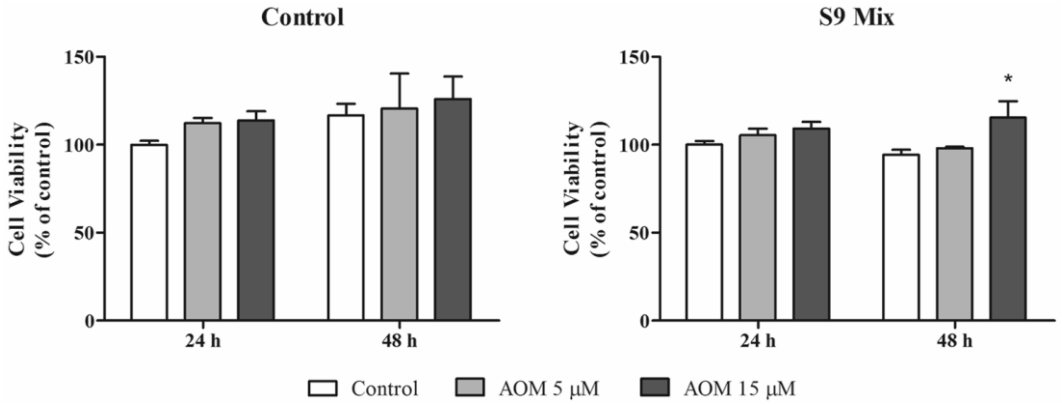


**Figure 2 –** The effect of different 5 and 15 µM of AOM on cell viability at 24 and 48 h, in Caco-2 cells. Control experiment was done in normal medium while S9 experiment was done adding the mixture of S9 liver fraction. Cell viability was measured by the MTT assay and results are the mean ± SEM of at least 3 independent experiments. *P≤ 0.05, when compared with respective control was determined by one-way ANOVA followed by Newman-Keuls multiple comparison test.

DNA damage is an important and initiating step in colon carcinogenesis and is a significant consequence of the production of reactive oxygen species (ROS) [Ramos, A.A., *et al.*, 2011]. The metabolism of procarcinogens can induce production of ROS, and these, in turn, induce damage in cellular molecules, such as DNA. The potential of AOM metabolism to induce ROS and DNA damage was evaluated by the DCFH oxidative stress assay and comet assay, respectively. ROS formation was measured after 30 min of incubation with AOM, although no effects were found on the production of ROS (Figure 4). A preliminary teste at 1 h incubation was also done to evaluate the necessity of a longer time point, but no effects were seen in these conditions as well (data not shown).

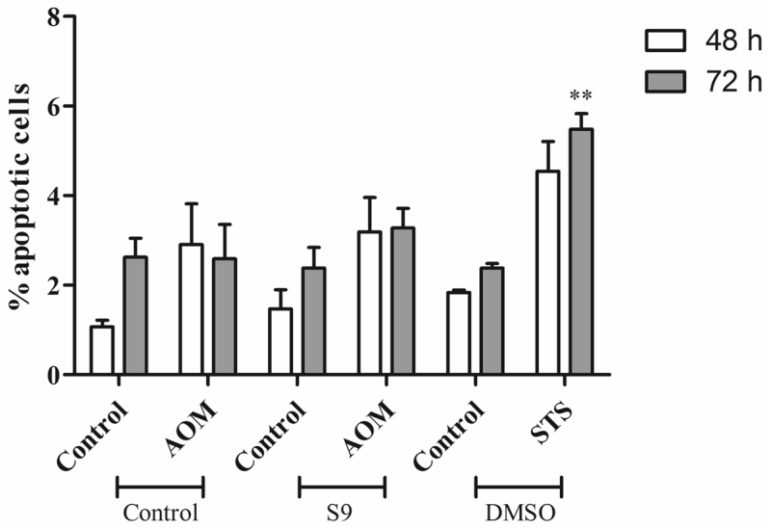


**Figure 3 –** Effects of 15 µM of AOM, with and without S9 liver fraction mixture, on apoptosis induction in Caco-2 cells. Apoptosis was measured by the nuclear condensation assay after 48 and 72 h incubation with the compound. Staurosporin (STS) at 0.5 µM was used as a positive control. Results are expressed as mean ± SEM of at least 3 independent experiments. ** $P \leq 0.01$, when compared with respective control, was determined by two-way ANOVA followed by Bonferroni post-tests.

As we found no ROS production with AOM tretament, we further evaluated the effect of AOM to induce DNA damage (Figure 5). We found no induction of DNA damage induced by AOM in any of the conditions tested. Not many studies have shown AOM-induced DNA damage. In the study with the IEC-6 cell line, they found increased DNA damage with AOM treatment alone, but not in cells treated with AOM and DCA [Sasaki, T., *et al.*, 2005]. In a study where the effect of beer drinking on colon carcinogenesis, they found an increase of DNA damage induced by AOM in isolated colonocytes, and this DNA damage was reduced with beer consumption [Nozawa, H., *et al.*, 2004]. In the first study, AOM was given during 40 weeks, while in the second study 16 h before animal sacrifice, the lack of results in our study could be due to a time issue, as we used short time periods (1, 2, 4 and 6 h). Studies with longer exposure times would help clarify this discrepancy.

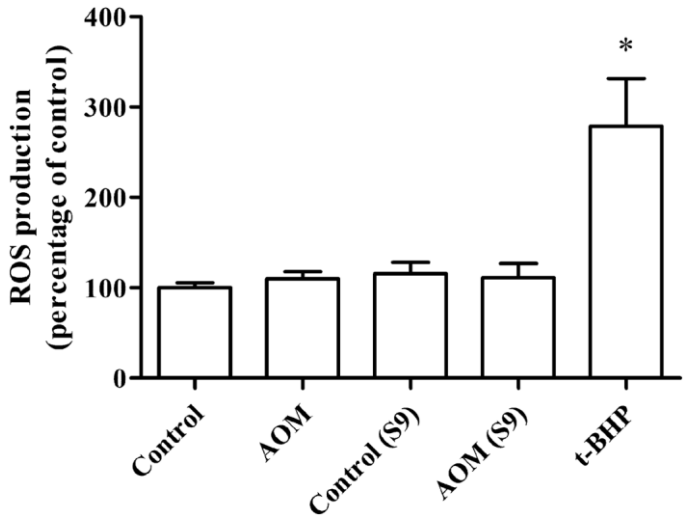


**Figure 4 –** Effects of 30 min incubation of AOM (15 µM), with and without S9 liver fraction mixture, on the production of ROS. Results are expressed as mean ± SEM of at least 3 independent experiments. ROS production was measured by DCFH oxidative stress assay and *tert*-butyl hydroperoxide (200 µM) was used as postive control. *P≤ 0.05 when compared with control was determined by student's *t*-test.

It is known that AOM cause DNA adducts that lead to induction of $O^6$meG [Fiala, E., 1975; Papanikolaou, A., *et al.*, 1998], and as the standard comet assay gives information on strand break type damage, we used our new adaptation of the comet assay (CoMeth) to assess the induction of this type of lesion (Figure 6). With this assay, we found no induction of $O^6$MeG with AOM with the tested conditions. The assay was functional as we found induction of this type of lesion with our positive control. So far in this study, it seems that, although we were able to induce a slight increase of proliferation with AOM, the other characteristics of the *in vivo* model have not been fulfilled.

The $O^6$MeG lesion gives rise to point mutations in key regulatory genes. It has been demonstrated that the most common genes altered by point mutations in the AOM-induced *in vivo* models are *Kras* and *β-catenin* [Perse, M., *et al.*, 2011]. With this in mind, and the fact that a slight increase in proliferation was found, the effect of AOM on the MAPK and WNT signaling was evaluated. No effects were found on KRAS and BRAF protein expression with any of the treatments (Figure 7). But, the downstream protein phospho-ERK was found slightly increased, although the values are not statistically significant with AOM treatment and S9 liver fraction mixture (Figure 8). This effect was found at 6 and 24 h incubation. So, it seems that the MAPK/ERK pathway is slightly activated, and could explain the slight increase in proliferation, although the activation is from 6 to 24 h incubation and the increase of proliferation is seen at 48 h incubation.

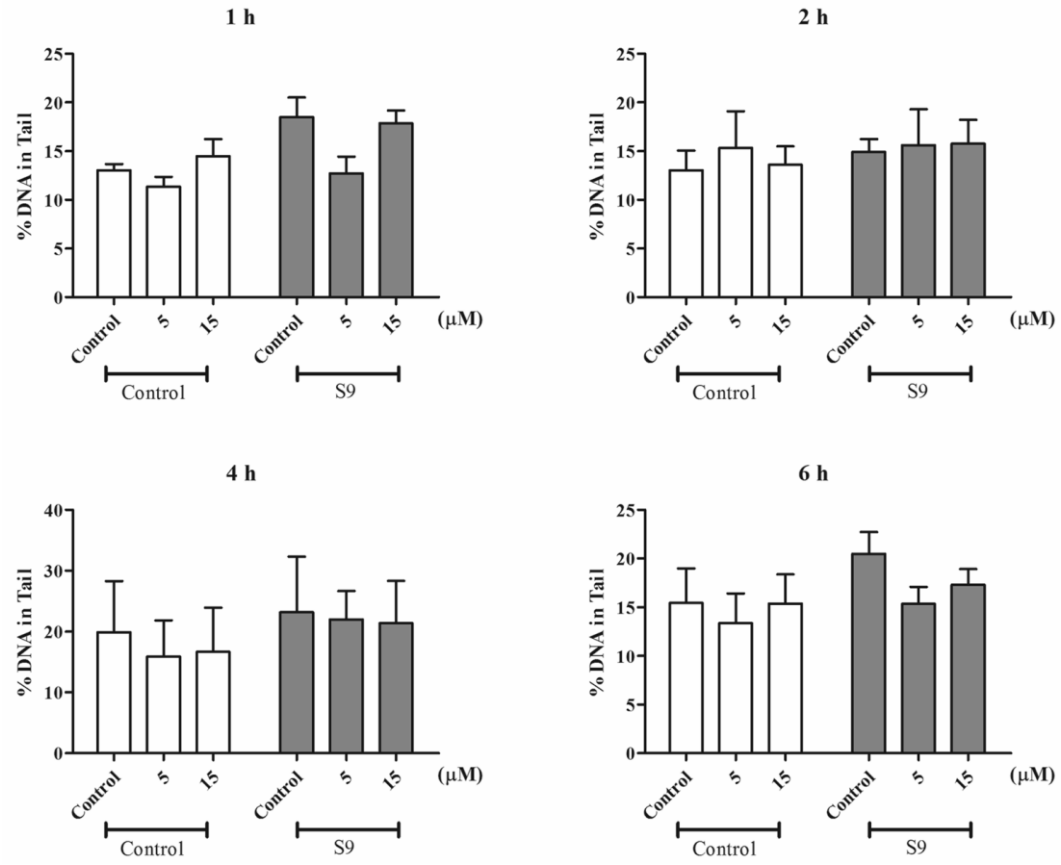


**Figure 5 –** Effects of AOM on DNA damage induction in Caco-2 cells using the comet assay. Cells were treated with 5 or 15 μM of AOM for 1, 2, 4, or 6 h of AOM, with and without S9 liver fraction mixture. Results are expressed as mean ± SEM of at least 3 independent experiments.

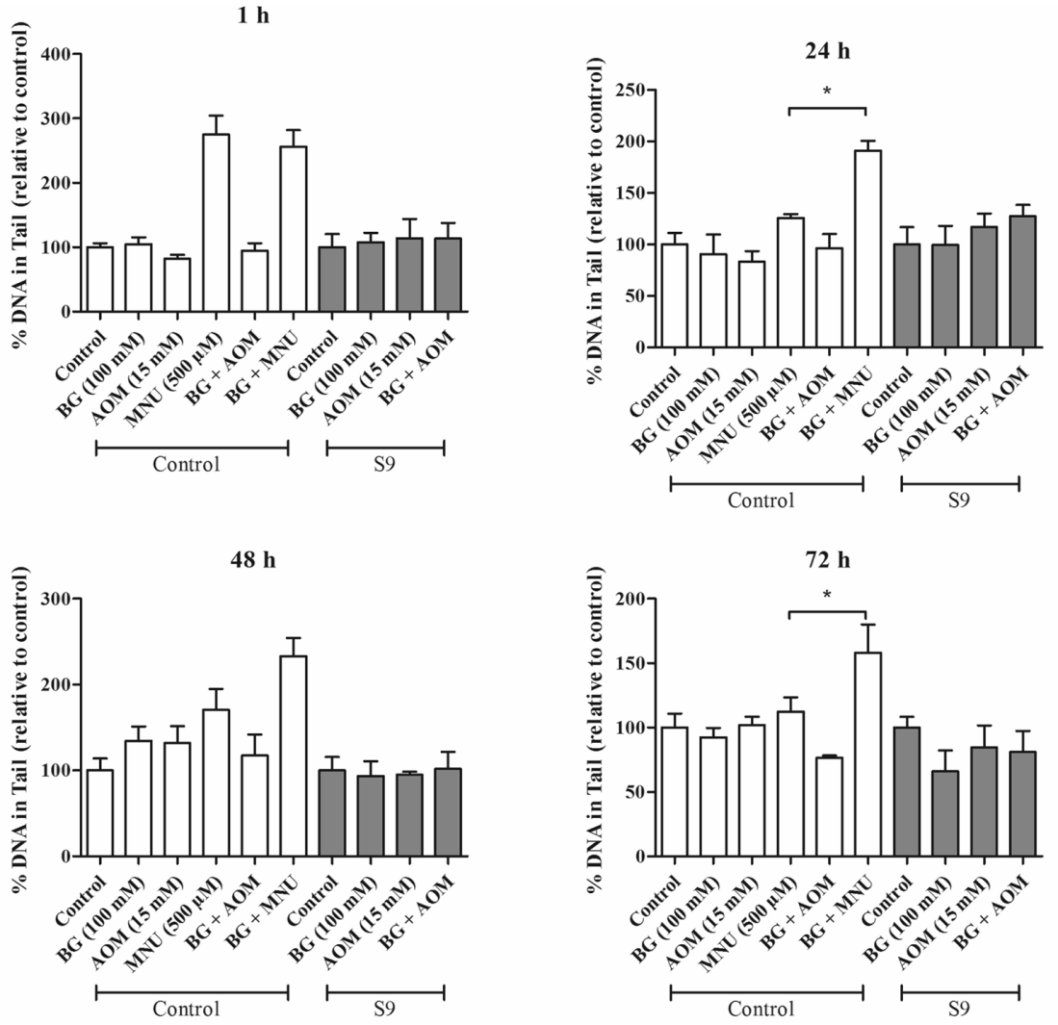


**Figure 6 –** Effects of AOM on $O^6$-MeG-type DNA damage induction in Caco-2 cells using the adapted version of the comet assay. Cells were pretreated with or without BG, 100 µM, for 2 h before treatment for 1, 24, 48 or 72 h of AOM, with and without S9 liver fraction mixture. MNU was used as a positive control. Results are expressed as mean ± SEM of at least 3 independent experiments. *P≤ 0.05 when compared with respective control was determined by one-way ANOVA followed by Newman-Keuls multiple comparison test.

As the $O^6$MeG lesion is corrected by the protein methylguanine methyltransferase (MGMT), or, if not repaired by this system, is corrected by the mismatch repair system, the expression of MGMT protein and MLH1 (intervenient in the mismatch repair system) was also evaluated. MLH1 expression was not significantly altered with the various treatments (Figure 9). On the other hand, AOM treatment with S9 mixture showed a tendency to increase MGMT expression at 24 h incubation. As MGMT is a suicide protein, it could be hypothesized that AOM induced $O^6$MeG lesion, but most of the cells had the capacity to correct this lesion. Although we did not find evidence of AOM inducing

$O^6$meG with our comet assay, it does seem that AOM induces a response that increases MGMT expression.

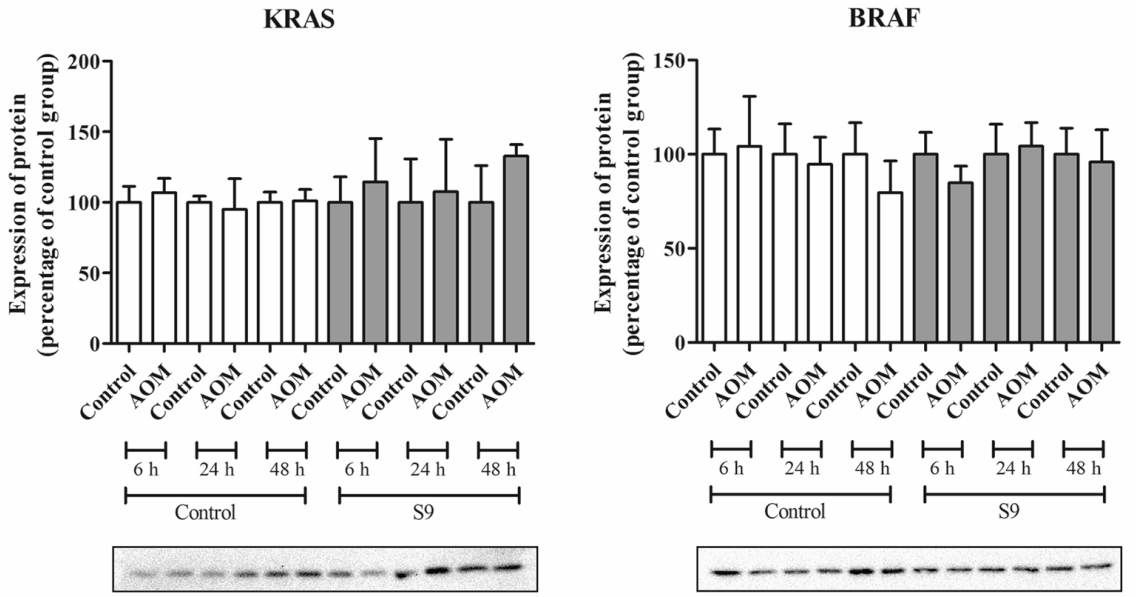


**Figure 7 –** Effects of treatment with AOM (15 μM) for 6, 24, and 48 h on BRAF and KRAS protein expression in Caco-2 cells, using western blot. A representative blot is shown under the quantification. β-actin was used as loading control. Values are mean ± SEM of at least three independent experiments.

Deoxycholic acid, a secondary bile acid, is considered a promoter of colon carcinogenesis [Flynn, C., *et al.*, 2007], while ursodeoxycholic acid is believed to be preventive of carcinogenesis. In the previous chapter, we showed the effects of DCA and UDCA on the expression of several proteins involved in DNA repair and cell signaling. With this in mind, we further investigated the possible effect of pretreatment with the bile acids on AOM effects on phospho-JNK, MLH1 and BRAF expression (Figure 10). We found no differences of expression with neither DCA nor UDCA on these proteins, although there is a slight decrease of BRAF and phospho-JNK expression with the bile acids and a slight increase of MLH1 expression. This slight increase in MLH1 was also seen with 6 h incubation of the bile acids alone (chapter 2).

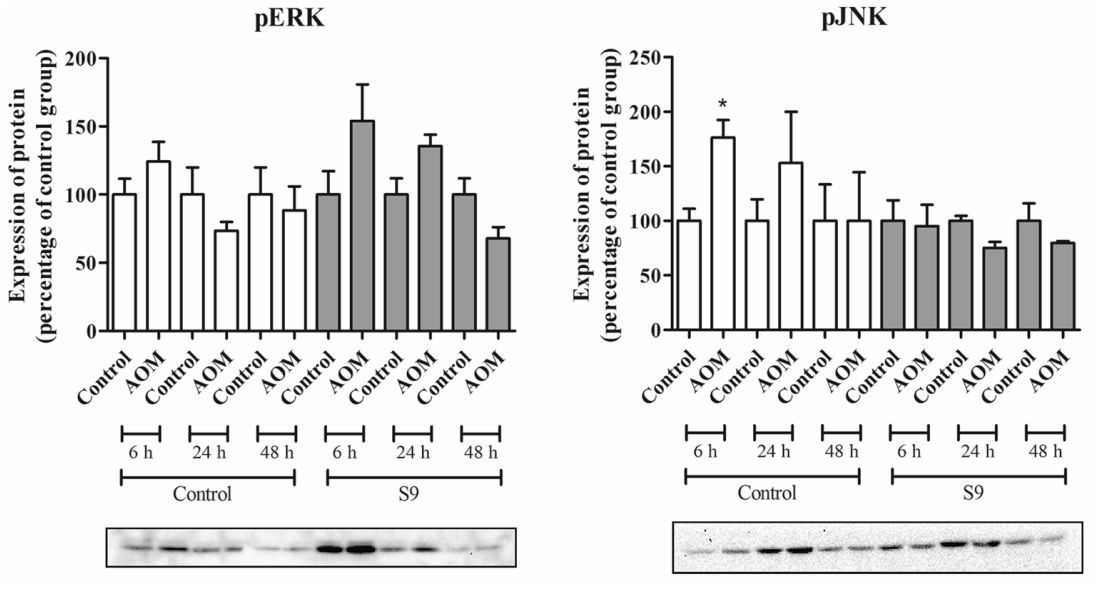


**Figure 8 –** Effects of treatment with AOM (15 μM) for 6, 24, and 48 h on phospho-ERK and phospho-JNK protein expression in Caco-2 cells, using western blot. A representative blot is shown under the quantification. The total protein was used to normalize the values. Values are mean ± SEM of at least three independent experiments. *P ≤ 0.05 when compared to control was determined by student's *t*-test.

Another pathway that can be altered in *in vivo* AOM-induced tumorgenesis is the WNT pathway [Perse, M., *et al.*, 2011]. The mutation in the *Apc* or *β-catenin* gene disrupt the regulation of β-catenin and this protein accumulates in the nucleus and acts as a transcription factor, regulating cellular processes, such as proliferation [White, B.D., *et al.*, 2012]. We analyzed the expression of nuclear β-catenin with AOM treatment (Figure 11). No increase in nuclear β-catenin was seen in Caco-2 cells with 24 h incubation of AOM. As *β-catenin* mutations usually appear in lesions with dysplastic features [Takahashi, M., *et al.*, 2004], the time point of the assay is probably not sufficient to see alterations in the WNT pathway.

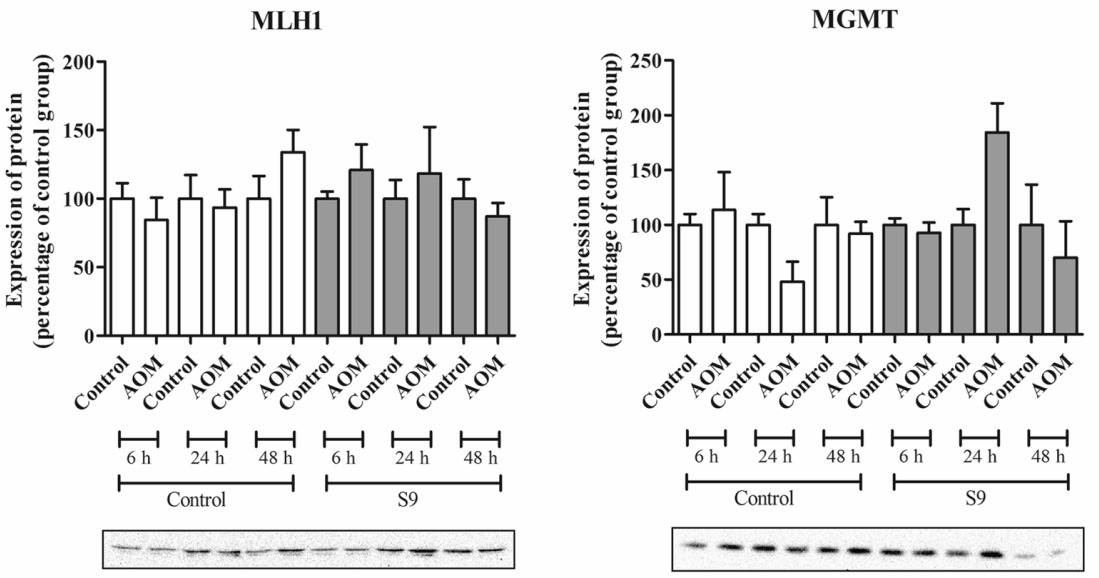


**Figure 9 –** Effects of treatment with AOM (15 µM) for 6, 24, and 48 h on MLH1 and MGMT protein expression in Caco-2 cells, using western blot. A representative blot is shown under the quantification. The total protein was used to normalize the values. Values are mean ± SEM of at least three independent experiments.

In conclusion, with this *in vitro* model, we were able to induce an increase in proliferation, which is typical of carcinogenic cells. We found no effects on induction of DNA damage by AOM. Different AOM incubation times should be tested. We found a slight increase in phospho-ERK expression although not statistically significant. This slight increase can give us some information that this signaling pathway is being altered, but more studies need to be performed. No effects were seen with β-catenin nuclear translocation, but the time point could be too soon. With these preliminary results, it appears that our model can be inducing some genetic alterations that could be comparable with the alterations seen in the *in vivo* model, but further work is needed to improve and validate this model.

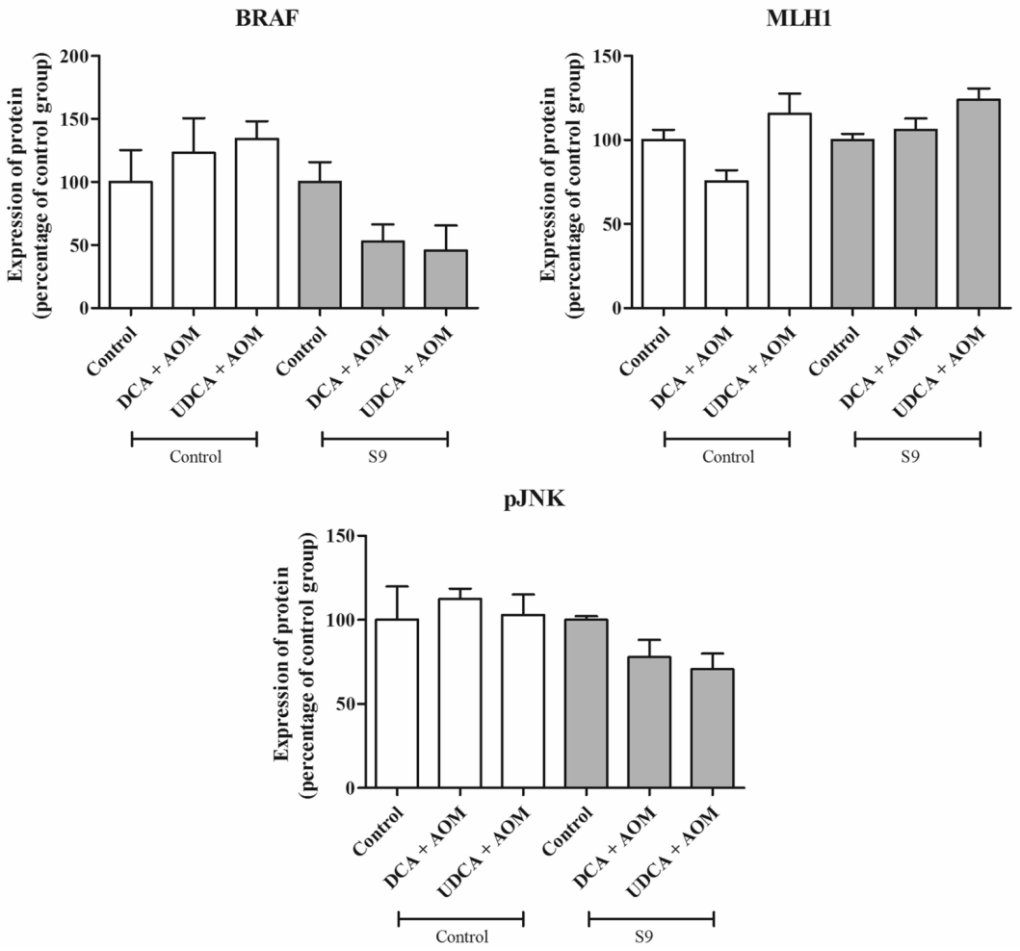


**Figure10 –** Effects of pretreatment with DCA or UDCA for 6 h prior to AOM (15 µM) treatment for 24 on BRAF, MLH1, and pJNK protein expression in Caco-2 cells, using western blot. The total protein was used to normalize the values. Values are mean ± SEM of at least three independent experiments.

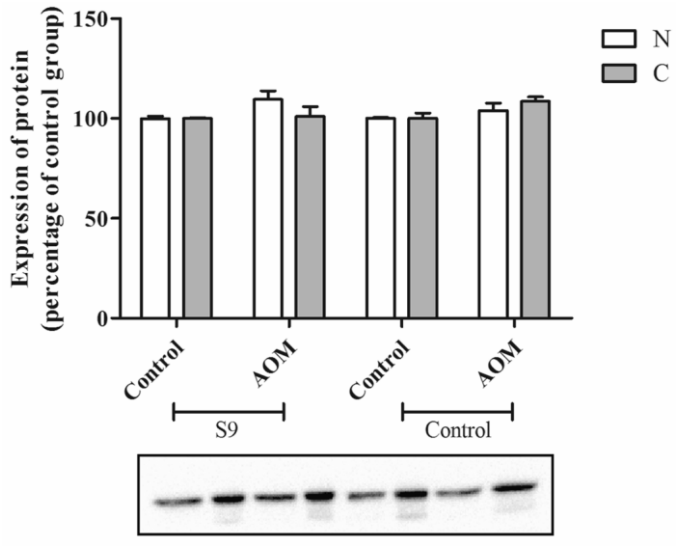


**Figure 11 –** Effects of treatment with AOM (15 μM) for 24 h on nuclear β-catenin expression in Caco-2 cells, using western blot. A representative blot is shown under the quantification. The total protein was used to normalize the values. Values are mean ± SEM of at least three independent experiments.

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*Chapter 4*

# *IN VIVO* EFFECTS OF NATURAL COMPOUNDS

# 1 – Colon cancer chemoprevention by sage tea drinking: decreased DNA damage and cell proliferation

## 1.1 – Introduction

Colorectal cancer (CRC) is one of the most common cancers worldwide and one of the leading causes of death by cancer in developed countries. Diet, in particular high fat foods, has been found to be an important risk factor in the development of this malignancy [Padidar, S., *et al.*, 2012; Tang, F.-Y., *et al.*, 2012]. A diet rich in fruits and vegetables has been shown, on the other hand, to have preventive effects on colon cancer [Pan, M.H., *et al.*, 2011]. In agreement with this, a variety of natural occurring plant compounds, such as phenolic acids, flavonoids, and carotenoids, have been found to prevent aberrant crypt foci formation, the precursor lesions of colon cancer [Mori, H., *et al.*, 2004; Pan, M.H., *et al.*, 2011]. *Salvia officinalis* (sage) is a native plant to southern Europe commonly used as a tea and condiment in Mediterranean countries that also has been attributed medicinal properties, antiseptic, anti-inflammatory and antihyperglycemic activities, due to the high content of biologically active compounds [Miura, K., *et al.*, 2002; Topcu, G., 2006]. The water extract of *Salvia officinalis*, used in the present study, contained rosmarinic acid and luteolin-7-glucoside, which are the major phenolic compounds of the species, while 1,8-cineole, cis-thujone, trans-thujone, camphor and borneol are the major volatile compounds [Lima, C.F., *et al.*, 2005]. Previous studies have shown antioxidant properties of *Salvia officinalis* (SO) extracts [Lima, C.F., *et al.*, 2005; Lima, C.F., *et al.*, 2006] and a recent study has shown chemopreventive effects of this extract *in vitro* by protection against oxidative DNA damage and stimulation of DNA repair [Ramos, A.A., *et al.*, 2010a]. Also, a study from our lab has shown that SO inhibited cell proliferation in HCT15, a colon cancer-derived cell line [Xavier, C.P., *et al.*, 2009].

Genomic instability is a common characteristic of cancer cells. A combination of induction of DNA damage, defective DNA repair systems, and failure to stop the cell cycle to correct DNA before it is passed on to the next generation of cells may be contributing factors [Lord, C.J., *et al.*, 2012]. DNA damage is constantly being inflicted to cells and, when the repair capacity is overwhelmed, it becomes the underlying cause of mutations. Critically, mutations may occur in key genes that control apoptosis and cell

proliferation, giving these cells growth advantage, eventually leading to cancer [Peltomaki, P., 2001]. The formation of aberrant crypt foci (ACF) is the reflex of increased cell proliferation which may result from DNA damage and occurrence of mutations in the colon mucosa. Many studies use induced ACF in rodents to study the chemopreventive or carcinogenic potential of natural or synthetic compounds [Johnson, R.L., *et al.*, 2013].

In the present study, we evaluated the potential effect of SO water extract (sage tea) as a chemopreventive agent against the initiation and/or promotion of colon cancer *in vivo*. The preventive mechanisms were further characterized at the level of drug metabolism, proliferative and inflammatory markers, as well as at the level of colonocyte DNA protection. It was also of our interest to study DNA protection of lymphocytes. These cells are more accessible than colonocytes and the possibility of their use as surrogate markers of effects at the colonocyte level is evaluated.

## 1.2 – Material and methods

### 1.2.1 – Animals

Female Fischer 344 rats, 3 weeks-old, were obtained from Charles River Laboratories and kept in quarantine during 1 week. Throughout the whole experiment, the animals had free access to food, an AIN76-based rat chow, and water or *S. officinalis* water extract (13.3 g/l). SO water extract was routinely prepared as an infusion by pouring 450 mL of boiling water onto 6 g of the dried plant material and allowing to steep for 5 min [Lima, C.F., *et al.*, 2005]. The composition of the extract has been published elsewhere [Lima, C.F., *et al.*, 2005], and the major phenolic compounds are rosmarinic acid and luteolin-7-glucoside (Table 1). Animals were weighed once a week during the whole experiment and food and water/sage tea consumption was registered 3 times a week. The experiments were carried out according to the regulations of national authorities for handling laboratory animals (Veterinary General Directive Board, Ministry of Agriculture, Rural Development and Fishing) and European Community Council Directive 86/609/EEC.

**Table 1 –** Main compounds found in SO water extract [Lima, C.F., *et al.*, 2005].

| **Component** | **µg/mL** |
|---|---|
| Water | |
| Phenolic acids | |
| Rosmarinic acid | 362.0 |
| Flavonoids | |
| Luteolin-7-glucoside | 115.3 |
| Other luteolin glycosides (3) | 48.5 |
| Volatile components | 4.8 |
| 1,8-cineole | 0.9 |
| *cis*-thujone | 1.7 |
| *trans*-thujone | 0.3 |
| Camphor | 0.5 |
| Borneol | 0.7 |
| Other (20) | 0.7 |
| Unknown | 2972.0 |

### 1.2.2 – Experimental design

Rats were divided into 6 groups (Figure 1A). Groups 2 (Sage (pre)) and 4 (Sage + AOM) had access to SO water extract instead of water (Groups 1 and 3) during the first two weeks. These two groups were used to evaluate the effects of sage water extract on initiating steps of the carcinogenic process. Groups 5 (Sage (post)) and 6 (AOM + Sage) had access to sage water extract during the last four weeks of the experiment. These two groups were used to investigate the potential of this extract to affect post-initiating steps of CRC progression. At the second and third week, groups 3 (Water + AOM), 4, and 6 received a s.c. injection of AOM (15 mg/kg) in saline (0.9%). The other three groups received an injection of the vehicle. Seven weeks later, the animals were sacrificed and the colons removed for ACF and immunohistochemistry.

For colonocyte and lymphocyte isolation, as well as for assessment of liver parameters, four additional groups of rats (Figure 1B) were sacrificed a day after AOM injection. For lymphocyte isolation, blood from all animals was collected in 5% EDTA and followed to the isolation protocol. The colon was also removed and washed in HBSS buffer (NaCl, 8.0 g/L; KCl, 0.4 g/L; $Na_2HPO_4.12H_2O$, 0.12 g/L; $KH_2PO_4.3H_2O$, 0.078 g/L; $NaHCO_3$, 0.35 g/L; HEPES, 4.8 g/L) for colonocyte isolation.

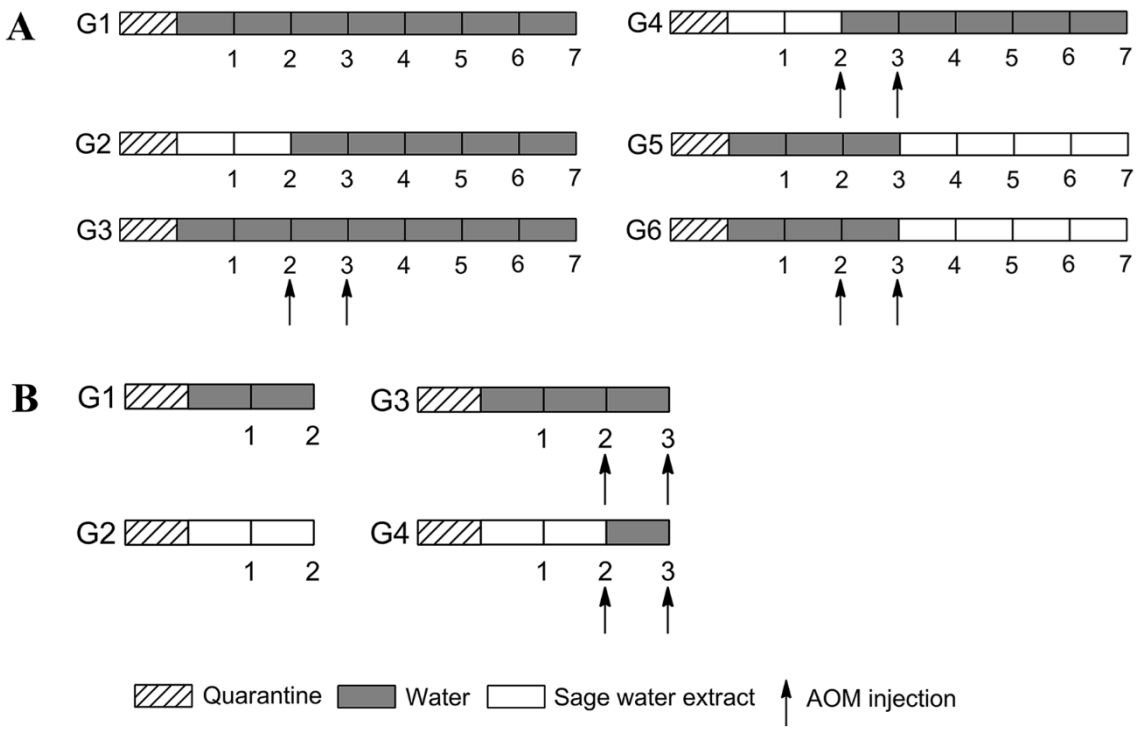


**Figure 1 –** Experimental plan 1 (A). G1 – Water, G2 – Sage (pre), G3 – Water + AOM, G4 – Sage + AOM, G5 – Sage (post), G6 – AOM + Sage. Experimental plan 2 (B). G1 - Water, G2 - Sage, G3 - Water + AOM, G4 - Sage + AOM All rats were kept in quarantine for one week. AOM injections were given to groups 3, 4, and 6 at weeks two and three. Saline injections were given to the other three groups. At the end of the seventh week, all animals were sacrificed by decapitation.

### 1.2.3 – Identification and quantification of ACF

The colons were washed with ice cold phosphate buffered saline (PBS), cut open longitudinally, fixed in 3% (w/v) paraformaldehyde for 24 hours and then stored in ethanol.

For ACF identification and quantification, colons were stained with 0.05% (w/v) methylene blue in PBS for 5 minutes. ACF number and multiplicity (number of aberrant crypts in each ACF) were scored blindly using a light microscope.

### 1.2.4 – Immunohistochemical analysis of ACF

Four µm sections were cut, deparaffinised and rehydrated. Antigen retrieval was performed using 10 mM citrate buffer solution, pH 6.0 at 98 ºC for 20 min and endogenous peroxidase inactivation was performed using 0.3% hydrogen peroxide for 10 min. Sections were incubated with a protein blocking solution for 10 min and then incubated with primary antibody: Ki67 (1:100), Abcam, Cambridge, UK and β-catenin (1:100), Santa Cruz Biotechnology, Heidelberg, Germany. Next, the sections were

incubated with Streptavidine Peroxidase for 10 min followed by development with 3,3′-diamino-benzidine (DAB + substrate System) during 10 min. Immunohistochemistry was performed using the Streptavidin-biotin peroxidase complex system (UltraVision Large Volume Detection System Anti-Polyvalent, Horseradish Peroxidase, Lab Vision Corporation, Fremont, CA, USA). Counterstaining was performed with hematoxylin and slides were permanently mounted. Ki67 positive-stained cells were expressed as percentage of positive cells per total number of epithelial cells per crypt.

### 1.2.5 – Microsomal fraction preparation for CYP2E1 evaluation

To measure the activity and expression of CYP2E1, liver microsomal fractions were isolated by differential centrifugation as previously described elsewhere [Barbier, O., *et al.*, 2000]. Aliquots were frozen in liquid nitrogen and stored at -80 ºC until further use.

### 1.2.6 – Evaluation of CYP2E1 activity

The activity of CYP2E1 can be measured spectrophotometrically by following, at 480 nm, the conversion of *p*-nitrophenol (PNP) to 4-nitrocatechol, as described by Allis *et al.*, 1994 [Allis, J.W., *et al.*, 1994].

### 1.2.7 – CYP2E1 protein expression

Protein concentration was quantified using with the Bradford Reagent purchased from Sigma using bovine serum albumin as a standard and 40 µg/well was separated by 12% SDS–PAGE and transferred to polyvinylidene difluoride membranes. A rabbit polyclonal antibody against CYP2E1 (StressGen, Ann Arbor, MI, USA) protein (1:5,000) was used overnight at 4 ºC and secondary antibody incubation was done with anti-rabbit antibody conjugated with IgG horseradish peroxidase. A chemiluminescence detection system (Chemi Doc XRS, Bio-Rad Laboratories, Inc, Hercules, CA, USA) and band area intensity was quantified using the Quantity One software (Bio-Rad).

### 1.2.8 – Glutathione-S-transferase (GST) activity

Glutathione-S-transferase (GST) activity was measured in liver homogenates and was determined by following the formation of glutathione (GSH) conjugate at 340 nm with 1-chloro-2,4-dinitrobenzene (CDNB) as previously described by our group [Lima, C.F., *et al.*, 2005].

### 1.2.9 – Colonocyte isolation

The colons were removed and washed with HBSS buffer and then with ice-cold incubation solution (glucose, 1 g/L; DTT, 1 mM; glutamic acid, 2.4 mM, dissolved in HBSS buffer). The colons were then cut open longitudinally and placed in 10 mL of incubation solution, with 2 mg of proteinase K and 1.5 mg of collagenase, for 10 min at 37 ºC, stirring slightly and gassed with carbogen (95% $O_2$ and 5% $CO_2$). The obtained cell suspension was filtered with 70 µm filters to separate the colonocytes. Next, 50 µL of 5.88% $CaCl_2$ was added for each 5 mL of cell suspension, following a centrifugation at 200 g for 10 min at 4 ºC. The pellet was ressuspended in culture medium and cells were counted and used to measure DNA damage by the comet assay.

### 1.2.10 – Lymphocyte isolation

For lymphocyte isolation, the blood collected in EDTA solution was diluted in an equal volume of PBS buffer. This volume was added to a tube containing Ficoll, also in equal proportion, following a centrifugation at 1,800 rpm for 18 min, 18 ºC. The lymphocyte layer was removed and transferred to another tube where PBS was added and another centrifugation was done at 1,800 rpm for 10 min, 18 ºC. The pellet was ressuspended in medium and cells were counted and used to measure DNA damage by the comet assay.

### 1.2.11 – Evaluation of DNA damage by comet assay and *in vitro* oxidative damage induction

The alkaline version of the single cell gel electrophoresis (comet) assay was used to evaluate DNA damage [Collins, A.R., 2004]. Briefly, 50,000 cells were centrifuged for 1 min at 5,000 rpm, ressuspended in low melting point agarose and spread onto agarose-coated slide using a cover slip. To study protection against AOM-induced DNA damage in colonocytes and lymphocytes, the standard comet assay was used. To study protection against *in vitro* $H_2O_2$-induced DNA damage, colonocytes from sage or water drinking animals were used. After spreading these colonocytes on the slides, the slides were placed in different concentrations of $H_2O_2$ solution (25, 50, or 100 µM) or PBS (control), on ice for 5 min. After 5 min at 4 ºC, the cover slip was removed and the slides were placed in lysis buffer (NaCl, 2.5 M; $Na_2EDTA$, 100 mM; Tris, 10 mM, pH 10) plus 1% Triton X-100 for 1 h at 4 ºC. Then, the slides were left in electrophoresis buffer (NaOH, 300 mM; $Na_2EDTA$, 1 mM, pH >13) for 40 min at 4 ºC. The electrophoresis was run for 20 min at

4 ºC, 21 V (0.8 V/cm) and 300 mA. After the electrophoresis, the slides were placed in neutralization buffer (Tris Base, 0.4 M, pH 7.4) for 15 min, washed with water and dehydrated with ethanol. To each slide, 20 µL of ethidium bromide was added and 100 cells were counted under a fluorescent microscope and quantified by visual scoring and using Comet 4 image analysis system (Perceptive Instruments).

#### 1.2.12 – Statistical analysis

Statistical significances were determined using Two-way ANOVA, followed by Bonferroni post-test for the effects of sage tea on protection against oxidative DNA damage, and Student's *t*-test for all other data. Data was expressed as means ± SEM. p values $\leq 0.05$ were considered statistically significant.

## 1.3 – Results and discussion

Diet is an important factor in colon carcinogenesis. It has been shown that fat and red meat can promote tumorigenesis. However, diet can also have a beneficial effect on colon cancer [Ramos, A.A., *et al.*, 2010b] and, in order to prevent this malignancy, dietary strategies to inhibit the formation or delay the progression of tumors are needed. In the present study, the chemopreventive potential of the water extract of sage, a plant with antioxidant properties [Lima, C.F., *et al.*, 2005; Lima, C.F., *et al.*, 2007b] and that has been shown to have *in vitro* ability to prevent DNA damage [Ramos, A.A., *et al.*, 2010b], was studied against ACF formation in the AOM-induced colon carcinogenesis model. As our group has various studies demonstrating the *in vitro* potential of this extract, the objective in this study was to test whether the *in vitro* effects could also be present *in vivo* and correspond to a cancer preventive effect. We used the water extract and not an isolated compound of the extract to give emphasis a dietary strategies with whole compounds, of easy access, and to study the food, in this case a tea, as a complex mixture. Treatment with sage tea before exposure to the carcinogen (AOM injection) significantly decreased the number of ACF formed, indicating a chemopreventive effect of this extract on the initiation phase of the carcinogenic process in the colon (Figure 2A). The lack of effect of the extract when given after carcinogen injection (Figure 2A) and on crypt multiplicity (average number of aberrant crypts in each focus) (Figure 2B) indicates that after mutations set in, the herbal tea does not affect molecular mechanisms involved in promotional phases, at least not this early in the process. More studies with tumors would

be necessary to evaluate the potential of the herbal tea in advanced stages of carcinogenesis. Genetic animal models are now used to obtain important information on familial and sporadic colon carcinogenesis, but the AOM-induced model has been very useful in explaining the molecular mechanisms involved in sporadic CRC due to the similarities in the pathophysiology of the tumors with human colon tumors [Johnson, R.L., *et al.*, 2013].

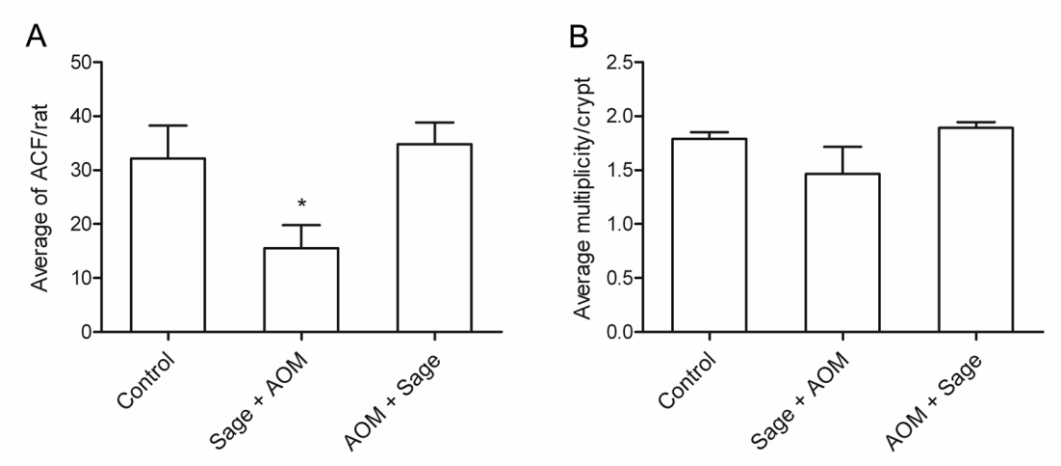


**Figure 13 –** Effect of sage drinking in AOM-induced ACF formation and multiplicity. A – Average number of ACF found in the whole colon after methylene blue staining. B – Average number of aberrant crypt per foci (multiplicity) per crypt in the colon. The results are expressed as mean ± SEM for n=6. * $P<0.05$ when compared with water control.

Effects of our herbal tea on initiation may reflect decreased AOM toxicity due to (1) decreased bioactivation through CYP2E1, (2) increased excretion through GST induction, (3) DNA damage protection, and/or (4) effects on cell proliferation. Beneficial effects of other plant foods have been found, for example, black tea and wine extracts, but not green tea, to protect against AOM-induced colon carcinogenesis in rats [Caderni, G., *et al.*, 2000] and theaflavin-2, a major compound found in black tea extract was shown to induce apoptosis in human colon cell lines [Gosslau, A., *et al.*, 2011]. In a study by Andersson *et al.*, 2008 [Andersson, D., *et al.*, 2008], ursolic acid, a pentacyclic triterpenoid also present in SO, was found to reduce the number of large ACF when given orally to rats during the initiation phase but not during the promotion/progression phase, results that are in agreement with our present study. Also, a study from our lab demonstrated that ursolic acid protects colon cells against DNA damage [Ramos, A.A., *et al.*, 2010c].

One of the possible mechanisms behind sage tea chemoprevention may be the modulation of enzymes involved in AOM metabolism. CYP2E1 enzyme is involved in the activation of AOM *in vivo* [Sohn, O.S., *et al.*, 2001], suggesting that agents that modify this enzyme's activity or expression levels may either promote or inhibit CRC through effects on formation of carcinogenic AOM derivatives. Based on this, effects of sage treatment on liver CYP2E1 activity and expression was evaluated, but no effects were found (Figure 3). A decrease of CYP2E1 activity with AOM injection was found, but this decrease was only significant in the water drinking group (Figure 3A). As demonstrated for other drugs, a decrease in CYP2E1 activity after a few hours (12-24 h) of AOM administration may indicate increased AOM metabolism and CYP2E1 inactivation due to the high amount of free radicals produced [Lima, C.F., *et al.*, 2007a; Weber, L.W., *et al.*, 2003; Zhukov, A., *et al.*, 1999]. Modulation of GST, a phase II enzyme, can increase carcinogen elimination before damage is done. In this study, and contrarily to what happened previously with mice [Lima, C.F., *et al.*, 2005], no effects on GST activity in any of the treatment groups were found (data not shown). Altogether, these results indicate that the preventive effect of sage tea on ACF formation was not due to decrease in AOM bioactivation or increase of AOM elimination.

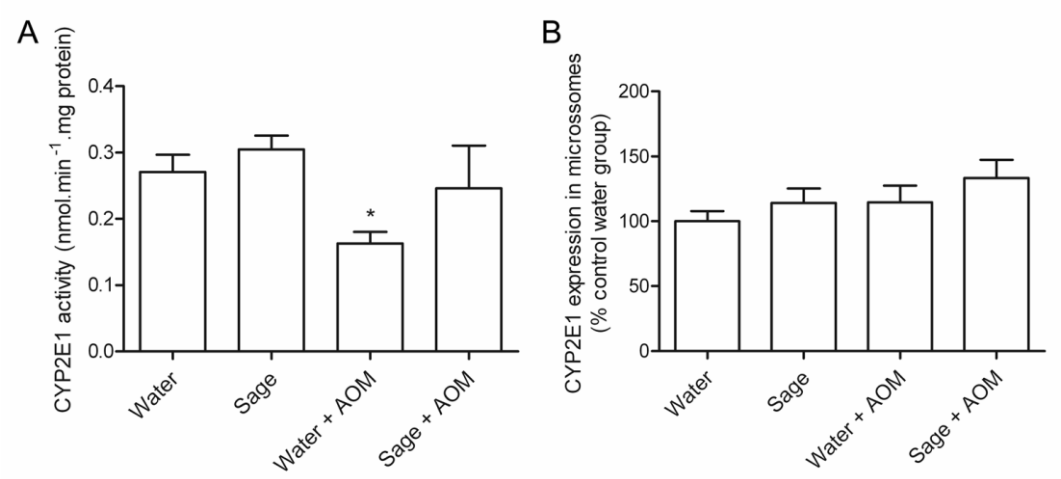


**Figure 3** – Effect of sage drinking (14 days) and/or AOM injection on CYP2E1 activity (A) and levels (B) measured in the microsomal fraction isolated from rat liver. CYP2E1 activity is expressed as nmol.min$^{-1}$.mg protein and protein levels expressed as percentage of water control group. The results are expressed as mean ± SEM for n=4. * $P<0.05$ when compared with water control.

The most important initiating factor in carcinogenesis is the occurrence of DNA damage and mutations. It is known that dietary mutagens and oxidative stress may lead

to DNA damage. Dietary mutagens may be present in foods through fungal contamination or methods of preservation and cooking. One example of food mutagens are the heterocyclic amines 2-amino-1-methyl-6-phenylimidazol[4,5-b]pyridine (PhIP) and 2-amino-3-methylimidazol[4,5-f]quinoline (IQ), which are produced from cooked protein-rich foods such as meat and fish. The mutagenicity of these heterocyclic amines is DNA damage caused by induction of an inflammatory response [Ferguson, L.R., *et al.*, 2008]. In our study, after bioactivation of AOM in the liver, methylazoxymethane (MAM) is transported to other organs where it will be further converted and finally, especially in the colon, induces DNA damage, suggesting that protection against DNA damage at the colonocyte level may be a relevant mechanism of chemoprevention.

Therefore, the effect of *in vivo* SO treatment on protection against *in vitro* $H_2O_2$-induced DNA damage in isolated colonocytes was investigated. Treatment with SO significantly decreased *in vitro* $H_2O_2$-induced DNA damage to colonocytes when compared with control (Figure 4), which is in agreement with our previous *in vitro* results [Lima, C.F., *et al.*, 2007b; Ramos, A.A., *et al.*, 2010b]. AOM-induced DNA damage to colonocytes was also evaluated. AOM was found to induce DNA damage detected by the comet assay, while SO treatment before AOM injection decreased DNA damage (Figure 5A). In a study by Dolara *et al*., a protective effect of DNA damage in colonocytes was also found using wine polyphenols [Dolara, P., *et al.*, 2005]. In the present study, the same protective effect of SO against AOM-induced DNA damage was found in lymphocytes isolated from the same animals (Figure 5B). This effect, as far as we know, has not been demonstrated in the literature and can be of great importance to further imply lymphocytes as surrogate markers in these kinds of studies. The protective effects of SO may be due, at least in part, to an increase in protection against DNA damage, which seems to be a global effect and not just localized in the colon, and may be the key to chemoprevention of colon cancer. Protection against DNA damage is not only beneficial for cancer prevention, but also for other diseases and the overall health of the individual.

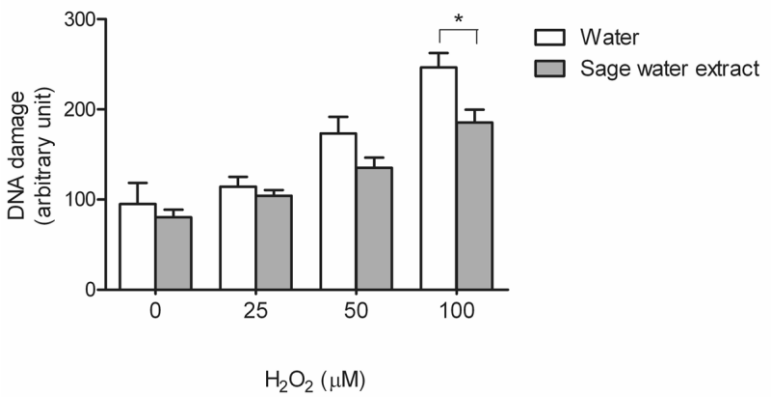


**Figure 4 –** Effect of 2 week SO treatment *in vivo* on $H_2O_2$-induced DNA damage in isolated rat colonocytes. DNA damage analyzed by the Comet assay. Mean ± SEM (n=3). Statistical significances were determined using a two-way ANOVA followed by Bonferroni's multiple comparison test. * $P<0.05$.

It is also known that there is a link between chronic inflammation, proliferation and CRC [Hull, M., 2008; Itzkowitz, S.H., 2006; Terzic, J., *et al.*, 2010]. The impact of SO on proliferation was evaluated in colon tissue, by Ki67 immunohistochemical staining of the colon mucosa. AOM increased the overall number of proliferating cells of the normal mucosa, but in animals treated with SO, this increase was significantly less pronounced (Figure 6A). The number of proliferative cells was naturally also higher in ACF when compared with the respective normal tissue (Figure 6B). Sage treatment was able to reduce the number of proliferating cells in the normal tissue, possibly as an effect of sage on different signalling pathways that regulate proliferation. A study from our lab showed that SO inhibits cell proliferation and inhibits the MAPK/ERK pathway in the KRAS mutated HCT15 cell line [Xavier, C.P., *et al.*, 2009]. With this study, we add that SO also reduces cell proliferation *in vivo*.

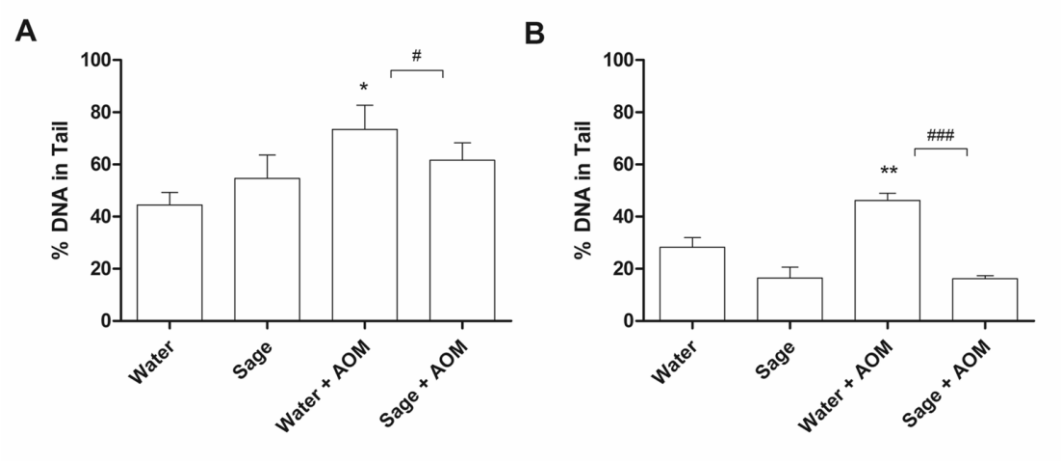


**Figure 5 –** Effect of 2 week SO treatment and/or AOM injection on DNA damage on colonocytes (A) and lymphocytes (B) measured by the comet assay. DNA damage expressed as percentage of DNA in tail. The results are expressed as mean ± SEM for n=4. * P<0.05 when compared with water control, # P<0.05 when compared with Water + AOM group, ** P<0.01 when compared with water control, ### P<0.001 when compared with Water + AOM group.

The WNT signalling pathway is important in the regulation of cell proliferation in the colon crypt [Pinto, D., *et al.*, 2005], and AOM may cause mutations in *β-catenin*, an intervenient in this pathway [Takahashi, M., *et al.*, 2004]. In the present study, no β-catenin translocation to the nucleus was found in normal tissue or in ACF and APC expression was the same in all groups (data not shown). AOM may not have caused alterations in *β-catenin* possibly due to the short duration of the study relative to the time these mutagenic lesions need to be expressed. So, SO chemoprevention appears to be, apart from DNA protection from damage, also due to regulation of proliferation but does not appear to be in the regulation of WNT signalling pathway [Takahashi, M., *et al.*, 2000; Takahashi, M., *et al.*, 2004]. The MAPK/ERK pathway is usually also altered in this AOM-induced model. As it was referred before, SO has shown to inhibit this pathway in HCT15 cell line. As the WNT pathway was not altered, we could assume that sage tea is acting on the MAPK/ERK pathway. Studies in the activation of this pathway would answer this question.

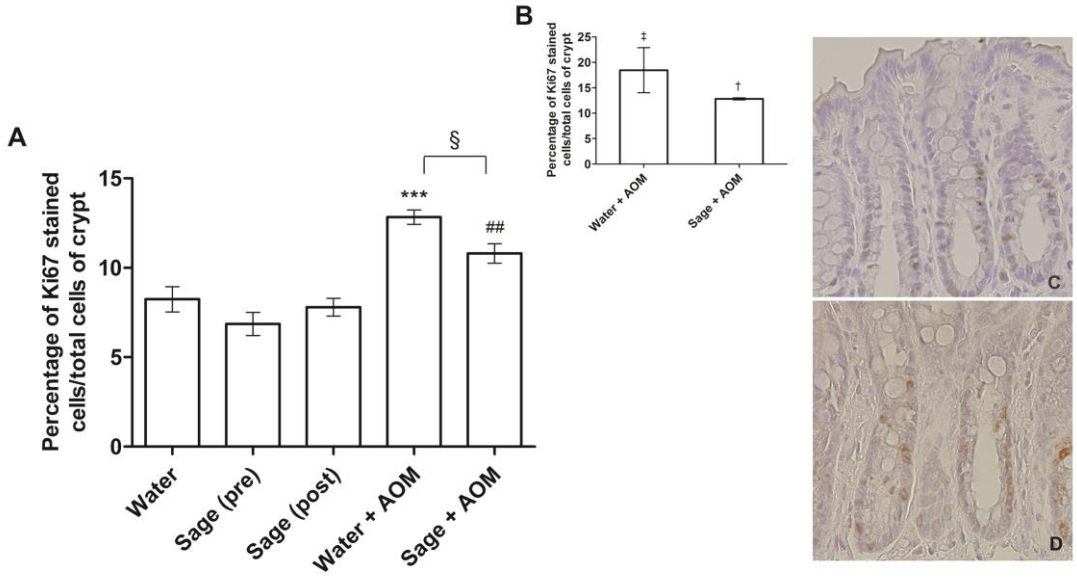


**Figure 6 –** Proliferating cells in normal colon crypts of rats from water, Sage (pre), Sage (post), water + AOM and Sage + AOM groups (A) and ACF from water + AOM and Sage + AOM groups (B) assayed by Ki67 stained cells. Representative image of Water group (C) and Water + AOM group (D). Results are expressed as percentage of Ki67 stained cells per total number of cells in a crypt ± SEM for n = 4. *** = P<0.001 when compared with water group, ## = P<0.01when compared with Sage (pre) group, § = P<0.05, ‡ = P<0.05 when compared with Water + AOM group of normal tissue, † = P<0.05 when compared with Sage + AOM group of normal tissue.

In conclusion, treatment with sage water extract (herbal tea) showed a chemopreventive effect against AOM-induced preneoplastic lesions of colon cancer (Figure 9). An effect on colonocytes was demonstrated as SO treatment *in vivo* confers DNA protection against AOM- and $H_2O_2$-induced DNA damage. The protective effect of SO against DNA damage induced by AOM was also observed in lymphocytes. In addition, sage treatment significantly lowered the increased expression of Ki67, a marker of the overall proliferation rate in the colon. No evidence was found that sage treatment affected AOM metabolism in the liver or inflammation. This study showed that the consumption of *Salvia officinalis* tea may contribute to prevention of colon cancer, and mechanisms of protection against DNA damage and modulation of cell proliferation are involved. Plant foods and beverages, such as *Salvia officinalis* tea, have shown great potential and they should be considered as possible chemopreventive agents in dietary strategies against colon cancer. This herbal extract could ameliorate the overall health of the individual, not just against cancer initiation, but also diseases associated with DNA damage.

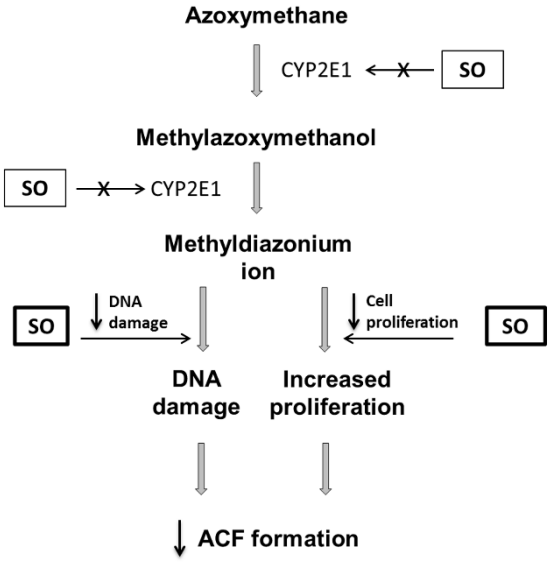


**Figure 7** – Overview of the relevant results obtained in this study. Possible mode of action of *Salvia officinalis* colon cancer chemoprevention.

## 2 – Impact of nutrition on DNA damage in rats

### 2.1 – Introduction

Colorectal cancer (CRC) is known to be caused by the accumulation of genetic and epigenetic alterations. Mutations and/or gene silencing may affect the expression or state of activation of key proteins involved in control of important cellular processes, such as cell proliferation, apoptosis, or repair of DNA damage [Arends, M.J., 2013]. Exposure to exogenous and endogenous agents results in an accumulation of DNA damage over time, and if not repaired, DNA damage contributes to carcinogenesis. Cells have a variety of DNA repair pathways to overcome the possible deleterious effects of DNA damage. Base excision repair (BER) is one of the major pathways involved in the repair of alkylation and oxidation DNA damage, repairing apurinic/apyrimidinic (AP) sites, DNA single-strand breaks and different types of base modifications [Maynard, S., *et al.*, 2009]. Briefly, this pathway involves a recognition and excision step by glycosylases (for example, 8-oxoguanine glycosylase (OGG1) recognizes oxidized bases, while methylpurine-DNA glycosylase (MPG) recognizes alkylated bases), generating an AP site. This AP site is cleaved by an AP endonuclease, human AP endonuclease (APE1), generating a 3´OH and 5´deoxyribose phosphate terminus. Finally, DNA polymerase fills the nucleotide gap and a DNA ligase seals the nick on the DNA strand [Maynard, S., *et*

*al.*, 2009]. The mismatch repair (MMR) system mainly corrects base to base mispairs which are caused by errors of the DNA polymerase. The recognition of these mispairs initiates a process that ends with the excision of the mispair and resynthesis of the DNA strand. Among the proteins important in this process, *MSH2* and *MLH1* are also found to be altered in CRC tumors [Lord, C.J., *et al.*, 2012].

It has been demonstrated that CRC risk is strongly associated with diet. Dietary habits can help prevent or promote this disease [Nystrom, M., *et al.*, 2009]. For example, western-type diet is thought to contribute to the development of colorectal cancer, while diets that are rich in fruits and vegetables have been found to be beneficial in the prevention carcinogenesis. Bile acids are considered as carcinogens in a variety of gastrointestinal cancers, including CRC. One of the effects of high bile acid exposure on cells is the DNA damage induced by reactive oxygen species (ROS) [Barrasa, J.I., *et al.*, 2013]. An increase of bile acid exposure increases ROS, which can increase DNA damage and consequently lead to mutations [Bernstein, H., *et al.*, 2009]. On the other hand, phytochemicals, found in fruits and vegetables, can inhibit, delay and/or reverse carcinogenesis. (-)-Epigallocatechin-3-gallate (EGCG) is a catechin found in green tea and has been extensively used in chemopreventive studies *in vivo* and *in vitro*. EGCG was found to reactivate the expression of silenced genes by downregulation of DNA methyltransferases (DNMT) and histone deacetylases (HDAC) activity in human skin cancer cell lines [Nandakumar, V., *et al.*, 2011]. Also, it has been found to suppress ACF formation when combined with sulindac and reduced the cytotoxicity induced by $H_2O_2$ and increase levels of enzymes related to oxidative stress in HepG2 cells [Lambert, J.D., *et al.*, 2010; Murakami, C., *et al.*, 2002; Ohishi, T., *et al.*, 2002]. Ursolic acid (UA) is a triterpenoid found in some fruits and herbs, with anti-cancer and anti-inflammatory properties. Previous work done in our lab has demonstrated various properties of UA, such as anticancer potential by induction of cell death in CRC cell lines and *in vivo [Xavier, C.P., et al., 2013]* and protection from oxidative damage and increase of DNA repair activity [Ramos, A.A., *et al.*, 2010c]. In another study, UA was found to have inhibitory effects on the formation of pre-neoplastic lesions of CRC, demonstrating a chemopreventive effect [Andersson, D., *et al.*, 2008].

The aim of our study was to evaluate the effect of diet on DNA damage and DNA repair systems *in vivo*. The potential chemopreventive effect of two natural compounds, UA and EGCG, was evaluated in lymphocytes and colonocytes and diet induced effects on *in vivo* protection from DNA damage and induction of repair. Also, the carcinogenic

potential of the bile acid, deoxycholic acid (DCA), was evaluated by induction of DNA damage and modulation of DNA repair systems. Blood samples are always easier to collect than colon samples, so another aim of this study was to evaluate the potential of lymphocytes to be surrogate markers of effects in colonocytes.

## 2.2 – Material and methods

### 2.2.1 – Animals

Male Fischer 344 rats, 3 weeks-old, were obtained from Charles River Laboratories and kept in quarantine during 1 week. Throughout the whole experiment, animals had free access to food, an AIN76-based rat chow, and water. The experiments were carried out according to the regulations of national authorities for handling laboratory animals (Veterinary General Directive Board, Ministry of Agriculture, Rural Development and Fishing) and European Community Council Directive 86/609/EEC.

### 2.2.2 – Experimental design

Rats were divided into 5 groups (Figure 8). Natural compounds were given to the animals, using chocolate as the administration vehicle, during two weeks. The first group was control group, the second group was administered UA (50 mg/kg rat), third group received DCA (100 mg/kg rat), while the forth group received a combination of UA and DCA. The last group was administered EGCG (40 mg/kg rat). During this experiment, animals were weighted once a week and treatment did not alter significantly the average weight between groups (Figure 9). After treatment, the animals were sacrificed and the colons removed for colonocyte isolation.

Blood was collected in 5% EDTA from all animals for lymphocyte isolation animals and followed to the isolation protocol.

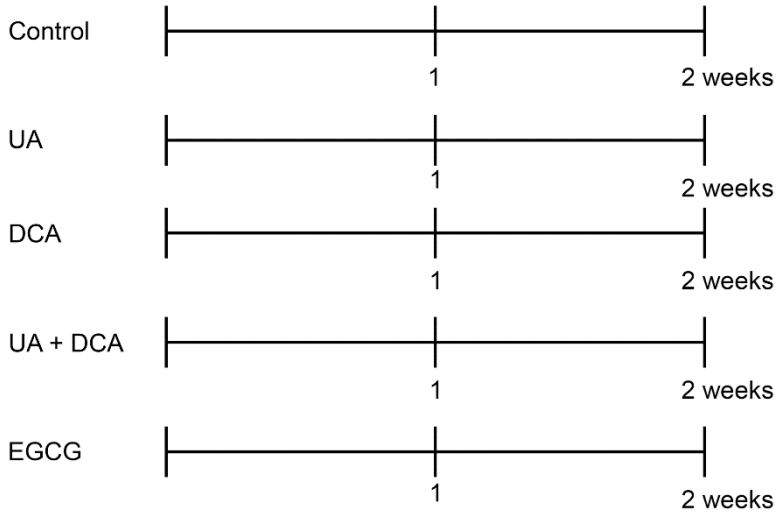


**Figure 8 –** Experimental plan. Fischer 344 male rats were given different compounds every day during 2 weeks. Control group received vehicle only, UA group was administered ursolic acid (50 mg/kg rat), DCA group received deoxycholic acid (100 mg/kg rat), UA + DCA received a combination of ursolic acid and deoxycholic acid, and finally the EGCG group was administered (-)-epigallocatechin-3-gallate (40 mg/kg rat). At the end of the two weeks, all animals were sacrificed by decapitation.

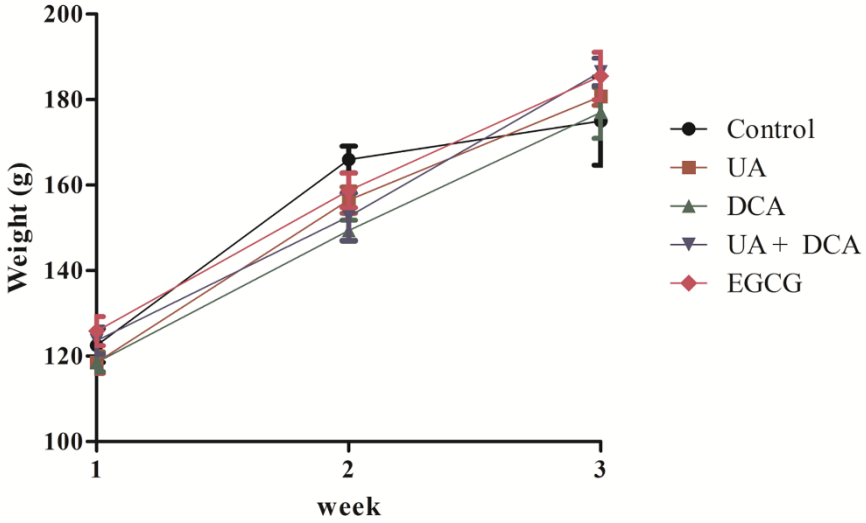


**Figure 9 –** The average weight (g) of the animals in each group. There seems to be no difference between the treatments on the growth of the animals.

### 2.2.3 – Colonocyte isolation

The colon was removed and washed in HBSS buffer (NaCl, 8.0 g/L; KCl, 0.4 g/L; $Na_2HPO_4.12H_2O$, 0.12 g/L; $KH_2PO_4.3H_2O$, 0.078 g/L; $NaHCO_3$, 0.35 g/L; HEPES, 4.8 g/L) and with ice-cold incubation solution (glucose, 1 g/L; dithiothreitol (DTT), 1 mM; glutamic acid, 2.4 mM, dissolved in HBSS buffer). The colons were then cut open longitudinally and placed in 10 ml of incubation solution, with 2 mg of proteinase K and 1.5 mg of collagenase, for 10 min at 37 ºC, stirring slightly and gassed with carbogen

(95% $O_2$ and 5% $CO_2$). The obtained cell suspension was filtered with 70 µm filters to separate the colonocytes. Next, 50 µL of 5.88% CaCl were added for each 5 mL of cell suspension, followed by a centrifugation at 200 g for 10 min at 4 ºC. The pellet was ressuspended in medium and cells were counted and used in the comet assay and western blot.

### 2.2.4 – Lymphocyte isolation

For lymphocyte isolation, the blood collected in a 5% EDTA solution was diluted in equal volume of PBS buffer. This volume was added to a tube containing Ficoll, also in equal proportion and centrifuged at 1,800 rpm for 18 min, 18 ºC. The lymphocyte layer was removed and transferred to another tube where PBS was added and another centrifugation was done at 1,800 rpm for 10 min, 18 ºC. The pellet was ressuspended in medium and cells were counted and used in the comet assay and western blot.

### 2.2.5 – Evaluation of DNA damage by comet assay and *in vitro* oxidative/alkylating damage induction

The alkaline version of the single cell gel electrophoresis (comet) assay was used to evaluate DNA damage in colonocytes and lymphocytes [Collins, A.R., 2004]. Briefly, 15,000 cells were centrifuged for 1 min at 5,000 rpm, ressuspended in low melting point agarose and spread onto agarose-coated slide. To measure basal DNA damage, slides were placed in lysis buffer (NaCl, 2.5 M; $Na_2EDTA$, 100 mM; Tris, 10 mM, pH 10) plus 1% Triton X-100 for at least 1 h at 4 ºC. Then, these were washed in PBS and then in buffer (40 mM HEPES, 0.1 M KCl, 0.5 mM EDTA, 0.2 mg/mL bovine serum albumin (BSA), pH 8) before treatment with formamidopyrimidine DNA glycosylase (FPG) for 20 min, 37 ºC. Afterwards, the slides were placed in electrophoresis buffer (NaOH, 300 mM; $Na_2EDTA$, 1 mM, pH >13) for 40 min at 4 ºC. The electrophoresis was run for 20 min at 4 ºC, 21 V (0.8 V/cm) and 300 mA. After the electrophoresis, the slides were washed with water and dehydrated with ethanol.

To measure prevention against oxidative and alkylating damage, cells were exposed to $H_2O_2$ (100 µM) or methyl methanesulfonate (MMS) (100 µM) for 5, on ice, and 15 min, at 37 ºC, respectively. Afterwards, the cells were centrifuged for 1 min at 5,000 rpm, ressuspended in low melting point agarose and spread onto agarose-coated slides, and followed the same protocol as described previously. For analysis of the comet images, slides were stained with SYBR Gold solution for 30 min at 4 ºC; after drying,

slides were analysed using a fluorescence microscope, and a Comet 4 analysis system (Perceptive software) was used to calculate the parameter percent tail intensity. Generally, 100 randomly selected cells were analysed per sample.

### 2.2.6 – Evaluation of expression of DNA damage-related proteins by western blot

MPG, APE1, and OGG1 expression was monitored by Western blotting in lymphocytes and colonocytes. Protein concentration was measured with the DC protein assay following manufacture instructions, and 20 μg/well was separated on 12% SDS-PAGE and transferred to polyvinylidene fluoride membranes. Membranes were blocked and incubated with the monoclonal anti-MPG (Abcam, Cambridge, UK), anti-APE1 (Abcam, Cambridge, UK) and anti-OGG1 (Abcam, Cambridge, UK). Immunoreactive bands were acquired using the Chemidoc camera (BioRad), and band area intensity was quantified by Quantity One software (BioRad). The results were expressed as percentage of control group.

### 2.2.7 – Statistical analysis

Data are expressed as means ± SEM. Statistical significances were determined using a Student's *t*-test and one-away analysis of variance using GraphPad Prism 5.0 software. P values $\leq 0.05$ were considered statistically significant.

## 2.3 – Results and discussion

Colon cancer is a major contributor to cancer–related morbidity, and cancer chemoprevention is becoming an important concept to implement through the use of nontoxic agents, specifically dietary sources, as a proper strategy for disease control. Natural products are now known to be promising agents in cancer prevention and therapy. Several reviews have compiled information on various dietary constituents and their possible contribution in the modulation of cancer development [Duthie, S.J., 2011; Lofano, K., *et al.*, 2013; Nolfo, F., *et al.*, 2013]. Recent studies from our group have also demonstrated the potential of natural compounds to protect against DNA damage and induction of DNA repair [Ramos, A.A., *et al.*, 2010a; Ramos, A.A., *et al.*, 2013; Ramos, A.A., *et al.*, 2012; Ramos, A.A., *et al.*, 2010c]. This study demonstrated that UA and EGCG in the diet protect colonocytes and lymphocytes from endogenous DNA damage

both *in vivo* and *in vitro* from MMS exposure. Bile acids are associated with high fat diets and DCA has been associated with increased risk of CRC [Barrasa, J.I., *et al.*, 2013; Bernstein, H., *et al.*, 2009]. We used DCA, also added in the diet, to evaluate the effect on DNA damage and DNA repair protein expression.

Unlike most studies that test DNA protection by dietary compounds in animals exposed to a genotoxic agent, in this study we used healthy individuals to evaluate the potential for cancer prevention by reduction of DNA damage. Endogenous DNA damage (strand breaks and FPG sites) in colonocytes and lymphocytes were evaluated by comet assay without and with FPG enzyme, respectively (Figure 10). Both cell types have more strand breaks than DNA damage recognized by FPG enzyme (FPG sites). Comparing endogenous DNA damage between colonocytes and lymphocytes, we found that the level of strand breaks are similar between the two cell types, while colonocytes present a tendency for higher levels of DNA damage recognized by FPG. This is in agreement with other studies that find higher levels of damage in colon tissue when compared with lymphocytes [Kager, N., *et al.*, 2010].

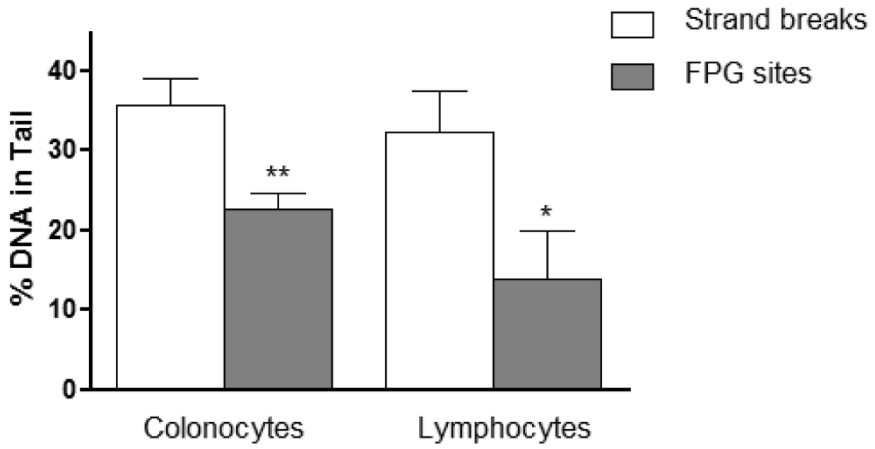


**Figure 10 –** Endogenous DNA damage in colonocytes and lymphocytes. DNA damage was measured by the comet assay. Results are expressed as percentage of DNA in tail and represent the means of at least 3 independent experiments. *P ≤ 0.05, ** P≤ 0.01 when compared with strand breaks was determined by student's *t*-test.

Oxidative DNA damage has been associated with being the cause of degenerative diseases and cancer. Several types of damaged bases result from ROS attack on DNA, many with mutagenic potential. 8-oxoguanine is a biomarker of oxidative stress and is believed to participate in carcinogenesis, possibly through G to T transversions through

mispairing during replication [Tudek, B., *et al.*, 2012] when it is efficiently repaired by the base excision repair system. Colonocytes treated with $H_2O_2$ showed an increase of strand breaks relative to endogenous strand breaks (Figure 11A). This increase was around 47% in comparison to respective control. $H_2O_2$ did not induced FPG sites in colonocytes. In lymphocytes, $H_2O_2$ (in the same concentration used for colonocytes) induced a high level of strand breaks (Figure 11B), most of the comets observed had more than 70% of the DNA in the tail of the comet. Therefore, lymphocytes showed more susceptibility to $H_2O_2$–induced DNA damage than colonocytes. The compounds tested in this study reduced the overall endogenous DNA damage (Figure 12A and 13A). In lymphocytes (Figure 13A), these natural compounds were found to reduce endogenous strand breaks. In a study by Brevik *et al.*, intake of a diet rich in phytochemicals by healthy human individuals during a total of eight weeks decreased significantly the levels of endogenous strand breaks in lymphocytes [Brevik, A., *et al.*, 2011]. On the other hand, reduction of DNA damage recognized by FPG, oxidative DNA damage, was seen by ursolic acid and EGCG in colonocytes (Figure 12A). The differences between the results in colonocytes and lymphocytes could be explained by the metabolization and absorption of the compounds.

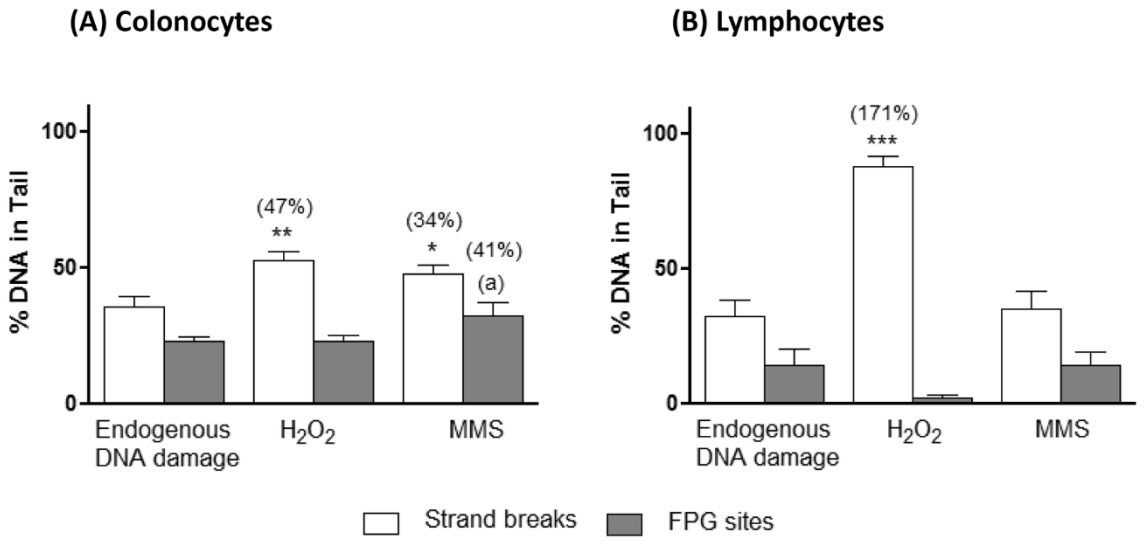


**Figure 11 –** Induced DNA damage in colonocytes (A) and lymphocytes (B): endogenous, $H_2O_2$, and MMS-induced DNA damage. $H_2O_2$ treatment was done 5 min on ice, while MMS was 15min at 37 ºC. DNA damage was measured by the comet assay. Results are expressed as percentage of DNA in tail. *$P \leq 0.05$, ** $P \leq 0.01$, ***$P \leq 0.005$ when compared with endogenous DNA damage was determined by student's *t*-test.

Alkylating DNA damage can result from exogenous, such as diet and environment, or endogenous (metabolism of enteric bacteria) sources. Alkylating damage include N-alkylating adducts ($N^7$-methylguanine, $N^3$-methyladenine, and $N^3$-methylguanine) and O-alkylating adducts. N-alkylating adducts are more abundant but are cytotoxic and less mutagenic than O-alkylating adducts. In our study, we used methyl methanesulfonate, which induces $N^7$-methylguanine and $N^3$-methyladenine, causing base mispairs that are recognized and repaired by base excision repair [Lundin, C., *et al.*, 2005; Ramos, A.A., *et al.*, 2013]. Since, FPG can also recognize alkylation damage, such as $N^7$-methylguanine, it was used to detect MMS-induced DNA damage. We found that MMS increased significantly the amount of strand breaks and FPG sites when compared with the control in colonocytes (Figure 11A). We found no effects of our natural compounds on MMS-induced DNA damage in lymphocytes (Figure 13B), but in colonocytes (Figure 12B) UA significantly decreased strand breaks and FPG sites, while EGCG only showed a slight tendency to decrease FPG sites induced by MMS. To our knowledge, few studies have studied the effect of UA on alkylating DNA damage. In a study with rats, it was found that administration of UA before treatment with an alkylating carcinogen, azoxymethane, reduced the number of preneoplastic lesions when compared with control group [Andersson, D., *et al.*, 2008]. UA was effective in reducing the oxidative stress mediated changes in liver of rats, showing chemopreventive potential in the liver [Gayathri, R., *et al.*, 2009]. Ramos *et al*. demonstrated that ursolic acid protects DNA from oxidative damage and increases repair activity *in vitro* [Ramos, A.A., *et al.*, 2010c]. EGCG is a flavonoid polyphenol found in green tea. It has been found to possess a variety of protective activities against cancer, such as inhibition of growth factor signaling and gene transcription and induction of tumor suppressors, but also the antioxidant activity of EGCG has been proposed as a potential anti-cancer mechanism [Lambert, J.D., *et al.*, 2010]. So, UA is able to decrease endogenous DNA damage and also decrease DNA damage induced by alkylating agents, in this case MMS, *ex vivo*, suggesting that diets with compounds rich in ursolic acid can ameliorate the individual's health, protecting against different types of DNA damage, reducing risk of DNA damage-associated diseases.

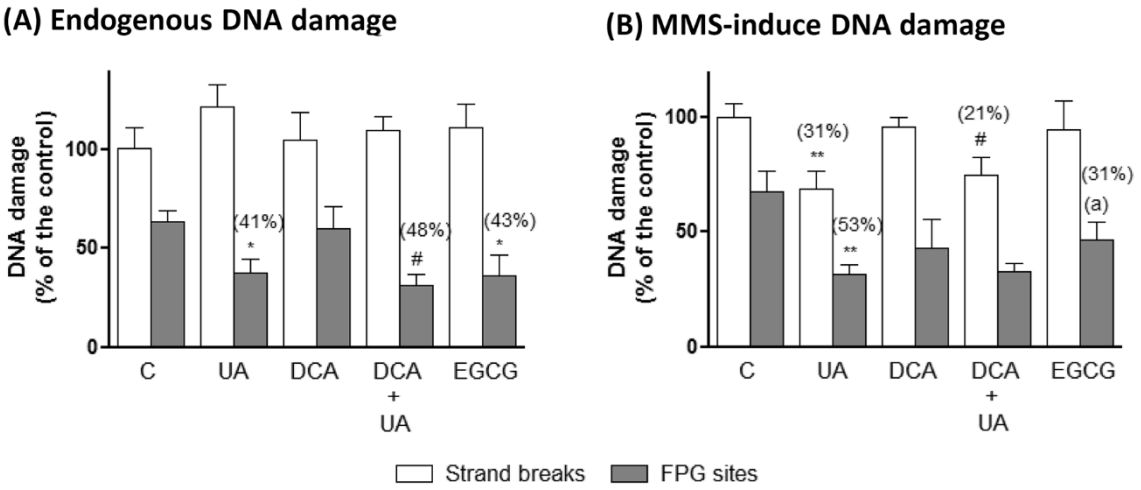


**Figure 12** – Endogenous (A) and MMS (B)-induced DNA damage in isolated colonocytes. Test compounds were given in the diet for two weeks, two natural compounds - ursolic acid (UA) and (-)-epigallocatechin gallate (EGCG), and deoxycholic acid (DCA). DNA damage was measured by the comet assay. Results are expressed as percentage of control. *P ≤ 0.05, ** P≤ 0.01, when compared with respective control, #P≤ 0.05 when compared with DCA treatment alone was determined by student's *t*-test.

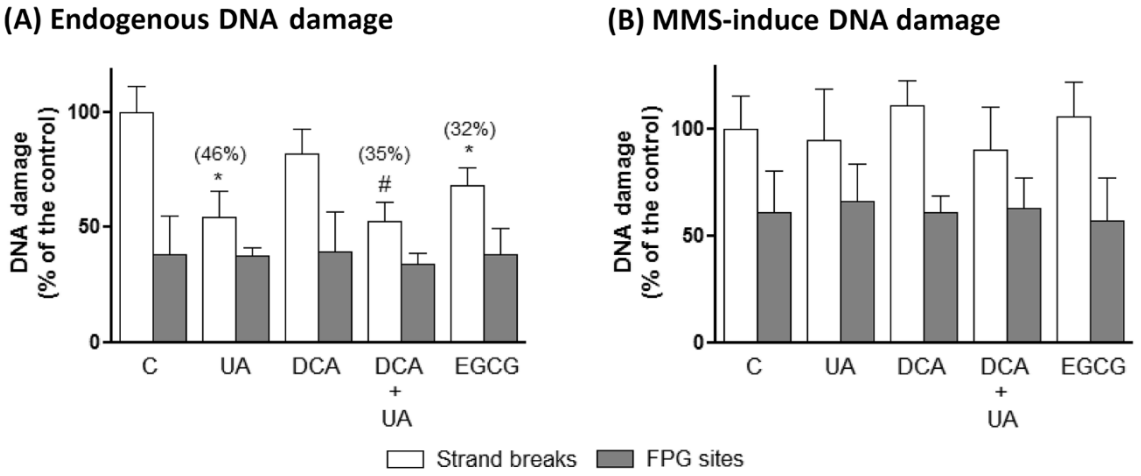


**Figure 13 –** Endogenous (A) and MMS (B)-induced DNA damage in isolated lymphocytes. Test compounds were given in the diet for two weeks, two natural compounds - ursolic acid (UA) and (-)-epigallocatechin gallate (EGCG), and deoxycholic acid (DCA). DNA damage was measured by the comet assay. Results are expressed as percentage of control. *P ≤ 0.05 when compared with respective control, #P≤ 0.05 when compared with DCA treatment alone was determined by student's *t*-test.

As pointed out previously, the types of DNA damage induced in this study are repaired by the base excision repair system. The protein expression of three intervenients in this repair system was evaluated in the isolated colonocytes and lymphocytes. We found no differences in the expression of APE1 and MPG proteins (data not shown), but found a decrease in OGG1 expression by UA and EGCG (Figure 14). OGG1 (the human

equivalent of FPG) recognizes oxidation damage, specifically 8-oxoguanine. In colonocytes, levels of 8-oxoguanine recognized by using the FPG enzyme were decreased by our natural compounds, so we can hypothesize that the ability of UA and EGCG to decrease OGG1 protein expression is related with the protective effect against DNA damage. It is known that transcription of BER genes is regulated by the cell cycle and by oxidative stress [Tudek, B., 2007]. If both compounds exert their antioxidant properties by reducing the levels of DNA damage, then OGG1 is not needed to recognize the lesions, therefore, the production of this protein is basal, hence explaining the decreased expression of OGG1 when compared with the control group. In lymphocytes, the levels of FPG sites are low to begin with (around 15% in the control group) possibly explaining the lack of protection by our natural compounds, nevertheless, the compounds are present and there can also be a reduction of expression of OGG1. Although bile acids are known to increase ROS production and therefore increase DNA damage, in our study deoxycholic acid did not affect DNA damage in any way, nor protein expression.

Although some of the effects observed in colonocytes were also found with lymphocytes, the susceptibility of the two cell types is different, so there are not enough results to authenticate lymphocytes as surrogate markers. Nevertheless, ursolic acid and EGCG were found to decrease DNA damage, possibly by their antioxidant potential, ameliorating the basic internal status of colonocytes and lymphocytes. Consumption of fruits and vegetables with these compounds could therefore benefit public health by countering oxidative stress factors and help prevent diseases mediated by ROS-induced DNA damage.

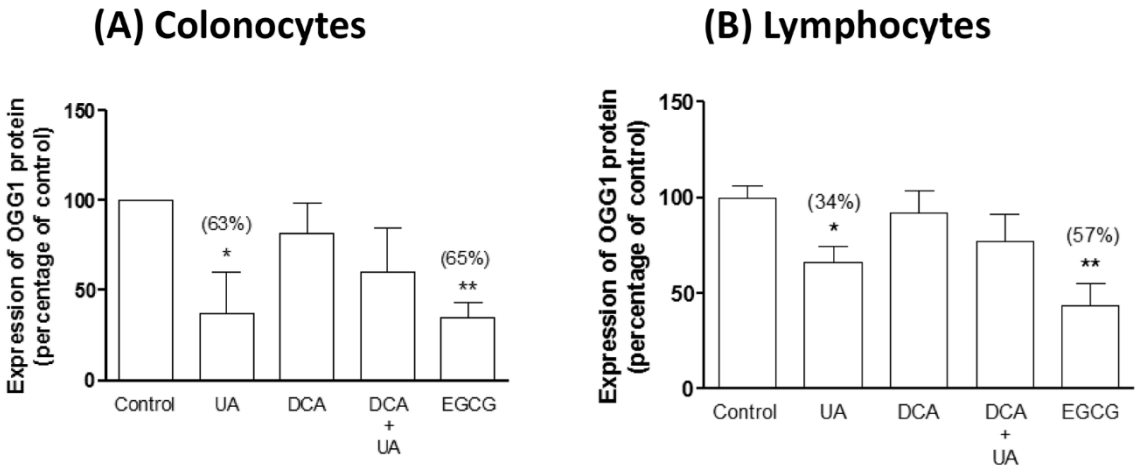


**Figure 14 –** Expression of OGG1 protein in colonocytes (A) and lymphocytes (B) isolated from rats. Protein expression was assayed by western blot. Results are expressed as percentage of control. *P ≤ 0.05 and **P ≤ 0.01 when compared with respective control determined by one-way ANOVA followed by Tukey's post-test.

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# *NEW APPLICATION FOR THE COMETH ASSAY*

*Chapter 5*

# 1 – An epigenetic application for the CoMeth assay

## 1.1 – Introduction

Colorectal cancer (CRC) arises from an accumulation of genetic and epigenetic alterations. Epigenetics is the regulation of gene expression without modification of the DNA sequence. This regulation can be done by DNA methylation in promoter regions, histone modifications regulating transcription or noncoding RNAs. DNA methylation is the most studied epigenetic modification in humans. Cytosine methylation consists of a covalent addition of a methyl group from a methyl donor to a cytosine within a CpG dinucleotide by a specific family of enzymes, the DNA methyltransferases (DNMTs). These CpG dinucleotides are usually found in small clusters, called CpG islands. In cancer cells, DNA methylation is affected by two major alterations: (1) a global hypomethylation of the DNA due to generalized demethylation in the scattered CpG islands in the genome, allowing transcription of otherwise silenced oncogenes, and (2) specific promoter regions of tumor-suppressor genes are hypermethylated, inhibiting gene transcription [Goel, A., *et al.*, 2012; Kulis, M., *et al.*, 2010; Sandoval, J., *et al.*, 2012].

Nowadays, the term "CpG island methylator phenotype" or CIMP, is a characteristic of tumors in which tumor suppressor genes are silenced by hypermethylation, and tumor progression occurred, at least in part, by progressive tumor silencing. The most common silenced genes in CRC are *PTEN*, *MLH1*, *MSH2*, *APC*, *RUNX3* (a transcription factor), *UNC5C* (involved in cell migration) and *MGMT*. The mismatch repair gene *MLH1* was one of the first important tumor suppressor genes to be seen hypermethylated in cancer. Lynch syndrome (formerly known as hereditary nonpolyposis colorectal cancer, or HNPCC) arises due to mismatch repair dysfunction and hypermethylation of *hMLH1* and *hMSH2*. *hMLH1* has also been found silenced in sporadic cases of CRC [Goel, A., *et al.*, 2012].

Different from irreversible gene mutations, epigenetic silencing can be reactivated by small molecules that act as epigenetic modifiers. These epigenetic modifiers can interfere with the activity of DNMTs, histone deacetylase (HDAC), and sirtuins, a specific class of histone acetyltransferases (HATs). Inhibiting DNMT's can reverse hypermethylation by covalently trapping DNMT after incorporation in the DNA strand, blocking the catalytic site of the enzyme, degradation of the enzyme, or suppression of

the enzyme expression. 5-azacytidine (5-aza) is incorporated in the DNA and DNMT activity is lost due to the enzyme becoming irreversibly bound to 5-aza residue in the DNA, and this leads to loss of methylation [Christman, J.K., 2002]. Some natural compounds have already been found to reverse hypermethylation in cells [Banno, K., *et al.*, 2012; Pezzuto, J.M., *et al.*, 2013]. For example, epigallocatechin gallate was found to bind to the catalytic site of DNMT, therefore inhibiting DNMT activity, and reactivating silenced genes in cell lines [Fang, M.Z., *et al.*, 2003]. Reversing hypermethylation of promoter regions by demethylating compounds is an interesting approach for cancer prevention as well as therapy. In therapy, epigenetic agents may be used as sensitization agents to overcome CRC drug resistance or to directly modulate an epigenetic alteration for therapeutic intervention [Bardhan, K., *et al.*, 2013].

In this study, we developed a new adaption to our CoMeth assay, for epigenetic agent screening that act on DNMT inhibition. We used a colorectal cancer cell line (RKO) with *hMLH1* silencing by promoter hypermethylation, reactivated the expression of the protein with a well-known demethylating compound, 5-azacytidine, and used the CoMeth assay to see strand breaks. After characterization of the method, by nuclear condensation and western blot analysis, we used some natural compounds in our adapted CoMeth assay to screen for potential epigenetic chemopreventive compounds.

## 1.2 – Material and methods

### 1.2.1 – Reagents, plant material and antibodies

*N*-Methyl-*N*-nitrosourea (MNU), $O^6$-benzylguanine (BG), thiazolyl blue tetrazolium bromide (MTT), 5-aza, (-)-epigallocatechin gallate (EGCG), curcumin, ursodeoxycholic acid, resveratrol, ursolic acid, rosmarinic acid, RPMI - 1640 medium, penicillin/streptomycin, and trypsin solution were purchased from Sigma–Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS) was purchased from Biochrom KG (Berlin, Germany). SYBR Gold (nucleic acid gel stain) was from Invitrogen Molecular Probes (Eugene, OR, USA). The protein quantification DC protein assay was purchased from Bio-Rad Laboratories (Hercules, CA, USA). Primary antibodies were purchased from the following sources: anti-actin was purchased from Sigma–Aldrich; anti-p53, anti-MGMT and anti-MLH1 Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Peroxidase-conjugated goat anti-mouse antibody and Immobilon Western blotting detection reagents were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and Millipore

(Billerica, MA, USA), respectively. All other reagents and chemicals used were of analytical grade.

*Salvia officinalis L*. plants was from an experimental farm in Arouca, Portugal. Plants were collected between 2001 and 2003 and the water extracts were prepared as described previously by our group [Lima, C.F., *et al.*, 2005]. The filtered water extract was then lyophilized to dryness, giving a yield of 25.8%, and stored at -20 ºC.

### 1.2.2 – Cell line and culture conditions

RKO cells, derived from human colon carcinoma were a gift from Dr. Raquel Seruca (IPATIMUP, University of Porto, Portugal) and were maintained as monolayer cultures in RPMI medium supplemented with 10% FBS and antibiotics (100 U/mL penicillin and 100 mg/mL streptomycin), under an atmosphere of 5% $CO_2$ at 37 ºC. Cells were seeded onto 6- (2 mL) or 12- (1 mL) well plates at a density of $0.2\times10^5$ cells/mL. Test compounds were added to culture medium to the desired concentration ensuring that the DMSO concentration did not exceed 0.5% (v/v); controls received vehicle only.

### 1.2.3 – Assessment of cell viability/proliferation by MTT reduction test

A MTT reduction assay was performed in order to evaluate the toxicity of 5-azacytidine in the RKO cell line. Cells were treated with different concentrations (0.5, 1, and 2 µM) of the 5-aza for 72 an 96 h, including 2 h incubation with MTT (final concentration 0.5 mg/mL). The formazan crystals were then dissolved in a solution of DMSO/ethanol (1:1). The number of viable cells in each well was estimated by the cell capacity to reduce MTT. The results were expressed as percentage relative to the control (cells without any compound).

### 1.2.4 – Adaptation of the CoMeth assay

The CoMeth assay [Ramos, A.A., *et al.*, 2013] was developed by our group to measure $O^6$MeG lesion in DNA. This assay can only be used in a cell line with a functional mismatch repair system, to be able to start repair and induce strand breaks. In a cell line that has a silenced *MLH1* gene, demethylating compounds revert this effect and the CoMeth assay is functional. So, we developed a new adaptation of the CoMeth assay to study demethylating compounds. We used a known demethylating compound, 5-azacytidine, to revert *MLH1* silencing in RKO cell line. Briefly, cells were treated with

5-azacytidine for 48 h before treatment with BG, 100 µM, for 2 h and MNU (500 µM) for 24 or 48 h. 5-azacytidine was also added in these incubations.

Afterwards, the alkaline version of the single cell gel electrophoresis (comet) assay was used to evaluate DNA damage [Collins, A.R., 2004]. Cells were collected by trypsinization and around 50,000 cells were centrifuged for 1 min at 5,000 rpm, ressuspended in low melting point agarose and spread onto agarose-coated slide using a cover slip. After 10 min at 4 ºC, the coverslips were removed and slides were placed in lysis solution (2.5 M NaCl, 100 mM $Na_2EDTA$, 10 mM Tris base, pH 10, plus 1% Triton X-100) for 1 h at 4 ºC. Slides were then placed in a horizontal electrophoresis chamber with electrophoresis solution (0.3 M NaOH, 1 mM $Na_2EDTA$, pH > 13) for 40 min at 4 ºC for the DNA to unwind before electrophoresis for 20 min at 0.8 V/cm and ∼300 mA. After electrophoresis, slides were washed twice with PBS and dried at room temperature. For analysis of the comet images, slides were stained with SYBR Gold solution for 30 min at 4 ºC; after drying, the slides were analyzed using a fluorescence microscope and the Comet IV analysis system (Perceptive Instruments Ltd, Haverhill, UK) was used to calculate the parameter percentage of DNA in the tail. About 100 randomly selected cells were analyzed per sample.

For the screening of natural compounds, the same protocol was used, substituting 5-azacytidine for the natural compounds tested: (-)-epigallocatechin gallate (EGCG) – 4 µM, curcumin - 3 µM, ursodeoxycholic acid - 500 µM, resveratrol – 20 µM, rosmarinic acid - 20 µM, ursolic acid - 4 µM, *Salvia officinalis* (SO) – 50 µg/mL. Cells were treated with the compounds for 48 h before treatment with or without BG, 100 µM, for 2 h and MNU (500 µM) for 48 h. Afterwards, the comet assay was used to evaluate DNA damage, as described before.

### 1.2.5 – Nuclear condensation assay

The effects of the conditions of the modified CoMeth assay on cell death in RKO cells. The number of apoptotic cells was counted after 48 and 72 h as previously described [Xavier, C.P., *et al.*, 2009]. At least 500 cells were counted and the number of apoptotic cells was divided by the total number cells counted to give the percentage of cell death.

### 1.2.6 – Western blot analysis

RKO cells were treated with 5-azacytidine for 48 h before treatment with or without BG, 100 µM, for 2 h and MNU (500 µM) for 24 or 48 h. 5-azacytidine was also

added in these incubations. Total cell lysates were prepared to measure expression of different proteins. The cells were washed with PBS 1x and lysed for 5 min at 4 ºC with ice-cold RIPA buffer (1% NP-40 in 150 mM NaCl, 50 mM Tris (pH 7.5), 2 mM EDTA) supplemented with 20 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 20 mM $Na_3VO_4$ and protease inhibitor cocktail (Roche, Mannheim, Germany). Protein concentration was quantified using the DC protein assay following the manufacturer's instructions, and 20 µg/well was separated by 12% SDS–PAGE and transferred to polyvinylidene difluoride membranes. Membranes were blocked and incubated with primary antibody overnight. After washing, membranes were incubated with secondary antibody conjugated with IgG horseradish peroxidase for 1 h, and immunoreactive bands were detected using the Immobilon solutions (Millipore, Billerica, MA, USA) under a chemiluminescence detection system (Chemi Doc XRS; Bio-Rad Laboratories, Inc.).

Band area intensity was quantified using the Quantity One software from Bio-Rad. β-Actin was used as loading control.

### 1.2.7 – Statistical analysis

Statistical analyses were done using *t*-test, one-way or two-way analysis of variance using GraphPad Prism 4.0 software, when appropriate (San Diego, CA, USA). P values $\leq 0.05$ were considered statistically significant. All results are presented as mean ± SEM of at least three independent experiments. Images are representative of three independent experiments.

## 1.3 – Results and discussion

In this study, a novel assay for screening compounds with potential demethylating capacity using the simple comet assay was developed. This method uses the adapted CoMeth version of the comet assay, developed by our group [Ramos, A.A., *et al.*, 2013]. The CoMeth assay is based on the principal that in a condition when MGMT is inhibited, for example with BG, and a compound, in this case MNU, induces the $O^6$methylguanine lesion, a functional mismatch repair system will recognize and start to repair the lesion, introducing nicks in the DNA strand. These nicks will be detected by the comet assay. In this new version, instead of using a mismatch repair functional cell line, as needed for the CoMeth assay, we use a *hMLH1* silenced cell line, that will not present comets in response to MNU due to the lack of functional mismatch repair. Theoretically, if we use a

demethylating agent and reactivate MLH1 expression, we have a functional mismatch repair system and will observe an increase in DNA strand breaks. The demethylating compound 5-azacytidine was used to test our hypothesis.

First, we tested for toxicity of the compound, as the objective is to increase strand breaks by activating the mismatch repair system and not due to direct toxicity of our test compound. For this, we measured cell viability using the MTT assay with 0.5, 1 and 2 µM of 5-azacytidine for 72 and 96 h incubation (Figure 1). This compound showed no decrease in cell viability at 72 or 96 h incubation, proving that these concentrations are not cytotoxic for these cells.

The concentration of 2 µM of 5-azacytidine was used in the subsequent assays. For the adapted epigenetic version of the CoMeth assay, cells were treated with 5-azacytidine for 48 h before incubation with BG and MNU. We found that at 24 h incubation of MNU there were no significant differences between treatment with BG + MNU and 5-aza + BG + MNU, but at 48 h incubation with MNU there is a significant increase of DNA damage with the 5-azacytidine treatment (Figure 2). The difference, at this time point, between BG + MNU and 5-aza + BG + MNU are cells that now have functional mismatch repair systems (*MLH1* is no longer silenced and there is transcription of the protein) and recognize the $O^6$-methylguanine lesion induced by MNU, generating strand breaks detected by the comet assay.

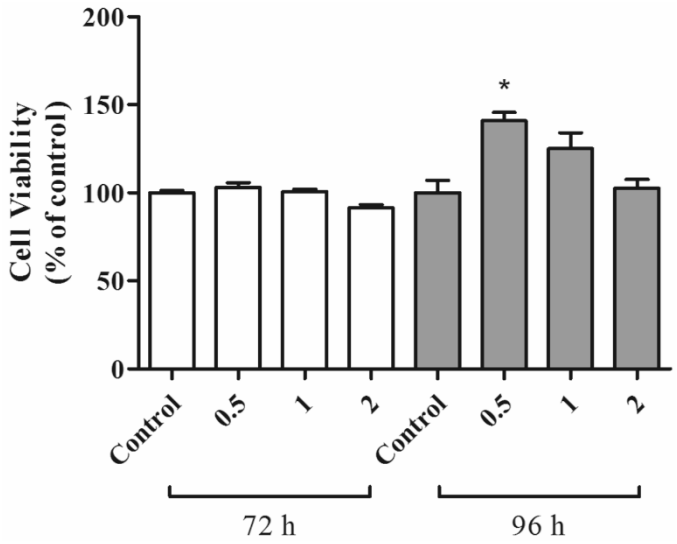


**Figure 1 –** The effect of different concentrations of 5-azacytidine on cell viability at 72 (white bars) and 96 (gray bars) h incubation, in RKO cells. Cell viability was measured by the MTT assay and results are the mean ± SEM of at least 3 independent experiments. *P≤ 0.05 when compared with respective control was determined by one-way ANOVA followed by Newman-Keuls multiple comparison test.

In this system, cell death is induced due to the recognition of unrepaired $O^6$methylguanine lesion by the mismatch repair system. This repair system introduces strand breaks by excising the mispair created by the lesion, but as long as the $O^6$methylguanine remains in the DNA template, this process will be repeated, creating a futile repair loop that results in double strand breaks and, consequently, cell death [Kaina, B., *et al.*, 2007; Kondo, N., *et al.*, 2010]. This only happens if the MMR system is functional. At 72 and 96 h incubation, it was possible to observe a significant increase in cell death with 5-azacytidine + BG + MNU when compared with control (Figure 3). This increase in cell death was not seen in the condition with BG + MNU alone because MLH1 is silenced and the mismatch repair is not functional. This increase in cell death is additional proof that the mismatch repair system is functional after 5-aza treatment. So, there is an increase of DNA damage when 5-azacytidine is used in the RKO cell line and this DNA damage leads to cell death at 96 h.

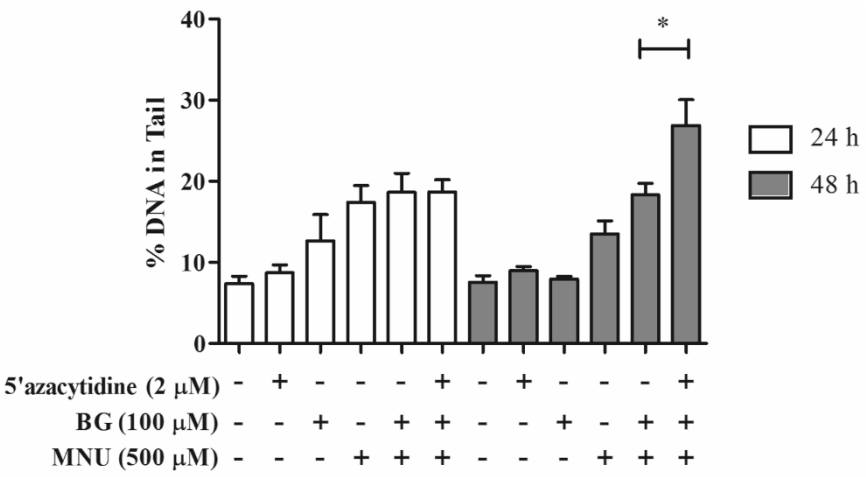


**Figure 2 –** Modified CoMeth assay for epigenetic screening of demethylating compounds. Cells were incubated with 5-azacytidine for 48 h before 2 h incubation with BG and 24 or 48 h incubation with MNU. DNA damage was then measured with standard comet assay and results are the mean ± SEM of at least 3 independent experiments.. *P≤ 0.05, ##P≤ 0.01, ### P≤ 0.005 when compared with respective control and + P≤ 0.05, were determined by one-way ANOVA followed by Newman-Keuls multiple comparison test.

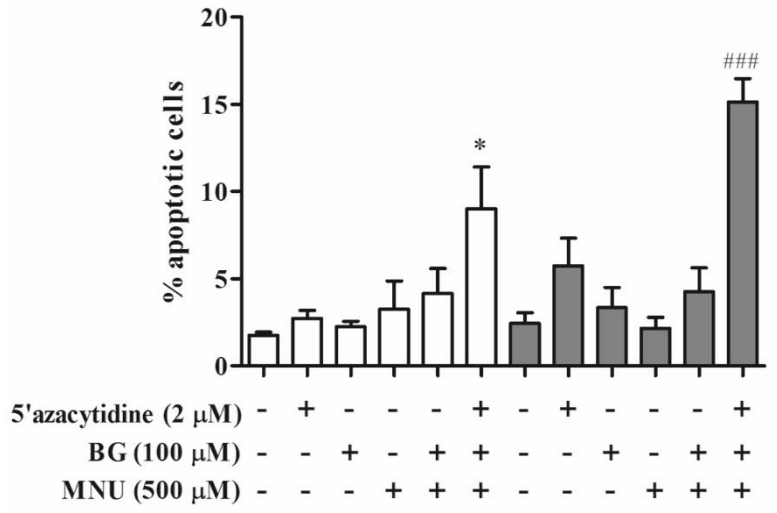


**Figure 3 –** Effects of the conditions of the modified CoMeth assay on cell death. Cells were incubated with 5-azacytidine for 48 h before 2 h incubation with BG and 72 (white) or 96 (gray) h incubation with MNU. Cell death was measured by nuclear condensation assay and results are the mean ± SEM of at least 3 independent experiments.. *P≤ 0.05 and ### P≤ 0.005 when compared with respective control were determined by one-way ANOVA followed by Newman-Keuls multiple comparison test.

MLH1 and MGMT protein expression were also evaluated. In this assay, BG is added to the cells for MGMT inhibition. MGMT is responsible for the repair of the $O^6$-methylguanine lesion, but this protein is a suicide repair enzyme, in which, after the transfer of the alkyl group from the DNA template to a cysteine residue in the active center of the molecule, this enzyme is inactivated and directed to the proteasome for degradation. BG acts as a pseudosubstrate of MGMT, thus inactivating the enzyme [Srivenugopal, K.S., *et al.*, 1996]. By western blot, we found that all the conditions that received BG had almost no expression of the protein, confirming that in this assay, this concentration of BG is sufficient to inhibit almost all of MGMT protein during 48 h incubations (Figure 4).

As MGMT is inactive, the mismatch repair system recognizes the unrepaired $O^6$-methylguanine lesion induced by MNU, as long as it is functional, which in this case is with 5-azacytidine incubation. Treatment with 5-azacytidine alone increase MLH1 expression when compared with the respective control at 24 h (Figure 4). When 5-aza treatment is not given, the levels of MLH1 protein are similar to the control. The demethylating agent is able to increase the expression of the silenced MLH1 protein, activating the mismatch repair system. These results suggest that treatment with 5-aza for 48 h reactivates the expression of MLH1 protein. When BG is used to inactivate MGMT

and MNU induces $O^6$-methylguanine lesion, in cells with reactivated MLH1, there is an increase of DNA damage observed by the comet assay, after 48 h treatment with MNU, and increase in cell death at 72 and 96 h incubations.

The p53 protein is referred to as the "guardian of the genome" as its role is to respond to DNA damage and pause the cell cycle, to allow repair, or upregulate proteins involved in cell death pathways. *TP53* mutations are very common in colorectal cancers, but usually appear in late stages of the carcinogenic process [Pezzuto, J.M., *et al.*, 2013]. The RKO cell line is *P53* wild-type and, in this study, the effect of the conditions used on the modified CoMeth assay on p53 protein expression were evaluated (Figure 5). Interestingly, we found a significant increase of p53 expression with the 5-azacytidine treatment alone at both time points. This increase could possibly be explained by the expression of another protein, the death-associated protein kinase (DAPK). DAPK is a serine/threonine kinase involved in the extrinsic death receptor-mediated apoptotic pathway. There is an autoregulatory feedback loop between *DAPK* and *p53*, as *DAPK* is induced by *p53* activation and DAPK expression increases p53 protein expression. It is known now that DAPK promoter is usually hypermethylated in many cancers [Gozuacik, D., *et al.*, 2006; Pezzuto, J.M., *et al.*, 2013], and the RKO cell line presents this promoter hypermethylation [Paz, M.F., *et al.*, 2003]. This increase of p53 expression could be explained by the re-expression of DAPK protein by 5-azacytidine and the regulatory feedback loop. There was also an increase of p53 protein expression with MNU treatment alone at 24 h incubation. As in this condition MGMT is not inhibited by BG, this increase could be due to normal molecular p53-dependent signaling due to the repair of DNA damage, induced by MNU, by MGMT [Roos, W., *et al.*, 2004]. P53 has been found to be involved in DNA repair in the cells. After the introduction of DNA injuries the level of p53 protein rises, which in turn induces a transient cell cycle arrest or apoptotic cell death. Also, some mismatch repair proteins are p53 target genes, including *MSH2* and *MLH1* [Gatz, S.A., *et al.*, 2006]. So, this increase of p53 expression can also regulate mismatch repair, inducing transcription of the mismatch repair proteins and increasing apoptosis, an effect that we also observed.

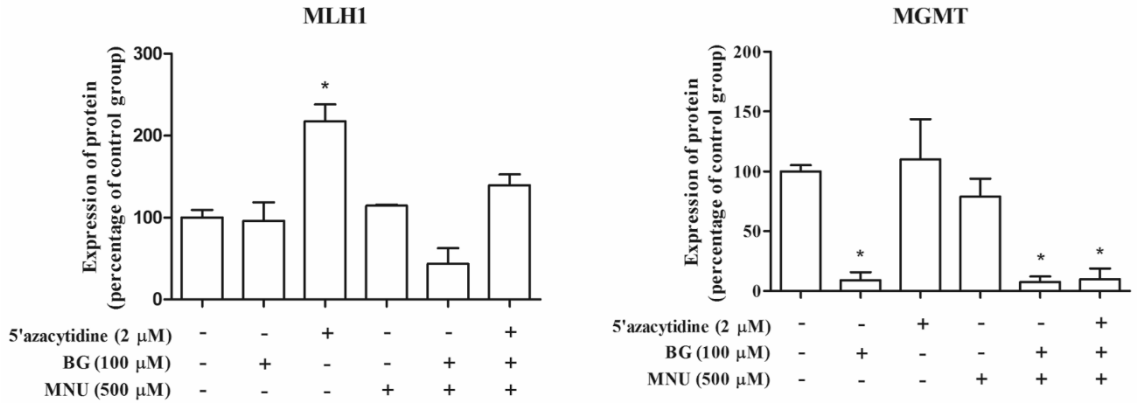


**Figure 4 –** Effects of treatment with the conditions of the modified CoMeth on MLH1 and MGMT protein expression in RKO cells, using western blot. Cells were incubated with 5-azacytidine for 48 h before 2 h incubation with BG and 24 h incubation with MNU. β-actin was used as loading control. Values are mean ± SEM of at least three independent experiments. *$P \leq 0.05$ when compared to control.

Until now, it seems that the assay is effective, so we used this method to investigate the potential of natural compounds to be demethylating compounds (Figure 6). Of all the compound, (-)-epigallocatechin gallate (EGCG) presented values very similar with our positive control, 5-azacytidine. It is known that EGCG has DNMT inhibiting capacity [Fang, M.Z., *et al.*, 2003; Nandakumar, V., *et al.*, 2011], so this result can confirm that our modified CoMeth assay can be used to screen demethylating compounds. Of the other natural compounds, resveratrol also showed results similar to our positive control and this compound has been shown to have demethylating potential in breast cancer cells [Paluszczak, J., *et al.*, 2010]. Further studies on MLH1 protein expression with these natural compounds are needed to validate the effects in the comet assay.

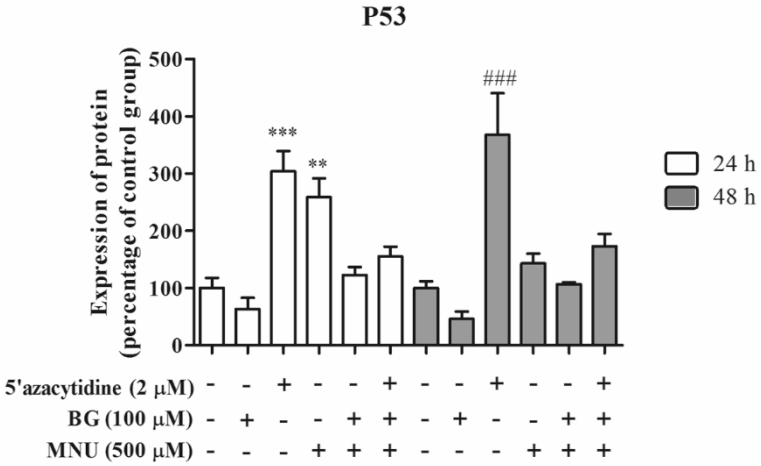


**Figure 5 –** Effects of treatment with the conditions of the modified CoMeth on p53 protein expression in RKO cells, using western blot. Cells were incubated with 5-azacytidine for 48 h before 2 h incubation with BG and 24 and 48 h incubation with MNU. β-actin was used as loading control. Values are mean ± SEM of at least three independent experiments. **P≤ 0.01, ***P≤ 0.005, and ### P≤ 0.005 when compared with respective control were determined by one-way ANOVA followed by Newman-Keuls multiple comparison test.

In conclusion, we developed a modified version of the comet assay to evaluate potential compounds with demethylating capacity. The comet assay is a simple, relatively cheap assay to evaluate DNA damage. The standard version is capable to evaluate strand breaks and different versions of this assay have been developed to increase the types of damage that are possible to assess. Our lab has developed a version to evaluate the alkylating DNA damage, $O^6$methylguanine. With this in mind, and using a mismatch repair deficient cell line in which MLH1 is epigenetically silenced, we were able to present a method to investigate potential demethylating compounds. We characterized this method using a well-known demethylating agent, 5-azacytidine, and also showed the application with natural compounds for screening purposes. This assay should be accompanied by a protein expression assays to ensure that the effects seen are due to the demethylating potential and reexpression of the protein. Further studies will be done to validate this method.

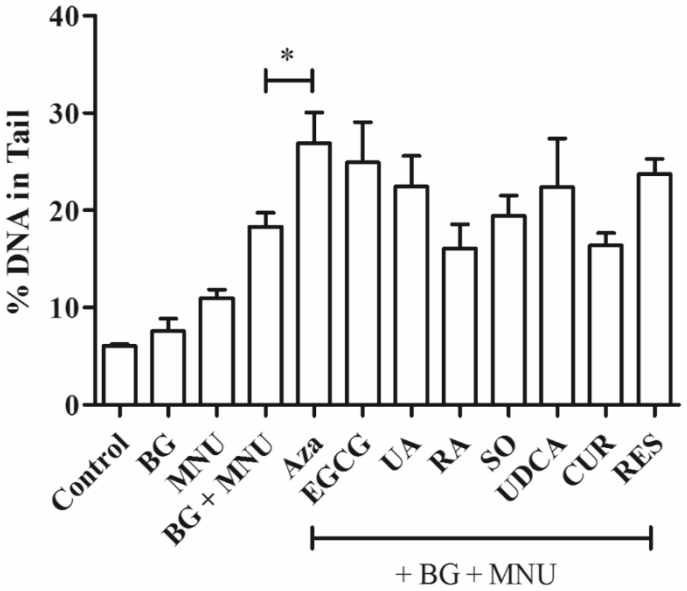


**Figure 6 –** Modified CoMeth assay for epigenetic screening of demethylating compounds. Cells were incubated with tested compounds or 5-azacytidine for 48 h before 2 h incubation with BG and 24 or 48 h incubation with MNU. . Concentrations used were: (-)-epigallocatechin gallate (EGCG) – 4 µM, curcumin (CUR) - 3 µM, ursodeoxycholic acid (UDCA) - 500 µM, resveratrol (RES) – 20 µM, rosmarinic acid (RA) - 20 µM, ursolic acid (UA) - 4 µM, Salvia officinalis (SO) – 50 µg/mL. DNA damage was then measured with standard comet assay and results are the mean ± SEM of at least 3 independent experiments.

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# GENERAL CONCLUSIONS AND FUTURE PERSPECTIVES

*Chapter 6*

# 1 – General conclusions

Colorectal cancer is a major health problem, as it is one of the most common types of cancer in Western societies. The accumulation of genetic and epigenetic alterations transforms normal colon cells and gives them growth advantage over their neighboring cells. These genomic alterations and mutations can alter the cell's behavior, in the initiation and/or progression process, and how the tumor will respond to therapy, in cancer treatment. Colorectal cancer is highly associated with lifestyle, such as obesity, lack of physical activity, smoking habits, and most importantly, diet. Diet has an important role not only on the initiation of the disease, but also in possible chemopreventive and therapeutic strategies. Diet may have adverse effects on colon cancer, being one of the main risk factors of this disease. High intake of saturated fat, red meat, and processed foods has been linked to increase in cancer risk. Fruits and vegetables, and isolated compounds of these foods, have shown to be promising agents that may play a role in cancer prevention as well as in cancer therapy. Modulation of signaling pathways associated with proliferation and apoptosis, protection against DNA damage, and induction of DNA repair are just some of the possible mechanisms of action for cancer prevention.

The main objective of this work was to evaluate the effect of some dietary compounds on colon cancer prevention and bile acids in colon cancer promotion. In Chapter 2, the effects of two bile acids, deoxycholic acid and ursodeoxycholic acid, were evaluated in colon cancer cell line with no mutations in the MAPK and PI3K/AKT pathways. Deoxycholic acid is considered a colon cancer promoter, while ursodeoxycholic acid is used in studies to evaluate chemoprevention. In this study, deoxycholic acid was found to decrease proliferation and induce apoptosis with high concentrations (500 µM). This induction of apoptosis could be due, in part, to formation of reactive oxygen species and/or induction of DNA damage that was observed with deoxycholic acid. Ursodeoxycholic acid did not induce any of these alterations. It is known that bile acids can regulate DNA repair proteins, so with this in mind, the effects of these bile acids on MLH1, a mismatch repair protein, and MGMT protein expression were evaluated. Both bile acids were able to decrease the expression of these proteins after 48 h incubation. This effect suggests that these two bile acids can promote carcinogenesis through reduction of DNA repair. The activation of three signaling

pathways was also determined. The bile acids were able to activate PI3K/AKT and MAPK/ERK by increase of phosphoAKT and phosphoERK expression, respectively, but not JNK signaling pathway. As ursodeoxycholic acid is believed to be chemopreventive, the effect of pretreatment of this compound before DCA treatment was also evaluated. In this study, we found an increase of cell death with the pretreatment of ursodeoxycholic acid when compared with deoxycholic acid alone. This increase of apoptosis was accompanied by a constant activation of the JNK signaling pathway, determined by phosphoJNK protein expression. Also, pretreatment with ursodeoxycholic acid significantly decreased expression of the repair proteins MGMT and MLH1. Deoxycholic acid induced typical alterations associated with cancer promotion. On the other hand, ursodeoxycholic acid did not show any effects of chemopreventive potential and even enhanced deoxycholic acid apoptosis and decreased DNA repair protein expression.

In chapter 3, we tried to develop an *in vitro* model of azoxymethane-induced colon carcinogenesis. The characterization of the effects induced were compared to the alterations induced in the *in vivo* model. For this, we used the Caco-2 cell line and added a S9 liver fraction mixture to enhance azoxymethane metabolization. Azoxymethane (15 µM) treatment for 48 h slightly increased cell proliferation and phosphoERK expression. On the other hand, no DNA damage was observed, oxidative or $O^6$meG, whereas azoxymethane induces the $O^6$meG lesion *in vivo*. Also, no nuclear translocation of β-catenin was observed. The lack of consistencies with the *in vivo* assay suggest the optimization of the *in vitro* assay.

Chapter 4 was dedicated to the *in vivo* effects of natural compounds on colon cancer prevention. In the first part, the effect of an herbal tea, *Salvia officinalis*, was evaluated on the prevention of colon carcinogenesis used the *in vivo* AOM-induced model. In this study, our herbal tea reduced the number of pre-neoplastic lesions induced by AOM. This reduction was accompanied by a decrease of Ki67 staining, suggesting an effect of *Salvia officinalis* on cell proliferation, an effect already seen *in vitro* by our group. Herbal tea drinking did not effect metabolization or elimination of azoxymethane in the liver, as there were no effects on CYP2E1 expression and activity, nor GSH and GST activity. Azoxymethane induced DNA damage in colonocytes and lymphocytes and *Salvia officinalis* drinking protect colonocytes and lymphocytes from this DNA damage. Also, $H_2O_2$ was used to induce DNA damage *ex vivo* to colonocytes and lymphocytes isolated from rats that drank the herbal tea and that drank water, and *Salvia officinalis* protected against this type of DNA damage. In the second part, we evaluated the effects

of two isolated compounds, ursolic acid and (-)-epigallocatechin-gallate, on protection against DNA damage in healthy animals. The comparison of the effects on lymphocytes and colonocytes for the possible use of lymphocytes as surrogate markers of effects on colonocytes, but the data collected was not sufficient to validate this hypothesis. The natural compounds tested protected against endogenous DNA damage, in colonocytes and lymphoctyes. When an alkylating agent was used *ex vivo*, MMS, ursolic acid and (-)-epigallocatechin-gallate protected against this type of damage in colonocytes, but not in lymphocytes. The expression of a base excision repair protein, OGG1, was evaluated by western blot and both natural compounds were found to decrease the expression of this protein. This could be explained by the fact that if the compounds exert their antioxidant properties by reducing the levels of DNA damage to begin with, then there is no need to increase the transcription levels of the protein. So in this section we demonstrated the potential of an herbal tea and two isolated dietary compounds to protect against colon carcinogenesis. In this study, DCA was also used, but it did not induce DNA damage itself or increase DNA damage induced by MMS.

In chapter 5, we adapted a version of the CoMeth assay to be used for screening of epigenetic compounds with DNMT inhibiting potential. We used a *hMLH1* silenced cell line and reactivated the expression of the protein by using 5-azacytidine. We then used BG and MNU, as in the CoMeth assay, to inactivate MGMT and induce the $O^6$methylguanine lesion. The difference of DNA damage we found between treatment with 5-aza + BG + MNU and BG + MNU alone is DNA damage induced by the reactivation of *hMLH1*, proving that the compound tested, in this case a well-known demethylating agent, has demethylating activity. We further characterized this model by observing the induction of cell death after 72 and 96 h incubation with the 5-aza + BG + MNU, typical phenotype of MMR proficient cells with inhibited MGMT and $O^6$methylguanine lesion corrected by the MMR system. By western blot, we observed efficient inhibition of MGMT by BG and transcription of MLH1 protein by 5-aza. We also observed an increase of p53 protein with 5-aza treatment. This increase could be explained by the reactivation of DAPK, also found silenced, and an autoregulatory feedback loop with p53. We further tested several natural compounds and their ability to act as demethylating agents. By the comet assay, we observed that EGCG showed DNA damage levels similar to 5-aza. It is known that EGCG is an inhibitor of DNMT activity, and therefore a demethylating agent, so this helps to further validate our assay.

In conclusion, this work demonstrates the chemopreventive effects of natural compounds, *Salvia officinalis* tea, ursolic acid and EGCG. These compounds protect against DNA damage and show colorectal cancer chemopreventive activity. We also demonstrated the deleterious effects of deoxycholic acid and ursodeoxycholic acid and characterized the molecular alterations induced by these bile acids. Not only does DCA promoter cell turnover, it inhibits DNA repair proteins, enhancing its tumor promoting ability. UDCA inhibits DNA repair proteins and enhances DCA-induced apoptosis. Finally, we developed a new adaptation of the comet assay to detect possible demethylating compounds. More studies are needed to safely add these compounds to dietary strategies for colorectal cancer prevention.

## 2 – Further perspectives

The studies presented in this thesis were carried out to increase our knowledge in dietary compounds that can be inserted into dietary strategies for cancer prevention. Despite all the work presented here, many questions remained unanswered and many more appeared.

The studies with DCA demonstrated increase of apoptosis, increase of DNA damage and increase of signaling pathways that control cell proliferation. It has been demonstrated elsewhere that low and high concentrations of DCA have different effects on cell proliferation. Studies with lower concentrations of DCA to compare with the effects of high concentrations would give more information on the effects of this bile acid in colon carcinogenesis. The preventive effects of UDCA before DCA needs further investigation. The effects presented in this thesis are different from the idea that UDCA is preventive. More studies with different concentrations and different time points could help clarify this inconsistency.

We tried to develop an *in vitro* model of the AOM-induced colorectal carcinogenesis using Caco-2 cell line. The effects found with the conditions tested did not correlate with the alterations found in the *in vivo* model. Evaluation of the effect of longer periods of incubation with AOM to verify if longer time points are needed to develop this *in vitro* model. Also, we can use BG before AOM exposure and evaluate MAPK pathway and if there is induction of alterations in this pathway. If it is possible to develop the model, using natural compounds to prevent AOM alterations *in vitro* for

screening processes. The most promising compounds can then be used in the *in vivo* model to verify if the effects seen *in vitro* are also seen *in vivo*.

In chapter V, we developed a method to study possible demethylating agents. Further studies are needed to validate this method. MS-PCR could be done to verify the reactivation of MLH1 expression in the model. Also, 72 and 96 h incubations for the comet assay could be done to see if the effect is more pronounced with longer periods of incubation. Also, western blot of the natural compounds + BG + MNU should be done to observe the expression of MLH1 protein.