UNIVERSITÀ DEGLI STUDI DI PALERMO
PhD Course in Biomedicine and Neuroscience
Department of Experimental Biomedicine and Clinical neurosciences
(BioNeC)
SSD BIO/10

Hidden biochemical action of Ethanol on colon carcinoma cell models

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XXXI CYCLE
ACHIEVEMENT YEAR TITLE 2018/2019

Hidden biochemical action of Ethanol on colon carcinoma cell models

by

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Dissertation

Presented for the requirements

toward the completion

for the Degree of

Doctor of Philosophy

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Abstract

Colorectal cancer (CRC) is one of the most widespread cancers in the world (Haggar FA et al 2009). Numerous risk factors have been correlated with the development of CRC, including genetic factors, inflammation, intestinal microflora composition, as well as lifestyle factors, such as smoking, high consumption of red meats and alcohol intake (Baan R et al 2007).

Epidemiological studies support the conclusion that chronic and heavy alcohol consumption increases the risk to develop CRC as well as favors the progression of this form of cancer. However, the biochemical mechanisms responsible for these events have not yet been fully clarified.

The aim of my doctoral project was to study the effects of ethanol in human colorectal carcinoma cells in culture and to evaluate its molecular action mechanism. In particular, my research focused on the identification of one or more molecules involved both in the survival of the tumor cells and, especially, in tumor progression and invasiveness. To this end, I investigated the effect of high doses of ethanol on survival and progression of three different colon cancer cells (HCT116 , HT29 and Caco2 cells). The results demonstrated that ethanol promotes oxidative and ER stress in colon cancer cells as demonstrated by ROS increase and upregulation of ER markers Grp78 and CHOP. Despite the activation of stress, colon cancer cells did not present sign of toxicity because they are able to activate an autophagic survival mechanism. Moreover, in response to oxidative stress, ethanol promoted nuclear translocation of Nrf2 and upregulated the level of the antioxidant enzymes SOD, catalase and heme-oxygenase (HO-1). Silencing Nrf2 in HCT116 cells abrogated the effect of ethanol on upregulation of SOD and HO-1, thereby suggesting that the induction of antioxidant enzymes is dependent on Nrf2 activation. Interestingly, ethanol also promoted HO-1 nuclear translocation. Preventing HO-1 nuclear translocation by addition of E64d, the activation of antioxidant response by Nrf2 was reduced. Finally, the results demonstrated that the activation of Nrf2/HO-1 axis induced by ethanol is also responsible for the induction of MMP-2 and VEGF, two well known factors favoring cellular invasiveness.

Introduction

Ethanol

Ethanol (CH_3CH_2OH, EtOH) commonly called alcohol or ethyl alcohol is a liquid, colorless substance that is formed by fermentation of some simple sugars or by distillation of fermented must. The etymology of the word is debated, some argue that it derives from the Arabic term "*alkukhul*", which literally translated means "the spirit", others as "spirit" in reference to the first distillation processes carried out in the Middle East in the 2nd millennium Before Christ. EtOH present in drinks is obtained by alcoholic fermentation of plant products such as grapes, cereals and potatoes; in particular, EtOH is the product of various microorganisms starting from the degradation of sugars with six carbon atoms such as glucose. Most yeasts produce EtOH by anaerobic fermentation. In absence of O_2, pyruvate (an α-ketoacid that is the final product of glycolysis) is decarboxylated to acetaldehyde from decarboxylase pyruvate and subsequently acetaldehyde is reduced to EtOH with CO_2 production (fig.1). Alcoholic fermentation does not occur in human beings because they do not contain the pyruvate decarboxylase enzyme. In some yeasts such as Saccaromyces cerevisae, the production of EtOH can also occur in the presence of O_2 through the Crabtree effect. This reaction occurs when there are high amounts of sugars that cause an excess of pyruvate with the consequent production of acetaldehyde (Piskur J et al 2006).

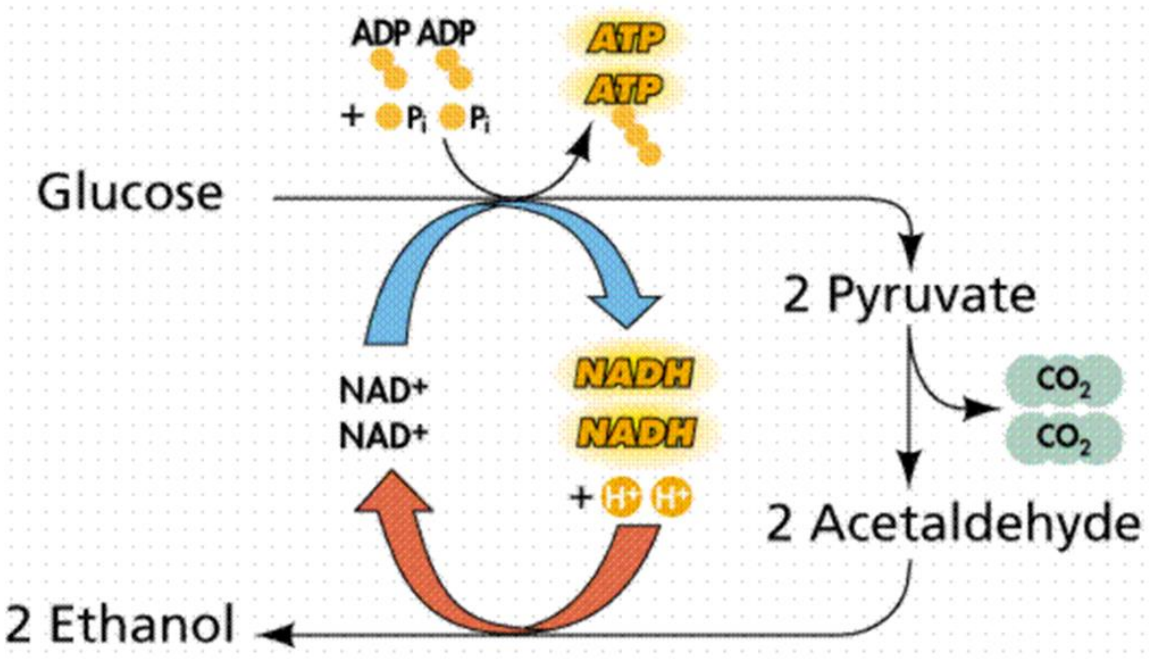

Figure 1 Fermentation of ethanol

EtOH is the main psychoactive component of alcoholic beverages. It is in fact able to act on the central nervous system and to alter states of consciousness.

In the course of human history, EtOH has been considered food, medical remedy, object of initiation rites, symbol of transgression and finally intoxicating drink. The consumption of EtOH took on different aspects in the history of humankind, the ancient Greeks worshiped the God Dionysus, the Americans forbade it in the early 1900s. While today in Western countries it is easily consumed even by very young people. Before the development of modern drugs, EtOH was used as a drug, suggested as a medicine for depression and anesthetic. It is also used as an antibacterial hand sanitizer (at a volume / weight concentration of about 70%), and as an antiseptic.

EtOH denatures proteins and dissolves lipids, so it is effective against most bacteria and fungi and many viruses, but it is ineffective against bacterial spores. The variety of these meanings probably depends on his own effects: alcohol can simultaneously make people feel strong and weak.

There are different drinks containing EtOH, some with low alcohol content, such as wine and beer, and others with a high alcohol content, such as hard liquors. The quantity of EtOH contained in the drinks is expressed in % vol. To calculate the alcohol content of a beverage, the total volume of the consumed drink must be multiplied with the ethanol conversion factor. The ethanol conversion factor differs slightly in the various countries but it usually falls within the range of 4-5%vol for beer, 12%vol for wine and about 40%vol for hard liquors. Therefore, in a glass of wine we will find about 12mL of EtOH (100mL x 0.12 = 12mL).

The World Health Organization (WHO) estimated that around 2 billion people consume EtOH every day with an average annual consumption of about 6.2 l for adult (Rehm J et al 2009). 73.6 million people have EtOH-related illnesses such as: alcoholic polyneuropathy, alcoholic cardiomyopathy, alcoholic gastritis, depression and other mental disorders; and still with hemorrhagic stroke, acute and chronic pancreatitis and liver cirrhosis (Thakker KD 1998). Furthermore, consumption of EtOH during pregnancy is associated with abortion, intrauterine growth retardation and fetal alcohol syndrome (Connor PD et al 1996). It has also been shown that EtOH consumption is correlated with more than 60 diseases in young adults, 1.8 million deaths a year, and only in Europe, a death toll is estimated at 55,000 cases per year (WHO, Department of Mental Health and Substance Abuse. Global status report on alcohol 2004).

Furthermore, it has been shown that there is a high correlation between EtOH consumption and the development of tumors. The incidence of EtOH-related cancers is higher in men

than in women, accounting for 5.2% in men and 1.7% in women respectively. In particular, ingestion of alcoholic drinks is involved in the development of cancer of the larynx, pharynx, esophagus, liver, breast cancer and colon (Baan R et al 2007). Recent studies identify a greater incidence of developing proximal colon cancer than distal colon, following consumption of EtOH (Thygesen LC et al 2008). Although several epidemiological studies show that regular consumption of EtOH is correlated with the development of different types of cancers, such as colorectal cancer, (in particular 45% for colon cancer and 49% for rectal cancer) it is still unclear and strongly debated the amount of EtOH associated with the development of colon cancer. There are different results based on the reference population. In a study conducted in North America and in Europe, there is a risk of developing colon cancer with average consumption of> 45g / day (Cho E et al 2004). Instead, a study conducted in Japan found a correlation consumption of EtOH and colon cancer with average daily consumption of about 23g / day (Munira A et al 2007). Other studies show that the carcinogenic effects associated with the consumption of EtOH seem to be attributable to an average daily consumption of more than 76g / day, especially if EtOH is consumed in the form of wine rich in polyphenols (Corrao et al 2000).

To date, the threshold value to be attributed to a greater probability of developing colon cancer remains unresolved. Evidence suggests that the

risk to develop colon cancer correlated with EtOH consumption is modulated by genetic factor like different variants in genes for EtOH metabolism.

Ethanol metabolism

EtOH is a water-soluble and fat-soluble molecule; this dual nature allows the EtOH to be rapidly absorbed by the intestine by passive diffusion. Only a small part of ingested EtOH is absorbed by the cells of the gastric mucosa, most of the EtOH is released into the bloodstream to reach the liver (organ involved in the metabolism of EtOH). A small amount of ingested EtOH (2%) is eliminated through the lungs and kidneys.

EtOH is considered a pseudo food because through the action of two enzymes it is first converted into acetic acid and then into acetyl-CoA, an important metabolite. In the liver, a cytosolic enzyme, Alcohol Dehydrogenase (ADH), which oxidizes EtOH to acetaldehyde

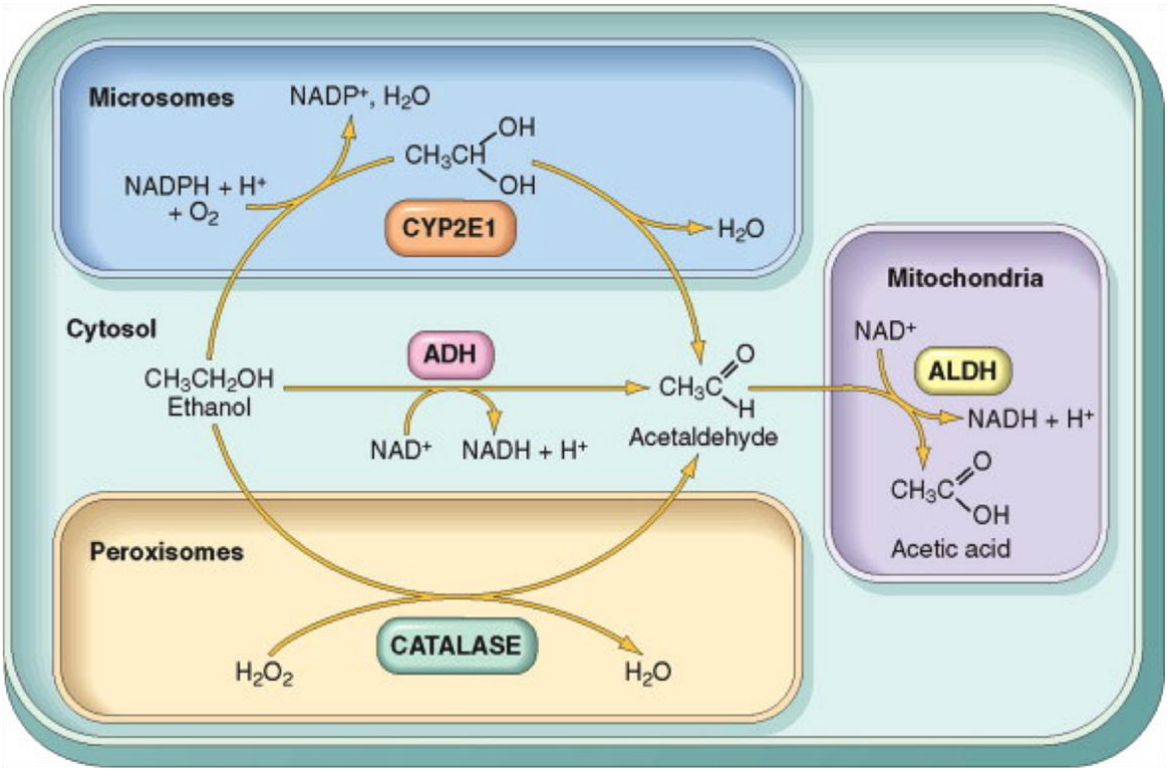

Figure 2 EtOH metabolism

with reduction of NAD $^{+}$ to NADH, metabolizes EtOH (Fig.2).

There are several ADHs belonging to different families, and among these ones, those belonging to the ADH1 family are those that show a greater affinity for ethanol and a lower Km. Within this family we find three genes each of which presents different allelic variants coding for different ADH isoforms. The ADH1 isoforms (ADH1A, ADH1B, ADH1C) are predominantly present in the liver (Table I). For this reason, they are defined hepatic alcohol dehydrogenases and, since they show high affinity for ethanol, they make the liver the main organ of ethanol metabolism, as well as the main site of production of acetaldehyde, highly toxic substance. Acetaldehyde is subsequently oxidized to acetate by mitochondrial acetaldehyde dehydrogenase (ALDH2), NAD $^{+}$ dependent enzyme. A small part of acetaldehyde is oxidized to acetate by the cytosolic acetaldehyde dehydrogenase. A small amount of acetate can be transformed, in the liver, to acetyl-CoA and become part of the fatty acid synthesis pathway; most of the acetate is released into the bloodstream, reaching other tissues, mainly heart and skeletal muscle, within which it will be activated to acetyl-CoA and used metabolically.

Many factors influence EtOH absorption, disposition and metabolism. These include gender, age, ethnicity and body weight. Polymorphisms in ethanol and acetaldehyde metabolizing enzymes, especially ADH and ALDH, have been closely associated with

ethnic and individual differences in susceptibility to ethanol related cancers.The functionally important polymorphic sites for ADH1B (formerly called ADH2) areArg48His in exon 3, and Arg370Cys in exon 9(Brennan P et al 2004). Histidine at the amino acid position 48 constitutes the * 2 allele (ADH1B * 2), while the cysteine at position 370 constitutes the * 3 Allele (ADH1B * 3). The allele * 1 is the wild type haplotype, which corresponds to the arginine in positions 48 and 370.he functionally important polymorphic sites for ADH1C (formerly called ADH3) are Ile350Val and Arg272Gln, valine at codon 350 and glutamine at codon 272 constitute the allele ADH1C * 1. This allele and the allele ADH1B * 2 code for enzymes that have a ' very high affinity for EtOH. ADH1C * 1 increases the oxidation of EtOH by about 2 to 5 times compared to ADH1C * 2 (isoleucine with amino acid position 350). Instead, ADH1B * 2 and ADH1B * 3 increase ethanol oxidation 40 times and 90 times, respectively, compared toADH1B * 1 (Bosron WF et al 1983).ADH1B and ADH1C are only 16 kb away on chromosome 4, but they have a different distribution among populations (Osier M et al 1999). The ADH1B isoform has not been associated with cancer development after consumption of EtOH in European populations. Studies on the Asian populations coherently associated the allele ADH1B * 1 with an increased risk of cancer of the esophagus (Lilla C et al 2005). With regard to mitochondrial aldehyde dehydrogenase (ALDH2), an allele is present that codifies for an inactive enzyme. This contains a lysine in position 487 (Glu487Lys) and is known as ALDH2 * 2. Homozygous * 2 carriers (Lys / Lys) are unable to oxidize acetaldehyde while heterozygotes (Lys / Glu) metabolize acetaldehyde slowly. The ALDH2 isoenzyme is a tetramer, only one in every 16 ALDH2 enzymes are functional in individuals heterozygous. Thus, homozygous or heterozygous individuals for ALDH2 * 2 have an accumulation of acetaldehyde, resulting in a toxic reaction that includes hot flashes, increased heart beats and nausea. ALDH2 * 2 is very common in Asian

populations, while almost all Europeans are homozygous for the ALDH2 * 1 (Gln / Gln) allele. Studies conducted in Japan have consistently reported an increased risk of oral,

Gene Name	Gene Class	Protein Name	K_M(mM) for Ethanol	Primary Tissue	Metabolic function
ADH1A	I	α	4.0	liver	Metabolize 70% of ethanol in the liver
ADH1B*1	I	β_1	0.05	liver, lung	
ADH1B*2	I	β_2	0.9		
ADH1B*3	I	β_3	40		
ADH1C*1	I	γ_1	1.0	liver, stomach	
ADH1C*2	I	γ_2	0.6		
ADH4	II	π	30	liver, cornea	Metabolize 30% ethanol at higher concentrations
ADH5 (also identified as ADHX)	III	χ	>1,000	widely expressed	Has a very low affinity for ethanol and used in methanol metabolism
ADH6	V	ADH6	unknown	stomach	Found in both fetal and adult liver; unknown function
ADH7	IV	μ or σ	30	liver, stomach	Involved in both ethanol and retinol oxidation
ALDH1				Cell cytosol, brain and other tissues	Eliminates acetaldehyde and keep its levels low and involved in the synthesis of retinoic acid
ALDH2				Mitochondria	Protective effect and main route of acetaldehyde elimination from the body

Table I: Mammalian Alcohol Dehydrogenases (ADH) and aldehyde dehydrogenases (ALDH) involved in alcohol metabolism (Bbosa G et al 2014).

pharyngeal, laryngeal and esophageal cancer related to the ALDH2 * 2 allele (Yokoyama A et al 2003).

At hepatic level, EtOH can be oxidized to acetaldehyde also by the "microsomal ethanol oxidation system" (MEOS). The enzymes belonging to this complex are isoenzymes of the cytochrome P450 family, in particular CYP2E1. Like all cytochromes P450, CYP2E1 are monooxygenases that use NADPH as an electron donor and O_2 as an electron acceptor.

The CYP2E1, compared to the ADH, has a low affinity for EtOH and a much higher Km. This means that the system intervenes only when the EtOH is present in the body in large quantities, then after high assumptions. Moreover, one of the most important characteristics of these enzymes is the inducibility, which is the ability to be induced by their own substrate, hence from EtOH. In fact, EtOH is able to bind a cytosolic receptor, induce its migration into the nucleus, where it promotes transcription of the gene coding for CYP2E1. In fact, the chronic consumption of EtOH does increase the hepatic levels of CYP2E1 by about 5-10 times. This causes an increase in endoplasmic reticulum size with a consequent increase in microsomal enzymes, including those not involved in the EtOH metabolism. The purpose of CYP2E1 induction is to stabilize and protect the same protein from degradation, as well as to increase the clearance of blood EtOH. There are several polymorphisms for CYP2E1 such as: RsaI, DraI and TaqI. For RsaI there are 2 different alleles with different enzymatic activity, the allele c2 has a lower enzymatic activity than the allele c1. The functionality of the enzymes encoded by DraI and TaqI is not very clear. A meta-analysis of 5 studies on the various polymorphisms of CYP2E1 found no correlation between EtOH consumption and colon cancer development (Yang C et al 2005).

Colorectal cancer

Colorectal cancer (CRC) is one of the most widespread cancers in the world. Despite progress in the surgical and therapeutic field, CRC still occupies the third place for cancer mortality, after that of breast and prostate cancer. Although it may occur at any age, it affects predominantly over six-year-old subjects of both sexes. In Italy, there are about 20 thousand new cases a year. CRC is a malignant neoplasm that affects the large intestine and is characterized by an uncontrolled proliferation of cells from the mucosa lining the intestinal walls(Fig 3). At the level of the large intestine, it is possible to distinguish colon

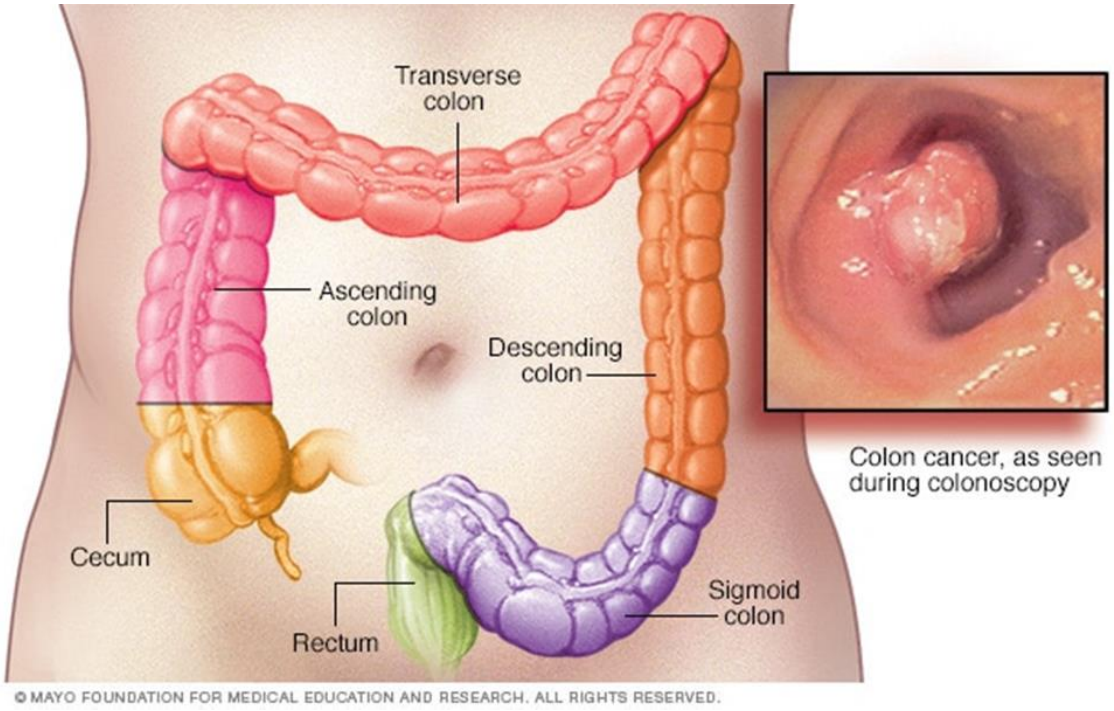

Figure 3 Colorectal Cancer

tumors from those of the rectum. Although the two tumor forms seem to have related causes, they are clearly different from each other both histologically and epidemiologically. The evolution of this neoplasm is closely related to the stage of the disease at the time of diagnosis. If the tumor is still limited to the basement membrane, there will be high chances of healing, if it exceeds the boundaries of the wall and metastasizes to the lymph nodes, the lethality will be high.

Although the mechanisms underlying carcinogenesis are not yet clear, CRC is certainly to be considered a multifactorial pathology involving genetic, epigenetic and environmental factors.

Hereditary genetic alterations can be determinants of genetic predisposition to the onset of CRC. The most frequent forms of hereditary CRC are Familial Adenomatous Polyposis (FAP) and Hereditary Non-Polyposis Colon Cancer (HNPCC).

In addition to hereditary genetic alterations, an important contribution to the onset of CRC can be attributed to environmental factors, such as a high-calorie diet rich in saturated fatty acids, obesity, ethanol abuse and smoking. Furthermore, it has also been observed that patients with other inflammatory colon diseases are more at risk of developing CRC.

The colorectal carcinoma is a multistep pathology in which there are well distinct morphological stages characterized by the presence of different mutations. In most cases of colorectal carcinoma, the initial stage is characterized by the formation of small polyps which, when enlarged, transform into benign tumors and which then, further increasing in size and acquiring various morphological characteristics, pass from the stage of early adenoma to that of carcinoma. Not all polyps, however, turn into a carcinoma; the transformation process, in fact, depends on the presence of some morphological characteristics, such as the presence of villi, and especially the size of the polyps. The acquisition of different mutations is associated at different stages of carcinogenesis and it have been seen to be a common event both in the form of sporadic and hereditary CRC (Ionov Y. et al 1993).

The evolution of CRC is associated with the acquisition of some precise mutations. The presence of these alterations and the order in which they acquired proves to be of considerable importance in determining the biological properties of the tumor (Fig. 4).

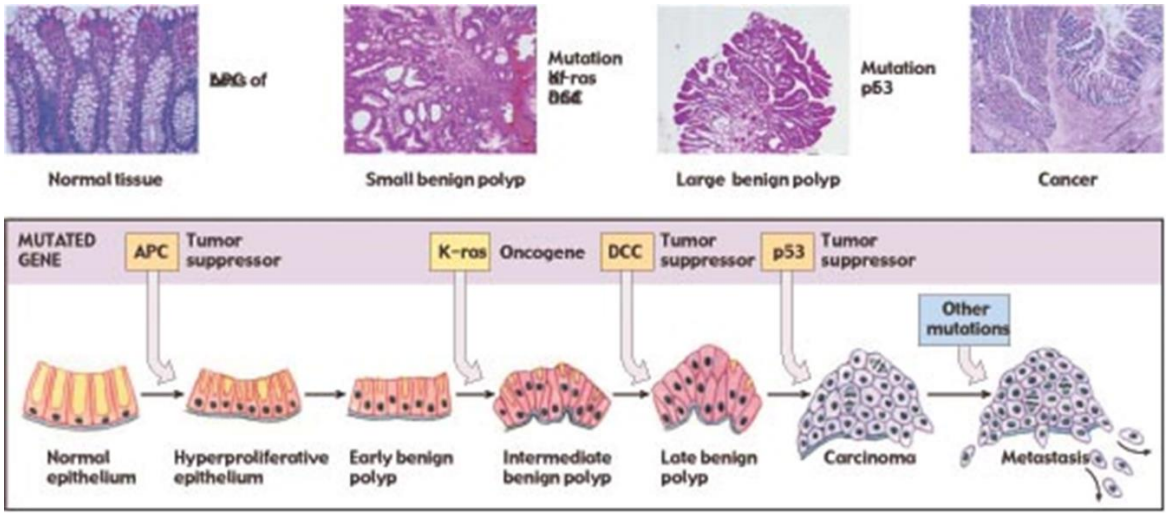

Figure 4 Model for multistep carcinogenesis in colorectal cancer development

One of the mutations acquired in the initial states of carcinogenesis is that of the APC tumor suppressor gene. Subsequently, during the development of the adenoma, mutations

occur on the K-ras oncogene, and finally, during the phase of transition from adenoma to carcinoma, further mutations are acquired in the DCC genes (Deleted in Colorectal Cancer), TP53, MET and deletions involving chromosome 18.

The succession of this series of events is representative of the so-called "CIS pathway", the traditional adenoma-carcinoma pathway first described in 1990 (Fearon ER et al 1990). This pathway is the basis of CRC onset in 85% of cases, and it is characterized by an accumulation of mutations in the key genes that control the cell cycle, intercellular communication and apoptosis, which are APC, K-ras and p53 genes (Armaghany T et al 2012).

The formation of adenomatous polyps is the initial step in the development of CRC and begins following the occurrence of mutations on the APC gene, mutation found in most patients with this disease.

The *APC* gene encodes a cytoplasmic protein, the APC protein, the product of a tumor-suppressor that intervenes in the inhibition of cell proliferation, in the control of cell adhesion and migration, and in the maintenance of chromosomal stability and cytoskeletal organization. The main function of APC is to control the cytoplasmic levels of beta-catenin and to prevent its translocation into the nucleus where it mediates proliferative effects supporting the Wnt pathway. In the absence of the Wnt ligand, beta-catenin interacts with a protein complex consisting of APC, AXIN1, AXIN2 and GSK3β. The GSK3β phosphorylates the terminal residues of serine and threonine of beta-catenin and promotes ubiquitination with the consequent proteasome dependent degradation. In presence of the Wnt ligand that interacts with the Frizzled membrane receptor, however, the protein complex is inhibited and beta-catenin is not degraded. This promotes the accumulation of beta-catenin and its translocation into nuclear sites where it interacts with many transcription factors including those of the TCF (T-cell factor) / LEF (lymphoid enhancing factor) family. The stimulation of TCF / LEF determines the activation of some genes, such as c-MYC and cyclin D1, with consequent stimulation of proliferation and inhibition of apoptosis. Mutations of APC are very frequent in CRC and they are associated with the production of a truncated protein that is unable to bind beta-catenin. Consequently, the levels of beta-catenin in the cytoplasm and the translocation in the nucleus increase with consequent stimulation of Wnt proliferative pathway (Choi SH et al 2013).

The adenoma-carcinoma progression is closely linked to the appearance of mutations concerning the *K-ras* gene, a proto-oncogene that encodes a membrane G protein (K-ras) with GTPase activity activated by extracellular growth factors.

The *K-ras* gene is constituted by a short gene sequence subject to point mutations, and within this, a single substitution of a base in a nucleotide, can generate an activating mutation found in 30-50% of CRC cases. This mutation provides the colonocyte of a growth advantage as the mutated K-ras protein loses its GTPase activity and is constitutively active. In this manner, it stimulates cell proliferation in a continuous way. K-ras is also responsible for transduction of mitogenic signals mediated by the EGF receptor (EGFR) (Dobre M et al 2013). The activation of the signal transduction cascade starting from the EGFR can also be favored by the extracellular binding of ligands other than EGF. Riboforin 2 (RPN2), for example, is one of these ligands that, by binding this receptor, enhances its proliferative activity by promoting the progression of CRC.

EGFR is a highly N-glycosylated surface glycoprotein which plays a crucial role in most human cancers as it is correlated with increased growth, proliferation and differentiation of cells (Ellina MI et al 2014). An increasing number of studies have indicated the importance of N-glycosylation of EGFR in the regulation of receptor functional properties. This includes the expression on the cell surface (Coskun Üet al 2011), ligand binding, conformational stability (Taylor ES et al 2016), dimerization (Wang XQ et al 2001), interaction with membranes (Prakash A et al 2010) and endocytosis (Lopez PH et al 2009). There are 11 potential N-glycosylation sites in the extracellular domain of the EGFR. It has been seen that in the presence of an N-glycosylation inhibitor, an immature EGFR protein that apparently does not reach the cell surface is synthesized and does not acquire the ability to bind EGF. One of the glycosylases of EGFR is riboforin 2 (RPN2). In some studies, it has been found that in CRC cases, the expression levels of RPN2 in tissues are significantly elevated and RPN2 is predominantly located in the cytoplasm of cells. These data therefore suggest that RPN2 has a potential role in CRC progression because, by activating EGFR glycosylation, it promotes cell growth and inhibits its differentiation.

The K-ras mutation, therefore, favors the adenoma-carcinoma transformation, however the sequence through which this mutation occurs, in relation to the APC mutation is particularly relevant. If this mutation occurs after an alteration of APC, dysplastic lesion will progress to cancer (Grady WM et al 2008). This is because K-ras mutated seems to promote greater aggressiveness of cancer cells.

However, the K-ras mutation alone is not sufficient to guide the complete adenoma-carcinoma transformation; in fact, the main event responsible for malignant transformation is represented by the acquisition of mutations concerning the *TP53* gene. This gene encodes p53, a 53 kDa protein present in all cells, which has the function of inhibiting the cell cycle and preserving the genomic stability (Suzuki Ket al 2011). When replication

errors or mutations occur, p53 stops or slows the cell cycle progression in the G1 / S phase and promotes DNA repair. If the damage to be repaired is too large, p53 activates caspase-dependent apoptosis (Amaral JDet al 2010). This protein, in addition to being activated by DNA damage, is also activated by other numerous factors such as ultraviolet radiation, oxidative stress, chemicals and viruses.

In non-tumor cells, p53 protein is maintained at low concentrations by its continuous degradation mediated by the MDM2 protein. Mutations in the *TP53* gene that result in functional protein inactivation were found in 75% of CRC cases (Bahnassy AAet al 2014).

The transition from intermediate to late adenoma is determined by mutations in the DCC gene. DCC (Deleted in Colorectal Cancer) is a tumor suppressor gene implicated in the development of colorectal cancer. DCC coding for the netrin-1 receptor (Patrick Mehlen, et al., 1998). In the presence of the ligand, netrin-1 activates pathway leading to cellular proliferation and migration. In the absence of the netrin-1 ligand, it promotes apoptosis, in fact an intracellular domain of netrin-1 is cut from caspase-3 and this seems to favor the activation of caspase-9-dependent apoptosis. Mutations in DCC that occur in the CRC result in reduced receptor expression with consequence failure to induce apoptosis. The DCC mutation is not considered a key genetic change in tumor formation, but one of the alterations that can promote the growth of existing cancer. The division of these cells takes place at the base of the villi and the old cells are pushed towards the luminal side from the successive cell divisions until they are eliminated by the activation of apoptosis. The netrin-1 is produced at the base of the villi, so there is a gradient of netrin that is lower in the luminal side. Physiologically, the presence of netrin-1 inhibits DCC-mediated cell death until the epithelial cell reaches the luminal side, where DCC not associated with the ligand promotes cell death. Mutations of DCC in the colonocytes associated with the absence of the receptor make it more likely that cells will continue to survive.

Other genes, which are interested in CRC, are the SMAD2 and SMAD4 genes, tumor-suppressor genes, which codes for proteins act as signal transducers involved in tumor suppression. The transition from invasive cancer to metastatic cancer is instead under the control of the *MET* gene. This is a protoncogene coding for a tyrosine kinase receptor called c-MET (mesenchymal epithelial transition factor) which binds to HGF factor (hepatocyte growth factor) activating different signaling pathways, including those involved in proliferation, in motility, migration and cellular invasion. In physiological conditions c-MET is important in the control of tissue homeostasis. The amplification of c-MET expression, found in most cases of human tumors (Comoglio PM et al 2008),

including CRC, is associated with tumor invasion and formation of lymph node metastases (Takeuchi Het al 2003).

Genetic factors and forms of hereditary CRC

Recently it has been found that at the base of Familial Adenomatous Polyposis (FAP) and of Hereditary Non-Polyposis Colorectal Cancer (HNPCC), both types of hereditary CRC, there are alterations of a single gene transmitted through germ line cells that increase the predisposition familiar to the CRC.

In both types of hereditary cancer, the tumor occurs at a much lower age than the average age of onset of sporadic CRC. Moreover, while in FAP the gene alteration determines the onset of the tumor; in the HNPCC it favors the progression of carcinogenesis. In the FAP, the first mutation that triggers tumor genesis concerns the APC tumor suppressor gene. Since the APC gene is responsible for controlling cell growth in the colonocytes, one mutation promotes the formation of a series of benign tumors within which, over time, other mutations accumulate that promote the adenoma-carcinoma transition.

At the base of HNPCC onset, however, mutations of genes responsible for DNA repair are found. In this type of tumors, in fact, the repair system of DNA damage repair Mismatch (MMR) is altered. The mutations that are most frequently found in this type of cancer affect the MLH1 genes, MSH2 and MSH6, each of which encodes a different protein belonging to the repair system that has the task of repairing at DNA level errors deriving from a wrong base matching that may occur during a normal replication cycle. Mutations involving only one of these genes favor the accumulation of replication errors, especially at the level of microsatellites that become unstable, which is why this form of colorectal cancer is classified as CRC with microsatellite instability.

Recently, it has been shown that the MMR repair system, in addition to repairing post-replication mismatches, also performs several other functions that are quite important in the process of carcinogenesis, one of which, for example, is the protection of DNA from damage induced by oxidizing agents. Usually in the initial phase of HNPCC cancer there is a mutation at the level of one of the alleles belonging to one of the MMR genes, which is then followed by the inactivation of the other allelic copy. This causes, as mentioned before, an altered function of the repair complex and, at the somatic level, leads to a consequent accumulation of mutations at the level of other genes, especially those involved in cell cycle control (Calabrese Pet al 2004). Here the cells of the adenomas that are

formed and that have mutations regarding the MMR, tend to accumulate mutations much more quickly than a normal cell, reason why the tumor progression turns out to be rather rapid. As previously reported, alterations in the MMR repair system lead to the inability to repair any replication errors and therefore, to an accumulation of these mutations especially at the level of microsatellites (Pedroni Met al 2001), or small DNA sequences containing repeats of tandem mono-, di-, or tri nucleotides that make this sequence particularly susceptible to replication errors. In most cases, microsatellites are placed within non-coding DNA sequences, but we can also find them inside coding regions of DNA belonging to genes mainly involved in the cell growth mechanism. Usually in cases of HNPCC with microsatellite instability, mutations regarding the APC and p53 genes are much less frequent and less incident. What is very important is that these types of cancer are very aggressive but at the same time show high chances of recovery, given by the instability of microsatellites that apparently appears to be associated with the possibility of survival.

Nutrition and onset of CRC

Incorrect food habits play an important role in the onset of CRC (Lynch HT et al 2003). Although cells are rapidly adapting to diet changes, during the adaptation process, colonocytes tend to accumulate genetic and epigenetic variations that often lead to genomic instability, favoring carcinogenesis.

The malignant transformation of the colonocytes may also be due to constant and prolonged exposure to carcinogens. Some data show that consumption of meat, tobacco and alcohol, considered carcinogenic substances, increase the risk of CRC, and how, instead, the consumption of dietary fibers reduces it. A diet rich in meat protein and low in fiber is considered a risky diet for CRC as it increases luminal pH facilitating the development of neoplastic cells. It has been shown that for every 30 gr of meat consumed per day there is an increase in the risk of CRC of about 10%. In a protein-rich diet, undigested protein residues and other nitrogenous compounds are degraded and fermented by the intestinal bacterial flora. These bacteria produce ammonia (NH3), phenols and hydrogen sulphides. The presence of these compounds in the colon causes inflammation and damage to the mucous membranes. For example, free ammonia is most toxic; it is easily absorbed by the colonocytes and induces inflammation, increases the proliferative rate and the intraluminal pH (Rao YK et al 2009). The presence of nitrogen, coming from

ammonia, facilitates the formation of nitrous compounds (NOC). The cooked meat provokes the formation of various carcinogens among which the NOC, closely related to inflammation and mucosal damage (Ben Qet al 2014). These compounds are mainly formed in the colon through nitrosylation of nitrosamines and starches by bacterial decarboxylation on amino acids in the presence of nitrosilating agents. Once the NOCs are formed, they can form DNA adducts and determine the onset of mutations in the main oncogenes and tumor suppressor genes (Bastide NM et al 2011).

The high consumption of animal fats, consequently to a high consumption of meat, causes an increased release of primary bile salts in the colon. These are metabolized by anaerobic intestinal bacteria and transformed into secondary bile salts through mechanisms of enzymatic deconjugation and dehydroxylation. Secondary bile salts, such as deoxycholic acid and lithocolic acid, are considered carcinogenic molecules as they alter the proliferative activity of mucosal cells by increasing the number of cells that synthesize DNA (Bernstein H et al 2009). The increase in cell proliferation predisposes to the risk of the onset of mutations and consequent malignant transformations. Furthermore, exposure of the colonocytes to high concentrations of secondary bile salts induces the production of ROS responsible for oxidative damage and mitotic aberrations that could induce changes in DNA and consequent gene instability. Therefore, greater is fats introduced into the body through food, greater is the production of secondary biliary salts and the predisposition to the onset of CRC. On the other hand, numerous studies show that a high consumption of dietary fibers, especially cereals fibers and whole grains, reduces the risk of CRC (Aune D et al 2011).When the fibers reach the colon they are partially or completely fermented with consequent production of short-chain fatty acids and gases that influence the gastrointestinal functions. Short-chain fatty acids reduce intraluminal pH by providing optimal conditions for the colonocytes and decreasing the conversion of bile acids into secondary bile acids. The dietary fibers also increase the volume by stimulating the growth of normal intestinal flora (Eswaran Set al 2013) and reduce the time and concentration of carcinogens in contact with the intestinal wall (Anderson JW et al 2009).There is also a well-established link between the high percentage of fats in the diet, inflammation and tumorigenesis. The high percentage of animal fats influences the microbiome favoring the expansion of pro-inflammatory microorganisms that lead to the development of intestinal inflammation (Candela M et al 2014). To support the relationship between CRC and inflammation there is the observation that the development of CRC is more frequent in patients with inflammatory bowel disease (Medzhitov R. 2008). In addition, the intestinal microbiome changes with age, as it tends to increase the opportunistic facultative

anaerobes and to decrease the anti-inflammatory species. Therefore, aging also contributes to a state of chronic inflammation that characterizes the whole organism. Intestinal inflammation could therefore elicit greater stimulation of the inflammatory response, allowing opportunistic pathogens to thrive at the expense of symbionts (Sansonetti PJ et al 2011).

Ethanol and Colorectal Cancer

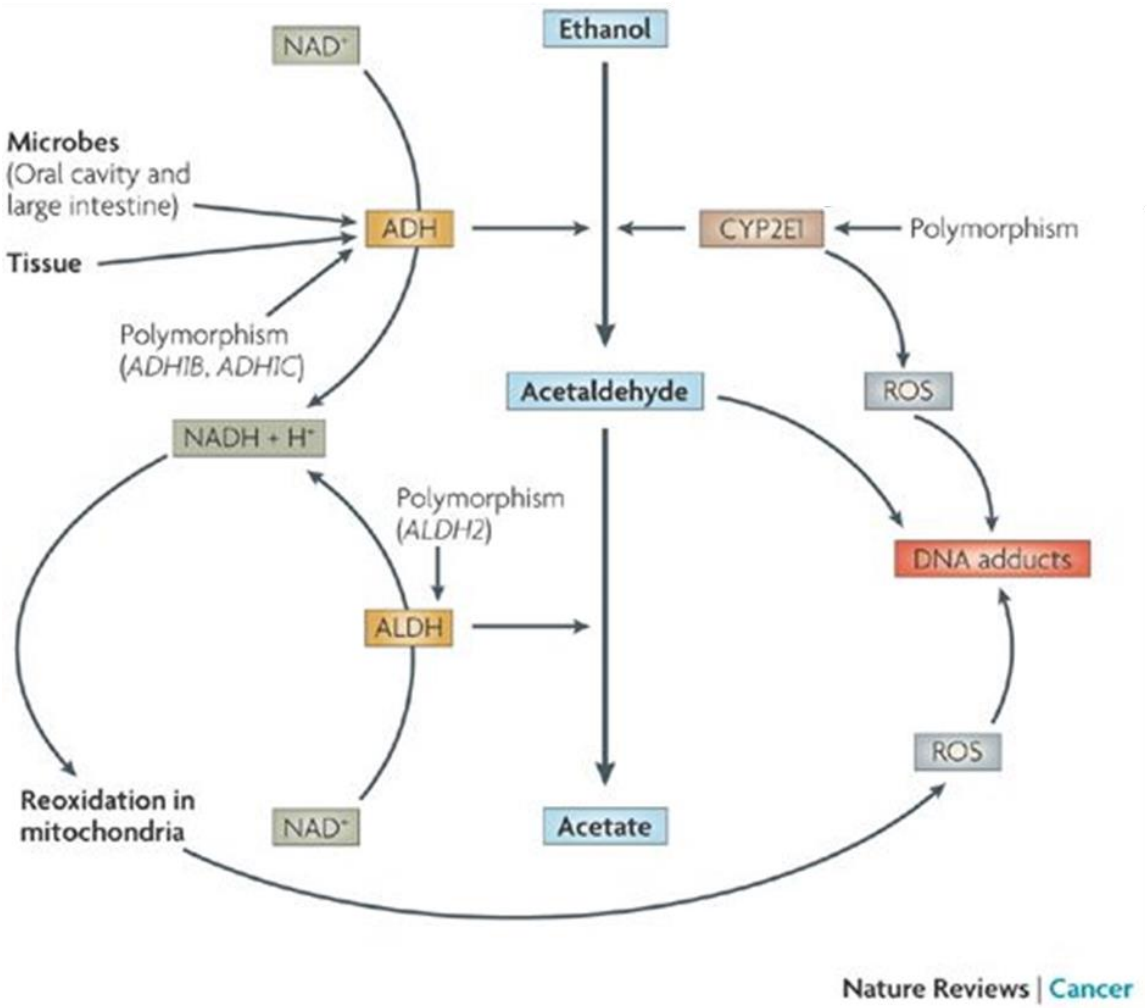

Figura 5 EtOH metabolism determines the production of ROS and acetaldehyde

While moderate EtOH consumption appears to have beneficial effects on cardiovascular disease, it is a common knowledge that a chronic and heavy consumption of EtOH exerts toxic effects and increases the risk of the occurrence of different types of cancer, including colorectal cancer (CRC) (Baan Ret al 2007). Most of the harmful effects due to the high consumption of EtOH can be attributed to the production of both acetaldehyde and oxygen reactive species (ROS) (Fig.5).

Infact, high consumption of EtOH results in a NADH / NAD^+ ratio increase at the cellular level, due to the high activity of both alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH). When the consumption of EtOH is moderate, the oxidation rate of NADH by the ADH is also moderate, and this is useful to maintain, within the cell, the right levels of NAD^+ that serve the ALDH for transform acetaldehyde into acetate. Instead, an increase intake of EtOH correlates with an increase in the NADH / NAD^+ ratio. In some cases the NADH will tend to accumulate because the rate at which this is oxidized within the cell is now far outweighed by the rate at which ethanol, present in large quantities, is metabolized. All this involves a malfunction of the ALDH with consequent accumulation of acetaldehyde inside the cell. Moreover, when high doses of ethanol are ingested, this is metabolized not only by hepatic ADH1, but also by ADH belonging to other families. These ADH normally are not very active given their high Km. Instead, when EtOH is present at high concentration in the stretch proximal gastrointestinal, it is also metabolized by gastric ADH (ADH4) with consequent production of large amounts of acetaldehyde on site. The high production of acetaldehyde in the intestine has been correlated with the increased risk of the occurrence of colorectal cancer. Under conditions of high EtOH assumptions, the amount of acetaldehyde in the bloodstream increases, also because the EtOH is metabolized by the microsomal ethanol oxidation system (MEOS). The latter, which has high Km, intervenes few at low doses of EtOH; a high consumption of EtOH determines the involvement of the MEOS system in the disposal of alcohol, also because EtOH itself induces an increase in the levels of the enzyme. The aim of the induction of the MEOS system, besides increasing the half-life of the CYP2E1 microsomal enzymes belonging to this oxidation system of EtOH, is to increase the clearance of the circulating EtOH. The increased clearance, despite representing a positive response of the organism in these cases, can also have negative implications. In these circumstances, in fact, acetaldehyde is produced faster than it is metabolised by acetaldehyde dehydrogenase. This involves its accumulation inside the liver cells but also a greater discharge of the same in the bloodstream where, through the blood flow, it will reach other organs, such as the digestive tract. The explicit toxic action of acetaldehyde at the cellular level is linked to the fact that it is a highly reactive molecule that tends to covalently bind to amino and sulfhydryl groups of proteins, nucleotides and phospholipids, forming adducts, leading to structural and functional changes of the molecules. The formation of adducts by acetaldehyde, especially at the level of certain proteins, inhibits the cellular defense mechanisms against oxidative stress. In particular, the interaction of acetaldehyde with GSH cysteine residues determines a drop in reduced glutathione and therefore deprives the

cell of an important system of defense against ROS. In a study of 24 heavy drinkers of EtOH it was shown that in their lymphocytes there was a quantity of DNA adducts 7 times higher than non-drinkers (Fang JL et al 1997). In addition to the metabolic activity of ADH in the colonic mucosa, the ADH of the bacteria present in the colon to produce acetaldehyde can also oxidize EtOH. To this end, it has been shown that the bacteria of the intestinal microflora incubated at 37 °C with different concentrations of EtOH produced high amounts of acetaldehyde (Jokelainen K et al 1994). This high production of acetaldehyde, both from colonocytes and intestinal bacteria, associated with a low enzymatic activity of ALDH of the colon mucosa, determines a substantial amount of acetaldehyde that accumulates in the colon. This would increase the probability of developing polyps of the colon and CRC that have been associated with a high consumption of EtOH (Salaspuro M, 1996). In fact, there is a statistically significant correlation between microbial ADH activity and production of acetaldehyde from EtOH in the colon (Jokelainen K et al 1996). More than 500 bacterial strains isolated from the faeces of Japanese alcoholics showed a high production of acetaldehyde following exposure of EtOH. In particular, among these bacteria some obligate anaerobes were large producers of acetaldehyde. The oxidation of ethanol by obligate intestinal anaerobes under aerobic conditions in the colon and rectum probably plays an important role in the pathogenesis of alcohol-associated colorectal cancer (Tsuruya A et al 2016). Alcohol administration gave rise to intracolonic levels of very high acetaldehyde in the rats, these levels were markedly reduced following the concomitant treatment with ciprofloxacin, an antibiotic (Homann N et al 2000). Furthermore, rats without bacteria had a significantly lower accumulation of acetaldehyde in the rectum and in the cecum than conventional animals, and this was directly proportional to the number of faecal bacteria present in the gut. Furthermore, individual variations in microflora of the human colon can influence the relative risk of alcohol-related colorectal cancer (Nosova T et al 1997).

An important function of the epithelial cells lining the gastrointestinal tract via the tight junctions is to provide a barrier against the hostile environment of the gastrointestinal lumen. Dysregulation of interactions between the intestinal epithelium and intestinal bacteria leads to loss of host immune tolerance, and thus promotes the development of colon cancer. Excessive intake of EtOH changes the composition of enteric microflora induces excessive growth of gram-negative bacteria and destroys the intestinal epithelial barrier. These results increase intestinal permeability and increase accumulation of proinflammatory cytokines, such as tumor necrosis factor (TNF) and interleukin (IL) -6 (Amin P.B et al 2009). EtOH facilitates the absorption of environmental carcinogens by

modifying the permeability and molecular composition of the gastrointestinal tract (Seitz H.K et al 2007). EtOH also acts as a solvent that improves the penetration of carcinogenic compounds into the mucosa.

Another possible mechanism of carcinogenesis related to alcohol consumption is attributable to the high production of ROS following ethanol metabolism. Oxidative stress determines the peroxidation of lipids, whose products are reactive electrophiles that react with DNA to form exocyclic DNA adducts (Ghissassi F et al 1995) and reactive aldehydes. The increase in ROS is also linked to an increased activity of the MEOS system. The latter, in fact, operates by transferring single electrons on oxygen and other organic substrates and therefore can generate reactive oxygen species, such as superoxide anion. The reaction catalyzed by MEOS also consumes NADPH, and this helps to reduce the antioxidant defenses of the cell. ROS production from ethanol metabolism activates signals that promote inflammation, angiogenesis and cell migration, as well as causing DNA damage.

The increased production of ROS and acetaldehyde following the intake of high doses of ethanol is considered the main cause of cellular alterations that may contribute to the development of colorectal cancer.

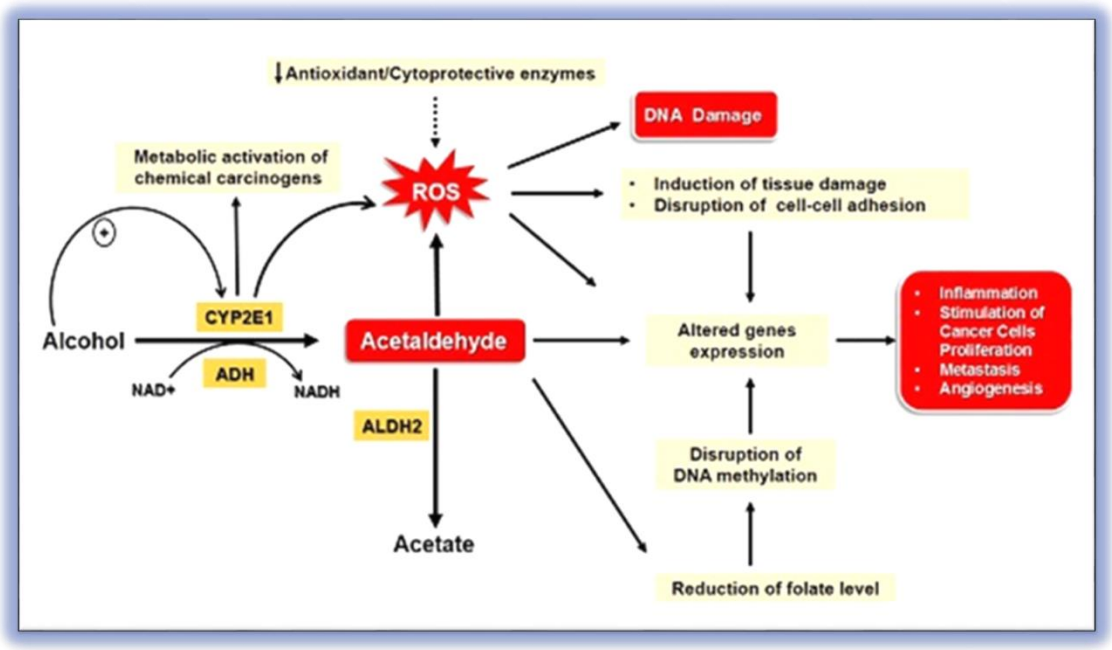

Figure 6 EtOH metabolism and high consumption-related cellular changes

The use of heavy alcohol could also lead to nutritional deficiencies by reducing the intake of foods rich in micronutrients, and altering intestinal absorption. The most relevant effect appears to be on folate metabolism (Lieber CS, 2003).

In fact the ethanol intake determines a reduction in the absorption of folic acid in the intestine (Kono S et al 2005). This has been attributed to the production of acetaldehyde in the colon by intestinal microflora. Indeed, it has been demonstrated in vitro that acetaldehyde determines a degradation of folic acid (Fig. 6) (Homann N et al 2000). A reduced availability of folic acid in cells compromises methionine metabolism and is associated with accumulation of homocysteine and S-adenosyl-homocysteine. The latter has been shown to inhibit DNA methyltransferase with the consequent presence in the cells of a hypomethylated state of DNA (Zakhari S. 2013).The methylation status of promoters of some tumor suppressor genes such as APC and p53 is more reduced in CRC associated with low folate / high levels of ethanol (Van Engeland M et al 2003).

Endoplasmic Reticulum Stress

The endoplasmic reticulum (ER) is an organelle consisting of a continuous compartment that extends from the nucleus to the cytosol and occupies about 10% of the cell volume. The ER carries out many important functions including

the synthesis and distribution of phospholipids and sterols, reserve and release of Ca^{2+} ions within the cytosol (Scheuner D et al 2008). Furthermore, ER is involved in the synthesis, folding and post translational modifications of membrane and secreted proteins (Ma Y et al 2004). Proteins synthesis inside the ER is monitored by different disulfide isomerases, which catalyze the formation of disulfide bridges. Protein synthesis is also regulated by molecular chaperones (GRP78, calreticulina, GRP94) which are associated with nascent peptides avoiding their aggregation and helping them to achieve correct folding in both physiological and pathological conditions (Kaufman RJ, 1999). Inside the ER, there is a real quality control, which ensures that only properly folded and functioning proteins leave ER. On the other hand, the non-folded or malfunctioning proteins are retained and finally sent to the 26S proteasome to be degraded, a process mediated by activation of the ER Associated Degradation (ERAD) system, or through the lysosomal mechanism, defined as macroautophagy, also commonly known as autophagy. Protein folding within the ER is highly regulated and susceptible to changes in intracellular energy levels, redox status and Ca^{2+} ion concentration. Under conditions of sudden or constant variations of one of these

three conditions, there is accumulation and aggregation of poorly folded proteins in the lumen of ER, a condition known as ER stress. ER stress is observed as a consequence of nutrients deprivation or differentiation of type B lymphocytes in plasma cells, as well as in pathological conditions, such as viral infections, ischemia, neurodegenerative diseases, diabetes and tumors (Bravo R et al 2013). In presence of ER stress, the cells activate a defense strategy, known as Unfolded Protein Response (UPR) (Fig.7). This cellular process is mediated by the activation of three ER membrane receptors, named respectively: Pancreatic ER Kinase (PERK), Activating Transcription Factor 6 (ATF6) and Inositol-Requiring Enzyme 1 (IRE1α). In absence of stress, the three receptors are kept inactive by the chaperone Glucose Regulated Protein 78 (GRP78).

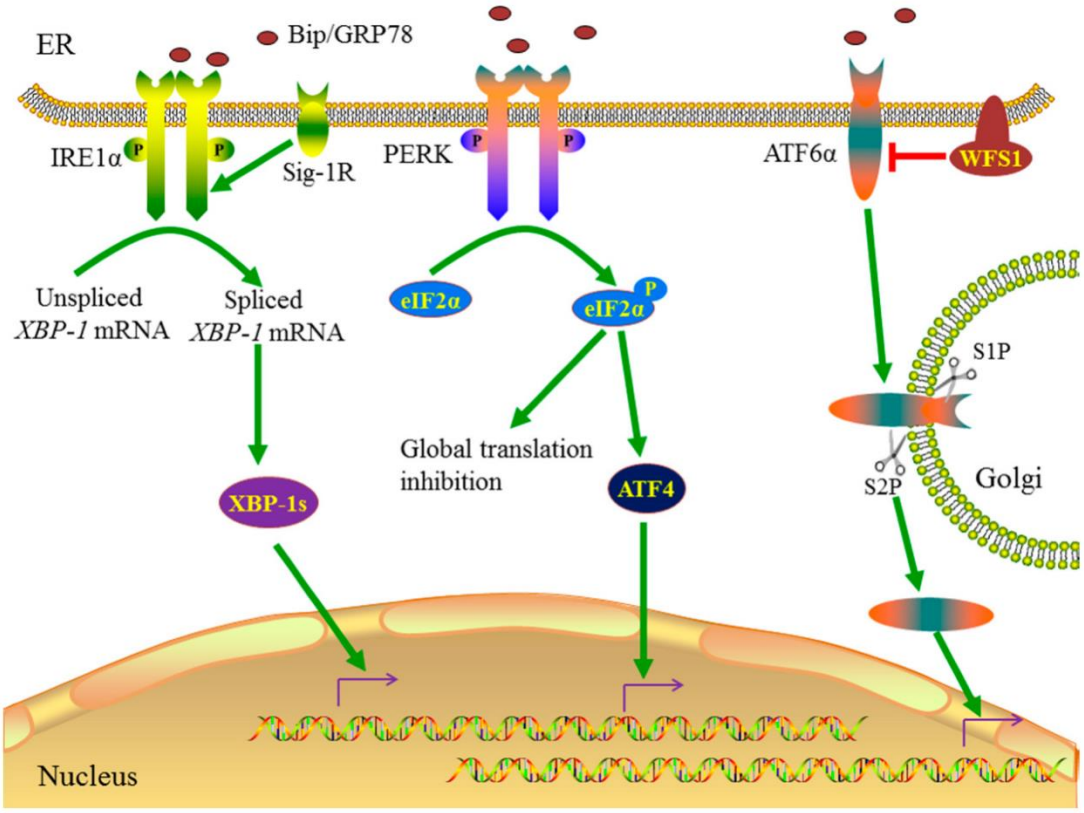

Figure 7 Endoplasmic Reticulum (ER) stress and Unfolded Protein Response (UPR) (Wang Y et al 2017).

Instead in the presence of stress, GRP78 dissociates from the three receptors by activating them and inducing UPR. The UPR, in the first phase, promotes cell survival with the consequent restoration of the normal functioning of the ER itself (Schroder M et al 2005). However, if protein aggregation is persistent and stress cannot be resolved, UPR causes cell cycle arrest and subsequently apoptosis. PERK is a type I transmembrane kinase, which is activated following the dissociation of GRP78 from its luminal domain. Under stress conditions of ER, PERK determines the phosphorylation of eIF2a, which, in turn,

determines the inhibition of protein translation (Wek RC et al2006). This event favors cell survival by inhibiting the accumulation of nascent proteins that arrive in the endoplasmic reticulum. However, eIF2a also activates proteins that are not involved in the blocking of protein translation. Among these there is the ATF4 protein encoding a cAMP respsonse element-binding transcription factor (C-EBP) (Schroder M et al 2005). ATF4 promotes cell survival through the activation of genes involved in the metabolism of amino acids, in redox reactions, and in protein secretion (Harding HP et al 2003). However, ATF4 can also activate C / EPB transcription factor homologous protein (CHOP), which promotes cell death by apoptosis (Wang XZ et al 1998). The second receptor involved in the UPR is ATF6, a type II transmembrane glycoprotein, whose luminal domain is responsible for the detection of misfolded proteins. The cytoplasmic portion of ATF6 is able to act as a transcription factor as it contains a DNA binding domain. Following its dissociation from the chaperone protein GRP78, ATF6 translocates into the Golgi apparatus, where it undergoes a proteolytic cut generating an active transcription factor (Chen X et al 2002). Among the ATF6 target genes there are those coding for some chaperone proteins, such as GRP78, GRP94, disulfide isomerase proteins (PDI), for the transcription factor GADD153 / CHOP and for X box-binding protein 1 (XBP1). Since the activation of ATF6 determines the induction of the expression of genes coding for the chaperones, its activation is responsible for the increase in the ability of the reticulum to replicate proteins, contributing to the restoration of initial homeostasis. Usually ATF6 mediates pro-survival signals in order to counteract the effect of ER stress (Wu J et al 2007). The last receptor / sensor of the activated UPR is IRE1α.It has a dual function, activates survival mechanisms by activating the transcription of chaperones as GRP78, but also activates pro apoptotic proteins under prolonged stress conditions. IRE1α is a sensor with double enzymatic activity, as it is equipped with both a serin-threonine kinase domain and an endoribonuclease domain (Tirasophon W et al 1998). Under ER stress conditions, the detachment from GRP78 determines the activation of IRE1α, through dimerization and autophosphorylation. Once activated IRE1α determines alternative splicing of the XBP1 mRNA. The generated splicing variant encodes a sXBP1 transcriptional factor that will activate ER chaperone transcripts such as $P58^{INK}$. This chaperone ends the block of translation activated by PERK and activates the transcription of pro apoptotic proteins (Ladiges WC et al 2005). Therefore, in presence of ER stress the activation of the IRE1α sensor plays a critical role in the initiation of pro-apoptotic signals, while the activation of the PERK and ATF6 sensors would seem to precede the activation of the IRE1α in the attempt to resolve the stress in the presence of a pro-survival action. If ER stress persists,

the pathways of PERK and IRE1α can converge, mediating the induction of the apoptotic process through mutual reinforcement. However, it has also been shown that in the presence of ER stress both IRE1α and CHOP can activate autophagy processes in human colon carcinoma cells (Shimodaira Y et al 2014).

Authophagy

Autophagy is a cellular mechanism discovered in mammals, but present in all the organisms starting from the yeasts. This process can play a double function inside cell, either promoting cell survival or triggering cell death. In particular, it exerts a survival function under metabolic stress enhancing cancer cells tolerability to different cell stresses as nutrient deprivation. However it can determine cell death, when the process in prolonged in time. In particular, when the cells are deprived from nutrients or in the

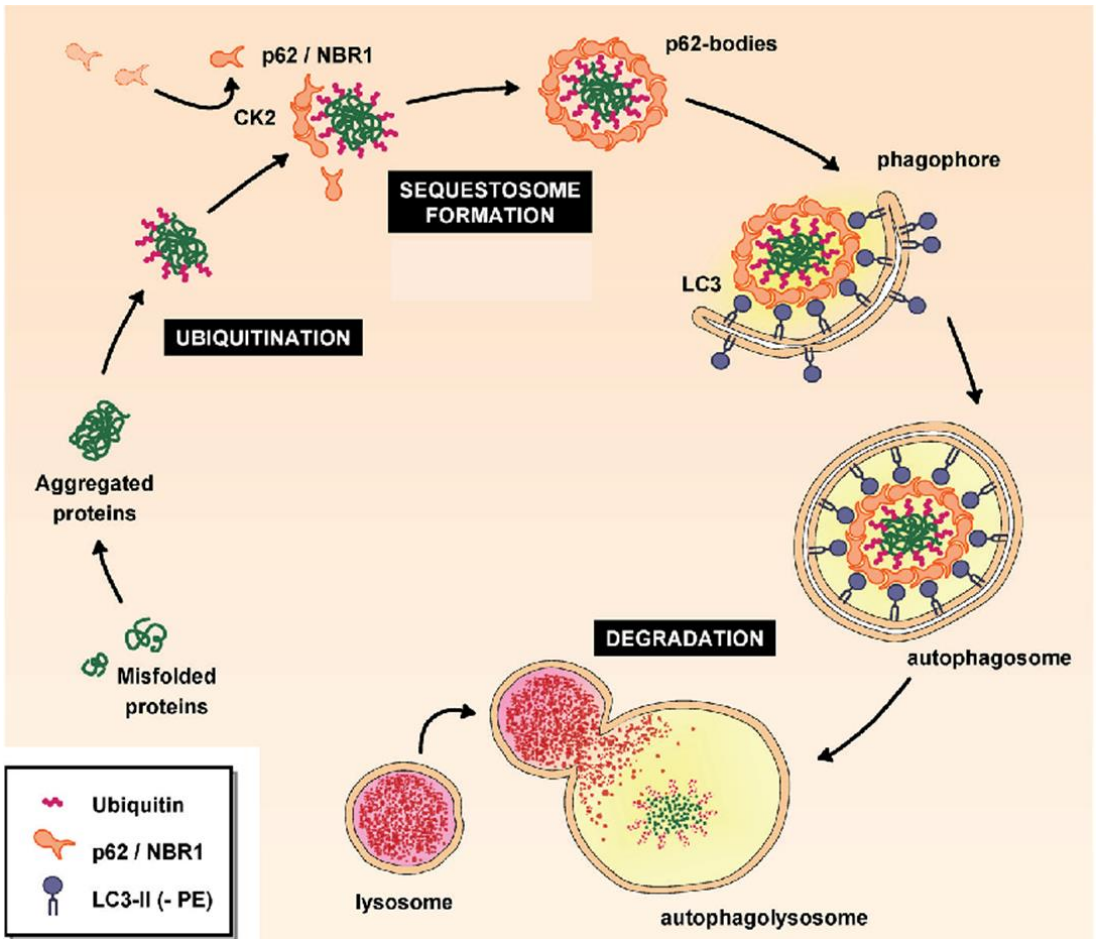

Figure 8 Autofagic process

presence of the autophagic signal, it has been observed the formation of double membrane vesicles, called autophagosomes, which incorporate portions of the cytoplasm (Levine B et al 2005). The membrane of these autophagosomes originates from intracellular organelles (eg mitochondrion) or from pre-existing intracellular molecules. Later these autophagosomes merge with the lysosomes, thus favouring the degradation of macromolecules and releasing monomers and ATP, available for a new synthesis of macromolecules. This process is called macroautophagy. However, there are two other types of autophagy such as microautophagy and chaperone-mediated autophagy. Autophagy also has a protective role because it allows the degradation of damaged organelles or of protein aggregates (misfolded proteins) that could damage cellular wellness. The main regulator of autophagy is the target kinase of rapamycin, mTOR. This kinase acts as a nutritional sensor, when there is abundantly nutrients is active and prevents the formation of autophagosome. In cases of stress or decreased nutrient availability (due to prolonged fasting) mTOR is inhibited and allows the formation of autophagosome. Generally, mTOR inhibits the kinase ULK1 (Gammoh N et al 2012). Akt following phosphorylation in Ser774 inhibits ULK1. In the same way, it can be activated by AMPK that phosphorylates it in different amino acid residues such as: Ser555, Ser467, Thr574 and Ser637. In the absence of nutrients, ULK1 undergoes a conformational change by an active state following autophosphorylation in the Thr180 residue. The ULK1 protein in the active form phosphorylates the proteins ATG13 and FIP200, with which it forms a fundamental complex in the maturation of autophagosomes, as it is able to recruit another protein complex consisting of Vps15, PI-3K of class III and the Beclin-1. This mega protein complex, as it is composed, recruits proteins encoded by ATG genes. ATG proteins are involved in the different phases of autophagy, performing functions both in the nucleation of the vesicles and in the maturation of autophagosomes (Kourtis N et al 2009). These ATG proteins also act on the cytosolic LC3-I protein converting it, following a proteolytic cut (by ATG4) and conjugation with phosphatidylethanolamine, in the LC3-II form (Liu Y et al 2010). Consequently, LC3-II produced is anchored to the autophagosome membrane where it remains even after fusion with lysosomes (Fig.8). The LC3-II protein is a marker of autophagy (Pyo JO et al 2012). In the last phase of autophagy, when the fusion of autophagosome with lysosomes occurs, the generation of autofagolisosomes, favors the degradation of the molecules. There is an interesting relationship between autophagy and ER stress since both mechanisms are aimed at restoring normal cellular functions following stress. In particular, CHOP activates autophagy mechanisms in colon carcinoma cells (Shimodaira et al 2014).

Project aim

In the western countries the colorectal cancer (CRC) is the third tumor for aggressiveness and incidence after the lung and breast/prostate cancer. The frequency of this cancer is higher in older age. There are different causes related to this disease, like genetics and lifestyle. However, the exact cause of colon cancer is not clear yet. Although the genetic alterations play a significant role in colon cancer development and progression, different studies support that lifestyle and environmental factors can increase the risk of developing this tumor. To this purpose epidemiologic studies correlating alcohol consumption with risk of developing colon cancer demonstrate that the incidence of the disease is 5-fold higher among drinkers compared to nondrinkers. Alcohol consumption is also associated with Colorectal Cancer patients' progression and poorer prognosis. Epidemiological investigations also indicate that chronic and heavy alcohol consumption is associated not only with the increased risk of developing CRC, but also with metastasis. Alcohol consumption is an independent risk factor for liver metastasis in colorectal carcinoma patients. The presence of tumor metastasis determines the failure of the chemotherapeutic treatment and a lower postoperative survival rate. The 5-year overall survival rates are around 68% for colorectal cancer. In the presence of concomitant metastases, the maximum survival rates after surgery do not exceed 20%. However, the relationship between alcohol consumption and tumor metastasis needs to be deepened further. Although it is now clear that the consumption of high doses of ethanol is an important risk factor for colorectal cancer and its tumor progression, the biochemical mechanisms responsible for these events have not yet been fully clarified. The aim of my doctoral project is to study the effects of ethanol in human colorectal carcinoma cells and to evaluate the biochemical mechanisms underlying survival and invasiveness in CRC.

Materials and Methods

Cell culture condition

The human colon cancer HCT116, HT29 and Caco-2 cells (Interlab Cell Line Collection, ICLC, Genova, Italy) were grown in monolayer in flasks of 75cm^2 in RPMI 1640 medium, supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin and 50 µg/ml streptomycin in a humidified atmosphere of 5% CO2 in air at 37°C. To study the effects of ethanol, cells were detached using trypsin-EDTA (2.5mg/ml trypsin and 1mg/ml EDTA) and plated in accordance to the experimental conditions, as described in the paragraphs below. Cells were allowed to adhere for 24h and then treated with different concentration of ethanol at different times.

All the reagents used for cell culture were purchased from Euroclone (Pero, Italy). Ethanol, E64d and all chemicals, except when stated otherwise, were supplied by Sigma-Aldrich (Milan, Italy).

MTT assay

To evaluate the effect of ethanol on cell viability the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) colorimetric assay was used. HCT116, HT29 and Caco2 cells (7x10^3/200µl/well) were plated in 96-wells and treated with various concentrations of ethanol (30-300mM) for different times. Then, 20 µl MTT (11mg/1ml) was added and cells were incubated at 37°C for 4 h. Finally, the medium was removed and 100µl of lysis buffer (20% sodium dodecyl sulfatein 50% N,N-dimethylformamide) was added. At the end, the absorbance of the formazan was measured directly at 490 nm with 630 nm as a reference wavelength using an automatic ELISA plate reader (OPSYS MR, Dynex Technologies, Chantilly, VA). Cell viability was expressed as the percentage of the OD value of ethanol-treated cells compared with untreated samples used as control. Each experiment was performed in triplicate.

Clonogenic assay

HCT116 cells were seeded in 6-well plates at a density of 200 cells/well with 3 ml culture medium and incubated for 10 days with or without 100 and 300 mM ethanol. The medium was changed every 3 days. There after medium was removed and cells were washed in cold PBS and then incubated on ice in cold methanol for 15 min. Then, the cells were washed in PBS, incubated for 1h in the presence of 0.01% crystal violet (Sigma-Aldrich, St. Louis, MO, USA). Representative views were photographed.

Measurement of ROS production

The production of reactive oxygen species (ROS) was measured using 2', 7' dichloro-dihydro-fluorescein diacetate (H2DCFDA, Molecular Probes Eugene, OR). This compound contains two acetyl groups and is able to cross the cell membrane by its lipophilic nature. After cell entry, cellular esterases remove acetyl groups by transforming H2DCFDA into the DCFH molecule that is oxidized by ROS and converted into dichlorofluorescein (DCF), a compound that emits a fluorescent green color.

HCT116 cells ($7x10^3$/200µl) were seeded in 96-well plates and incubated with 100 or 300 mM ethanol for different times. After treatment, cells were washed with PBS and incubated with 10 µM H2DCFDA for 15 minutes at 37° C in the dark. Finally, cells were resuspended in PBS and analysed by fluorescence microscopy using a Leica DMR (Leica Microsystems S.r.l., Wetzlar, Germany) inverted microscope equipped with a FITC filter system (excitation wavelength of 485 nm and emission wavelength of 530 nm). Images were acquired by computer-imaging system (Leica DC300F camera).

Western blotting analysis

Cells were seeded in 6-well plates ($1.5x10^5$/2 ml culture medium) and treated with ethanol for the indicated times. Then, cells were washed in PBS and harvested with trypsin-EDTA (2.5mg/ml trypsin and 1mg/ml EDTA), and centrifuged at 800 rpm for 8minutes. The pellets were lysed in RIPA buffer (1% NP-40, 0.1% SDS and 0.5% sodium deoxycholate in PBS), containing protease inhibitor cocktail (25µg/ml aprotinin, 1mM PMSF, 25 µg/ml leupeptin and 0.2 mM sodium pyrophosphate). After sonication (3 cycles of 10 seconds, at an intensity of 10 REV in Soniprep 150), protein content of cell extracts was determined by the Bradford method. Equivalent amounts of proteins (30-50 µg) were separated by SDS–polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Bio-RadHercules, CA, USA). Equal proteinloading was ascertained by Ponceau-S staining of blotted membranes. Then, the filter was incubated with "blocking" solution (1% or 5% milk in TBST (20mM Tris-HCl, 150mM NaCl, 0.005% Tween-20, pH 7.5) for 1h. So, the filter was incubated overnight in a solution containing specific primary antibody (1µg/ml TBST). Primary antibodies used for catalase, lamin B, MnSOD, Nrf2, iNOS, Cox2, Grp78, CHOP, MMP-2 and MMP-9 were purchased from Santa Cruz Biotechnology (St. Cruz, CA); β-actin from Sigma Aldrich; Hsp60, Hsp90 and HO-1 from Enzo Life Sciences, (Milan, Italy). After incubation, the filter was incubated for 1h with secondary antibody (1µg/3ml of TBST; Pierce, Thermo Fisher Scientific) conjugated with

horseradish peroxidase (HRP). Immunoreactive signals were detected using enhanced chemiluminescence (ECL) reagents (Cyanagen, Bologna, Italy). The correct protein loading was confirmed by stripping the immunoblot and reprobing with primary antibody for β-actin. The signal obtained through ECL development was detected through CHEMIDOC and processed using the "Quantity One" software (BioRad).

Cytosol and nuclear extraction

HCT116 cells were seeded in 100-mm tissue culture dishes ($1x10^6$ cells/5 ml culture medium) and, after treatment with ethanol, washed in PBS and harvested with lysis buffer (250 mM Sucrose, 20 mM HEPES, 10 mM KCl, 1.5 mM MgCl2,1 mM EDTA, 1 mM EGTA, 1 mM DTT, and protease inhibitors,pH7.4). Next, cells were passed 10 times through a needle of 25 G on ice for 20 minutes. The homogenates were centrifuged at 1,000 g for 10 minutes at 4°C. The pellets (nuclear fraction) were resuspended in lysis buffer and passed 10 times through a needle of 25 G and centrifuged at 1,000 g for 10 minutes at 4°C. The pellets were lysed with RIPA buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, inhibitors of proteases: 25μg/ml aprotinin, 1mM PMSF, 25 μg/ml leupeptin and 0.2 mM sodium pyrophosphate) and sonicated. The supernatants obtained from the two centrifugations were united, centrifuged at 10,000 g for 30 minutes at 4°C. The supernatants obtained were considered as cytosolic fraction.

Nuclear and cytosolic fractions were used to evaluate Nrf2 and HO-1; β-actin and lamin-B were used as cytoplasmic and nuclear markers, respectively.

Monodansylcadaverine test

To evaluate the formation of autophagic vacuoles Monodansylcadaverine (MDC) test was employed. HCT116 cells ($7x10^3$/200μl culture medium) were plated in 96-wells and treated with ethanol. After the treatment, cells were incubated with 50 μM MDC for 10 min at 37°C in the dark. Then, cells were washed with PBS and analysed by fluorescence microscopy using a Leica DMR (Leica Microsystems) microscope equipped with a DAPI filter system (excitation wavelength of 372 nm and emission wavelength of 456 nm). Images were acquired by computer-imaging system (Leica DC300F camera) using SMX software.

Transient down-regulation of Nrf2 by short interfering RNA (siRNA)

HCT116 cells were seeded (2×10^5 cells/well) in 6-well plates and cultured in RPMI 1640 medium, supplemented with 10% FBS, without antibiotic, for 24 h to reach approximately 60–80% confluence before transfection. Specific siRNAs directed against Nrf2, obtained by Qiagen, Hilden, Germanyas a pool of double-stranded RNA oligonucleotides, (SI03246950, SI03246614) were transfected for 5 h into the cells. Each siRNA was transfected into cells at 25 nM. A non silencing siRNA (SI03650318, Qiagen) was used as a negative control (50nM). For transfection, specific siRNAs and negative control were transfected in the presence of 5µl Lipofectamine 2000 (Invitrogen, Carlsbad, US) in a final volume of 1 ml serum-antibiotic free RPMI 1640 medium. At the end the reaction was stopped replacing the culture medium with complete RPMI 1640 medium plus FBS and antibiotic. After 24h of transfection, cells were treated with ethanol for other 24h. Then, the cells were examined for Nrf2 down-regulation and other proteins by western blotting analysis.

Gelatin zymography

HCT116 cells were seeded in 100-mm tissue culture dishes ($5x10^5$ cells/ 5 ml culture medium). After 48h of ethanol treatment, cells were washed in PBS and harvested with lysis buffer (25 mM Tris-HCl pH 7.5, 100 mM NaCl, 1% (vol/vol) IGEPAL CA-630, Protease inhibitors: 10 µg/mL aprotinin, 2 µg/mL leupeptin, and 4 mM benzamidine). The lysates were centrifuged at 800 g for 10 minutes. The samples (50µg of proteinsprepared in sample Buffer: 50 mM Tris-HCl, 2% SDS, 0.1% Bromophenol Blue, 40% Glycerol, pH 6.8) were loaded on polyacrylamide gels (10%) with 10X gelatin and subjected to electrophoresis. Then, the gel was washed for 1 hour with enzyme renaturing buffer (200mM NaCl, 5mM CaCl2, 5µM ZnCl2, 2,5% (v/v) Triton X-100 and 50 mM Tris HCl, pH 7.5) and incubated overnight at 37°C with developing buffer (50mM Tris base, 200mM NaCl, 5mM CaCl2, pH 7.5). Then, the gel was incubated for 30 min at RT with Staining solution (0.125% Coomassie brilliant blue R-250, 50% methanol, 20% acetic acid) and washed with Destaining solution (30% methanol, 0,01% formic acid) until clear bands of MMP activity are visible in the blue background.

Immunofluorescence

HCT116 cells were plated on coverslips ($8x10^3$) and treated with 300 mM EtOH for different times. After washing two times with PBS, cells were fixed in methanol for 30 minutes at room temperature. After fixation, cells were washed three times in PBS for 5 minutes and treated with a blocking solution (3% BSA in PBS) for 30minutes. Subsequently, the cells were washed two times with PBS and incubated with primary antibody against HO-1 (anti-rabbit, Enzo Life Sciences) or against Nrf2 (anti-rabbit, Santa Cruz Biotecnology) at dilution 1:100, overnight at 4°C. Then, cells were washed three times with PBS for 5 minutes and incubated for 1h with a conjugated secondary antibodies: anti-rabbit IgG-FITC produced in goat (Sigma-Aldrich) at dilution 1:200. Nuclei were stained with Hoechst Staining Solution (1:1,000, Hoechst 33258, Sigma-Aldrich). The images were captured using a Leica Confocal Microscope TCS SP8 (Leica Microsystems).

Statistical analysis

Data, represented as mean ± S.D., were analyzed using the Student t test for densitometric analyses. The Graph Pad Software (GraphPad Software 7825 Fay Avenue, Suite 230 La Jolla, CA 92037 USA) was used for statistical calculations. Differences were considered significant when $p< 0.05$ or $p< 0.01$.

Results

Effects of EtOH in colon cancer cell viability

Although epidemiological studies have shown that heavy and chronic alcohol consumption increases the risk to develop colon cancer and favors tumor progression (Baan R et al 2007), the underlying molecular mechanisms are not still clear.

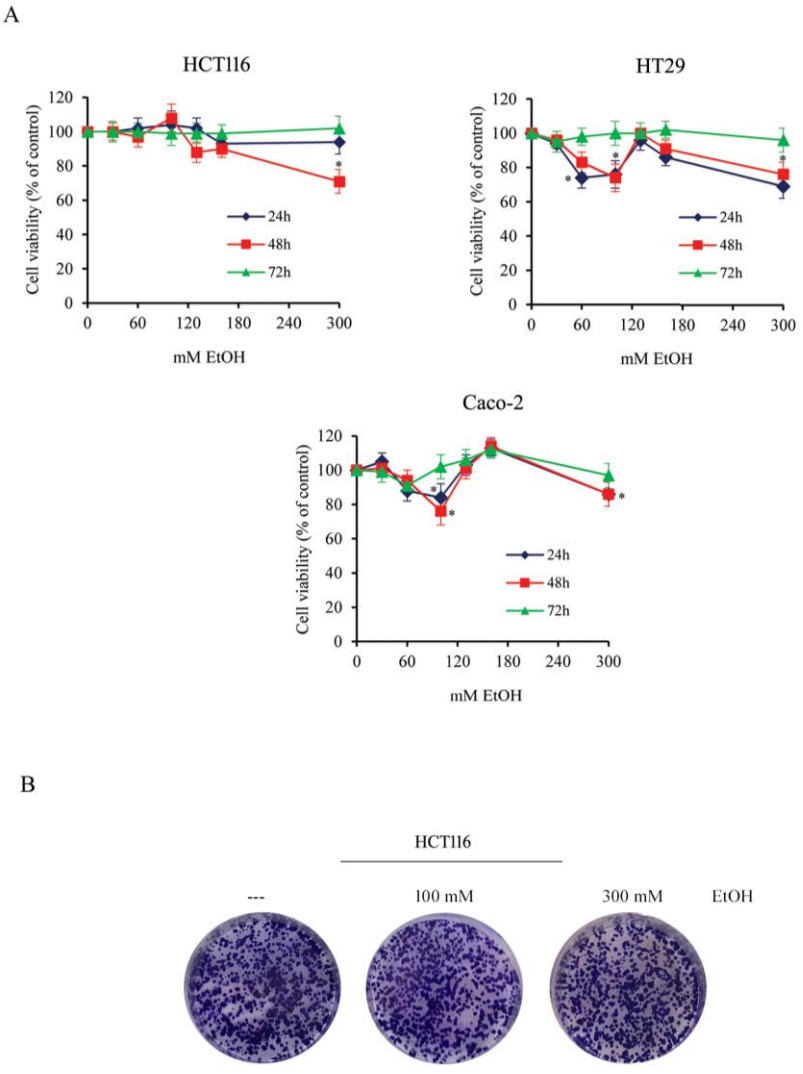

Fig. 1. **Effect of Ethanol on colon cancer cell viability and colony generation ability (**A) HCT116, HT29 and Caco-2 cells ($7x10^3$) were incubated in the presence of various doses of EtOH for different periods. Cell viability was assessed by MTT assay as described in Materials and Method section. Values are the means of three independent experiments ±S.E. (*) $p<0.05$ compared to the untreated sample. (B) Effect of EtOH on colony generation ability of HCT116 cells. Clonogenic assay was performed seeding a single cell suspension (200 cells/well) in 6-well plates and after 48h was treating with different doses of EtOH. The ability of cells to generate colonies was evaluated after 10 days as reported in Methods. Photographic images of cells after staining with 0,1% crystal violet were reported. Three different visual fields were examined for each condition. Results are representative of three independent experiments.

To shed light on this aspect, I studied the effects of Ethanol (EtOH) on HCT116, HT29 and Caco-2 cells, three human colon cancer cell lines. HCT116 cells are derived from a colon carcinoma line characterized by a mutation in codon 13 of the K-ras gene (Schroy PC et al

1995). The HT29 cells were isolated from a primary tumor in 1964 by J. Fogh and are characterized by mutations in the oncogenes myc +, ras +, myb +, fos +, sis +, and p53 + (Trainer DL et al 1988). Caco-2 cells form moderately well differentiated adenocarcinoma consistent with colonic primary (grade II) in nude mice. This cell line expresses heat stable enterotoxin (Sta, E. coli) and epidermal growth factor (EGF) as reported by the American Type Culture Collection (ATCC) (Cohen MB et al 1993).

To perform my studies, circulating levels of EtOH in the bloodstream were taken into account after alcohol consumption. Considering that the concentrations ranging from 10 to 100 mM correspond to the circulating levels of EtOH in the blood following moderate to heavy consumption (Singletary et al., 2001), the HCT116, HT29 and Caco-2 cells were exposed to a range of concentrations of EtOH from 10 to 300 mM for different times. As shown in figure 1A, compared with untreated cells the viability of HCT116, Caco-2 and HT29 cells was not significantly affected by EtOH treatment for 72 h, also with higher doses of EtOH (300mM).

To explore the ability of EtOH to form colonies, HCT116 cells were plated with and without the addition of increasing doses of EtOH. Control and treated cells were maintained in culture for an additional 10 days to allow formation of colonies. The results demonstrated that there are no difference between control and EtOH-treated cells (figure 1B), leading to the conclusion that high doses of EtOH favor survival of colon cancer cells, although not increase cell proliferation. To evaluate the biochemical mechanisms linking high doses of alcohol consumption and colon carcinogenesis, subsequent experiments were performed in colon cancer cells using two different high concentrations of EtOH, 100 mM and 300 mM.

EtOH treatment induced oxidative stress in HCT116 colon cancer cells

I further evaluated whether in my experimental conditions EtOH induced oxidative events. It is well known that EtOH metabolism by CYP2E1 produces acetaldehyde and ROS, which have been correlated with the toxic effects of the compound in several cell types and tissue (Yang C et al 2005). To ascertain ROS generation in my experimental system, I performed immunofluorescence analysis by employing the fluorochrome H_2-DCFDA, a general indicator of the level of the reactive oxygen species (ROS). This compound crosses the cell membrane thanks to its lipophilic nature; inside the cell it is metabolized by cellular esterases and, in the presence of radical species, it is oxidized by emitting green fluorescence. As shown in figure 2A, in HCT116 cells EtOH treatment rapidly increased intracellular ROS level. The effect appeared at 10 minutes of exposure with 100 and 300 mM EtOH and then rapidly decreased with the time of treatment.

Further analysis were performed to assess the levels of other stress markers. iNOS (inducible Nitric Oxide Synthase) and COX2 (cyclooxygenase-2) are two important enzymes that mediate inflammatory processes and favor tumor progression. Western blotting analysis showed that EtOH upregulated iNOS and COX2 levels in HCT116 cells. In particular, in comparison with control, treatment for 3h with 300 mM EtOH increased the levels of iNOS and COX2 by 2.2 and 2.7 fold respectively. I also evaluated the effect of EtOH on the levels of HSP90 and HSP60, two heat shock proteins, whose levels increase after cellular stress. As showed in figure 2B, 300 mM EtOH at 3h of treatment increased the level of HSP90 (1.7 fold) and HSP60 (2.4 fold) compared to control.

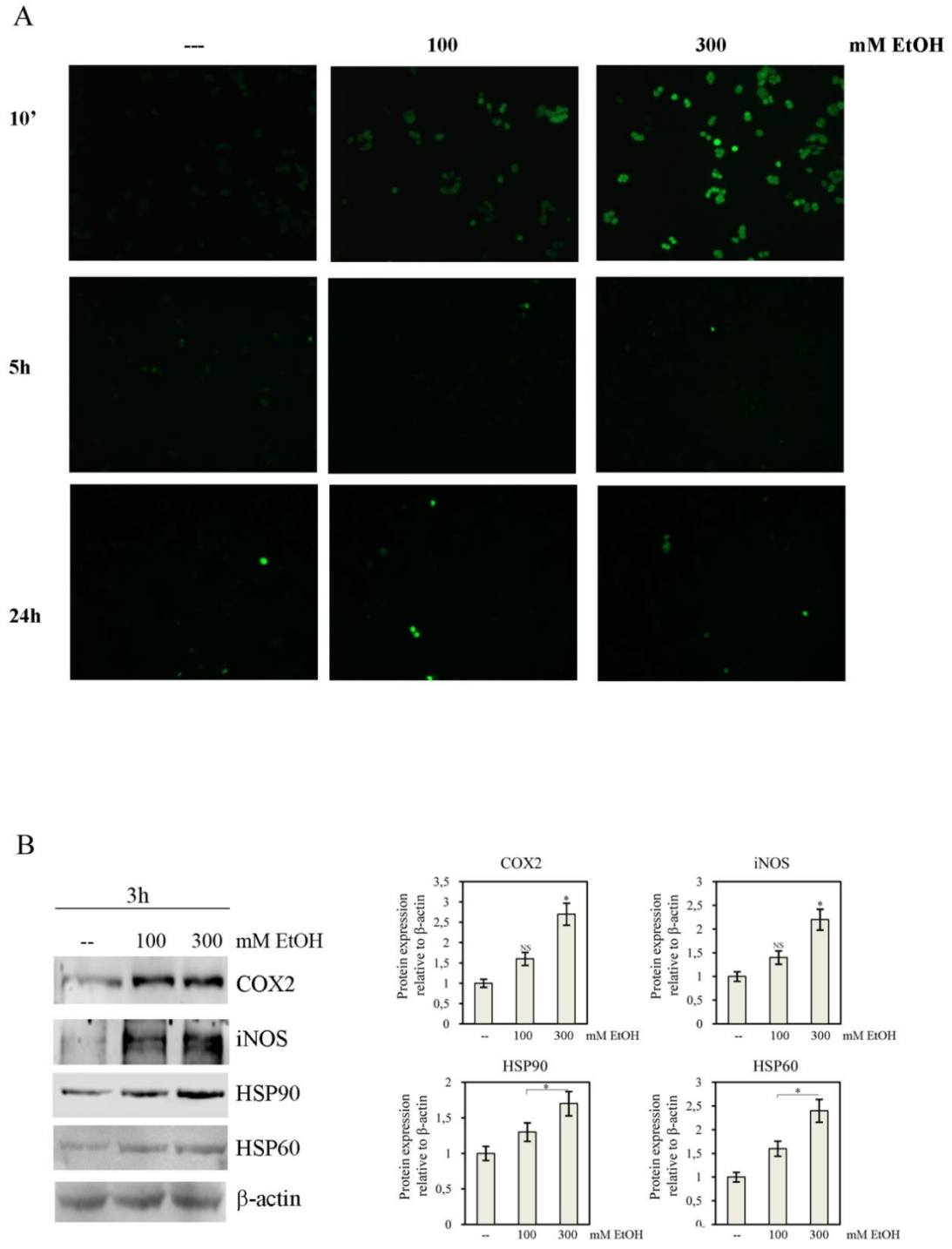

Fig. 2**. Ethanol induces oxidative stress in colon cancer cells.** (A) HCT116 cells ($7x10^3$) were treated with 100 or 300 mM EtOH for different times. ROS production was assayed by H_2DCFDA staining under a Leica DC300F microscope (100X original magnification) using a FITC filter. Three different visual fields were examined for each condition. Results are representative of three independent experiments. (B) Western blotting of iNOS, COX2, HSP60 and HSP90 in EtOH-treated HCT116 cells. All analyses were performed after 3h of treatment with 100 or 300 mM EtOH. The correct protein loading was ascertained by immunoblotting for β-actin. Representative blots of three independent experiments and densitometric analysis are shown. (*) $p<0.05$ compared to the untreated sample.

Treatment with EtOH induces ER stress in colon cancer cells

The endoplasmic reticulum (ER) is an organelle that plays multiple roles but in particular, it is specialized in the folding of proteins destined to the cellular membrane or to other organelles. ER is very sensitive to changes in intracellular homeostasis and extracellular stimuli. Alterations in the protein-folding environment cause accumulation of misfolded proteins in the ER and this profoundly affects a variety of cellular signaling processes, including reduction–oxidation (redox) homeostasis, energy production, inflammation, differentiation, and apoptosis. Under ER stress, ER actives a response known as Unfolded Protein Response (UPR) in order to resolve protein misfolding and reestablish an efficient protein-folding environment (Bravo R et al 2013). Current advances assert that production of reactive oxygen species (ROS) has been related to ER stress and the UPR. ROS show a critical role in many cellular processes and can be produced in the cytosol and in several organelles, including ER and mitochondria. Studies suggest that altered redox homeostasis in the ER is sufficient to cause ER stress, which could, in turn, induce the production of ROS in the ER and mitochondria (Siyan Cao S et al 2014).To evaluate the possible induction of endoplasmic reticulum stress the levels of ER stress markers, as Grp78 (BiP) and CHOP, were analyzed by western blotting. Grp78 is one of the best-described ER chaperones, which is also indicated as BiP. Grp78 is involved in many cellular processes, as translocation of new synthesized polypeptides across the ER membrane to facilitate protein folding and assembly, targeting of misfolded proteins for ER-associated degradation (ERAD), regulation of calcium homeostasis; moreover, it serves as an ER stress sensor. Grp8 is a master regulator of ER stress; it plays an antiapoptotic role and control the activation of UPR signaling (Wang M et al 2009).The transcription factor CHOP (CCAAT / enhancer binding protein (C / EBP) homologous protein) is considered one of the main sensors of endoplasmic reticulum stress. In particular, this factor is known to mediate cell death in response to stress; in fact, over-expression of CHOP has been reported to lead to cell cycle arrest and/or apoptosis. Furthermore, recent findings support that CHOP can promote autophagy by activating IRE1α (Shimodaira Y et al 2014). The densitometric analysis of the bands obtained in western blotting experiments showed that in HCT116 cells, EtOH up-regulated Grp78 levels already after 3h and the levels increased after 24h of treatment. In particular, compared to the control, in cells treated for 24h with 300 mM EtOH, the Grp78 level increased by 1.7 times (figure 3A). Data also showed that treatment with EtOH up

regulated CHOP already after 3h. After 24h its level remained higher (2.2-fold) than control in cells treated with 100 mM EtOH, while it lowered (0.8 fold) in cells treated with

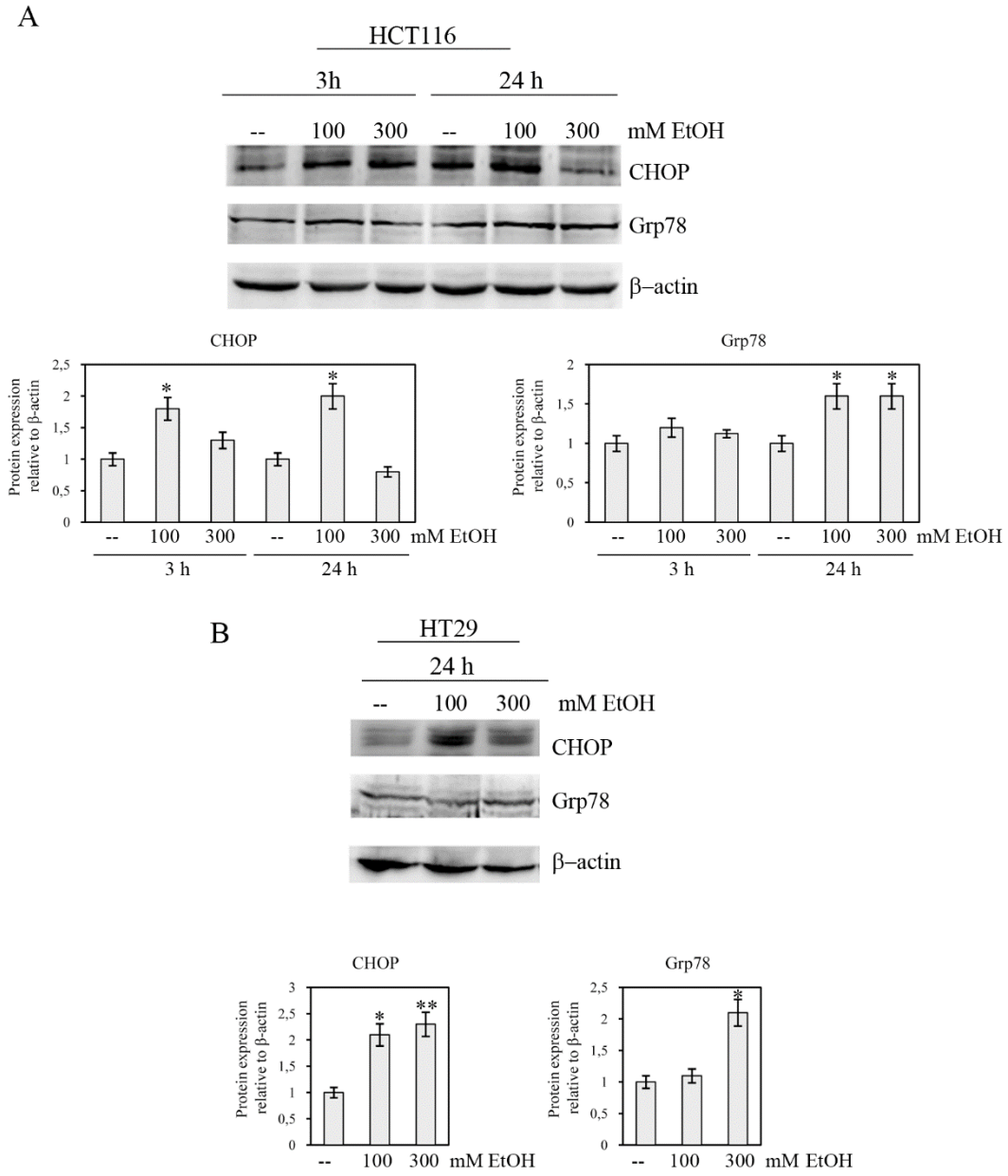

Fig. 3. **Ethanol induces ER stress in colon cancer cells**. HCT116 and HT29 cells ($1.5x10^5$) were treated for different times with 100 or 300 mM EtOH. Western blotting analyses of CHOP and Grp78 in HCT116 cells (A) or HT29 cells (B). The correct protein loading was ascertained by immunoblotting for β-actin. Representative blots of three independent experiments and densitometric analysis are shown. (*) $p<0.05$, (**) $p<0.01$ compared to the untreated sample.

300 mM EtOH. In the same way, EtOH increased the levels of ER markers also in HT29 cells, although the effects were observed after 24h of treatment (figure 3B).

Ethanol stimulates a prosurvival effect sustained by an autophagic flux in colon cancer cells

Under ER stress conditions, autophagy can be activated to degrade unfolded/aggregated proteins and maintain cell survival (Shimodaira et al 2014). To evaluate the possible induction of the autophagic process, the expression levels of the main markers, Beclin, LC3 and p62 were analyzed. The expression of the autophagic microtubule-associated protein light chain (LC3) was determined by western blotting. LC3 may be present in two forms: a cytosolic form (LC3-I) and a lipidated form (LC3-II). During the autophagic process, LC3-I is converted into LC3-II, which is the autophagosome membrane-bound form. As shown in figure 4A, in HCT116 cells both 100 and 300 mM EtOH induced the conversion of LC3-I into LC3-II.

In addition to LC3, the level of Beclin, a protein that plays an essential role in the formation of autophagosome, has been evaluated. The results indicated that the relative Beclin/β-actin ratio increased already after 3h of treatment with 100 or 300 mM EtOH and remained high after 24h.

The p62 protein is an ubiquitin-binding scaffold protein that is colocalized with ubiquitinated protein aggregates. The protein is able to polymerize through an N-terminal PB1 domain and can interact with ubiquitinated proteins through the UBA C-terminal domain. Furthermore, p62 is directly bound to the proteins of the LC3 family and it is degraded by autophagy. p62 has the role of connecting ubiquitinated proteins to autophagic machinery to allow their degradation in the lysosome. p62 is a marker of autophagic flow as it accumulates when autophagy is inhibited, while it is degraded when autophagy is induced. In my experimental conditions the level of p62 increased after 3h in EtOH-treated cells and then lowered after 24h, thereby indicating that the autophagic process is completed. The induction of the autophagic process was confirmed by fluorescence microscopic observations following staining the cells with monodansylcadaverin (MDC), a fluorescent molecule that highlights the presence of acidic vesicular organelles. As figure 4B shows, compared with control cells, in EtOH-treated HCT116 cells a large number of autophagic vacuoles appeared in the cytoplasm. MDC-positive fluorescent cells were already observed after 24h of treatment with either 100 or 300 mM EtOH. Furthermore the addition of the antioxidant N-acetylcysteine (NAC) to EtOH-treated HCT116 cells markedly reduced the presence of autophagic vacuoles (figure 4B) as well as cell viability (figure 4C). This result suggests that autophagy is activated in response to oxidative stress as a survival mechanism.

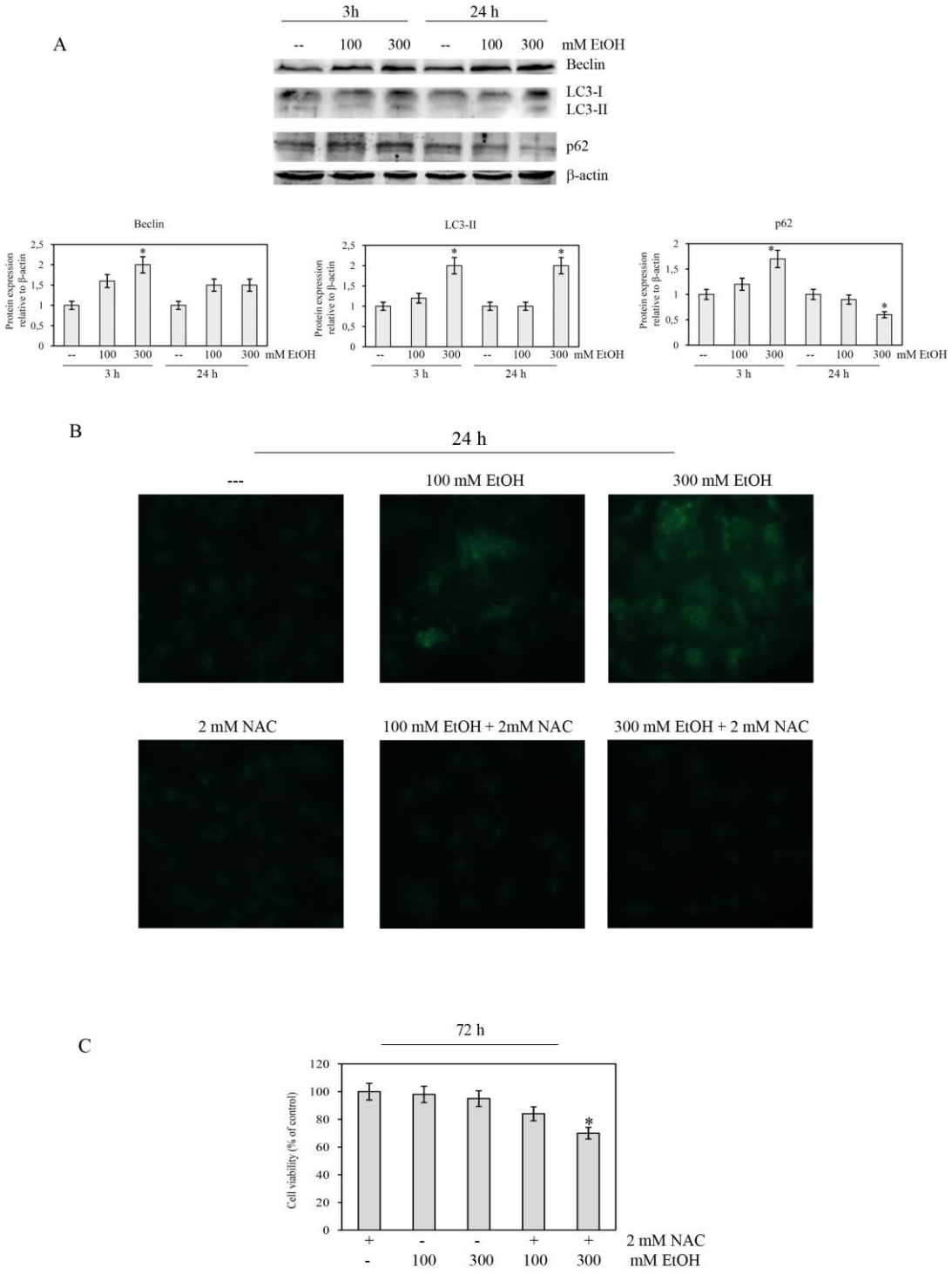

Fig. 4. **Ethanol induces authophagy in colon cancer cells. (**A) Western blotting analysis of Beclin, LC3 and p62 in HCT116 cells treated for different times with 100 or 300 mM EtOH.The correct protein loading was ascertained by immunoblotting for β-actin. Representative blots of three independent experiments and densitometric analysis are shown. (*) $p<0.05$ compared to the untreated sample. (B) HCT116 cells ($7x10^3$) were treated with 100 or 300 mM EtOH for 24 h in the presence or absence of 2 mM NAC . Autophagic vacuoles production was assayed by MDC staining under a Leica DC300F microscope (400X original magnification) using a DAPI filter. Three different visual fields were examined for each condition. Results are representative of three independent experiments. (C) HCT116 ($7x10^3$) were incubated in the presence of various doses of EtOH for different periods in the presence or absence of 2 mM NAC. Cell viability was assessed by MTT assay as described in Materials and Method section. Values are the means of three independent experiments.

EtOH increases antioxidant enzymes levels

Although EtOH induces ER and oxidative stress in colon cancer cells, no cytotoxic effects were observed, thus suggesting the activation of an antioxidant response. The nuclear factor erythroid 2–related factor 2 (Nrf2) is a member of the cap 'n' collar (CNC) subfamily of basic region leucine zipper (bZip) transcription factors, which is activated in response to oxidative stress. Nrf2 is a short-lived medium protein subject to proteolytic degradation catalyzed by the ubiquitin-proteasome proteolytic pathway (Kobayashi A et al 2005). Nrf2 is present in the cytosol in an inactive state linked to the Keap1 protein (Kelch-like erythroid cell-derived protein with CNC homology-associated protein 1), a component of the ubiquitin ligase complex. Two molecules of Keap1, once bound to Nrf2, form a trimeric complex whose structure accelerates the ubiquitination of lysine residues of Nrf2 and its subsequent proteasomal degradation (Zhang DD. et al. 2004). This proteolytic mechanism, under physiological conditions, serves to keep the concentrations of Nrf2 low in the cell. When oxidative stress increases in the cells, this result in a stabilization and activation of Nrf2. ROS, in fact, induce the oxidation of some cysteine residues of Keap1 and its consequent degradation. As consequence, Nrf2 dissociates from Keap1 to accumulate in the cytosol and subsequently migrate to the nucleus. At the nuclear level, Nrf2 forms transcriptionally active complexes with other proteins, such as the Mafs (musculoaponeurotic fibrosarcoma). The result is an increase in the transcription of cytoprotective and antioxidant genes, such as mitochondrial superoxide dismutase (MnSOD), catalase and hemeoxygenase-1 (HO-1). All these genes contain in their promoters the sequence ARE (antioxidant response element) recognized by the transcription factor Nrf2. As shown in figure 5A, EtOH increased Nrf2 levels in HCT116 cells compared to control cells. The effect is observed in cells treated with both doses of EtOH (100 and 300mM). In fact, in comparison with control cells the level of Nrf2 protein increased with 300 mM approximately by 1.5-fold after 3h and by 1.2 fold after 24h of treatment. Concomitantly, the same figure shows a decrease in the level of Keap1 in EtOH-treated HCT116 cells. I further evaluated whether EtOH promoted the Nrf2 nuclear localization in HCT116 cells. As shown in figure 5B, Western blot analysis revealed that EtOH treatment increased the expression of Nrf2 in nuclear fraction and concomitantly decreased the expression of Nrf2 in cytosolic fraction. Nuclear translocation of Nrf2 was further evaluated by confocal microscopy in which an increase of nuclear Nrf2 is clearly seen in cells exposed to EtOH (figure 5C). In light of these data, I evaluated if EtOH

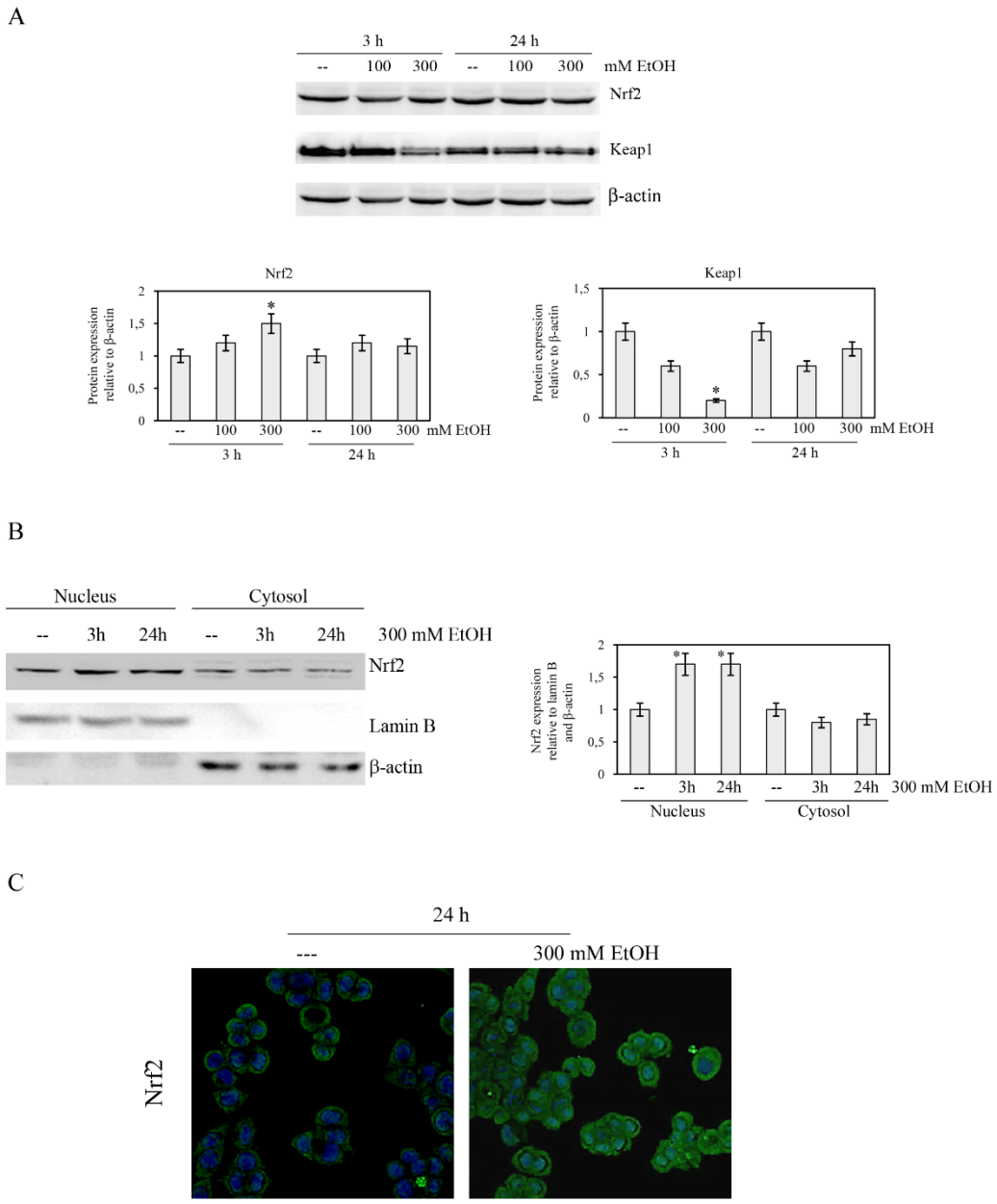

Fig. 5. **Ethanol induces activation of Nrf2 in HCT116 cells (**A) Western blotting analysis of Nrf2 and Keap1 in HCT116 cells treated for different time with 100 or 300 mM EtOH. The correct protein loading was ascertained by immunoblotting for β-actin. Representative blots of three independent experiments and densitometric analysis are shown. (*) $p<0.05$ compared to the untreated sample. (B) Western blotting analysis of Nrf2 in nuclear and cytosolic fractions after 3h and 24h of 300 mM EtOH treatment. The correct protein loading was ascertained by immunoblotting for Lamin B or β-actin. Representative blots of three independent experiments are shown. (*) $p<0.05$ compared to the untreated sample. (C) EtOH-induced nuclear translocation was observed under confocal microscope. Nrf2 detection was performed by incubating the cells with Nrf2 specific antibody followed by incubation with a TRITC-conjugated secondary antibody (red). The cells were also stained with DAPI (blue fluorescence) to visualize nuclear morphology. Each image shown is representative of 10 random fields observed. Original magnification 400X. The results are representative of three independent experiments.

influences the levels of MnSOD, catalase and HO-1, antioxidant enzymes, which are target of Nrf2. MnSOD, is an oxidoreductase that catalyzes the following reaction: $2O_2^- +2H^+ \rightleftharpoons O_2+H_2O_2$, removing the superoxide anion O_2^-, a highly toxic radical species.

Catalase is instead an enzyme devolved to the detoxification of hydrogen peroxide according to the following reaction: $2H_2O_2 \rightleftarrows O_2 + 2H_2O$.

The hemeoxygenase-1 is part of a family of enzymes responsible for the degradation of EME, a prosthetic group present in several important proteins such as hemoglobin, myoglobin and cytochromes. Degradation of EME catalysed by hemeoxygenase produces biliverdin (then converted into bilirubin, a powerful antioxidant),

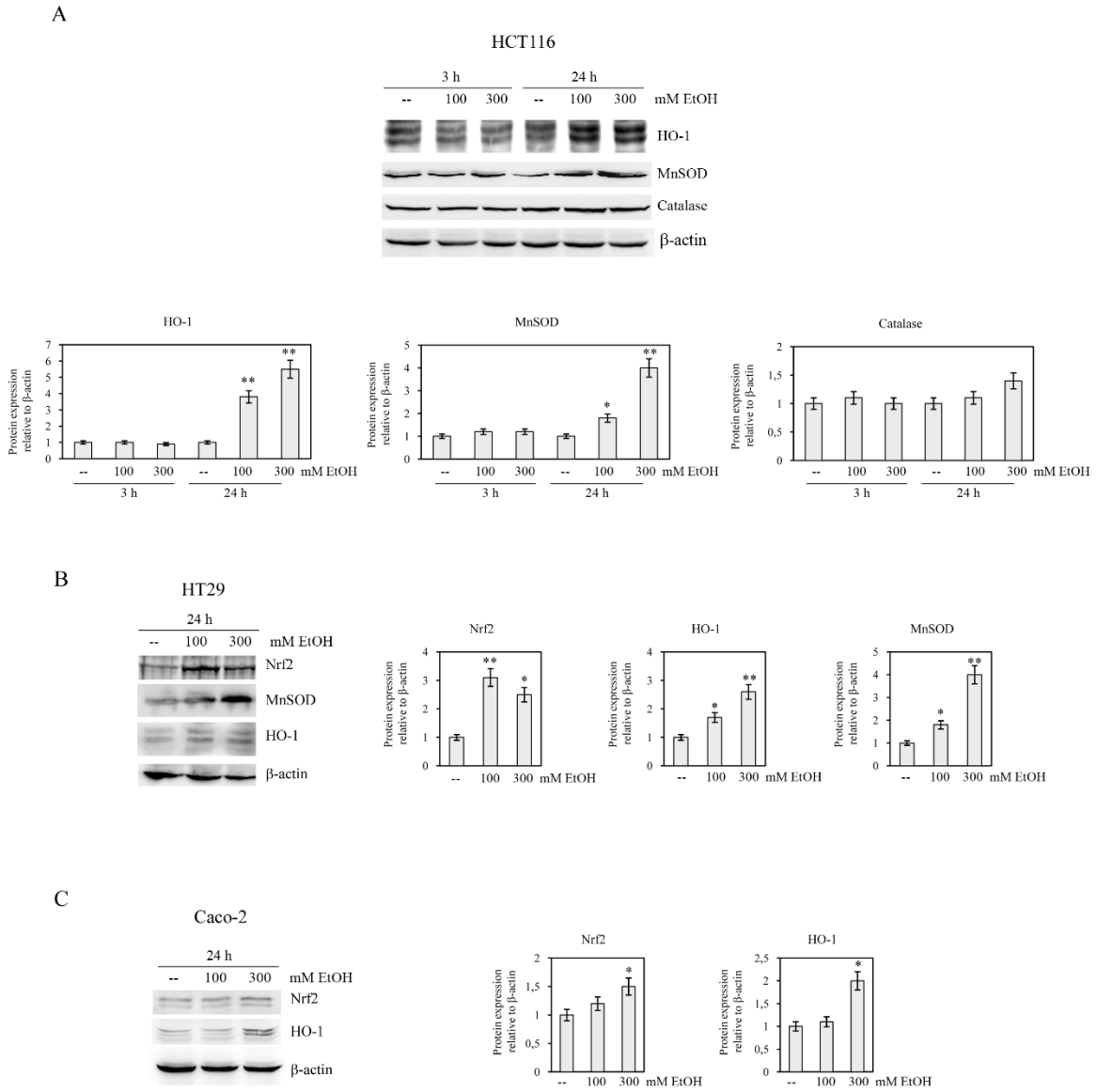

Fig. 6. **Ethanol increases the levels of antioxidant enzymes in HCT116 cells**. (A) Western blotting analysis of HO-1, MnSOD and Catalase in HCT116 cells treated for different times with 100 or 300 mM EtOH. (B) Western blotting analysis of Nrf2, MnSOD and HO-1 in HT29 cells treated for 24h with 100 or 300 mM EtOH. (C) Western blotting analysis of Nrf2 and HO-1 in Caco-2 cells treated for 24h with 100 or 300 mM EtOH. (A, B and C) The correct protein loading was ascertained by immunoblotting for β-actin. Representative blots of three independent experiments and densitometric analysis are shown. (*) $p<0.05$, (**) $p<0.01$compared to the untreated sample.

Fe^{2+} and carbon monoxide (CO). Fe^{2+} released from EME induces an increase in ferritin, which prevents the production of ROS by sequestrating the ion inside the cells.

To assess whether treatment with EtOH in HCT116 cells up regulated the levels of these enzymes, Western Blotting analysis was performed. As shown in figure 6A, treatment with EtOH increased, after 24h, the levels of MnSOD, catalase and HO-1. In particular, the increments for 300 mM EtOH-treated cells ranged by from 1.4 fold for catalase to 4 fold for MnSOD and to 5 fold for HO-1, respectively. The level of Nrf2 and its transcriptional targets were also evaluated in HT29 and Caco-2 cells. As shown in figure 6B an increase of Nrf2, MnSOD and HO-1 is observed in HT29 cells, whereas Caco-2 cells showed an increase of Nrf2 and HO-1(figure 6C). Overall, these results indicate that EtOH induces Nrf2 nuclear translocation and increases the levels of antioxidant enzymes MnSOD, catalase and HO-1. These events represent a cell response to oxidative stress induced by EtOH.

EtOH promotes HO-1 nuclear translocation

Further analysis were performed to assess whether EtOH also promotes HO-1 nuclear translocation. Although HO-1 is an ER-anchored protein, some studies showed HO-1 localization in other subcellular compartments (Nitti M et al 2017). There are several isoforms of hemeoxygenase. The isoform 1 (HO-1), is an inducible form of 32 kDa, which is induced in response to various stimuli, such as oxidative stress, hypoxia and inflammatory cytokines. Hemeoxygenase-1 plays its catalytic role in a membrane-bound form of the smooth endoplasmic reticulum. The binding of the HO-1 to the ER membrane occurs through its transmembrane carboxy-terminal segment (TMS). The increase in ROS as well as the presence of ER stress are events that favor the detachment of HO-1 from the endoplasmic reticulum and its migration into the nucleus in a truncated and enzymatic inactive form (Tibullo D et al., 2016). The protein responsible for the release of HO-1 from the ER membrane is the signal peptide peptidase (SPP), a 42 kDa glycoprotein belonging to the family of aspartyl proteases. SPP is also present in the endoplasmic reticulum (ER) membrane and catalyzes the intramembrane proteolysis of various substrate proteins including HO-1 in the presence of ER stress. It has been referred that in the nucleus HO-1is able to bind Nrf2 and protecting it from the action of glycogen synthase kinase 3β (GSK3β). This kinase phosphorylates Nrf2 at the level of its serine residues leading to its degradation through the ubiquitin ligase complex. Inhibition of GSK3β by HO-1 keeps active Nrf2 in the nucleus and consequently increases the transcriptional activation of all those genes coding for antioxidant proteins and for the same hemeoxygenase-1.To assess whether EtOH induced the nuclear translocation of HO-1, the level of this protein has been evaluated in nuclear and cytosolic extracts. Overall, the results demonstrated that EtOH promoted HO-1 translocation in the nucleus. In fact, as shown in figure 7A, the nuclear HO-1 levels increased after treatment with 300 mM EtOH already after 3h of treatment and concomitantly decreased the levels of the cytosolic fraction. HO-1 nuclear translocation after EtOH treatment was confirmed by confocal microscopy analysis. As observed in figure 7B, in the HCT116 cells treated with EtOH, the analysis of the image shows a significant presence of the HO-1 in the nucleus. Overall, the results obtained show that, following treatment with EtOH, HO-1 protein translocates into the nucleus.

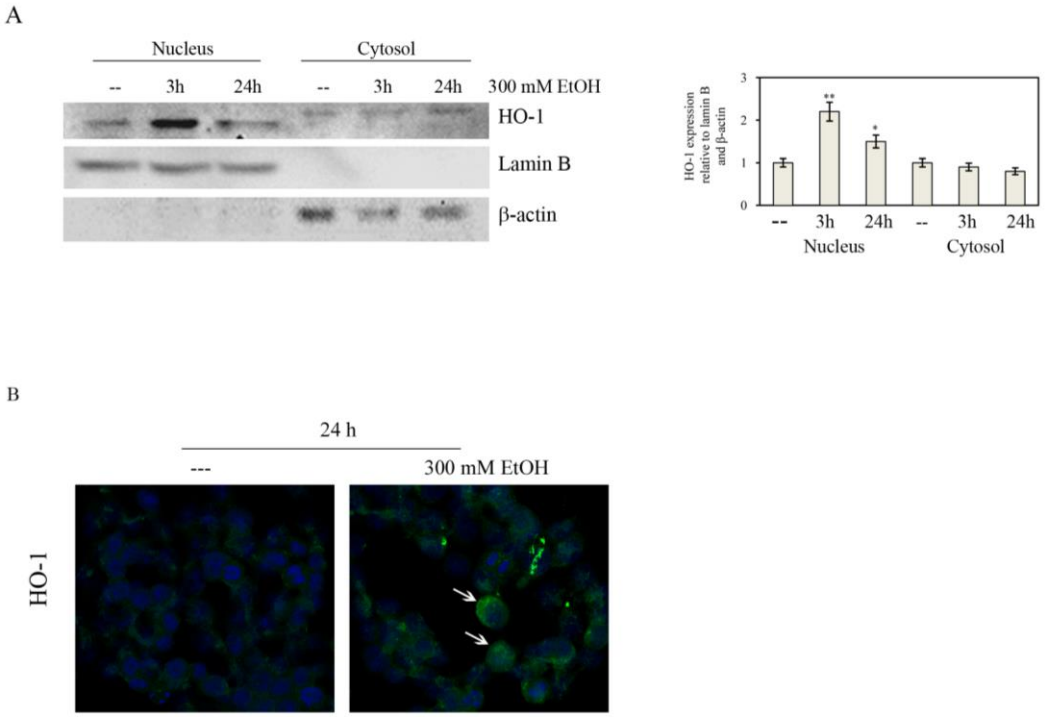

Fig. 7. **Ethanol induced translocation of HO-1 into the nucleus in HCT116 cells.** (A) Western blotting analysis of HO-1 in nuclear and cytosolic fractions after 3 and 24h of 300 mM EtOH treatment. The correct protein loading was ascertained by immunoblotting for LaminB or β-actin. Representative blots of three independent experiments are shown. (*) $p<0.05$, (**) $p<0.01$ compared to the untreated sample. (B) EtOH translocation of HO-1 from cytosol to nucleus were observed under confocal microscope. HO-1 detection was performed by incubation with specific antibody followed by secondary antibody conjugated to TRITC (red). The cells were stained with DAPI (blue fluorescence) to visualize nuclear morphology. Each image shown is representative of 10 random fields observed. Original magnification 400X. The results are representative of three independent experiments.

EtOH increased MMPs and VEGF in colon cancer cells

It has been shown that activation ofNrf2/HO-1 axis at the nuclear level is associated with tumor progression and invasiveness (Hsu FF et al 2014). In fact, activation of Nrf2 has been associated with up regulation of MMP9 in hepatocellular carcinoma (Zhang M et al, 2015) and hemeoxygenase-1 (HO-1) has been reported to promote angiogenesis (Dulak Jet al 2004). In light of the results obtained, the effect of EtOH on the levels of matrix

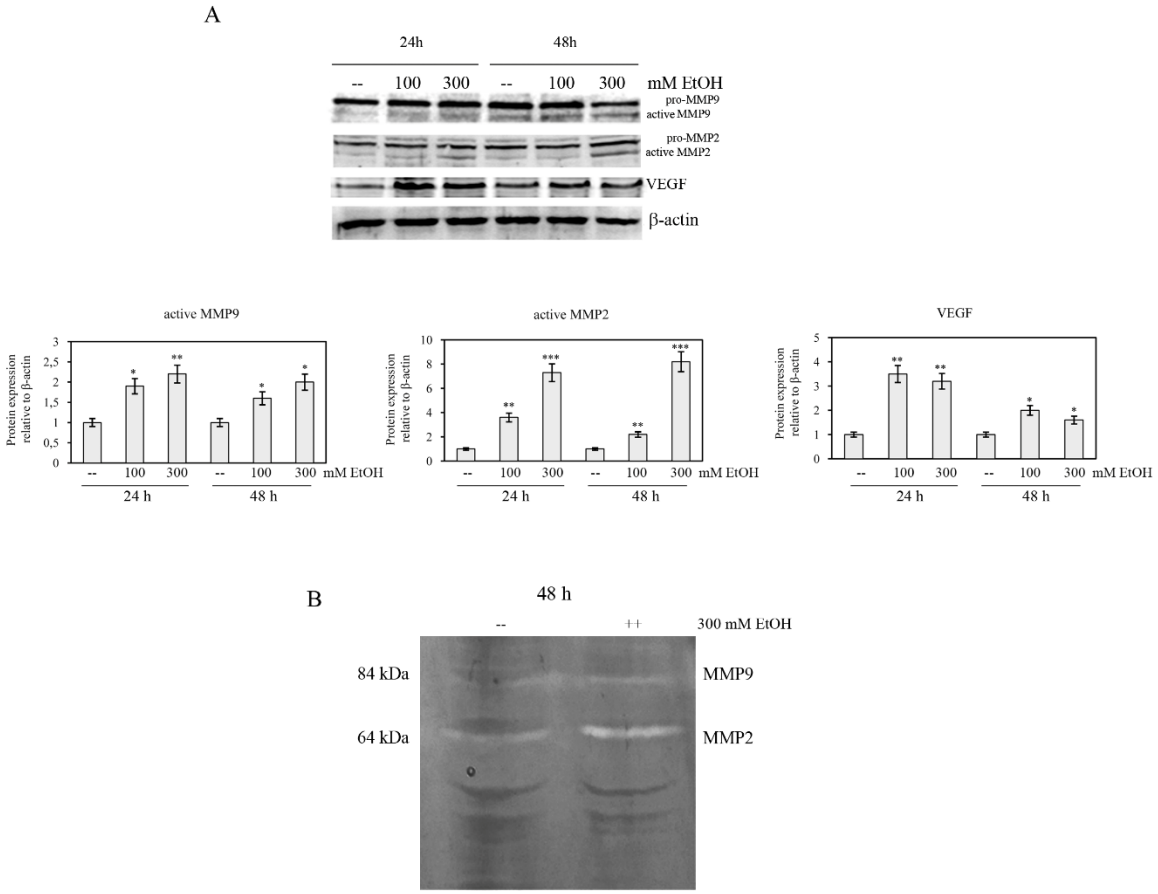

Fig. 8.**Ethanol increases the levels of VEGF and activates metalloproteases.** (A) Western blotting analysis of MMP-2, MMP-9 and VEGF in HCT116 cells treated for different times with 100 or 300 mM EtOH. The correct protein loading was ascertained by immunoblotting for β-actin. Representative blots of three independent experiments and densitometric analysis are shown. (**) $p<0.01$, (***) $p<0.001$compared to the untreated sample. (B) The activity of MMP-2 and MMP-9 in HCT116 cells treated with 300 mM EtOH for 48h was examined by gelatin zymography assay, as described in Materials and Method. The results are representative of three independent experiments.

metalloproteases 2 and 9 (MMP-2 and MMP-9) was assessed. The matrix metalloproteases are zinc dependent endopeptidases that degrade many components of the extracellular matrix, in particular the collagen IV and the lamin, allowing the cells to penetrate inside

and to reach other tissues. Although MMPs allows the leukocytes to easily reach the damaged tissues, activation of MMPs in tumor cells favors their migration.

Given that MMPs are critical to cell invasion and metastasis, the expression and activity of MMP-2 and MMP-9 of HCT116 cells treated with different concentrations of EtOH were examined by western blotting. The results showed that there is a significant increase in the protein expression of MMP-2 (7-fold) in HCT116 cells treated with 100 or 300 mM EtOH already after 24h of treatment (figure 8A). A minor increase was observed for MMP-9 (2-fold) after 48h. Subsequently, the activity of MMPs was explored following treatment with EtOH by gelatin zymography assay. As shown in figure 8B, there is a remarkable increase in the activity of MMP2 after 48h of exposure with 300mM EtOH. Finally, the effect of EtOH in HCT116 cells on the production of Vascular endothelial growth factor (VEGF) has been evaluated, since it has been shown that HO-1 is involved in the production of VEGF (Huang S et al 2015). VEGF is involved in angiogenesis and its production is more consistent in tumor epithelial cells to vascularize the growing tumor mass that would otherwise be in hypoxic conditions. Western blotting results show that treatment with 100 and 300 mM EtOH in HCT116 cells resulted in an increase in cytoplasmic VEGF levels after 24 and 48h of treatment (figure 8A).

Role of Nrf2/HO-1 axis in colon cancer survival and invasiveness

Recent studies suggest that in the nucleus HO-1 has no enzymatic activity but acts as a regulator of Nrf2 activity for the activation of antioxidant enzymes (Biswas C et al 2014). To determine the role of Nrf2 in protecting the cells by oxidative damage induced by EtOH in colon cancer cells, loss-of-function experiments were performed by transfecting HCT116 cells with siNrf2. The transfection efficiency was identified by western blot that showed a marked decrease in Nrf2 levels in HCT116 silenced cells both in control and in cells exposed to 300 mM EtOH for 24h. Moreover, I evaluated in Nrf2 silenced cells the levels of Nrf2 transcriptional targets: MnSOD and HO-1, as well as that of MMP2 and VEGF. As shown in figure 9, Nrf2 silencing markedly reduced the level of HO-1 both in control and in EtOH-treated cells.

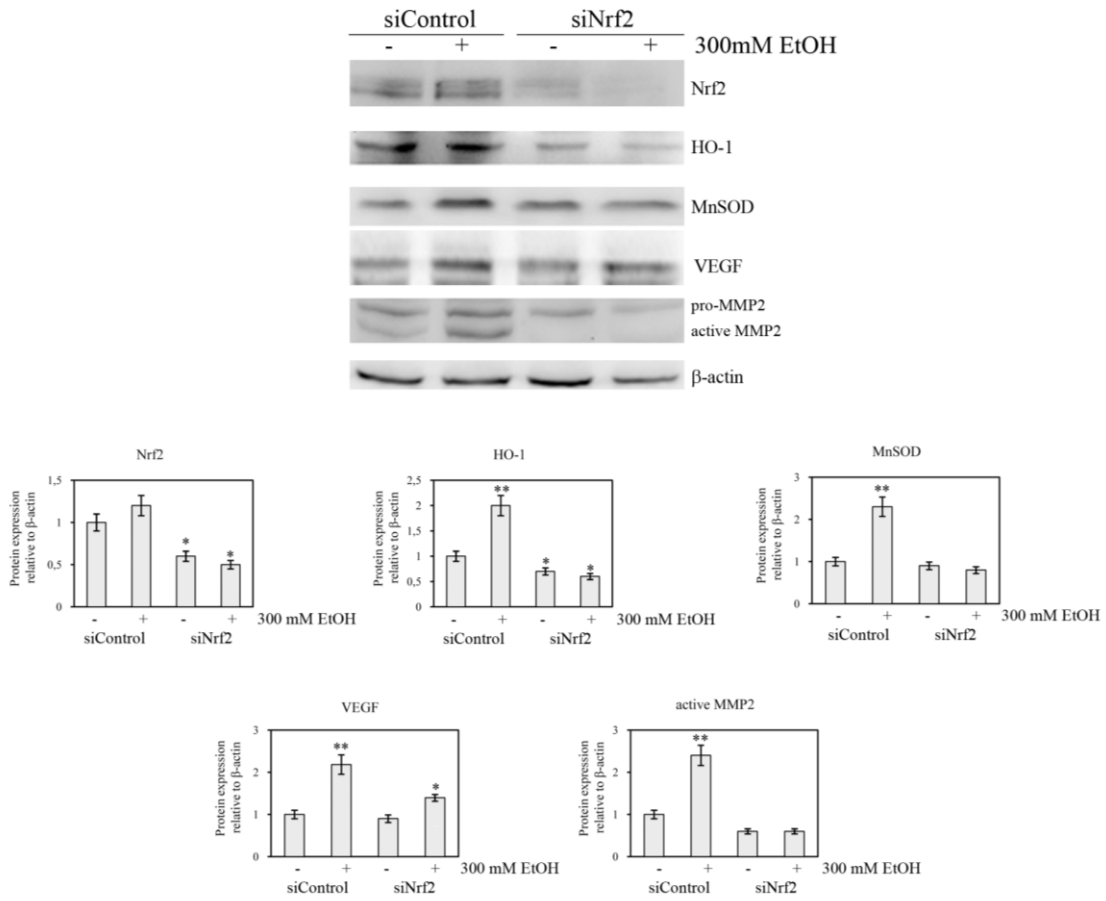

Fig. 9. **Nrf2-silencing represses the increase of antioxidant enzymes and MMP2 induced by EtOH treatmentin HCT116 cells**. HCT116 were transiently transfected with a nonspecific siRNA- or with Nrf2 specific siRNA as described in Material and Method. Nrf2-silencing prevented the effect of 300 mM EtOH on the increase of MnSOD, HO-1 and MMP2. Nrf2-silencing was ascertained by measuring the level of Nrf2 after transfection. The correct protein loading was tested by immunoblotting for β-actin. Representative blots of three independent experiments and densitometric analysis are shown. (*) $p<0.05$, (**) $p<0.01$ compared to the untreated sample.

In addition, a minor decrease in the level of the MnSOD was also observed in silenced cells compared to the control cells both in the presence and in absence of EtOH. Interestingly, I also demonstrated a drastic reduction of MMP2 levels after Nrf2 silencing both in control and in EtOH-treated cells. Simultaneously, the increase of VEGF induced

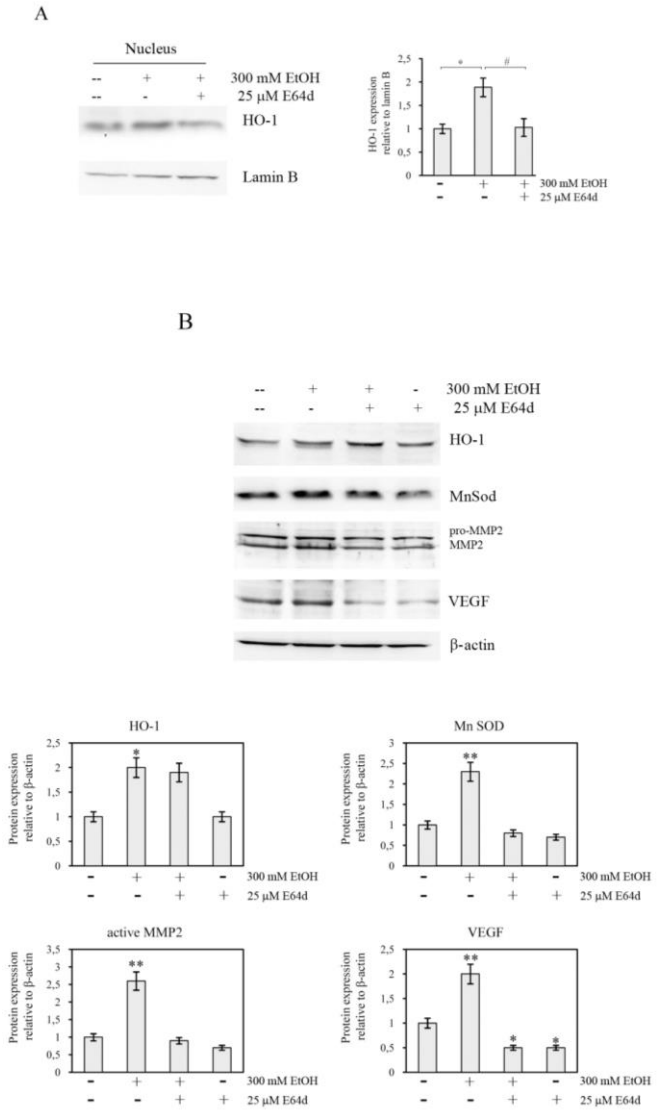

Fig. 10. **E64d represses the increase of antioxidant enzymes, MMP2 and VEGF induced by EtOH treatment in HCT116 cells**. HCT116 cells were pre-treated for 3h with E64d, then 300mM EtOH was added and the treatment was continued for another 24h. Nuclear and cytosolic fraction were prepared as reported in Material and Methods section. Western blotting analysis showed that E64d inhibited HO-1 nuclear translocation (A) and the appearance of the active forms of HO-1 as well as prevented the increase of MMP2, MnSOD and VEGF induced by 300 mM EtOH (B). The correct protein loading was ascertained by immunoblotting for β-actin. Representative blots of three independent experiments and densitometric analysis are shown. (*) $p<0.05$, (**) $p<0.001$ compared to the untreated sample.

in EtOH treated cells was reduced by Nrf2 silencing. Moreover, to evaluate if nuclear translocation of HO-1 translocation favor EtOH survival in colon cancer cells, HCT116 cells were treated with E64d, a compound that specifically inhibits the proteolytic cut and

nuclear translocation of HO-1. Combination of E64d with 300 mM EtOH was accompanied by a reduction in nuclear localization of HO-1 (figure 10A). In addition, E64d significantly reduced the cleaved form of HO-1 as well as suppressed the increase of MnSOD protein level induced by 300 mM EtOH treatment (figure 10B), thereby suggesting that nuclear HO-1 could regulate MnSOD expression. Interesting co-treatment with E64d and EtOH also causes a considerable reduction in MMP-2 and VEGF levels (figure 10B), thus suggesting that nuclear HO-1 could cooperate with Nrf2 to promote invasiveness of colon cancer cells.

Discussion

Many studies support the conclusion that chronic and heavy alcohol consumption not only increases the risk to develop CRC, but also promotes tumor progression (Zhao H et al 2018). My results provided evidence that in colon cancer cells activation of Nrf2/HO-1 axis in response to high doses of ethanol promotes cell survival and induces the acquisition of markers of aggressiveness.

Some evidence suggest that EtOH is able to increase ROS in different cell lines, including colon cancer cells, through the activity of ADH and CYP2E2 (Yang C et al 2005). In accordance with these observations, I showed that EtOH rapidly increased ROS production in different colon cancer cells (Caco-2, HCT116 and HT29 cells). In addition, I demonstrated an increase in the level of both iNOS and COX2, two enzymatic markers of inflammation, as well as the stress chaperones HSP90 and HSP60, thus suggesting that EtOH stimulates the production of oxidative stress.

Accumulation of unfolded/misfolded proteins in the ER stimulates a response known as Unfolded Protein Response (UPR) that is mediated by three types of ER transmembrane receptors: protein kinase RNA-like ER kinase (PERK), activating transcription factor 6 (ATF6) and inositol-requiring enzyme 1 (IRE1α). ER stress is known to contribute to alcoholic damage of major organs such as the liver, pancreas, and brain. The mechanisms responsible for alcoholic ER stress response are either directly or indirectly related to alcohol metabolism, including the production of acetaldehyde protein adducts and oxidative modification of proteins (Ji C 2012). My results provided evidence that high doses of EtOH up-regulate both Grp78 and CHOP, two ER stress markers. Grp78 is an ER chaperone protein that binds to ATF6, PERK, and IRE1α in healthy cells. When unfolded proteins accumulate, Grp78 dissociates from these proteins that activate down-stream signaling genes to initiate UPR. CHOP is a transcription factor whose expression markedly increases in response to ER stress and which mediates induction of apoptosis under prolonged ER stress. The observation that the level of CHOP lowered after 24h of treatment with 300 mM EtOH suggest that in my conditions ER stress has not been correlated with the induction of apoptotic cell death. Despite the induction of oxidative and ER stress in my system, MTT and clonogenic assay showed that colon cancer cells survive also after prolonged treatment with high doses of EtOH, thereby suggesting the activation of survival mechanisms. Accumulating evidence suggest that activation of autophagy, which degrades proteins in organelles such as the mitochondria and the ER, can play a

protective role against the toxic effects of ER stress (Shimodaira Y et al 2014). Interestingly, recent studies showed that alcohol consumption activates in CRC patients' autophagy and that this event increased risk of tumor metastasis (Zhao H et al 2018).

It is now known that ER stress can activate autophagy and there is considerable crosstalk between the ER and autophagy. It has been reported that ER stress activates PERK, a kinase which in turn promotes the phosphorylation of eIF2α to induce the activation of LC3, an autophagosome marker. Moreover, Shimodaira et al. (Shimodaira Y et al 2014) demonstrated that activation of CHOP, an ER marker, CHOP promotes autophagy in colon cancer cells by activating inositol-requiring enzyme 1α (IRE1α). My results provided evidence that in HCT116 colon cancer cells EtOH activated LC3 by promoting the cleavage of its cytosolic form LC3-I to LC3-II, as well as increased the level of Beclin, a molecule involved in the initiation of autophagosome formation. The involvement of autophagy was also confirmed by the augmented accumulation of acidic intracellular compartments. Moreover, the observation that the level of p62 protein, a marker of autophagic degradation, decreased after 24h of EtOH treatment, suggested that the compound triggered in my conditions a complete autophagic response. This effect is prevented by the addition of the antioxidant N-acetylcysteine, thus suggesting that autophagy is activated as a survival mechanism in response to oxidative stress.

Concomitantly to oxidative and ER stress production, I showed a significant activation of Nrf2, a transcription factor which acts as a key regulator of antioxidant-responsive genes (Kim J et al 2016). Under my experimental conditions, EtOH promoted translocation of Nrf2 into the nucleus, as suggested by the increase of nuclear Nrf2 content already at 3h following EtOH treatment. This event was associated with a reduction in the level of Keap1 (Kelch-like ECH-associated protein1), a protein which in unstressed condition sequester Nrf2 in a cytoplasmic complex leading to its ubiquitination and consequent proteasomal degradation (Dodson M et al 2015). Nuclear activation of Nrf2 by EtOH could be promoted by oxidative and ER stress. In line with this conclusion, it has been reported that induction of oxidative stress triggers Nrf2 nuclear import through the oxidation of redox-sensitive cysteines within Keap1. Moreover, phosphorylation of Nrf2 by PERK, a kinase activated following the accumulation of unfolded proteins in the endoplasmic reticulum (ER), promotes dissolution of Nrf2/Keap1 complexes and Nrf2 nuclear import. Finally, a relationship between autophagy and Nrf2 activation has been also demonstrated. In fact, recent evidence revealed that the autophagic protein p62 interacts with Keap1 favoring its dissociation from Nrf2 (Katsuragi Y et al 2016).

Nrf2 is the main antioxidant system in the cells. After nuclear translocation, Nrf2 form transcriptionally active complexes with other proteins, such as Mafs, thereby transactivating the expression of a battery of genes encoding antioxidant enzymes such as MnSOD, catalase and HO-1. My data indicated that the level of both SOD and HO-1 was significantly up-regulated after EtOH treatment in colon cancer cells, suggesting a protective role of these enzymes against EtOH-induced oxidative stress. The observation that by silencing Nrf2, EtOH-induced HO-1 and SOD increased expression was significantly reduced, suggested that this effect was mediated by activation of the transcription factor Nrf2.

Interestingly, my data also provided evidence that EtOH promotes HO-1 nuclear translocation already after 3h of treatment. HO-1 is a stress-inducible enzyme localized in the endoplasmic reticulum which exerts anti-oxidant and anti-inflammatory effects through the production of carbon monoxide (CO) and biliverdin. Recent evidences suggest that HO-1 can translocate in a truncated form into the nucleus where it binds to transcription factors favoring gene transcription (Biswas C et al 2014). Nuclear expression of HO-1 has been detected in several tumors and it has been correlated with tumor growth and invasion (Nitti M et al 2017). To demonstrate whether nuclear translocation of HO-1 is involved in cell survival of EtOH-treated colon cancer cells, I treated HCT116 cells with EtOH in combination with E64d, a cysteine protease inhibitor. Inhibition of the proteolytic cleavage necessary for HO-1 nuclear translocation significantly reduced the EtOH-induced HO-1 nuclear translocation increase as well as prevented EtOH-induced MnSOD increase, showing that HO-1 nuclear cooperates with Nrf2 to stimulate antioxidant response and colon cancer cell survival. This suggestion is in accordance with some recent evidence which demonstrated that nuclear HO-1 modulates the activation of Nrf2, leading to activation of antioxidant genes. Interestingly, it has been shown that nuclear HO-1 prevents GSK3β induced phosphorylation of Nrf2 leading to its stabilization and activation (Biswas C et al 2014).

Activation of the Nrf2/HO-1 axis is a double-edged sword in cancer. The decrease of oxidative stress by activation of antioxidant response by both Nrf2 and HO-1 can prevent the development of tumors by counteracting the genotoxic damage induced by ROS. In line with this consideration, several dietary phytochemicals exert cancer preventive effect by activating Nrf2/HO-1 axis. Moreover, activation of Nrf2 reduced chronic inflammation which has been correlated with CRC induction (Ahmed SM et al 2017). On the other hand, the activation of Nrf2 antioxidant response in tumor cells can promote the survival of cancer cells by creating an optimal environment for cell growth.

Overexpression of Nrf2 has been detected in primary CRC and metastatic tissue relative to normal colon and contributes to chemo-resistance in CRC cell lines (Sadeghi MR et al 2017). In addition, it has been reported that Nrf2 increased CRC risk by promoting colonic inflammation or promoting angiogenesis and uncontrolled proliferation. Moreover, HO-1 overexpression has been associated with a more aggressive behavior of tumors and poor prognosis in various cancers.

Western blotting and zymography analyses demonstrated that EtOH increased both the levels and the activity of MMP-2 and 9, two enzymes involved in tumor progression. Moreover, EtOH up-regulated VEGF, the main angiogenetic factor. To clarify whether the activation of MMPs and VEGF was regulated by Nrf2/HO-1 axis, I evaluated the effect of EtOH after Nrf2 silencing or in the presence of E64d, the cysteine protease inhibitor able to inhibit either the proteolytic cut or the nuclear translocation of HO-1. Interestingly, Nrf2 silencing prevented EtOH-induced MMP-2 increase and reduced VEGF increase. Moreover, inhibition of HO-1 nuclear translocation by E64d addition counteracted the effect of EtOH on both MMP-2 and VEGF, thus suggesting a role of Nrf2/HO-1 axis in colon cancer progression.

Collectively, my findings demonstrate that high doses of EtOH enhanced in colon cancer cells autophagy and activation Nrf-2/HO-1 axis. These events sustain the survival of cancer cells by protecting the cells from oxidative and ER stress induced by EtOH. Moreover, the activation of Nrf2/HO-1 axis seems to be also responsible for colon cancer progression through the acquisition of markers of invasiveness, like MMP-2 and VEGF.

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