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Chemopreventive effects of eicosapentaenoic acid free fatty acid in a murine model of colitis-associated colorectal cancer

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AA	Arachidonic acid
ACF	Aberrant crypt foci
AFAP	Attenuated familial adenomatous polyposis
AOM	Azoxymethane
APC	Adenomatous polyposis coli
BHT	Butylated hydroxytoluene
CAC	Colitis-associated colorectal cancer
CD	Chron's disease
CGI	CpG island hypermethylation
CIMP	CpG islands methylator phenotype
CIN	Chromosomal instability
COX-2	Cyclooxygenase-2
CRC	Colorectal cancer
DAB	3,3'-Diaminobenzidine
DHA	Docosahexaenoic acid
DMH	Dimethyl hydrazine
DPA	Docosapentaenoic acid
DSS	Dextran sodium sulfate
DTT	Dithiothreitol
EPA	Eicosapentaenoic acid
EPA-FFA	Eicosapentaenoic acid-free fatty acid
FAP	Familial adenomatous polyposis
FFA	Free fatty acid
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GC-MS	Gas chromatography–mass spectrometry
H & E	Hematoxylin & Eosin
HGD	High-grade dysplasia
HNPCC	Hereditary nonpolyposis colorectal cancer
HRP	Horseradish peroxidase
HTF	High taxonomic fingerprint

IBD	Inflammatory bowel disease
IFN-γ	Interferon-γ
IHC	Immunohistochemistry
IL-10	Interleukin 10
IL-17	Interleukin-17
IL-1β	Interleukin-1β
IL-6	Interleukin-6
iNOS	Inducible nitric oxide synthase
KO	Knockout
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LC-PUFAs	Long chain polyunsaturated fatty acids
LDR	Ligation detection reaction
LGD	Low-grade dysplasia
LOH	Loss of heterozygosity
LOX	Lipoxygenase
LTs	Leukotrienes
LXs	Lipoxins
MAP	MUTYH-associated polyposis
MINT	Methylated-in-tumor
miRNAs	microRNAs
MMP	Metalloproteinase
MMR	Mismatch repair
MSI	Microsatellite instability
MUFAs	Monounsaturated fatty acids
NICD	Notch intracellular domain
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PGE2	Prostaglandin E2
PGE3	Prostaglandin E3
PGE-M	PGE metabolite
PGs	Prostaglandins
PUFAs	Polyunsaturated fatty acids

qRT-PCR	Quantitative real time PCR
ROS	Reactive oxygen species
SFAs	Saturated fatty acids
TBS	Tris-buffered saline
TNF-α	Tumor necrosis factor-α
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick-end labeling
TXs	Thromboxanes
UC	Ulcerative colitis
UDG	Uracil-DNA-Glycosylase
WT	Wild-type

ABSTRACT

Inflammatory bowel diseases are associated with increased risk of developing colitis-associated colorectal cancer (CAC). Epidemiological data show that the consumption of ω-3 polyunsaturated fatty acids (ω-3 PUFAs) decreases the risk of sporadic colorectal cancer (CRC). Importantly, recent data have shown that eicosapentaenoic acid-free fatty acid (EPA-FFA) reduces polyps formation and growth in models of familial adenomatous polyposis. However, the effects of dietary EPA-FFA are unknown in CAC. We tested the effectiveness of substituting EPA-FFA, for other dietary fats, in preventing inflammation and cancer in the AOM-DSS model of CAC. The AOM-DSS protocols were designed to evaluate the effect of EPA-FFA on both initiation and promotion of carcinogenesis. We found that EPA-FFA diet strongly decreased tumor multiplicity, incidence and maximum tumor size in the promotion and initiation arms. Moreover EPA-FFA, in particular in the initiation arm, led to reduced cell proliferation and nuclear β-catenin expression, whilst it increased apoptosis. In both arms, EPA-FFA treatment led to increased membrane switch from ω-6 to ω-3 PUFAs and a concomitant reduction in PGE2 production. We observed no significant changes in intestinal inflammation between EPA-FFA treated arms and AOM-DSS controls. Importantly, we found that EPA-FFA treatment restored the loss of Notch signaling found in the AOM-DSS control, resulted in the enrichment of Lactobacillus species in the gut microbiota and led to tumor suppressor miR34-a induction. In conclusion, our data suggest that EPA-FFA is an effective chemopreventive agent in CAC.

1.INTRODUCTION

1.1 Colorectal cancer

Colorectal cancer (CRC) identifies a malignant transformation affecting the colon or the rectum, which are the first and the final sections of the large intestine, respectively. In most cases (~ 95%) colorectal cancer occurs as adenocarcinoma originated by glandular epithelium, while rare types of CRC include melanoma, sarcoma, carcinoid, squamous cell carcinoma or lymphoma (DiSario et al., 1994). Constituting the third most commonly diagnosed cancer in men and the second in females, CRC represents one of the major type of cancer worldwide (Jemal et al, 2011). In 2014 the estimated number of newly diagnosed CRC cases in the United States was 136.830, of which 50.310 dead (Siegel et al., 2014). Colorectal cancer incidence and death rate increase with the age. It has been estimated that approximately 90% of new cases were diagnosed in adults 50 years old or older (Haggar and Boushey, 2009). Interestingly, there is a disparity in CRC incidence in relation to the geographical distribution characterized by the highest frequency in economically developed countries, in particular Australia/New Zealand, United States and Europe, respect to developing countries with a lower incidence in Africa and South-Central Asia (Figure 1). Indeed, the probability to develop CRC in the more developed areas results to be higher respect the less developed areas (Jemal et al., 2011) This disparity in CRC incidence rates among the countries suggest the crucial role of lifestyle and environment as crucial risk factors in CRC development. Among the modifiable factors occurring in the promotion of CRC, the dietary habits, obesity, cigarette smoking and heavy alcohol consumption represent the main determinants (Huxley et al., 2009). However, although environmental factors play a critical role in the etiology of CRC, inheritance or genetic predisposition, as well as a bowel chronic inflammatory state also importantly contribute to the onset and progression of CRC. According to the disease background there are three main subsets of CRC: sporadic, hereditary or familial, and inflammatory-driven CRC.

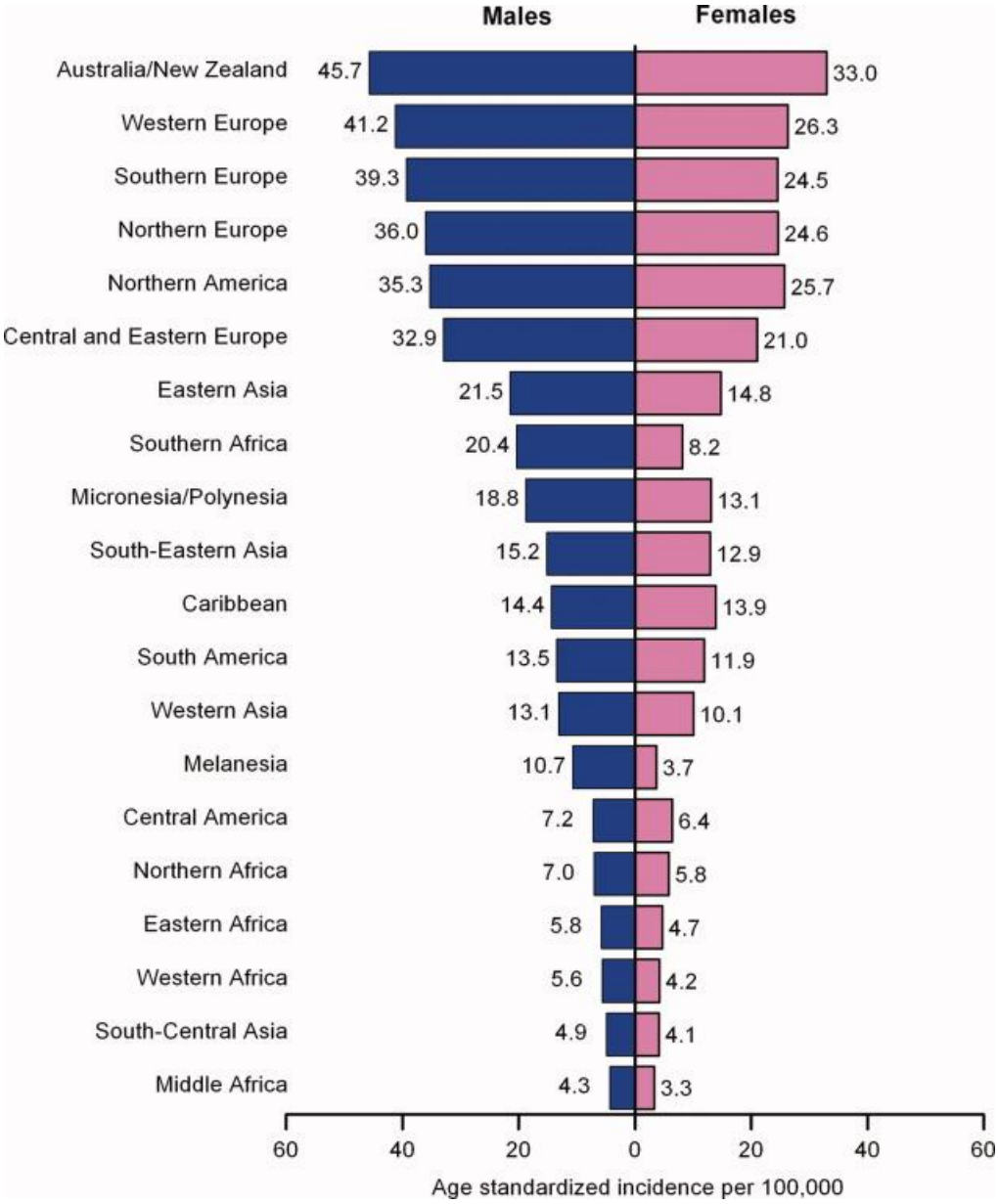

Figure 1 Colorectal cancer incidence rates by sex and world area

(Jemal et al, 2011.)

1.1.1 Sporadic colorectal cancer

CRC is a heterogeneous disease which can occur sporadically in absence of a defined inherited and familial component or a predisposing disease, in the 75-80% of cases (Morán et al., 2010). The development of sporadic CRC evolves through a multistep process called 'adenoma-carcinoma sequence' in which a carcinoma develop from a preexisting benign adenoma through morphological changes accompanied by an accumulation of genetic and epigenetic alterations (Fearon and Vogelstein, 1990). Aberrant crypt foci (ACF) were identified as the early detectable dysplastic lesion in the colon (Siu et al., 1997) and defined as colonic crypts which appear microscopically elevated, with a circumscribed lumen and increased epithelial thickness (Alrawi et al., 2006). Sustained by a protumorigenic microenvironment which promote the acquisition of additional mutations, only a small fraction of ACF, can evolve in adenoma and then to colorectal carcinoma (Alrawi et al., 2006).

Three main pathways resulted primarily involved in the onset of CRC: chromosomal instability (CIN), microsatellite instability (MSI) and CpG islands methylator phenotype (CIMP). The traditional CIN pathway follows the model proposed in 1990 by Fearon and Vogelstein, which pioneeringly described the temporal sequence of specific genetic alterations in the context of the 'adenoma-carcinoma' sequence (Fearon and Vogelstein, 1990) (Figure 2a). In this model inactivating mutations in Adenomatous polyposis coli (APC) gene or loss of chromosome 5q which include the APC gene are an early event in the initiation of CRC. The importance of APC gene in the pathogenesis of CRC is due to its fundamental role in the suppression of the Wnt signaling pathway (Cadigan and Liu, 2006). Different events including truncating mutations, deletions, loss of heterozygosity (LOH) or epigenetic silencing may cause the inactivation of APC impairing its binding with β-catenin. In this condition, β-catenin is able to escape from the degradation pathway with a subsequent nuclear accumulation and transcription of oncogenes such as CYCLIN-D1 and C-MYC (Cadigan and Liu, 2006). Mutations in APC are followed by mutations in the oncogene KRAS which generally occur in the intermediate adenoma stage (Fearon and Vogelstein, 1990). KRAS encode for a guanosine triphosphate/guanosine diphosphate binding protein whose aberrant activation led to constitutive signaling of the downstream Ras/Raf/Mek/Erk pathway which critically regulate cell proliferation, differentiation, invasion and apoptosis

(Smith et al., 2002), (Smith et al., 2010). In addition, in the advanced adenoma, LOH which affect SMAD2,SMAD4 and *DCC* genes characterize the CIN phenotype (Colussi et al., 2013). Mutations in p53 gene or deletions of chromosome 17p harboring this gene, constitute a later event occurring during the malignant transformation from adenoma to carcinoma in situ (Fearon and Vogelstein, 1990). As a consequence of chromosomal alteration CIN tumors were typically characterized by aneuploidy and the prevalent localization of these tumors is the left, or distal, side of the colon (Grady and Carethers, 2008). Although the model proposed by Fearon and Vogelstein has laid the foundation for the understanding of the molecular mechanisms underlying the development of sporadic CRC, subsequently it has been demonstrated that only 6.6% of CRC tumors was characterized by concurrent mutations in APC, KRAS and p53, while 38% of tumors had mutations in only one of these three genes (Smith et al., 2002). Moreover, it was also found that more frequently (27.1%) there is a combination of APC and p53 mutations, whereas the occurrence of mutations in both p53 and KRAS genes is a rare event (Smith et al., 2002). These observations have led to consider the development of CRC in a more complex view. Indeed, alterations in additional pathways, as well as epigenetic changes in cancer related genes and modulation of non-coding RNA importantly contribute to the malignant transformation (Pancione et al., 2012). Shortly after the description of the CIN model of CRC, another model of genetic instability the so called 'MSI pathway' was introduced to explain the genetic alterations which occur during CRC development (Thibodeau et al., 1993) (Figure 2b). Microsatellites consist of repetitive units of 1-6 bases flanked by unique sequences, which are scattered around the genome (Thibodeau et al., 1993). The MSI phenotype arise from germline mutations in one or more of the genes encoding for components of the DNA mismatch repair (MMR) system (hMLH1, hMSH2, hMSH6, and hPMS2) (Thibodeau et al., 1993). Due to an improper recognition and elimination of mispaired base-pairs, defects in these genes are responsible for several insertions or deletions which affect microsatellites sequences within DNA (Ward et al., 2001). MSI account for approximately 15-20% of sporadic CRC and is the genetic cause of Lynch syndrome (Ward et al., 2001). CRCs characterized by the MSI phenotype frequently appear in the right colon and are generally diagnosed at a lower pathological state compared with CIN cancers (Pancione et al., 2012). Noteworthy, the MSI phenotype is associated with

a survival improvement and morphologically characterized by the presence of tumor infiltrating lymphocytes (Michael-Robinson et al., 2001).

More recently, Toyota et al. proposed the term CIMP phenotype to define a different subset of CRCs which consistently show widespread CpG island hypermethylation, at seven different loci defined methylated in tumors (MINT) (Toyota et al., 1999). In this model, epigenetic events rather than changes in the DNA sequence, promote the development of CRC. Importantly, methylation at different MINT loci resulted significantly correlated with hMLH1 and CDKN2A (p16) methylation (Pancione et al., 2012) Then, further loci appeared to be involved in the CIMP phenotype and the CIMP panel was expanded including: CACNA1G, IGF2, NEUROG1, RUNX3, and SOCS1 (Weisenberger et al., 2006). Importantly, Weisenberger and colleagues found that the CIMP phenotype frequently occur in presence of BRAF V600E mutations, while the relationship with KRAS mutations is uncertain (Weisenberger et al., 2006). An overlap between CIMP positive tumors and MSI-CRCs was also observed. In particular, hMLH1 methylation, which is the main mechanism of MMR genes silencing, frequently occur during CIMP tumorigenesis. CIMP CRCs have frequent proximal localization, often in the right colon, and are associated with the female sex and aging (Hawkins et al., 2002). Recent interesting evidences further revised the classical sequence of CRC development emphasizing the malignant potential of hyperplastic lesions which can evolve in a serrated polyp neoplasia and finally in an MSI-carcinoma (O'Brien et al., 2006). In these tumors, BRAF is viewed as a 'gatekeeper' gene and its mutations are considered as a specific marker for the serrated polyp pathway. Importantly, the occurrence of the CIMP phenotype is an early event in this sequence, while MSI develops later (O'Brien et al., 2006) (Figure 2c).

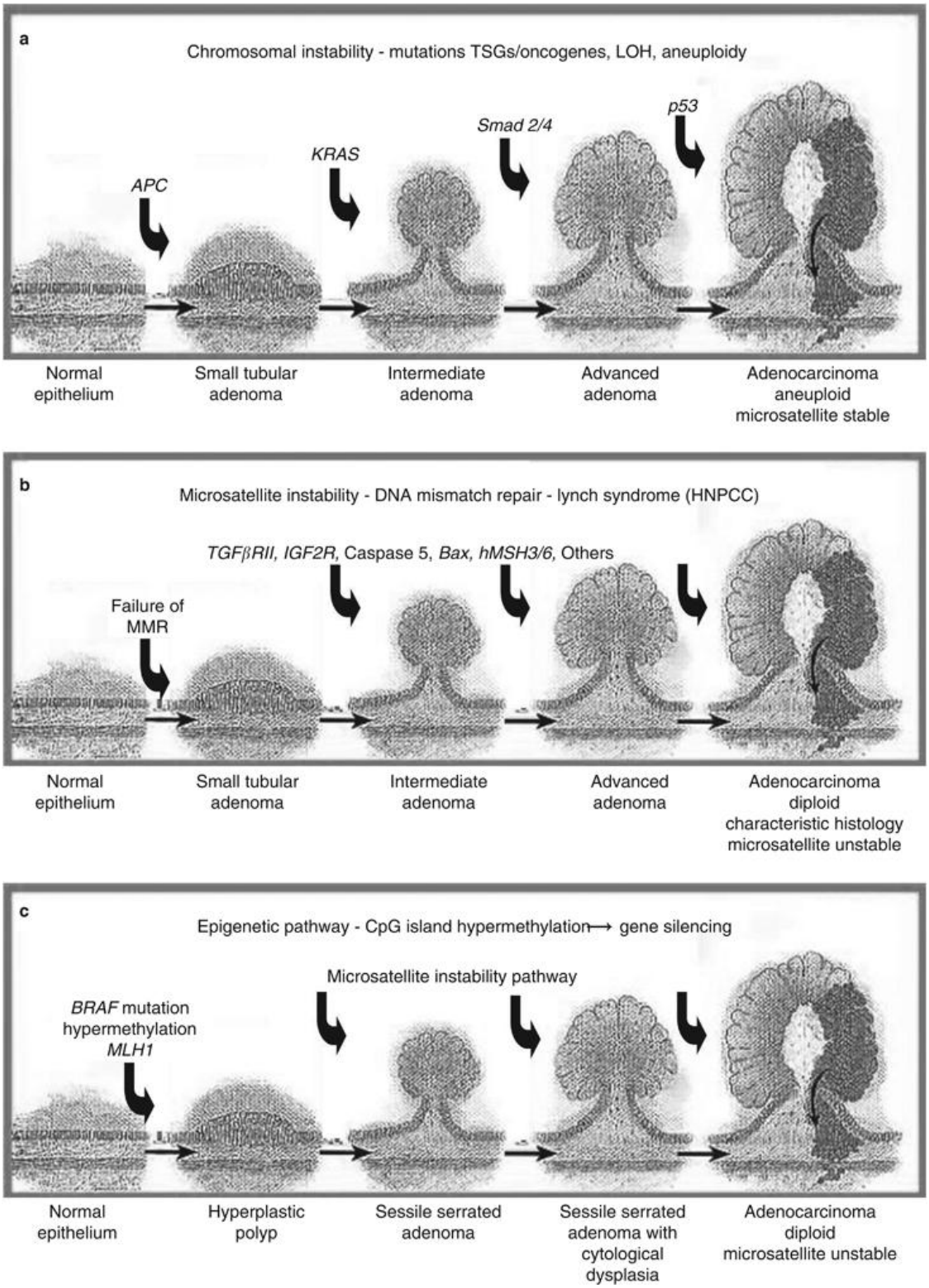

Figure 2 Molecular pathways involved in CRC development

(Ahnen, 2011)

1.1.2 Hereditary colorectal cancer

Although most CRC occurs sporadically (approximately 95–99% of cases), a lower percentage of cases (2% to 5%) are hereditary or familial forms of this disease (Stoffel et al., 2014), (Jasperson et al., 2010). According to the absence or presence of polyposis, we can distinguish the hereditary forms of CRC in two main categories, Lynch syndrome (previously known as hereditary nonpolyposis colorectal cancer or HNPCC) and the polyposis syndromes, respectively. The latter can be further divided in subtypes including familial adenomatous polyposis (FAP), attenuated FAP (AFAP), MUTYH-associated polyposis (MAP) and other rare hereditary syndromes such as Peutz-Jeghers syndrome, Cowden syndrome and juvenile polyposis (Allen and Terdiman, 2003). All these syndromes were characterized by germ-line mutations which confer a major risk to develop CRC with an early age of onset respect to the sporadic forms (Burt and Neklason, 2005).

1.1.2.1 Lynch Syndrome

Lynch Syndrome (previously known as HNPCC), identifies an autosomal dominant disorder characterized by an early onset of colorectal cancer (approximately 44 years) which typically occur without polyposis, with a predilection for the right colon. Lynch syndrome is the most common form of hereditary CRC accounting for the 15% of all CRC (Umar et al., 2004). Patients with Lynch syndrome have an 80% lifetime risk to develop CRC (Umar et al., 2004). A distinctive feature of Lynch syndrome is the presence of extracolonic malignancies in addition to colorectal cancer, including endometrial, ovarian, gastric, pancreatic, small bowel, brain, hepatobiliary and urothelial neoplasms (Lynch and Lynch, 2000). Mutations in each of the mismatch-repair (MMR) genes MLH1, MSH2, MSH6, and PMS2, can be responsible for this phenotype, although mutations in MLH1 and MSH2 gene occur more frequently accounting for the 90% of all MMR gene mutations (de la Chapelle, 2004). In a subset of cases, Lynch syndrome is due to 3' end deletion of the EPCAM gene, with subsequent epigenetic silencing of MSH2 (Sehgal et al., 2014). In most cases, patients inherited a germinal mutation in one copy of the MMR gene, while the inactivation of the remaining wild-type allele occur later and represents a somatic event. Given the

fundamental role of the MMR system to recognize and correct mispaired bases, as well as insertion/deletion events that may arise during DNA replication, mutations in these genes leads to an accumulation of replication errors in microsatellites sequences giving arise to an MSI phenotype, which is the hallmark of Lynch syndrome (de la Chapelle, 2004).

1.1.2.2 Familial Adenomatous Polyposis

The FAP is as rare autosomal dominant disease with a penetrance close to 100%. The clinical phenotype of FAP, defined as the prototype of hereditary polyposis syndrome, is characterized by the presence of multiple (hundred to thousand) adenomatous polyps along the colon and the rectum. The age of onset is early and the symptoms generally occur during childhood or adolescence (Shussman and Wexner, 2014). Although the risk to develop CRC in FAP patients is near to 100%, it is estimated that less than 1% of all CRCs are due to FAP. This condition arises in presence of germline mutations in the tumor suppressor gene APC. The APC protein is a fundamental component of the scaffold complex implicated in the degradation of β-catenin and is a central negative regulator of Wnt signaling in the malignant transformation of colonic epithelial cells (Morin et al., 1997). Most mutations are nonsense or frameshift resulting in the synthesis of a truncated protein with subsequent accumulation of nuclear β-catenin and induced expression of tumor promoter genes (Chung, 2000), (Fearnhead, 2001). A distinct and less aggressive form of FAP is the AFAP, which is diagnosed when the number of colorectal adenomas ranges from ≥ 20 to ≤ 100. In this form adenomatous polyps occur later and there is a minor lifetime cancer risk respect to the classic FAP. The genetic basis of AFAP are less characterized and mutations in APC or MUTYH genes were found only in some cases (Knudsen et al., 2003).

1.1.2.3 MUTYH-Associated Polyposis

MAP, differently from FAP and other polyposis syndromes, is an autosomal recessive disease which appears in individuals with biallelic mutations in MUTYH gene, encoding for a DNA glycolase implicated in the Base Excision Repair system. In this

context, MUTYH is important to prevent DNA transversion (G:C to T:A) excising guanine mispaired with 2-hydroxyadenine and adenine opposite 8-oxoguanine (8-oxoG), produced as effect of oxidative stress (Goodenberger and Lindor, 2011). In patients with MAP, a phenotype similar to AFAP was generally observed with the formation of ten to hundreds colorectal polyps in adulthood. A MAP syndrome is evaluated in patients with a suspected FAP missing mutations in APC gene or in absence of a family history (Morán et al., 2010). A 28-fold increase in CRC risk was found in carriers of biallelic mutations of MUTYH with a high predisposition for proximal disease. The risk appears to be age dependent and importantly increases in patients not subjected to regular endoscopic surveillance (Lubbe et al., 2009).

1.2 Colitis-associated Colorectal Cancer (CAC): an overview

It is known that an inflammatory setting is an unfavourable condition predisposing and sustaining the development of a protumorigenic microenvironment which contribute to the onset of cancer (Mantovani et al., 2008). In this context, the inflammatory state may be a preexisting condition that encourages the onset of a malignant process, or otherwise, oncogenic transformation can induce inflammatory mediators which contribute to create a tumor-promoting context. The effect of inflammation on sporadic CRC is essentially a promoting effect (Terzić et al., 2010). Differently, in Colitis-associated colorectal cancer (CAC), a subtype of colorectal carcinoma occurring in patients with Inflammatory Bowel Disease (IBD), a chronic inflammatory disorder led to tumor formation acting both on initiation and promotion of CRC (Terzić et al., 2010). CAC represents one of the clearest evidence of the correlation between inflammation and cancer. The appearance of CRC as a consequence of IBD was first observed by Chron and Rosenberg in 1925 (Crohn and Rosenberg, 1925). Subsequently, this observation was strengthened and confirmed by several studies (Rubin et al., 2013), (Eaden et al., 2001) (Gyde et al., 1988). Although IBD-associated colorectal neoplasia account for only 1-2% of all CRCs, a preexisting condition of intestinal chronic inflammation constitute an important predisposing factor to develop CRC with a mean age of onset lower than sporadic CRC (40-50 years *vs*. 60 years) (Mattar et al., 2011). Both the main forms of IBD, Ulcerative colitis (UC) and Chron's disease (CD), confer an increased risk for developing CAC, in particular when inflammation extensively involve the colorectal tract (Ullman and Itzkowitz, 2011) (Farraye et al., 2010). However, while Ulcerative Colitis (UC) immediately appeared clearly involved in the pathogenesis of CAC, the existence of a consistent correlation with Chron's disease (CD) has been unclear for a long time (Waldner and Neurath, 2015). The difficulty to exactly estimate the risk of CRC in patients with CD is mainly due to the heterogeneity of the disease which can affect different parts of the gut (Waldner and Neurath, 2015). Different factors affect CAC incidence in IBD patients. In particular the duration and the severity of the disease, as well as the extent of colonic involvement are critical in CAC initiation (Rutter et al., 2004), (Ekbom et al., 1990). Indeed, the probability to develop CAC increases of 8% and 13% respectively after 20 and 30 years of the disease (Eaden et al., 2001). Although sporadic CRC and CAC share several molecular

mechanisms and both require accumulation of mutations for cancer development, important pathogenic differences discriminate these two types of CRC.

1.2.1 Clinical and molecular features of CAC

The development of CAC can be clinically distinguished from sporadic CRC since they arise and evolve on a different genetic and clinical background. In sporadic CRC we observe the 'adenoma-carcinoma' sequence previously described in which the malignant transformation begin from adenomatous polyps (Fearon and Vogelstein, 1990). Differently, in CRC occurring in IBD patients we can recognize an 'inflammation-dysplasia-carcinoma' sequence in which the tumor formation often derive from a flat and nonpolypoid dysplasia which frequently appears as a multifocal condition with the involvement of a larger area of the colon (Ullman and Itzkowitz, 2011). The multifocality of CAC is thought to be due to the broad stimulus to which the colonic mucosa is subjected during a persistent inflammatory state (Xie and Itzkowitz, 2008). For the assessment of epithelial dysplasia in IBD a standardized classification system is adopted which allow the identification of three main conditions: 1) the absence of dysplasia; 2) a state of indefinite dysplasia, with epithelial changes that exceed the limits of ordinary regeneration, but are not clearly classifiable as dysplastic areas or lesions; 3) a condition of positive dysplasia (Riddell et al., 1983). The positive dysplasia is further microscopically differentiated in low-grade dysplasia (LGD), in which the nuclei are still confined in the basal part of cells, and high-grade dysplasia (HGD) in which the nuclei are mainly localized in the apical region. This sequence of dysplastic conditions is generally observed in the evolution of CAC, although carcinoma can occur also skipping one of more of these steps (Farraye et al., 2010) (Figure 3). Regarding the molecular events that underlie and support the tumor formation, there is an overlap in the genomic alterations which are observed in sporadic CRC and CAC. For instance, the genomic instability in CIN and MSI, occurs approximately at the same rate in both types of CRC appearing with a frequency of 85% and 15%, respectively. Interestingly, the temporal sequence of genetic changes between sporadic CRC and CAC results opposite, showing important differences in their tumorigenic background (Ullman and Itzkowitz, 2011), (Xie and Itzkowitz, 2008). In sporadic CRC, mutations in APC gene are determinant constituting one of the first events responsible of the transition from normal mucosa to early adenoma. Otherwise,

the loss of APC function is usually a rare and a late event in IBD-associated neoplasia intervening in the shift from HGD to CAC (Ullman and Itzkowitz, 2011), (Aust et al., 2002). In addition, in sporadic CRC p53 inactivation is detected in the final steps of the multistage process, while in CAC, p53 alterations occur early and are sometimes observed in absence of dysplasia or in presence of indefinite dysplasia indicating that this event can be induced by a chronic inflammatory state preceding the onset of malignant transformation (Hussain et al., 2000a), (Yin et al., 1993). Moreover, the loss of a p53 allele (p53 LOH) correlates with the evolution of dysplasia and it is found in 85% of biopsies from UC patients histologically classified as carcinoma (Burmer et al., 1992).

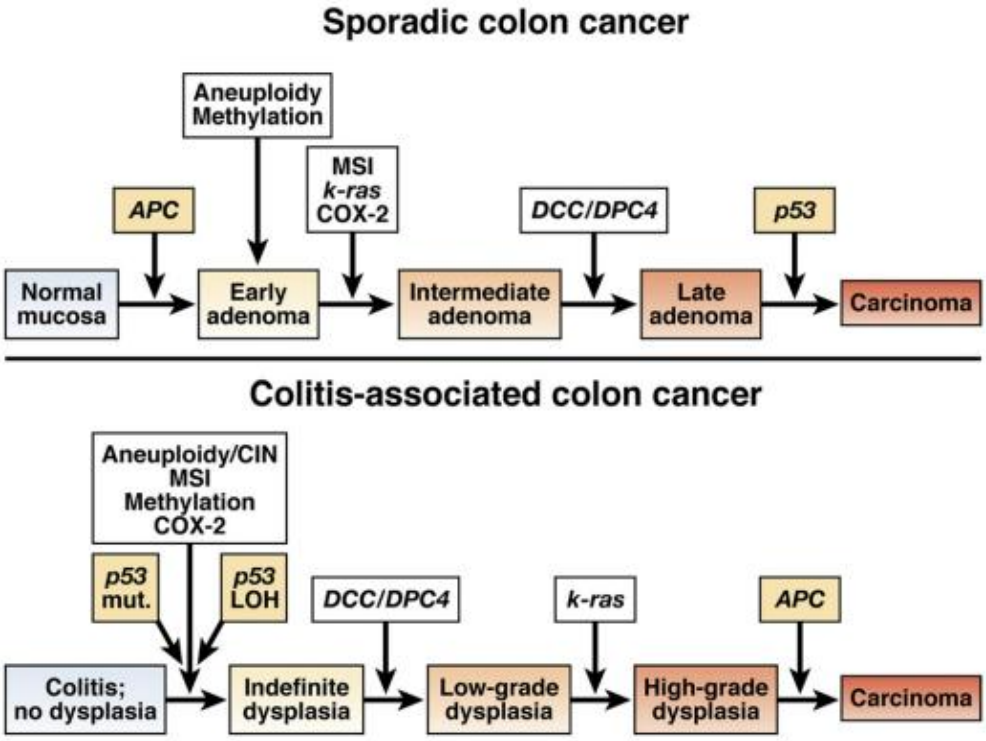

Figure 3 Molecular pathogenesis of sporadic CRC and CAC

(Ullman and Itzkowitz, 2011)

Gene silencing by CpG island (CGI) hypermethylation represents another important molecular mechanism involved in sporadic CRC as well as in CAC setting, although this event results to be more frequent and relevant in the first case (Olaru et al., 2012).

An aging-related hypermethylation was observed in precancerous colonic lesions of patients with IBD, although the global CGI methylation pattern in CAC was also influenced by the CIMP status (Issa et al., 2001). In fact, standardization for age revealed a lower methylation rate in IBD patients with no CIMP phenotype respect to CAC occurring on CIMP positive background, the characteristics of which are similar to those of sporadic CRC (Olaru et al., 2012). In addition, hypermethylation of hMLH1 promoter gene was detected in CAC characterized by a high-frequency MSI (MSI-H) inducing a defective DNA mismatch repair system (Fleisher et al., 2000). However, it is believed that alterations of DNA methylation are uncommon in CAC with a greater overall importance of genetic abnormalities than those epigenetic changes (Konishi et al., 2007).

1.2.2 Mouse models of Colitis-associated colorectal cancer

In order to understand the pathogenesis of cancer and to test new preventive as well as therapeutic options, the animal models are valuable tools to define the molecular mechanisms which underlie the onset of the disease in different settings. For the study of the hereditary forms of CRC different mouse models were generated. The Apc $^{Min/+}$ mouse, which is created to have a truncating mutation in the APC gene, is a successfully used model for reproducing the clinical condition observed in patients with FAP (Yamada and Mori, 2007). Several other models which are based on the germinal inactivation or a conditional knock-out of APC were also developed (Nandan and Yang, 2010). Mouse models hosting mutations in genes of the MMR system constitute the main strategy used for the study of Lynch syndrome (Nandan and Yang, 2010). To simulate the onset of sporadic CRC many chemicals pro-carcinogenic agents have been used including dimethyl hydrazine (DMH) and azoxymethane (AOM) whose efficiency in tumor induction strongly depends on the strain susceptibility, the dosage and the duration of the exposition (Karim and Huso, 2013). To best characterize the etiology and the behaviour of CAC, both genetic as well as chemical and pro-inflammatory protocols were developed, some of which will be discussed in detail.

1.2.2.1 IL-10 -/- Mice Model

IL-10 is an essential immunoregulatory and anti-inflammatory cytokine produced by regulatory T cells which plays a crucial role in the pathogenesis of IBD. Mice with

targeted deletion of IL-10 gene represents a genetically engineered mouse model which develop spontaneous colitis and colorectal cancer following the dysplastic sequence observed in patients with IBD-related CRC (Kühn et al., 1993). A massive production of pro-inflammatory mediators by macrophages and the induction of an important pathogenic Th1 response represents the main effects of IL-10 knock out (Berg et al., 1996). Localized histopathologic signs of chronic colitis early appear in 3 weeks-old mutants mice, while multifocal dysplastic lesions appear in all regions of the large intestine in 3 months-old mutants associated with epithelial hyperplasia, crypt abscesses, inflammatory infiltrates in mucosa and submucosa (Berg et al., 1996). The adenocarcinoma incidence was of 25% at 3 months of age reaching the 60% at 6-months of age (Berg et al., 1996). Importantly, although IL-10 KO develop colitis and CAC similarly to IBD patients, no alterations in p53, APC, K-RAS and MSH genes were found in this mouse model indicating the implication of additional mechanisms in the development of these cancers and placing a limit on the use of this animal model for the study of CAC (Sturlan et al., 2001).

1.2.2.2 AOM-DSS Mouse model

The azoxymethane-dextran sodium sulphate (AOM-DSS) model represents a chemical-induced mouse model of CAC which rapidly recreate the inflammation-dysplasia-carcinoma sequence occurring in patients with IBD-driven CRC. This model relies on the combined use of the pro-carcinogen (AOM) as tumor-inducing agent followed by repeated administrations of the proinflammatory agent (DSS). Irregularities and disorders of epithelial crypts, associated with inflammatory and immune infiltrating cells in the colonic mucosa, are early identifiable in this model with evident signs of extensive colonic inflammation detectable after 3-4 weeks of treatment (Neufert et al., 2007), (Tanaka et al., 2003). This two-stage colitis-related protocol led to a rapid development of multiple colonic tumors within 10 weeks, although the tumor incidence was different depending on the strain susceptibility. Indeed, the colonic adenocarcinoma incidence changes from 100% in Balb/c mice to 50% in C57BL/6N strain with no formation of colorectal adenocarcinomas observed in C3H/HeN mice and the DBA/2N mice (Suzuki et al., 2006). Moreover, the tumor-promoter activity of DSS in this model appeared to be dose-dependent in ICR mice reaching a 100% of tumor incidence in animals receiving AOM plus 2% of DSS (Suzuki et al., 2005). Colorectal

adenocarcinoma in AOM-DSS are mainly located in the distal or middle region of the colon and, interestingly, recapitulate crucial molecular alterations occurring in human colorectal cancer (Tanaka, 2012). Indeed, tumors in AOM-DSS model present alterations of the Wnt signaling pathway and are associated with a nuclear translocation of β-catenin, differently from IL-10$^{-/-}$ mice in which β-catenin localization is mainly membranous (Tanaka et al., 2003), (Gerling et al., 2011). However, despite the relevance of p53 alterations in CRC occurring on colitis background in humans, no mutations of p53 were generally found in AOM-DSS treated animals, while COX-2 and iNOS upregulation were commonly detected in the same murine model (Tanaka et al., 2003). Therefore, given the great feasibility of the experimental protocol, as well as the high repeatability associated with reduced cost, AOM-DSS represents one of the models of choice when the study is focused on inflammatory-driven CRC.

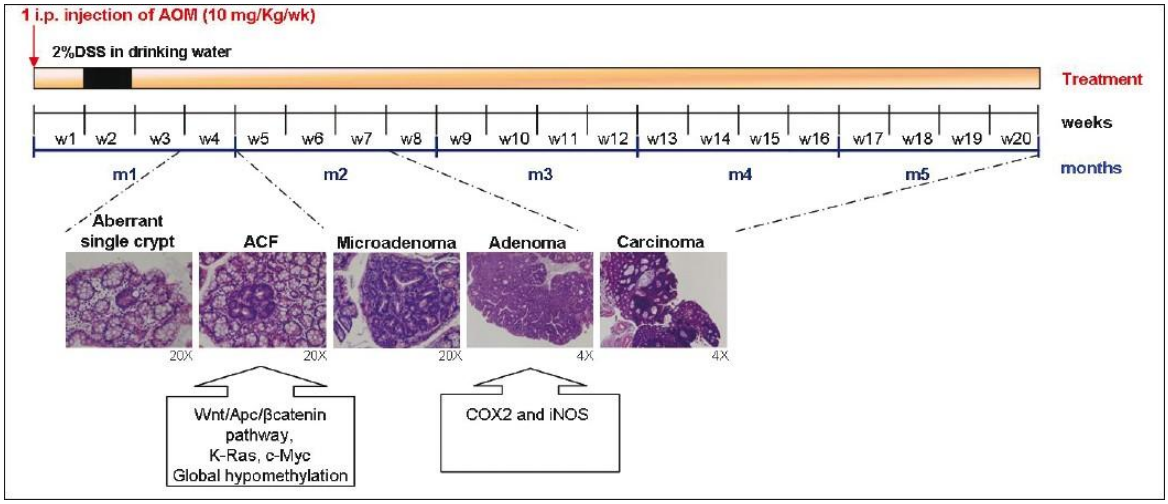

Figure 4 Representation of tumor progression in the AOM/DSS murine model (De Robertis et al., 2011)

1.2.3 Oncogenic mechanisms in chronic inflammation

In CAC, differently from sporadic CRC, tumor arises on a chronic inflammatory background which importantly contribute to the initiation of malignant transformation. Although the mechanisms which drive the onset of CAC in IBD patients need to be further defined, it is known that immune and inflammatory cells activated during colitis were able to produce reactive oxygen and nitrogen species and sustain a localized condition of oxidative stress which promote DNA damage (Terzić et al., 2010). The mutagen role of the oxidative stress in the pathogenesis of CAC was supported by Hussain and colleagues which demonstrated that the acquisition of p53 mutations in inflamed intestinal cells was related to an increased activity of iNOS (Hussain et al., 2000). Enforcing this hypothesis, iNOS transcript levels resulted significantly upregulated at early stage of neoplasia development in the colonic mucosa of UC patients (Svec et al., 2010). There are also evidences that the ROS produced by inflammatory cells were able to inactivate members of MMR system at protein level as well as to induce an upregulation of DNA methyltransferase enzymes with a consequent epigenetic silencing of hMLH1 (Colotta et al., 2009). Moreover, the massive induction of proinflammatory cytokines and chemokines is a critical event for the neoplastic transformation which occur during the evolution from an IBD disease to the acquisition of a CAC phenotype. In addition, cytokines and chemokines are essential for sustaining carcinogenesis through the stimulation of cell proliferation and angiogenesis, as well as impairing the beneficial immune function (Terzić et al., 2010). The analysis of cytokines profile in the DSS mouse model of colitis performed at different times during the study, revealed that there are distinctive cytokine patterns in relation to the duration and the severity of the disease (Alex et al., 2009). In particular, during the transition from acute to chronic inflammation, a switch from Th1-Th17 derived cytokines to a prevalent Th2 inflammatory mediated response occur. According to these results, serum levels of TNF-α, IL-6, IFN-γ, IL-12, IL-17, MIP-1α and Keratinocyte-derived chemokine (KC) increased after 7 days of DSS administration, while during the chronic phase, IL-10 and IL-4 resulted upregulated at the expenses of TNF-α, IL-6, IL-17 and KC which resulted downregulated (Alex et al., 2009). Many cytokines were involved in the promotion of CAC, some of which seems to have a crucial role. The importance of the signaling mediated by TNF-α, a potent pro-inflammatory cytokine, in the onset of

CAC has been proven by several studies. In the AOM-DSS mouse model a significant lower number of tumors were observed in mice deficient in TNF-Rp55 gene (encoding for the receptor TNFR1 of TNF-α) compared with their WT AOM-DSS-treated counterpart (Popivanova et al., 2008). In addition, the treatment with the specific antagonist of TNF-α etanercept in AOM-DSS animals, showed less immune and inflammatory infiltrations and an important reduction of tumor numbers and size (Popivanova et al., 2008). Moreover, the activation of TNF-α receptor 2 (TNFR2) appeared to be critical in CAC development. (Onizawa et al., 2009). The beneficial effects of anti-TNF-α monoclonal antibodies were also proved in IBD patients, although the effect of TNF-α inhibition in humans with CAC remain to be defined (Waldner and Neurath, 2015). Another important cytokine implicated in the pathogenesis of CAC is IL-6. The inhibition of IL-6 signaling or IL-6 trans-signaling, respectively mediated by a membrane-bound IL6-receptor (IL6R) or by a soluble form of IL6R, led to a reduction of tumor development in AOM-DSS mouse model (Becker et al., 2004) The importance of IL-6 in the development of CAC was further confirmed by Matsumoto and colleagues which proved, using a colitis-associated premalignant cancer (CApC) model, the central role of both colonic and soluble IL-6 produced by colonic inflammatory macrophages in the induction of the transcription factor STAT3 and supported the anticancer effects arising through the inhibition of the IL-6 trans-signaling (Matsumoto et al., 2010). Also, Grivennikov and colleagues investigated the impact of IL-6 deficiency in the AOM-DSS treated mice founding a decreased number, size and load of tumors in IL-$6^{-/-}$ mice respect to their WT counterpart with a concordant reduction of colonic STAT3 expression (Grivennikov et al., 2009). Importantly, in the same study, the critical role of STAT3 hyperactivation for the evolution of CAC was enforced demonstrating a significant lower number and size of adenomas in mice with a conditional deletion of STAT3 (*Stat3*$^{\Delta IEC}$) (Grivennikov et al., 2009). Nuclear factor-kappaB (NF-kB) is also thought to have an outstanding role in CAC. Different tumor promoter cytokines were able to activate the transcription factor NF-kB which in turn can mediate the synthesis of pro-inflammatory cytokines including TNF-α, IL-6, IL-8 and IL-1β (Tak and Firestein, 2001). Greten and colleagues demonstrated that in the AOM-DSS mouse model the suppression of NF-kB activation, obtained through a conditional knockout of IKKβ in enterocytes, significantly reduced tumors number

while not affecting inflammation (Greten et al., 2004). In addition, they also encountered protective effects when IKKβ deficiency was induced in myeloid cells (lamina propria macrophages and dendritic cells) probably due to a downregulation of pro-inflammatory cytokines production which functions as growth factor for premalignant enterocytes (Greten et al., 2004)' (Terzić et al., 2010). Moreover, a hyperactivation of NF-kB was found in macrophages and epithelial cells in the inflamed mucosa of patients with IBD compared to the normal colonic mucosa (Rogler et al., 1998). However, opposite functions were proposed for NF-kB signaling during acute or chronic intestinal inflammation. Indeed, the inhibition of IKKβ-dependent NF-kB activation attenuate chronic intestinal inflammation, but exacerbate the acute phase of inflammation (Greten et al., 2004). The role of the transforming growth factor-β (TGF-β) in CAC development appear to be dichotomic showing both promoting and suppressive effects. The tumor suppressive functions of TGF-β were supported by the evidence that mutations in TGF-β pathway including SMAD 3 and SMAD 4 in colonic epithelial cells promoted tumor development and progression in both sporadic and chronic colitis setting (Terzić et al., 2010). However, at later stage of carcinogenesis TGF-β showed a protumorigenic effect inducing an immunotolerant microenvironment and driving the epithelial mesenchimal transition (EMT) phenomenon leading to metastatic invasion (Terzić et al., 2010).

MicroRNAs (miRNAs) represent an additional class of regulatory molecules importantly involved in the mechanisms linking inflammation to cancer (Iliopoulos, 2014). miRNAs are small (18-24 nucleotides) non-coding, single-stranded RNA molecules which finely regulate gene expression at post-transcriptional level by targeting specific mRNAs for cleavage or translational repression (Bartel, 2004). Several miRNAs can be induced or downregulated upon inflammatory stimuli intervening in inflammation-associated carcinogenesis (Schetter et al., 2010). Oralu et al. evaluated the miRNA profiles in humans with IBD-associated neoplasia showing 22 miRNAs significantly upregulated and 10 miRNAs significantly downregulated in inflamed-dysplastic tissues compared to non dysplastic inflamed mucosa (Olaru et al., 2011). In particular, this study showed that miR-31 levels linearly increased with the malignant transformation which occur during CAC development (Olaru et al., 2011). Recently, a significant upregulation of miR-21,which is an IL-6 induced microRNA,

was detected in AOM-DSS mouse model supporting the oncogenic role of this microRNA in CAC (Gao et al., 2013), (Löffler et al., 2007). Differently, miR-143 and miR-145 have been proved to function as tumor suppressor in colon cancer and resulted significantly downregulated in patients with chronic UC, as well as in mice during DSS-induced chronic inflammation (Pekow et al., 2012), (Josse et al., 2014)

1.2.4 Intestinal microbiota and its role in CAC

A complex symbiotic microbial community, including up to trillions of microorganisms (10^{14}-10^{15}) and more than 9 million of bacterial genes, widely exceeding the number of human cells and genes, is hosted in the human gastrointestinal tract, collectively constituting respectively the human gut microbiota and microbiome (Qin et al., 2010). There is a prevalence of strict anaerobic bacteria in the normal gut microbiota of the colon, and the two dominant bacterial phylogenetic divisions in the healthy adults are represented by Bacteroidetes and Firmicutes. Otherwise, relative minor proportion of Verrucomicrobia, Actinobacteria, Proteobacteria, Fusobacteria and Cyanobacteria phyla are recognized (Eckburg et al., 2005). However, while in terms of divisions healthy adults are less dissimilar, there is a great inter-individual variability in the relative proportion of these main components and these differences are more evident at lower taxonomic levels (Tap et al., 2009). There are strong evidences that our indigenous microbial community coevolves with us and that changes in the composition of the gut microbiota may have an important harmful or otherwise beneficial effect on human health (Qin et al., 2010). Indeed, the host physiology is critically modulated by these commensal bacteria defining the intestinal microbiota as an additional metabolic organ (Gill et al., 2006). Colonization resistance against intestinal pathogens and invading microbes, as effect of a competition among bacterial genes, is one of the major function of the host-associated microbiota (Buffie and Pamer, 2013). This phenomenon, counteracting overgrowth of indigenous pathobionts and harmful pathogens, provide an important defense against microbial infection of the gut (Kamada et al., 2013). The gut microbiota appeared also to be fundamental for a proper development and differentiation of the intestinal epithelium as well as for the maintenance of a normal intestinal homeostasis and maturation and function of the host immune system (Sommer and Bäckhed, 2013). There is a growing number of evidences supporting a crucial role

of the intestinal microbiota in the onset of CAC. One of the assumption underlying the role of the gut microbiota in bowel related diseases concerns the establishment of an imbalance condition which is referred to as dysbiosis in which the healthy ratio between protective, and harmful (pro-inflammatory, protumorigenic or damage-inducing) bacterial strains results to be impaired (Tamboli et al., 2004), (Ullman and Itzkowitz, 2011). In addition, it is known that microbial pathogens can induce intestinal inflammation promoting CAC development through the activation of pattern recognition receptors, toxins secretion as well as through the production of short chain fatty acids which are the main products of colonic fermentation (Terzić et al., 2010). Uronise and colleagues demonstrated that germ-free AOM-treated IL $10^{-/-}$ mice appeared to be resistant to tumor development indicating a tumor-promoting role of the intestinal bacteria (Uronis et al., 2009). In addition, the absence of the intestinal microflora, seems to confer resistance to tumor development in AOM-DSS mouse model of CAC, as well as the Apc Min/+ model of CRC (Li et al., 2012),(Zackular et al., 2013). There are also clear experimental evidences regard the existence of a colitogenic microbiota which is able to induce intestinal inflammation in normal mice. Importantly, the T-bet$^{-/-}$RAG2$^{-/-}$ ulcerative colitis (TRUC) animal model, showed that this colitogenic flora can be vertically transmitted to WT pups nursed from TURC mice and horizontally transmitted, as demonstrated by co-housing studies (Garrett et al., 2007). The involvement of luminal bacteria in the pathogenesis of DSS-induced colitis was also proved by the amelioration of intestinal inflammation consequent to the treatment with selected antibiotics (Rath et al., 2001). However, other evidences highlighted a major susceptibility to DSS-induced colitis in germ-free mice and in mice treated with wide-spectrum antibiotics (Ayres et al., 2012). Interestingly, it has been recently demonstrated that, during the induction of CAC on AOM-DSS mouse model, important changes in the community structure of the gut occur, however not affecting the community membership indicating a variability in the relative abundance of the bacterial phylotypes rather than the appeareance of different phylotypes. Importantly, the authors of this study showed a correlation between the gut microbiota composition and the oncogenic molecular events that flanked the development of CAC in its various phases over the time generating a longitudinal model which show the phylotype shifts as a complex and dynamic process (Liang et al., 2014).

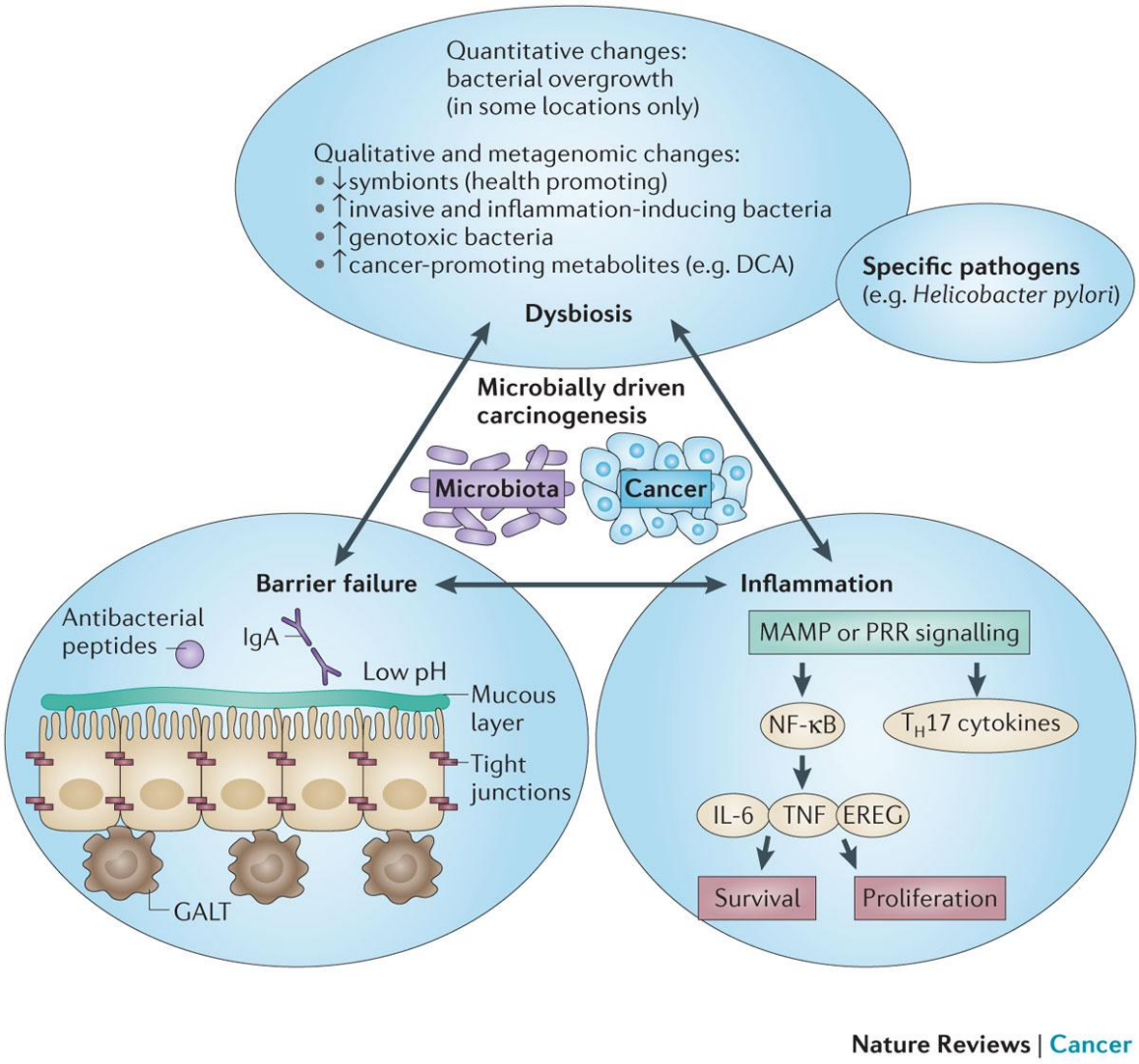

Figure 5 Intestinal microbiota and cancer

(Schwabe and Jobin, 2013)

1.3 The Notch signaling

The Notch signalling pathway is an evolutionary conserved pathway which appears as a paradigmatic simple network, while intervening in a wide range of physiological and developmental cellular processes, as well as in different human diseases (Bray, 2006). Among the pleiotropic functions of Notch it is possible remark the regulation of cell fate determination in different cell types, the self-renewal of stem cells, as well as its implication in tumorigenesis through a modulation of important processes including cell death and proliferation (Andersson et al., 2011). Despite the functions mediated by this signalling can be extremely complex, most of the Notch-dependent processes were mediated by the same core pathway (Bray, 2006). In human and mice the core components of this pathway includes the five different Notch ligands (Jagged1, Jagged2, DLL1, DLL3 and DLL4) and four distinct Notch receptors (Notch1-4). Both Notch receptors and ligands are single-pass transmembrane proteins with extracellular domains characterized by multiple epidermal growth factor (EGF)-motifs which enable a cell-cell communication which is essential to trigger the signaling mediated by the canonical Notch pathway (Rebay et al., 1991). Importantly, it has been demonstrated that of the 36 EGF repeats present on the extracellular portion of Notch receptors, only the repeats 11-12 of Notch are essential and sufficient to promote the physical interaction with both the specific ligands Delta and Jagged (Rebay et al., 1991). The Notch receptors are synthesized in a larger precursor form of 300 KDa which need to be further processed in order to obtain a mature form. The first post-translational modification occur in the endoplasmic reticulum mediated by the enzyme O-fucosyltransferase 1 (Ofut1) (Kopan and Ilagan, 2009). This enzyme works directly transferring fucose onto serine or threonine residues of EGF-like domains present on Notch receptors, a critical step to ensure the proper folding of the protein (Kopan and Ilagan, 2009). The furin-like convertase enzyme subsequently perform the cleavage of the glycosylated precursor in the trans-Golgi network generating respectively the N-terminal extracellular domain of Notch (NECD) and the transmembrane-Notch intracellular domain named (NICD) which are non-covalently linked to form a heterodimer which constitute the mature receptor (Bray, 2006). This complex is further glycosylated during the passage through the Golgi by Fringe glycosyltransferase enzymes. In mammals three different fringe proteins were expressed: Lunatic Fringe

(LFng), Manic Fringe (MnFg) or Radical Fringe (RFng). There are evidences that all these fringe proteins were able to potentiate the signaling of Notch1 induced by Delta1 enforcing the binding between the receptor and its ligand. However, although none of these enzymes impaired the binding of Jagged1 to Notch1, the signalling deriving by this interaction appeared to be compromised by LFng and MnFg indicating the importance of this step in the modulation of Notch activity (Yang et al., 2005). Reached the mature form Notch receptors were translocated on cell surface where, upon binding with the specific ligand expressed on an opposing cell surface, the signaling is activated. The receptor-ligand binding triggers two consecutive proteolytic cleavages in the receptor. The first proteolityc event, catalyzed by the TACE metalloproteinase (ADAM17) cleaves the extracellular portion of the receptor (NECD) which was after internalized through endocytosis by the ligand-expressing cell for lysosomal degradation (Guruharsha et al., 2012). The remaining membrane-anchored fragment which derive from the first cleavage, is further processed by the γ-secretase enzyme releasing the active intracellular domain of Notch (NICD) (Kopan, 2012). In canonical Notch signaling, NICD translocate into the nucleus and interact with the transcriptional repressor protein CSL/RBP-J. Following the recruitment of co-activators Mastermind-like (MAML) and the histone acetyltransferase (HAT) p300, CSL/RBP-J is converted to a transcriptional activator leading to the induction of downstream target genes including Hes1 and c-Myc (Figure 6) (Kopan, 2012). Importantly, over the years a non-canonical role for Notch signaling was reported which functions in a CSL/RBP-J-independent manner and can be either ligand-dependent or independent(Andersen et al., 2012). An important effect of the non-canonical signaling is the regulation of the Wnt signaling pathway affecting the stability of β-catenin (Andersen et al., 2012). Noteworthy, representing an alternative crucial role of the Notch pathway, the non-canonical activation of this signaling, occurring with a ligand independent mechanism, resulted frequently associated with antagonism of Wnt/ β-catenin, a negative regulation evident also at the transcriptional level (Andersen et al., 2012).

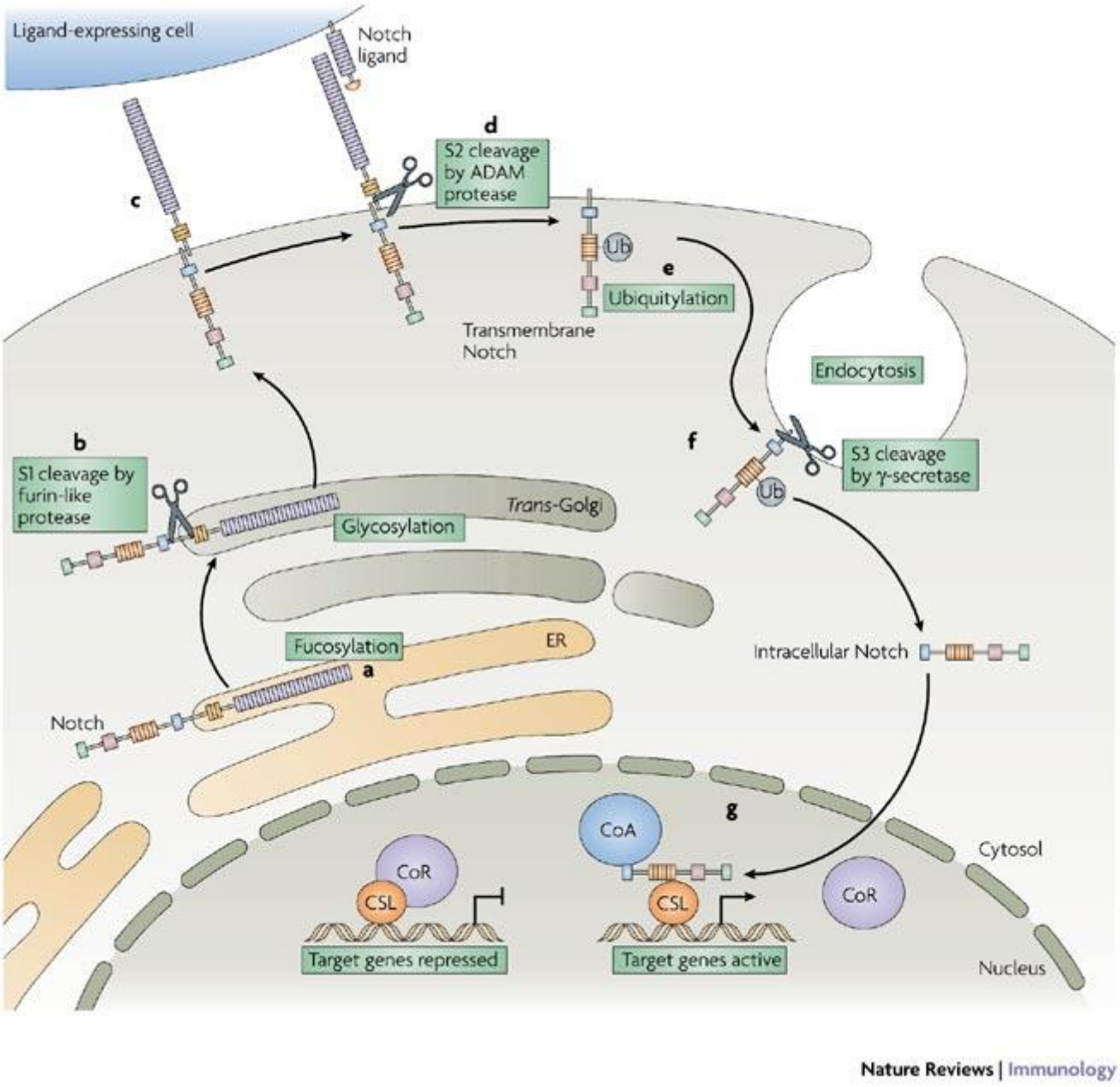

Figure 6 The Notch signaling

(Osborne and Minter, 2007)

1.3.1 Notch signaling in CRC

In the normal intestine, Notch signaling is crucial for the maintenance of stem cell phenotype as well as for the regulation of cell fate determination (Miyamoto and Rosenberg, 2011). The implication of Notch system in the development of CRC has been proved in several studies. In Apc $^{Min/+}$ mouse model of FAP it has been demonstrated that knocking down the Notch transcription factor CSL/RBP-J or blocking the signaling with an inhibitor of γ-secretase, a massive conversion of crypt proliferative cells in post-mitotic goblet cells was induced indicating the critical role of the Notch activation in the definition of the colon crypt compartment and adenoma formation (van Es et al., 2005). The gene expression profile of HES1, a downstream target of Notch signaling, appeared to be constant, although variable in tumors of patients with sporadic or familial CRC, supporting the concept that Notch signaling is a consistent feature of human CRC (Reedijk et al., 2008). Sonoshita et al. recently reported that the knock-out of Aes (or Grg5), a negative regulator of Notch signaling, in Apc $^{Min/+}$ was associated with local tumor invasion and intravasion suggesting that Notch signaling inhibition could be a promising strategy for treatment of colon cancer metastasis (Sonoshita et al., 2011). Noteworthy, differently from other studies which reported an increased tumor formation in Apc $^{Min/+}$ mice as effect of Notch signaling activation, in this study the genetic depletion of Aes did not result in enhanced tumor formation and didn't affect cell proliferation in colon cancer cells or grafting experiments (Sonoshita et al., 2011). Although there are proof relative to an oncogenic role of the Notch signaling, conflicting evidences support a more complex framework for this pathway in the pathogenesis of CRC. Recently, Kim et al. showed that inducing the activation of Notch1 in Apc $^{Min/+}$ mice a phenotype conversion from intestinal high-grade adenoma into low-grade adenoma was observed suggesting a negative effect on CRC progression. This tumor suppressor function of Notch1 in CRC resulted directly linked to a downregulation of the Wnt/β-catenin pathway and importantly, in this study the Notch1 downstream target gene NRARP was proposed as reliable indicator of Notch1 activity in intestinal tumors (Kim et al., 2012a). The complex function of Notch signalling in CRC was further clarified by Garg et al. They previously demonstrated that MMP-9 functions as a protease able to cleaves and activates Notch1regulating goblet cell differentiation in colon (Garg et al., 2007). In a later study performed in

AOM-DSS mouse model of CAC they reported that activation of Notch1 downstream target $p21^{W\ AF1/Cip1}$ was impaired in mice deficient for MMP-9 (MMP $9^{-/-}$), while increased β-catenin was detected compared to the WT counterpart. Importantly, these events observed in MMP $9^{-/-}$, resulted correlated with increased susceptibility to CAC (Garg et al., 2010). In addition, they tested *in vitro* the effects of MMP-9 overexpression showing an increased activation of Notch1 concomitant to a reciprocal downregulation of β-catenin (Garg et al., 2010). Concluding they proposed a tumor suppressor function of MMP-9 and Notch1 in CAC which occur through an intriguing interplay with the Wnt signalling in accordance with the results of Kim et al. previously discussed.

1.4 Polyunsaturated fatty acids

Fatty acids (FAs) are carboxylic acids consisting of a long hydrocarbon chain with a carboxylic (-COOH) and a methyl group (-CH3) located at the two ends respectively According to the nomenclature system widely adopted, the last carbon of the hydrocarbon chain is identified as n or ω. In addition, depending on the presence and the number of double bonds in the chain, FAs can be distinguished in three main classes : saturated fatty acids (SFAs) which have exclusively single bonds saturated with hydrogens atoms, monounsaturated fatty acids (MUFAs) which present only one double bond along the chain, and polyunsaturated fatty acids (PUFAs) characterized by the presence of multiple double bonds not saturated with hydrogen atoms. Given the importance of PUFAs for human health, and given the inability of the organism to produce them, they are classified as essential fatty acids, so they need to be introduced with the diet (Kaur et al., 2014). On the basis of the position of the first double bond in the chain starting from the methyl group we can discriminate ω-3 and ω-6 PUFAs as two main families of essential PUFAs (Wall et al., 2010). The main sources of Linoleic acid (LA; C18:2 ω-6), the precursor of ω-6 series fatty acids, are vegetable oils including corn, safflower and soybean oil, but a high content of LA is also found in nuts and sunflower seeds. Differently, ω-3 series fatty acids derive from α-linoleic acid (ALA; C18:3 ω-3) which can be introduced with the diet feeding fish oil, flaxseeds, rapeseeds, hempseed, canola oil (Das, 2006) The two essential short chain PUFAs, LA and ALA, were *in vivo* elongated and further unsaturated to long chain (LC) PUFAs in the endoplasmic reticulum through a biosynthetic pathway involving the Δ6- and Δ5-desaturase enzyme and the elongase enzyme (Elovl-5 and Elovl-2). A β-oxidation reaction for fatty acid shortening finally occur in the peroxisomes (Emery et al., 2013). From the metabolism of LA mediated by these enzymes derive arachidonic acid (AA 20:4 ω-6), while the ω-3 LC-PUFAs eicosapentaenoic acid (EPA 20:5 ω-3) and docosahexaenoic acid (DHA 22:6 ω-3) are the product of ALA conversion (Figure 6). The ability to convert PUFA in LC-PUFA among the species is not constant, but there is a variability in relation to the presence, the abundance and the activity of the enzymes involved in the metabolic pathway. In particular the activity of desaturase enzymes is known to be lower in humans compared to other species and is negatively regulated by multiple factors such as cholesterol, saturated fat and aging (Das, 2006). It is important

to underlie that the two main classes of LC-PUFAs described are not interconvertible and present distinct and often opposite physiological functions. There is a competition for accessing the Δ6-desaturase and the fatty acid elongase by ω-6 and ω-3 PUFAs for the bioconversion into LC-PUFA. In presence of both substrate the affinity of Δ6-desaturase for ALA is higher than AA (Rodriguez et al., 1998). However, since LA constitute the most common source of PUFA introduced with the diet, in particular in the Western diet lifestyle, accounting for approximately 90% of dietary ω-6 intake, it represents the more frequent substrate of Δ6-desaturase enzyme (Harris et al., 2009). Hence, in order to reduce the synthesis of AA and the production of eicosanoids derived from ω-6 PUFAs metabolism, the dietary intake of ω-3 PUFAs should be implemented eating foods containing a high content of EPA and DHA, in particular oily fish such as salmon, mackerel and sardines (Hull, 2011).

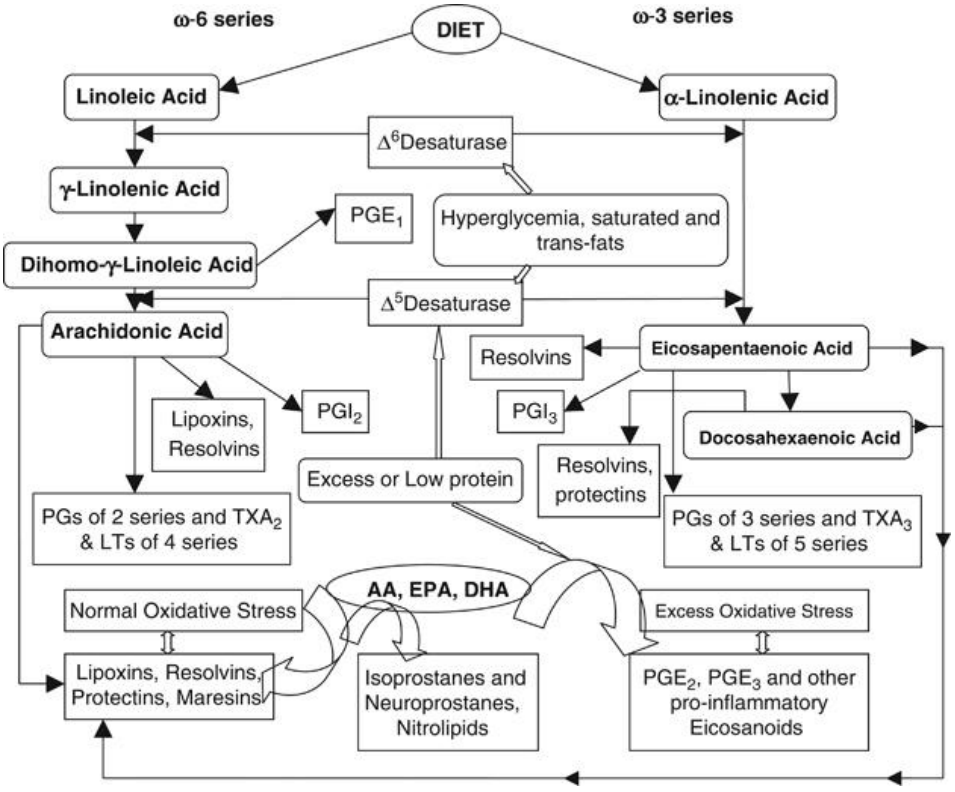

Figure 7 The ω-6 and ω-3 PUFAs metabolism

(Das, 2010)

1.4.1 Eicosanoids biosynthesis from PUFAs

Once ingested and absorbed PUFAs were incorporated as lipid component into the phospholipids of many cellular membranes influencing their physical and chemical ,properties as well as their function (Serhan and Chiang, 2008), (Mitchell et al., 2003). The composition of lipidic microdomains including lipid rafts and caveolae also resulted to be modified after PUFA incorporation (Stillwell et al.). Given the inability of the body to synthesize ω-3 and ω-6 PUFAs *de novo*, their content into the cellular membranes is not constant, but highly determined by their dietary intake. The biologically activities of these compounds extends well beyond the influence on the membrane properties, but also intervening in the productions of bioactive lipids, named eicosanoids, which have pleiotropic and a complex function over the body (Larsson et al., 2004). The biosynthesis of eicosanoids occur upon activation of phospholipase A2 (PLA2) enzyme resulting in LC-PUFA esterification, cleavage and release from phospholipids membrane. Subsequently, the 20-carbon PUFAs, including AA and EPA were further used as precursor for the eicosanoids synthesis which are bioactive hormon-like lipids with a short life, but a potent impact on many pathophysiological processes including the modulation of inflammatory and immune response, the regulation of cellular growth and differentiation, as well as platelet aggregation (Larsson et al., 2004). The eicosanoids biosynthesis is mediated by three main classes of enzymes which use both AA and EPA as substrates: the constitutive and inducible cyclooxygenases enzymes, respectively represented by COX-1 and COX-2 enzymes, the lipoxygenases (LOX) enzymes and cytochrome P450 monooxygenases (CYP). From the activity of COX enzymes derive the production of prostaglandins (PGs) and thromboxanes (TXs), while the LOX enzymes give rise to lipoxins (LXs), leukotrienes (LTs) and hydroxy fatty acids. At last hydroxy fatty acids, dihydroxy fatty acids, and epoxy fatty acids are the products generated from CYP enzyme (Larsson et al., 2004). Although AA and EPA uses the same enzymes for eicosanoids production, the families of eicosanoids produced from the different LC-PUFA is dissimilar. From the metabolism of AA were generated the 2-series PGs and TXs, the 4-series LTs and the hydroxy and hydroperoxy derivatives (5-HETE and 5-HPETE). Differently, when EPA is used as a substrate for COX and LOX enzymes, occur the production of the 3-series

PGs and TXs and the 5-series LTs (Lands, 1992). In addition, new classes of potent bioactive eicosanoids derived from both EPA and DHA metabolism have been recently identified including E- and D-series resolvins (RvE1, RvE2, RvD1, RvD2) protectins and docosatriens with important protective and anti-inflammatory properties (Serhan and Chiang, 2008) (Figure 6)

1.4.2 Omega-3 PUFAs intake and CRC development

The existence of a correlation between the dietary intake of PUFAs and the risk to develop CRC emerged from preliminary epidemiological data which reported a lower rate of CRC incidence in countries with a dietary lifestyle characterized by an increased fish consumption including Greenland and other Northeast countries respect to the Western populations (Byers, 1996). However, over the time epidemiological and observational human data appeared to be inconsistent regard this hypothesis reporting conflicting data probably as effect of heterogeneity of the studies, the difficulty to obtain appropriate values of ω-3 and ω-6 PUFA dietary intake as well as other confounding elements (Cockbain et al., 2012a), (Hull, 2011). Despite the inconsistency of the epidemiological evaluations, the anticancer effects of ω-3 PUFAs on CRC onset were importantly revealed by animal studies and begins to be also confirmed in different phases of clinical trials. Although there are experimental evidences of beneficial effects from a combination of both EPA and DHA intake (Cockbain et al., 2012), we focused our attention on studies describing the effect of EPA alone. One of the first evidences become from rats treated with the carcinogen AOM to induce CRC in which the tumor incidence and multiplicity resulted significantly decreased in the group feeding a diet containing EPA 4.7% compared to the arm fed with linoleic acid diet (Minoura et al., 1988). These evidences were subsequently confirmed and enforced in Apc $^{Min/+}$ mouse model of FAP which develop multiple intestinal tumors at 5 weeks of age. In this study, Barone and colleagues observed a reduction of tumors number and size in mice feeding a salmon oil-enriched diet characterized by a high percentage of ω-3 PUFAs compared with animals feeding a standard diet (Barone et al., 2014). In the same mouse model, our research group previously demonstrated that diets containing a different percentage (2.5% or 5%) of free-fatty acid (FFA) formulation of EPA are highly protective in CRC development compared to the control diets in which the corn oil, which is ω-6-PUFAs predominant, was substituted for soybean oil that contains

both ω-3- and ω-6-PUFA. They observe that the number of and load of tumors was dramatically decreased (over 70% and 80% respectively) in the animals feeding both EPA-FFA diets with a stronger effect of that containing 5% of EPA-FFA (Fini et al., 2010). The efficacy of EPA-FFA observed in Apc $^{Min/+}$ mouse model led to a Phase III clinical trial in 55 patients with FAP which showed a significant lower number and size of rectal polyps (22.4% and 29.8% respectively) in the group feeding EPA-FFA 2 g daily for 6 months compared to the placebo group (West et al., 2010). Similar results were obtained on patients with a familial history of colorectal adenomas feeding EPA-FFA (2 g/day) for 3 months in which a significant reduction of crypt cell proliferation associated with an increased apoptosis rate was observed (Courtney et al., 2007). Noteworthy, it has been also demonstrated that these effects are directly attributable to the increased uptake of ω-3 PUFA and not only due to the reduction of the ω-6 PUFA content (Davidson et al., 2004). Importantly, since ω-3 PUFA are natural compounds, they appear to be safe and well tolerated, so they could represent efficient chemopreventive agents against CRC.

1.4.3 Anti-neoplastic properties of omega-3 PUFAs on CRC

The anti-neoplastic properties of ω-3 PUFAs can be recognized in different molecular and cellular mechanisms, many of which were characterized through *in vitro* studies. One of the proposed anticancer activity of ω-3 PUFAs is the inhibition of COX-2 activity, the inducible form of COX enzymes, which is detected in many inflammatory processes, as well as in different types of cancer including CRC (Calviello et al., 2007). This effect is due to the ability of EPA to function as alternative substrate for COX-2 enzymes stimulating the synthesis of anti-inflammatory and anti-tumorigenic '3-series' PGs (PGE-3) rather than the pro-tumorigenic and pro-inflammatory '2 series' PGs (PGE-2). A reduction of COX-2-driven PGE2 synthesis leading to a "PGE2-to-PGE3 switch" was detected treating CRC cell lines with the ω-3 PUFA EPA in the FFA form (Hawcroft et al., 2010) In the same study, it has been also demonstrated that in presence of PGE-2, PGE-3 were able to antagonize the protumorigenic signaling mediated by the EP4 receptor (Hawcroft et al., 2010). In *fat-1* transgenic mice, which convert endogenous ω-6 to ω-3 PUFAs, treated with AOM-DSS to induce CAC, a significant increase of PGE-3 production was appreciated at the expenses of ω-6 derived

eicosanoids including PGE-2 compared with the WT counterpart (Jia et al., 2008). Also the DHA appeared to be an alternative substrate for COX-2 enzyme and both EPA and DHA significantly decreased PGE-2 synthesis and tumor growth in nude mice inoculated with HT29 cells (Vecchio et al., 2010)' (Calviello et al., 2004). The modulation of eicosanoids synthesis observed in presence of ω-3 PUFAs does not affect exclusively the PGs production, but also the induction of other lipid mediators as resolvins and protectins. Indeed, from the metabolism of EPA derive a COX-2 dependent production of RvE1, while D-series resolvins and protectins are generated from DHA. These downstream effectors of both EPA and DHA showed important anti-inflammatory and pro-resolution properties in animal models of acute inflammation contributing to explain the beneficial effects of these natural compounds (Serhan et al., 2008). The pleiotropic effects of ω-3 PUFAs also occur through an alteration of the membrane organization. In particular, it is known the ability of ω-3 PUFAs to change the composition, structure and function of lipid rafts or caveolae. These lipids microdomains, enriched in cholesterol, sphingolipids and saturated acyl chains, compartmentalize proteins and lipids with specific function acting as dynamic signaling platforms. Therefore, their modulation could have an important meaning for the anti-cancer activity of ω-3 PUFAs (Turk and Chapkin, 2013). For instance in splenic T cells of *fat-1* mice larger rafts domains were observed compared to WT mice (Kim et al., 2008). Moreover, it has been demonstrated an alteration of caveolae microenvironment in mouse colonic mucosa feeding ω-3 PUFAs which led to a significant reduction of cholesterol content, as well as of caveolin-1. In addition, also the distribution of caveolae signaling proteins such as H-RAS and eNOS resulted strongly modulated by ω-3 PUFAs (Ma et al., 2004). Importantly, ω-3 PUFAs not only modulate the size and the composition of lipid rafts, but also affect the localization of cell surface receptors such as the epidermal growth factor receptor (EGFR), which is a key receptor in colonic transformation. A displacement of EGFR was found in Young adult mouse colonic (YAMC) cells treated with DHA with a consequent impairment of transduction signal which is reflected in a modulation of cell proliferation. These results were also confirmed *in vivo* in which the suppression of EGFR signaling induced by DHA was also associated with a reduction of tumor incidence in AOM-DSS mouse model. This effect appear to be exclusively due to DHA since no variations in EGFR

phosphorylation were found in YAMC cells treated with EPA or AA (Turk et al., 2012). Although not direct effects of EPA in the modulation of EGFR signaling were reported, it is known that the migration and invasion induced by PGE-2 are dependent on EGFR transactivation indicating that EPA may be a similar functional effect of DHA impairing the PGE-2 synthesis (Hull, 2011),(Buchanan et al., 2003). There are also evidences of an anti-tumorigenic effect of ω-3 PUFAs due to an alteration of cellular redox state. Indeed, LC-PUFAs, as effect of their insaturation degree, appear to be particularly prone to lipid peroxidation. So, their incorporation in the cellular membrane can stimulate the generation of reactive oxygen species (ROS) and the establishment of a condition of oxidative stress. An enhancement of oxidative stress induced by the fermentation product butyrate was observed in mouse colonocytes treated with DHA which appear to be concomitant with an induction of apoptotic pathways (Ng et al., 2005). In addition, it has been demonstrated that arsenic trioxide (As2O3) used in combination with DHA have strong, but selective cytotoxic effects on 7 solid tumor cell lines including SW620, HT-29 and LS147T CRC cell lines. Indeed, they didn't found toxic effects on cell deriving from healthy donor such as skin fibroblast, pheripheral blood mononuclear cells and microvascular endothelial cells. Interestingly, this effect on cell viability resulted associated with an increase of intracellular lipid peroxidation and induction of apoptosis (Baumgartner et al., 2004). Although a similar effect of oxidized ω-PUFAs could be hopeful, both beneficial and harmful effects were described for lipid peroxidation in cancer development. The protective effects of ω-3 PUFAs in cancer appeared to be also linked to the ability of these fatty acids to modulate gene expression though the activation of the peroxisome proliferator-activated receptors (PPARs). This nuclear receptor family includes three isoforms of PPAR, named α,β/δ, and γ which all heterodimerize with retinoid X receptor α (RXRα) (Lemberger et al., 1996). Both PPAR γ and δ were expression in the colon and EPA and DHA constitute natural ligands for these receptors. In this context, the anti-neoplastic activity of omega-3 could be related to the inhibition of cell proliferation and induction of apoptotic processes through a mechanism which is dependent of PPAR γ activation (Edwards and O'Flaherty, 2008).

2. AIM OF THE WORK

Recently, our research group successfully tested the chemopreventive properties of a highly purified (99%) formulation of EPA supplied as the free fatty acid (EPA-FFA) in Apc $^{Min}/^{+}$ mouse model of FAP supporting an important tumor suppressor role of this bioactive compound (Fini et al., 2010) . In addition, EPA-FFA, has been demonstrated to reduce adenoma number and size in patients with familial adenomatous polyposis in a subsequent clinical trial conducted by another research group (West et al., 2010). However, the effect of dietary EPA-FFA on the onset of CAC remains to be elucidated. On the basis of these knowledge, this thesis investigates the potential chemopreventive effect of highly pure EPA-FFA on colitis-associated colorectal tumorigenesis with the following specific aims:

- To evaluate the impact of highly pure EPA-FFA dietary supplementation on the initiation and promotion of colorectal tumors in AOM-DSS mouse model of CAC

- To assess the effects of EPA-FFA dietary intake on colonic fatty acid composition and inflammation

- To best characterize the molecular mechanisms affected by EPA-FFA on inflammation-driven CRC and to identify new molecular pathways targeted by EPA-FFA

3.MATERIALS & METHODS

3.1 Animals

3.1.1 Mice

Five-week-old male C57BL/6J mice were obtained from Harlan Laboratories (San Pietro al Natisone, Udine, Italy) and housed in a temperature and humidity controlled animal facility with 12 hour light/dark cycle. All animal procedures were performed according to the animal protocol approved by the Institutional Animal Care committee of the University of Naples Federico II (Naples Italy).

3.1.2 Establishment of the Colitis-associated Cancer model

In order to obtain a mouse model of colitis-associated colorectal cancer (CAC) sixty mice were subjected to a well-defined chemically inflammation based colorectal cancer protocol (Tanaka et al., 2003), (Neufert et al., 2007). This model combines the use of the chemical genotoxic agent azoxymethane (AOM) with the pro-inflammatory agent DSS resulting in a pro-carcinogenic setting which promote the experimental onset of inflammation-driven CRC. In this study sixty mice were intraperitoneally injected with a single dose of AOM 7.5 mg/Kg (Sigma-Aldrich, Milan, Italy) diluted in phosphate buffered saline (PBS) followed by three cycles of 2.5% Dextrane sulfate sodium (DSS) in drinking water (Microtech, Naples, Italy) each consisting of four days. Each DSS cycle was followed by 16 days of regular drinking water (Figure 8).

3.1.3 Feeding protocol

At the beginning of the study the animals were randomized in three arms (20 mice each) to be fed either the control diet (Control) or diet enriched with Eicosapentaenoic acid free fatty acid (EPA-FFA) (ALFA, SLA Pharma AG, Switzerland). As Control diet the AIN-76A diet (Charles River Laboratory, Lecco, Italy) was used. EPA-FFA diets were obtained on the basis of the Control diet substituting 1% of the corn oil, which is ω-6 predominant, with 1% of a highly purified (99%) formulation of EPA-FFA. On the basis of previous experience (Fini et al., 2010), EPA-FFA stability was preserved enriching the food with antioxidant vitamins, sealing it and nitrogen flushed in foil bags. The diets were stored at 4°C and used within 3 days after opening. Being a protocol based on the diet, in order to minimize the variability among the arms, a fixed equal

amount of food was provided daily. Mice had free access to drink tap water. For the initiation protocol EPA-FFA diet was started four weeks before the AOM injection, while for the promotion protocol, which was designed in order to recreate the clinical scenario of patients with long-standing inflammatory bowel diseases, EPA-FFA diet was started after the first DSS cycle (Figure 7). Since it has been reported an exacerbation of DSS-induced colitis with a high rate of mortality and morbidity in presence of fish oil supplementation, during DSS cycles EPA-FFA diets were switched on Control diet. Eight C57BL/6J male mice were fed the Control diet without any additional treatment to be used as reference for some experiments (Healthy mice). Food intake was tracked daily and body weight was tracked three times per week. Rectal bleeding and diarrhea were also monitored during the study.

3.1.4 Sacrifice of mice and colon harvesting

Mice were euthanized after 15 weeks from the beginning of the protocol. Blood was collected by intracardiac puncture, serum samples were obtained after centrifugation at 2.500 r.p.m at 4°C and stored at -80°C. Urine was taken through bladder aspiration and immediately stored at -80°C. Stools were also collected and stored at -80°C. During the sacrifice the colon from each mice was removed immediately, opened longitudinally and washed with cold PBS. At the sacrifice tumors macroscopically analysis was performed in blind evaluating the number and the location of visible tumors. Tumors size measurements were carried out using a digital caliper evaluating the largest and the perpendicular diameter. Fresh tissue samples from colon tumors and normal mucosa were collected and stored at -80°C. Given the low number of tumors encountered in EPA-FFA treated groups compared to the Control arm, all the molecular analysis were performed on adjacent normal mucosa in order to have homogeneity among groups.

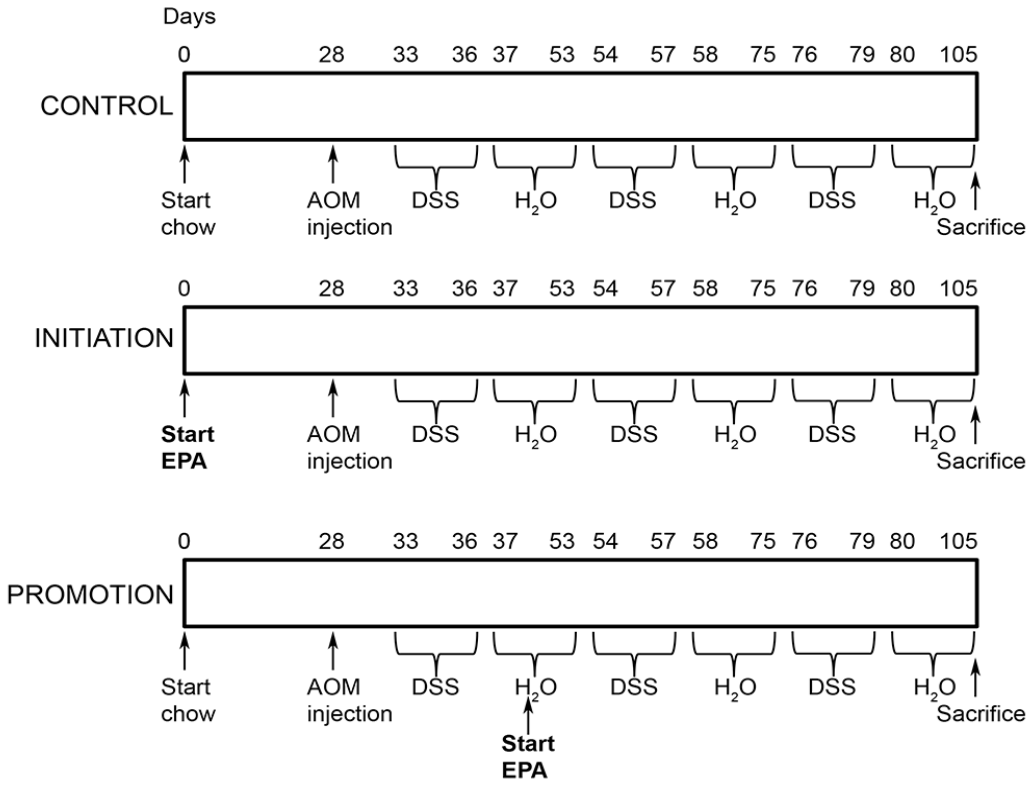

Figure 8 Experimental protocol of tumor induction and feeding in C57BL/6J mice. After the AOM injection, animals were subjected to three cycles of DSS. The Control arm fed a Control (Control) diet for the entire duration of the protocol. The Initiation arm started 1% EPA-FFA diet 4 weeks before AOM, while for the promotion protocol EPA-FFA was started during the recovery after the first DSS cycle. EPA-FFA diet was switched on Control diet during each DSS cycle.

3.2 Histological analysis for colon

3.2.1 Sample preparation

For histological analysis colon tissues were fixed in 10% formalin (DIAPATH srl, Martinengo (BG), Italy) solution at RT overnight. The samples were then washed with distilled H_20 and tissues dehydration obtained through ascending series ethanol (70%, 90%, 100%). After tissue dehydration slides were diafanized in Toluene for 20 minutes and samples embedded in paraffin blocks. Colon tissues were finally sectioned with a microtome at 4µm thickness and stained with hematoxylin and eosin (H&E) or used for immuno-staining.

3.2.2 Hematoxylin & Eosin staining

For histological analysis, formalin-fixed and paraffin-embedded colon sections of 4 µm thickness were de-waxed in toluene for 10-15 min and then rehydrated in descending series of ethanol solutions (100%, 95%, 70% and then in water). The de-waxed, rehydrated colon sections were stained with Hematoxylin & Eosin (H&E) using standard protocol and analyzed by pathologists in blinded fashion.

3.2.3 Histological assessment of colonic inflammation

Sections from each mice were microscopically examined for histopathologic changes associated with inflammation using the following scoring system. For the analysis of inflammatory cell infiltration: 0) none 1) minimal 2) moderate 3) severe. For inflammation extent: 0) none 1) mucosa, 2) mucosa and confluence of cells in submucosa 3) transmural inflammation. Cryptitis, which was defined as the presence of neutrophils within the crypt epithelium, and crypt loss were also evaluated according the following criteria: 1) 1/3 of affected crypts 2) 2/3 of affected crypts 3) crypt lost with intact epithelium surface 4) crypt lost with changes of epithelial surface structures. Percent area involvement was defined as: 0) none 1) 1-25%, 2) 26-50%, 3) 51-75%, 4) 76-100%. Histology score was determined by multiplying the percent involvement for each of the three previous described histological features by the percent area of colonic involvement (Poudyal et al., 2012).

3.2.4 Immunohistochemistry

For immunohistochemical (IHC) analysis, formalin-fixed and paraffin-embedded colon sections were de-waxed in toluene for 20 min, re-hydrated in 100% Ethanol for 5 min and subjected to endogenous peroxidase inhibition for 20 minutes at 4°C in methanol/H_2O_2 mixture. Slides were after re-hydrated with a 96% to 70% ethanol gradient and washed in distilled water for 2 min. Antigen retrival was performed in pressure cooker with citrate buffer (pH 6.0) at 120°C for 15 min. For Ki-67 slides were washed in PBS and unspecific background was blocked with blocking buffer (10% normal goat serum in PBS). The staining was performed with an avidin-biotin reaction applying a diluted primary mouse monoclonal antibody (1:400 in PBS o/n at 4° C) (clone MM1, Leica Microsystems, Milan, Italy). After washing primary antibody, a biotinylated secondary antibody (Vector Laboratories, Burlingame, CA) was subsequently applied (1:500 for 1h RT) and the reaction was revealed with DAB (3,3' Diaminobenzidine) (Sigma-Aldrich), using hematoxylin as a counterstaining. For β-catenin, slides were processed using the non-biotin-amplified complex (NovoLink Polymer Detection System, Leica Microsystem). Briefly, according to the manufacturer's instruction slides were blocked with Novocastra™ Protein Block for 5 minutes in a wet chamber in order to minimize the non-specific binding of primary antibody, rinsed twice with TBS 1X, and then incubated with a primary mouse monoclonal anti-beta-catenin antibody (1: 2.500 o/n at 4°C) (clone 14, BD Biosciences, Buccinasco, Italy). After washing with TBS, the slides were incubated with Post primary Block (Rabbit anti-mouse IgG) for 30 min at RT, rinsed in TBS and then incubated with Novolink polymer for 30min. The peroxidase activity was revealed using DAB as substrate, prepared from DAB Chromogen and Novolink™ DAB substrate buffer. To stop the reactions slides were rinsed in distilled water, counterstained with hematoxylin, dehydrated through an ethanol series and coverslipped. The Ki-67 proliferation index was calculated as the ratio between positive nuclei and total number of nuclei per crypt analyzed. β-catenin nuclear positivity was quantified on at least 50 crypts per sample.

3.3 TUNEL assay

Apoptosis was evaluated through the DeadEnd^{TM} Fluorimetric TUNEL System (Promega, Milan, Italy). This system allows the identification of apoptotic cells through the evaluation of DNA fragmentation, a biochemical hallmark of late programmed cell death. During apoptosis the cleavage of genomic DNA generate double-stranded DNA fragments (DSBs) with accessible 3'-hydroxyl (3'-OH) groups making it possible the enzymatic addition of fluorescently labeled nucleotides by Terminal Deoxynucleotidyl transferase (TdT) that can be revealed by fluorescence microscopy. For carrying out the TUNEL assays, according to the manufacturer's recommendations, tissues sections were de-waxed in xylene and rehydrated through a reverse ethanol series (100%, 95%, 70%). The slides were subsequently fixed in formaldehyde (4% in PBS) for 15 minutes at RT, permeabilized with Proteinase K solution incubation (20 µg/ml diluted in Tris-HCl 50 mM pH 8.0) at RT for 10 minutes and subsequently washed twice with PBS for 5 minutes. After repeating fixing and washing step, tissues sections were treated with equilibration buffer at RT for 10 minutes and incubated with nucleotide mix and rTdT buffer for 1 hour at 37°C in a humified chamber. As a positive control, slides were treated with DNaseI (GE HEALTHCARE) for 10 minutes to cause DNA fragmentation and prepared as previously described . As a negative control slides were incubated with nucleotide mix without rTdT enzyme. To stop the reaction, slides were incubated in SSC 1X and washed with PBS to remove unbound fluorescent dNTPs. All the slides were mounted adding the DAPI (Prolong Gold) nuclear stain in mounting medium and analyzed by fluorescence microscopy. Tunel positive cells were counted in at least 10 randomly selected fields (magnification 40X).

3.4 Mucosal fatty acid analysis

The PUFAs introduced with the diet were able to be incorporated in cell membranes as lipid component modulating the biological features (e.g., fluidity, thickness and deformability) and subsequently the biological functions of the membrane components (Rajamoorthi et al., 2005). These fatty acids, competing as substrates for cyclooxygenases and lipoxygenases enzymes, may affect their activity and subsequently the production of signalling molecules, named eicosanoids, with opposite physiological

functions. The membrane fatty acid composition was analyzed through gas-chromatography mass spectrometry (GC-MS) on mice colonic tissues as described below .

3.4.1 Tissues homogenization

For tissues homogenization, frozen colonic tissues fractions were pulverized in 12x75 glass tubes in 500 µl of PBS in ice and the tissues on the homogenizer blades were washed with 1 ml of chloroform-methanol mixture (CHCl3/MetOH 1:2 v/v) in the same tube. In order to avoid cross contaminations among specimens, the homogenizer blade was washed with ethanol, water and PBS for each sample.

3.4.2 Lipid extraction

Subsequently, in order to proceed with lipid extraction, the samples were transferred with Pasteur pipettes in SOVIREL extraction tubes, washed with 3 ml of chloroform-methanol mixture (CHCl3/MetOH 1:2 vol/vol) in presence of the antioxidant butylated hydroxytoluene (BHT 0.01%) and agitated under shaking for 30 minutes at room temperature. After thorough mixing, samples were centrifuged for 10 minutes at 2.000 x G at room temperature and the upper solvent layer was transferred in a new SOVIREL tube. The extraction was repeated with 2 ml of solvent and the supernatant layer combined. The solvent fraction was then re-extracted with 3 ml of CHCl3/H2O (1:1 - BHT 0.01%), vortexed and spinned, (2000 x G, 5 min, 25°C); the inferior solvent phase was transferred to a new SOVIREL tube and frozen at –20°C up the transmethylation.

3.4.3 Transesterification

The solvent phase was evaporated under a stream of nitrogen and 2 ml of KOH/MetOH solution (0.5M + 0.01% BHT) were added, the tubes carefully closed and heated at 80°C for 10 minutes in a dry thermal block. When the solution was at room temperature 1 ml of BF3: MetOH solution was added to each sample and the tubes wormed at 80°C in the dry thermal block for 10 minutes to develop a Methyl Ester mix.

3.4.4 Extraction of Methyl Esters

Methyl Esters were extracted adding 3 ml of hexane to each sample. The samples were then vortexed for 30 sec and spinned at 2000xG for 10 min at room temperature. The

supernatant solvent phase has been carefully recovered and transferred to a new glass tube. This extraction process was after repeated with other 2 ml of hexane. The hexane was evaporated under a stream of nitrogen to reduce the volume to 1 ml which was then transferred to a glass auto sampler vial. The tube was washed with 500 µl of hexane and the sample evaporated to dryness, redissolved with 20 µl of ciclohexane for the analysis and frozen at –20°C until injected.

3.4.5 GC-MS conditions

GC MS analysis was carried out using an Agilent HP6890 GC with PTV Injector and MS is an HP5973 mass detector; The column is SUPELCO SPTM-2330 (30mt x 0.25 mm x 0.2 µm film thickness) column. Carrier gas is Helium at 0.5 ml/min, in constant pressure mode; Injection cycle is in Solvent Vent mode, starting from 60° and ramps to 220°C ; column temperature was from100°C for 1.25 min, then was programmed to rise to185°C at 3 0 °C and to 205°C at 5 °C/min; total GC run time was 32min 1-4 µl samples was injected to GC-MS analysis. MS operates in EI mode, at 70 eV; Quadrupole temperature is 150°C; Ion source and GC interface were respectively 230°C, 280°C; Full data scan from m/z 40 to m/z 550.

3.4.6 Data processing and quantification

Retention times and peak area of analytes were determined by the Enhanced Data Analysis integration system of G1701 DA Agilent software. The percentage of analytes were calculated from the sum of the peak area of all analytes of interest using the following equation:

$$\textbf{Analyte percentage} = \frac{\text{Analyte Peak area} * 100}{(\Sigma \text{ areas of all identified peaks})}$$

3.5 Urinary PGE-M excretion analysis

PGE-2 is one of the prevalent eicosanoids produced from arachidonic acid by cyclooxygenases (COX-1 and COX-2) enzymes in presence of an inflammatory milieu. Given the very short half-time of PGE-2 in the circulatory system, the tetranor 11α-hydroxy-9, 15-dioxo-2, 3, 4, 5-tetranor-prostane-1, 20-dioic acid (known as PGE-M), the major urinary metabolite of PGE-2, represents a well validated marker for the PGE-2 biosynthesis determination *"in vivo"* (Murphey et al., 2004). In this work PGE-M levels were assayed on pooled urinary samples deriving from each animal in a given treatment using a liquid chromatography (LC)/tandem MS (LC-MS/MS) system according to the protocol described by Murphey et al (Murphey et al., 2004).

3.5.1 Samples preparation, PGE-M extraction and purification

Pooled urine samples (1ml) were acidified to pH 3 using 1M HCl. Endogenous PGE-M was derivatized incubating the sample with 0.5 ml of 16% (w/v) methyloxime HCl in 1.5 M of sodium acetate buffer (pH 5) for 1 hour. The assay internal standard was prepared converting the chemically synthesized PGE-M to a [$^2H_6$] *O*-methyloxime derivative by treatment with [$^2H_3$] *O*-methoxyamine HCl. The resulting methoximated PGE-M was extracted with 10 ml of water (pH 3). Samples were purified by solid phase extraction using a C-18 Sep-Pak cartridge (Waters, Milford, MA, USA) preconditioned with 5 ml methanol and 5 ml of water (pH 3). The samples were applied to the cartridge and then washed with 20 ml of water (pH 3) and 10 ml of heptane. The analytes were then eluted from the cartridge using 5 ml ethyl acetate and dried under a continuous stream of nitrogen at 37°C. The internal standard of [$^2H_6$] *O*-methyloxime PGE-M was then added and the eluate collected and dried under the same conditions. The resulting fraction was reconstituted in 50 µl of mobile phase A (95:4:9:0:1 (v/v/v) 5 mM ammonium acetate: acetonitrile: acetic acid) and filtered through a 0.2-micron Spin X filter (Corning, NY, USA).

3.5.2 Sample analysis by LC/MS/MS

Separation by LC was performed using an Acquity Ultra Performance LC™ (UPLC ™) BEH C8 Column, 2,1 X 100 mm, 1.7 µm (Waters, Milford, USA). Mobile phase A was 95:5, 0,1% (v/v) acetic acid: acetonitrile, and mobile phase B was 50:50, 0,1% (v/v)

acetic acid: acetonitrile. Samples were separated by a gradient of 98-40% mobile phase A over 15 min at flow rate of 300µl/min prior to delivery to a Quattro Premier ™ XE (Waters Corp, Milford, USA) bench-top tandem quadrupole mass spectrometer operated in multiple reaction monitoring (MRM) mode. Samples were ionized through ESI using nitrogen as source for both sheath and auxiliary gas setted at 60 psi and 7L/min, respectively. The mass spectrometer was operated in the negative ion mode with a capillary temperature of 210°C, a spray voltage of 3.0 kV, and a cone voltage of 19V. MRM channels were established for PGE-M (m/z 385.2 > 336.2) and the internal standard PGE-Md6 (m/z 391.2 > 339.2) (Cayman Chemical, Ann Arbor, MI). Quantification of endogenous urinary PGE-M was performed against the internal standard with calibration using 10-100 ng/ml authentic PGE-M. The limit of detection was 1ng/ml. Data were expressed after correction for urinary creatinine concentrations and reported as nanongram (ng) of PGE-M per milligram (mg) of creatinine. The urinary creatinine concentration was measured by HPLC using the method by Hewavitharana and Bruce (Hewavitharana and Bruce, 2003).

3.6 Circulating cytokine analysis

Circulating cytokine levels were determined in serum samples using a Bio-Plex multiplexed bead-based sandwich immunoassay (Bio-Rad Laboratories, Milan, Italy). This system relies on the calculation of the fluorescence emission signal resulting from the binding of a biotinylated antibodies with the complex constituting by an antibody-coupled fuorescent dyed bead and a specific cytokine. The reaction is revealed using streptavidin-phycoerythrin (streptavidin-PE), which binds to the biotinylated detection antibodies. The Bio-Plex Manager™ software automatically calculate cytokines concentrations in the sample using a standard curve derived from a recombinant cytokine standard. The use of the different colour beads-set allows the discrimination and simultaneous quantification of multiple cytokines in the same sample. In our experiments we used a multiplex cytokines assay (Bio-Plex Bio-Rad Laboratories, Milan, Italy) which allow the detection of six mouse cytokines: IL-6, IL-10, IL1β, IL-17,IFN-γ and TNF-α. Serum samples were obtained from whole blood after

centrifugation at 1.000 x g for 15 min at 4°C. The analysis was performed pooling serum samples from animals in each arm. According to the manufacturer instructions, the lyophilized cytokines standards were diluted with 500 µl of the appropriate standard diluent, vortexed gently and incubated on ice for 30 min. Reconstituted cytokine standards were used to produce an eight-point standard curve with a fourfold (1:4) dilution between each point. Serum samples were also diluted with the specific Bio-Plex mouse sample diluent (1:4). 50 µl of cytokine standards or samples were added to each well of a 96-well filter plate and incubated with 50 µl of coupled beads for 30 min at room temperature under shaking (850 +/- 50 rpm). Plates were then washed by vacuum filtration three times with 100 µl of Bio-Plex wash buffer, 25 µl of diluted detection biotinylated antibody were added, and plates were incubated for 30 min at room temperature with shaking. After three filter washes, 50 µl of streptavidin-PE were added, and the plates incubated for 10 min at room temperature under shaking. Finally, plates were washed by vacuum filtration three times, beads suspended in Bio-Plex assay buffer, and samples analyzed on a Bio-Rad 96-well plate reader using the Bio-Plex Suspension Array System and Bio-Plex Manager software (Bio-Rad Laboratories, Hercules, CA). Each sample was tested in duplicate, and cytokine standards supplied by the manufacturer were run on each plate.

3.7 Gene expression analysis

3.7.1 RNA extraction

Total RNA was extracted from distal colon segments. Briefly, tissues were homogenized in Trizol® Reagent (Life Technologies, Monza, Italy) and RNA extraction was performed following the manufacturer's instructions. RNA concentration and purity was evaluated by measuring the absorbance (optical density, OD) at 260 nm in relation to the absorbance at 280 nm (OD 260/280) or 230 nm (OD 260/230) respectively for protein or ethanol contamination using NanoDrop spectrophotometer (Thermo Scientific).

3.7.2 qRT-PCR: Taqman Gene Expression Assay

Total RNA (2 µg) was reverse transcribed to cDNA using a GoScript ™ Reverse Transcription System (Promega) according to the manufacturer's suggested protocol.

Briefly, after heating of combined RNA and Oligo(dT)15 primers at 70°C for 5 minutes followed by a step on ice for other 5 minutes, the reverse transcription mix was prepared and added to primer/RNA mix in a final volume of 20 µl. The reverse transcription mix was composed of 4.0 µl of GoScript™ reaction buffer, 4.0 µl of $MgCl_2$ 25 mM, 1.0 µl of PCR nucleotide mix 10mM, 0.5 µl of Recombinant RNasin® Ribonuclease Inhibitor (40 U/ µl), 1.0 µl of GoScript™ Reverse Transcriptase and 4.5 µl of nuclease free water. After annealing at 25°C for 5 minutes, the extension reaction was performed at 42°C for 60 minutes and reverse transcriptase was then inactivated at 70°C for 15 minutes. Finally, the obtained cDNA was stored at -20°C until the use. For quantitative real-time PCR the cDNA was diluted 1:3 and 2 µl of diluted cDNA was used in duplicate in 20 µl total volume using TaqMan Gene Expression Master Mix and TaqMan Gene Expression Assays (Life Technologies, Monza, Italy) for genes listed in Table 1 . The qRT-PCR reaction was performed on Stratagene MX3000P QPCR thermal cycler machine (Agilent Technologies, Milan, Italy) as follows: 50ºC for 2 min for Uracil-DNA Glycosylase (UDG) incubation, 95°C for 10 min for AmpliTaq Gold enzyme activation followed by 45 cycles of denaturation at 95°C for 15s and annealing/extension at 60°C for 1 min. Fold induction levels for each gene were obtained using the $2^{-\Delta\Delta Ct}$ method by normalizing against GAPDH used as endogenous control. Fold changes values were compared to the healthy mice group.

Table 1 TaqMan assays employed for qRT–PCR analysis

TaqMan Assay ID	Gene symbol	Gene name (mouse)
Mm00496902-m1	**Jag1**	Jagged1
Mm01342805-m1	**Hes1**	Hairy and enhancer of split 1
Mm00476035-s1	**Atoh1**	Atonal homolog 1
Mm00482529-s1	**Nrarp**	Notch-regulated ankyrin repeat protein
Mm00446190-m1	**IL-6**	Interleukin 6
Mm00443260-g1	**TNF-α**	Tumor necrosis factor-alpha
Mm00434228-m1	**IL-1β**	Interleukin 1 beta
Mm01168134-m1	**IFN-γ**	Interferon gamma
Mm99999915-g1	**Gapdh**	Glyceraldehyde-3-phosphate dehydrogenase

3.7.3 qRT-PCR: Taqman miRNA Expression Assay

Reverse transcription reaction for the Taqman miRNA Assays (Life Technologies) was performed using the Taqman microRNA Reverse Transcription Kit (Life Technologies) with 150 ng of total RNA, per 15 µl reaction. The 1X reverse transcription mix was composed of 0.15µl 100mM dNTPs, 1µl Multiscribe Reverse Transcriptase 50U/µl, 1.5 µl 10X reverse transcription buffer, 0.19µl RNAse inhibitor 20U/µl and 4.16µl of nuclease free water, 3µl of 5X specific miRNA reverse transcription primers, and 5µl of sample RNA, making a total volume of 15µl. Specific supplied primers for snoRNA202 and miR-34a were used. Reverse transcription thermocycling parameters were as follow: 16°C for 30min, 42°C for 30min, 85°C for 5min and a final holding temperature of 4°C. The resultant cDNA were quantified in qRT-PCR in duplicate in 20 µl total volume using TaqMan® Universal PCR Master Mix, no AmpErase® UNG (Life Technologies, Monza, Italy) and TaqMan® MicroRNA Assays (Life Technologies, Monza, Italy) with the specific probes for each miRNA assay (snoRNA202 Assay ID: 001232; mmu-miR-34a Assay ID: 000426). The qRT-PCR reaction was performed on Stratagene MX3000P QPCR thermal cycler machine (Agilent Technologies, Milan, Italy) as follows: 95°C for 10 min for AmpliTaq Gold enzyme activation followed by 45 cycles of denaturation at 95°C for 15s and annealing/extension at 60°C for 1 min. Fold induction levels for miR-34a were obtained using the $2^{-\Delta\Delta Ct}$ method by

normalizing against snoRNA202 used as endogenous control. Fold changes values were compared to the healthy mice group.

3.8 Gut microbiota analysis

The mouse intestinal microbiota composition was characterized on pooled faecal samples deriving from each animal in a given treatment using a validated phylogenetic DNA microarray platform High Taxonomic Fingerprint microbiota Array (HTF-Microbi.Array) (Candela et al., 2010). This approach is based on Ligase Detection Reaction-Universal Array (LDR-UA) allowing the identification of 16S rDNA of 33 phylogenetically related groups corresponding to the 95% of the mammalian gut microbiota. For the LDR reaction two pairs of oligonucleotide probes and a thermostable DNA ligase were used with previously PCR-amplified DNA products. The two probes are designed as a pair of adjacent oligonucleotides specific for each target sequence: a probe specific for the variation, called "Discriminating Probe" or DS which carries a fluorophore Cy3 at 5' and a second probe, named "Common Probe" or CP, starting one base 3'-downstream of the DS and carries a 5'-phosphate group and a unique sequence named cZipCode at its 3'end. This LDR technology is based on the high discriminating power of the DNA ligation enzyme which will occur only in presence of a perfect match between a specific template and a particular pair of probes DS and CP.

3.8.1 DNA extraction from faecal samples

Total DNA from faecal samples obtained at sacrifice was extracted by using QIAmp DNA Stool Mini Kit (Qiagen, Milan, Italy) with µa modified protocol as described by Candela et al. (Candela et al., 2010). 250 mg of pooled faeces for each arm were suspended in 1 ml of lysis buffer. Then 0.5 g of Zirconia beads and four 3 mm glass beads were added to the samples and placed into the instrument FastPrep24 (MP Biomedical, Irvine, CA, USA) performing 3 cycles of rotation at a speed of 5.5 m / s for 3 minutes allowing cell lysis and recovery of nucleic acids. All samples were heated at 95 °C for 15 minutes and then centrifuged at maximum speed for 5 minutes to pellet stool particles. Supernatants were collected, supplemented with 260 µl of 10 M ammonium acetate, incubated on ice for 5 min and centrifugated at maximum speed for

10 minutes. One volume of isopropanol was added to each supernatant and then samples were incubated on ice 30 minutes. The precipitated nucleic acid were collected by centrifugation at full speed for 15 minutes and washed with EtOH 70%. Subsequently, the pellets were resuspended with 100 µl of TE buffer and incubated with 2 µl of DNase-free-RNase (10 mg/ml) at 37°C for 15 minutes. DNA purification with QIAmp Mini Spin columns and protein removal by Proteinase K treatment were performed following the kit protocol. Purificated DNA were quantified by using NanoDrop ND-1000 (Thermo Scientific, Wilmington, DE).

3.8.2 PCR amplification of 16S rDNA gene

A nearly full- lenght portion of 16S ribosomal DNA was performed using universal forward primer 16S27F (5'-AGAGTTTGATCMTGGCTCAG-3') and reverse primer r1492 (5'-TACGGYTACCTTGTTACGACTT-3'). PCR amplifications were performed with Biometra Thermal Cycler T Gradient (Biometra, Germany). The reaction mixtures included 500 nM concentrations of each primer, 200 µM concentrations of each deoxynucleoside triphosphate, 10 mM Tris-HCl (pH 8.8), 1.5 mM MgCl2, 50 mM KCl, 0.1% (wt/vol) Triton X-100, 0,5 U of DNAzyme DNA polymerase II (Finnzymes, Espoo, Finland), and 50 ng of genomic DNA in a final volume of 50 µl. PCR conditions are as follows: 95°C for 5 min, 35 cycles of denaturation at 95°C for 60 s, annealing at 60°C for 30s, elongation at 72°C for 90 sec and one final step at 72°C for 10 min. PCR products were purified using the High Pure PCR Cleanup Micro Kit (Roche, Mannheim, Germany) and eluted in 30 µl of sterile water and quantified with NanoDrop.

3.8.3 Ligation Detection Reaction

The PCR products were used as template for the Ligation Detection Reaction (LDR). The LDR was carried out in a final volume of 20 µl containing 20 mM Tris-HCl (pH 7.5), 20 mM KCl, 10 mM MgCl2, 0.1% NP-40, 0.01 mM ATP, 1 mM dithiothreitol, 250 fmol of each discriminating probe, 250 fmol of each common probe, 10 fmol of the hybridization control, and from 0.5 to 100 fmol of purified PCR products. After the reaction mixture was preheated for 2 min at 94°C and centrifuged for 1 min, 4 U of *Pfu* DNA ligase (Stratagene, La Jolla, Calif.) was added. The LDR was performed using a thermocycler Thermal Cycler Gradient T (Biometra) by setting the following

program: initial denaturation at 94 ° C for 5 minutes, 40 cycles at 94 ° C for 30 seconds, 60 ° C for 4 minutes and a cycle at 94 ° C for 2 minutes.

3.8.4 Array hybridization of the LDR products

The hybridization mixture was prepared in a final volume of 65 µl containing 20 µl of LDR mixture, 5× SSC, and 0.1 mg of salmon sperm DNA/m and distilled H_2O. After heating at 94°C for 2 min and chilling on ice, the hybridization mixture was applied to the slide, on which the sixteen arrays were separated by Press-To-Seal silicone isolators (1.0 × 9 mm; Schleicher & Schuell BioScience, Dassel, Germany). The hybridization was carried out at a temperature of 65 ° C in the dark with shaking at 40 rpm for an hour and 30 minutes. Afterwards the slides were washed at 65°C for 15 minutes in prewarmed SSC 1X preheated to 65 ° C and dried in a JA14 tube by centrifugation for 3 minutes at 800 rpm.

3.8.5 DNA Array

The HTF-Microbi.Array allows the identification and approximate relative quantification of 16S rDNA of 33 phylogenetically related microbial groups phylogenetically of the intestinal microbiota . The targeted intestinal bacteria revealed by HTF-Microbi.Array were showed in Table 2

3.8.6 Data Acquisition

The fluorescent signals were acquired at a 5-µm resolution by using a ScanArray 4000 laser-scanning system (PerkinElmer Life and Analytical Sciences, Boston, Mass.) with a green laser for Cy3 dye (λex, 543 nm; λem, 570 nm). ScanArray Express v 4.0, was used to quantify the fluorescent intensity of the spots. Fluorescence intensity were normalized on the basis of the synthetic ligation control signal. Relative abundance of each bacterial group was obtained by calculating the relative fluorescence contribution of the corresponding HTF-Microby Array probe as a percentage of the total fluorescence.

Table 2 Microbial groups detected by HTF-Microbi.Array

Bacterial group	Taxonomic level	Cluster	Order	Division
Bacteroides/Prevotella	Cluster	Bacteroides/Prevotella	Bacteroidales	Bacteroidetes
Ruminococcus bromii	Sub cluster	Cl IV	Clostridiales	Firmicutes
Ruminococcus albus	Sub cluster	Cl IV	Clostridiales	Firmicutes
Faecalibacterium prausnitzii	Sub cluster	Cl IV	Clostridiales	Firmicutes
Oscillospira guillermondii	Sub cluster	Cl IV	Clostridiales	Firmicutes
Clostridium IX	Cluster	Cl IX	Clostridiales	Firmicutes
Veilonella	Species	Cl IX	Clostridiales	Firmicutes
Clostridium XIVa	Cluster	Cl XIVa	Clostridiales	Firmicutes
Eubacterium rectale	Species	Cl XIVa	Clostridiales	Firmicutes
Bifidobacteriaceae	Family	Bifidobacterium	Bifidobacteriales	Actinobacteria
B. longum	Species	Bifidobacterium	Bifidobacteriales	Actinobacteria
Lactobacillaceae	Family	Lactobacillaceae	Lactobacillales	Firmicutes
L. plantarum	Species	Lactobacillaceae	Lactobacillales	Firmicutes
L. casei	Species	Lactobacillaceae	Lactobacillales	Firmicutes
L. salivarius	Species	Lactobacillaceae	Lactobacillales	Firmicutes
Bacillus clausii	Species	Bacillaceae	Bacillales	Firmicutes
Bacillus subtilis	Species	Bacillaceae	Bacillales	Firmicutes
Streptococcacea	Family	Streptococcaceae	Lactobacillales	Firmicutes
Fusobacterium	Genus	Fusobacteriaceae	Fusobacteria	Fusobacteria
Akkermansia	Genus	Verrucomicrobiae	Verrucomicrobiae	Verrucomicr obia
Cyanobacteria	Family	Cyanobacteria	Cyanobacteria	Cyanobacteria
Clostridium XI	Cluster	Cl XI	Clostridiales	Firmicutes
Clostridium diffcile	Species	Cl XI	Clostridiales	Firmicutes
Clostridium I e II	Cluster	Cl I e II	Clostridiales	Firmicutes
Clostridium perfringens	Species	Cl I e II	Clostridiales	Firmicutes
Enterococcus faecalis	Species	Enterococcales	Lactobacillales	Firmicutes
Enterococcus faecium	Species	Enterococcales	Lactobacillales	Firmicutes
Staphylococcus	Genus	Staphylococcaceae	Bacillales	Firmicutes
Bacillus cereus	Species	Bacillaceae	Bacillales	Firmicutes
Enterobacteriaceae	Family	Enterobacteraceae	Enterobacterales	Proteobacteria
Yersinia enterocolitica	Species	Enterobacteraceae	Enterobacterales	Proteobacteria
Proteus	Genus	Enterobacteraceae	Enterobacterales	Proteobacteria
Campylobacter	Genus	Campylobacteraceae	Campylobacterales	Proteobacteria

3.9 Statistical analysis

Data were reported as mean ± SEM. One-way ANOVA was used to analyze continuous variables followed by Turkey's for comparison among groups. Tumor incidence was evaluated by Fisher's exact test. Data analysis was performed using Graph Pad Prism 5(GraphPad software, La Jolla, CA). For the gut microbiota experiments, statistical analysis was performed using SigmaStat v 3.5 (Systat Sofware, San Jose, CA) and R statistical software. P values less than 0.05 were considered statistically significant.

4. RESULTS

4.1 Food intake, body weight and mortality rate during the experimental protocol

Food intake was monitored daily. Animal body weight was evaluated three times per week. No statistical differences were appreciated in term of food intake (Figure 9) and body weight (Figure 10) among the diets arms during the AOM-DSS experimental protocol.

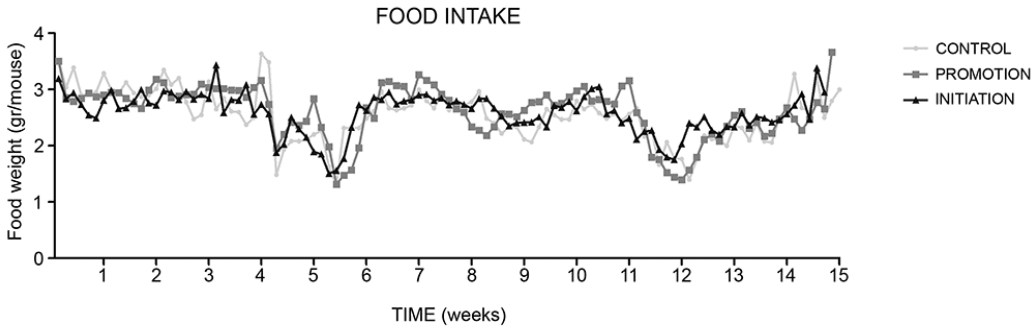

Figure 9 Food intake in the AOM-DSS protocol No significant changes were observed in terms of food intake among the three different groups. Data are means ± SEM.

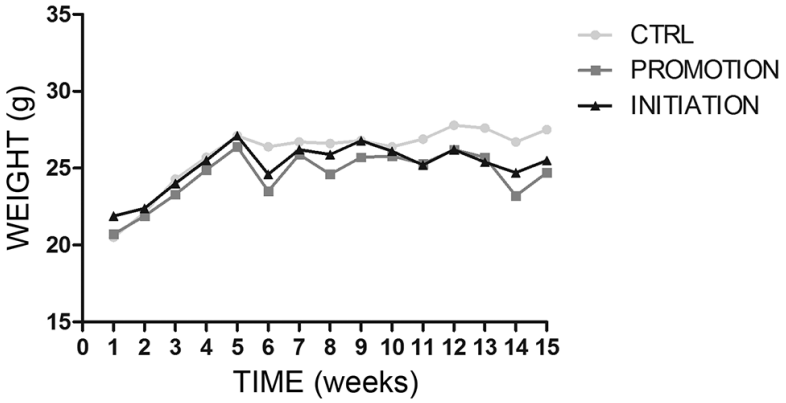

Figure 10 Body weight during the AOM-DSS protocol No significant changes in body weight were observed among the three different groups during the protocol Data are means ± SEM.

Importantly, no statistical differences were found in terms of animal mortality among the three different groups of treatment (7/20 in the control group and 9/20 in the promotion and initiation arms).

4.2 EPA-FFA protect from inflammatory carcinogenesis in AOM-DSS mouse model

In this study, colitis associated colorectal cancer (CAC) was induced by a single injection of AOM followed by repeated administration of DSS according to a well established protocol (Tanaka, 2012), (Neufert et al., 2007a). In order to investigate the effect of EPA-FFA on the initiation and promotion of CAC, we performed a feeding protocol in AOM-DSS mouse model background. Control group fed a balance diet (AIN76A) for the entire duration of the study. Promotion and Initiation arms were fed with a balance diet (AIN76A) in which 1% of corn-oil was substituted with 1% EPA-FFA. Our results showed an important chemopreventive effect of EPA-FFA on tumor development in both arms fed with EPA-FFA diet respect to the Control arm. Indeed, macroscopic and histologically analysis revealed that all mice except one fed with the Control diet, developed colon adenocarcinoma at the end of the study, while only three animals in the promotion and four in the initiation groups developed cancers. Lesions were mainly located in the distal to middle colon, while no tumors were found in the proximal section of the colon in either group of animals. The reduction of tumor incidence encountered was respectively of 72.7% in the promotion group ($p = 0.0003$ *vs.* Control arm) and 63.6% in the initiation group respect to the Control arm ($p = 0.0013$ *vs.* Control arm) (Figure 11A). Moreover, we found that EPA-FFA diet was able to significantly decrease tumor multiplicity (number of lesions/mouse) when compared to the Control diet. We counted a reduction of 78.2% and 69.8% in the number of lesions for each mouse respectively in the promotion and initiation arms respect to the Control group ($p < 0.0001$ for promotion *vs.* Control and $p < 0.001$ for initiation *vs.* Control) (ANOVA $p = 0.002$) (Figure 11B). In addition, the maximum polyps size (mm) was also significantly higher in mice feeding the Control diet respect the promotion and initiation groups in whom the average tumor size resulted decreased by 75.7% and 63.9% respectively ($p < 0.001$ for promotion *vs.* Control and $p < 0.05$ for initiation *vs.* Control) (ANOVA $p = 0.002$) (Figure 11C) (Table 3). Representative

images of H&E stained colon sections at the end of the AOM/DSS protocol were reported in Figure 12.

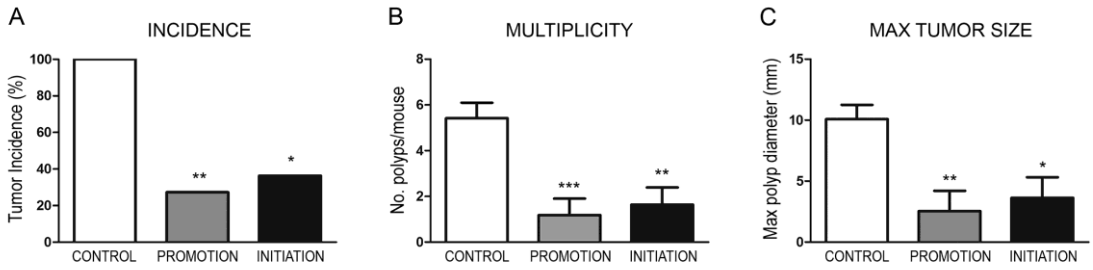

Figure 11 Effect of EPA-FFA on tumor incidence, multiplicity and size A) Tumor incidence, B) Tumor multiplicity and C) Max tumor size in Control group and in EPA-FFA feeding groups at the end of the AOM/DSS protocol. Polyp incidence was calculated as percentage of animals with tumors in each arm using Fisher's exact test. Tumor multiplicity (number of lesions/ mouse in each arm) and max tumor size (mm) were reported as means ± SEM and analyzed using one-way ANOVA followed by Turkey's for multiple comparison. * P < 0.05; ** P < 0.001; *** p < 0.0001.

Table 3 Effect of experimental diets on tumor incidence, multiplicity and size

Group	N	% Animals with colon tumors	N° tumors per animal	Average size of tumors (mm^2)
Control	13	92,3	5 ± 0,75	10,08 ± 1,17
Promotion	11	27,3	1,18 ± 0,72	2,55 ± 1,66
Initiation	11	36,4	1,64 ± 0,75	3,64 ± 1,68

Values are reported as means ± SEM.
Statistical variations among the feeding protocols were reported in Figure 11

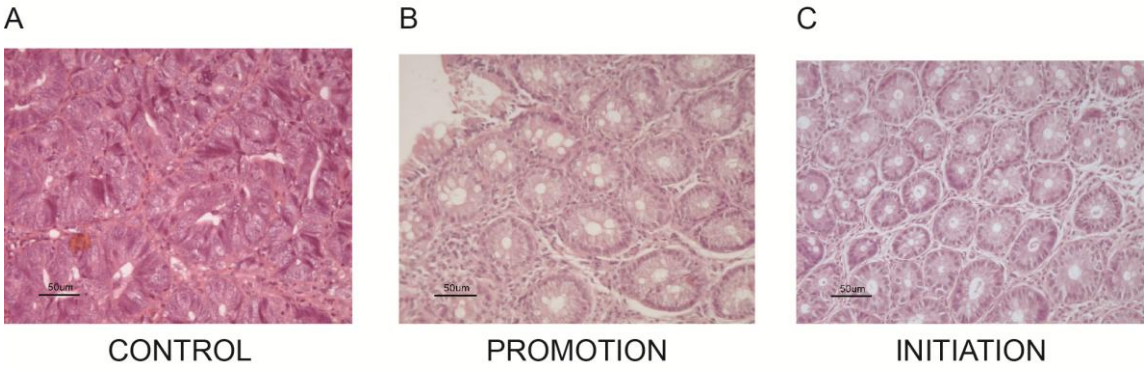

Figure 12 Representative images of H&E stained colon sections at the end of the AOM/DSS protocol A) Control B) Promotion C) Initiation (Ctrl n = 13, Promotion and Initiation n = 11).

4.3 EPA-FFA increases apoptosis, reduces cell proliferation and nuclear β-catenin in AOM-DSS-treated mice

Given the important chemopreventive effect encountered in mice feeding EPA-FFA diets, we speculate that changes in apoptosis or cell proliferation may contribute to explain the differences in tumor development among the diets groups. Apoptotic cells were evaluated using *in situ* terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining. Both arms feeding the EPA-FFA diet showed a significant increase of apoptosis rate compared to the Control group. We found an increase in the number of tunel positive cells of 43.1% in the promotion and 55.1% in the initiation groups, respectively ($p < 0.05$ for promotion *vs.* Control; $p < 0.001$ for initiation *vs.* Control) (ANOVA $p = 0.003$) (Figure 13A). Next, to determine the impact of EPA-FFA on cellular proliferation, Ki-67 immunoistochemistry (IHC) was performed. Ki-67 positive cells on total number of nuclei/crypt were counted and averaged. We observed that nontumorigenic colonic epithelium in the promotion and initiation protocols was characterized by a marked reduction of cell proliferation as compared to the Control arm. The percentage of Ki-67 positive cells reduction detected was 24% and 27.7% in the promotion and initiation arms, respectively (both groups *vs.* Control $p < 0.0001$) (ANOVA $p < 0.0001$) (Figure 13B). Subsequently, since it is known that β-catenin signaling activation is a critical event in colorectal carcinogenesis (Sellin et al., 2001) and the upregulation of nuclear β-catenin was reported in AOM-DSS mouse model(Lu et al., 2014), we decided to evaluate whether EPA-FFA is able to prevent this phenomenon. Immunohistochemical analysis of β-catenin was carried out in each arm of treatment quantifying nuclear positivity on at least 50 crypts for sample. Quantitative analysis revealed a significant lower nuclear translocation of β-catenin in both EPA-FFA treated arms showing a reduction of 58% in the promotion and of 68.2% in the initiation arms when compared to the Control group ($p < 0.0001$ for both groups *vs.* Control) (ANOVA $p < 0.0001$) (Figure 13C).

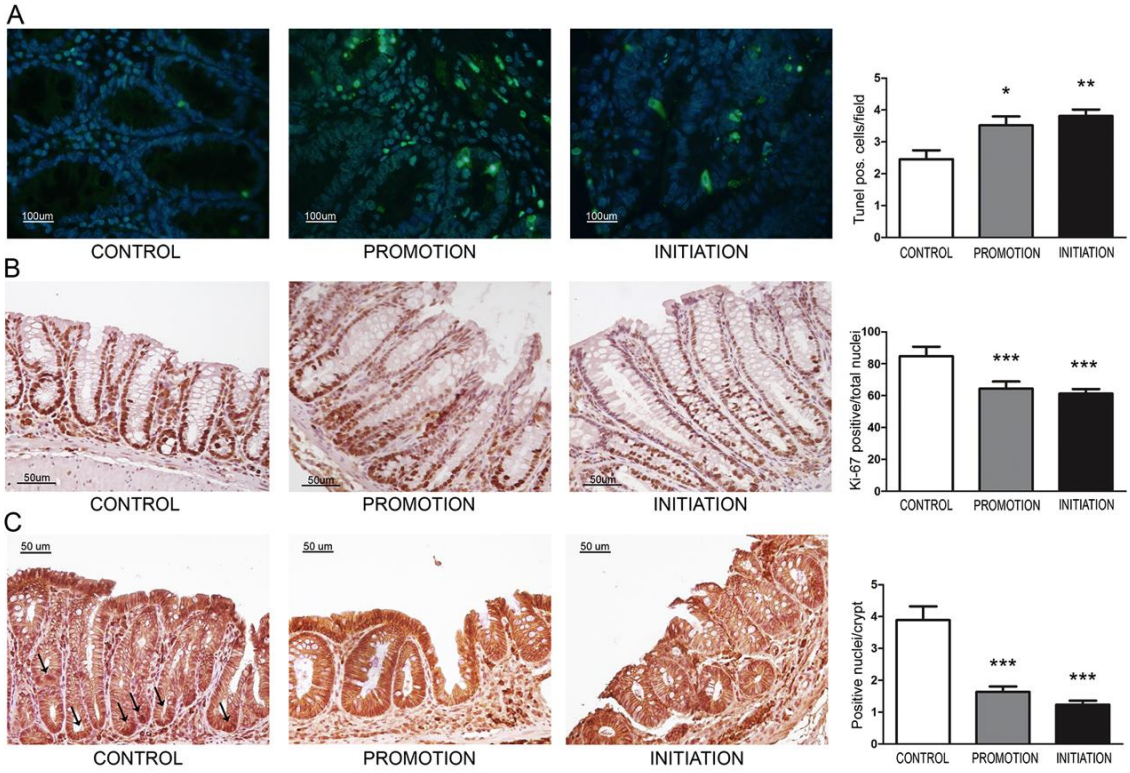

Figure 13 Effects of EPA-FFA on apoptosis, cell proliferation and nuclear β-catenin

A) Representative TUNEL staining of colon sections from mice feeding the Control diet or EPA-FFA diets in the AOM-DSS protocol. EPA-FFA significantly increases tunel positive cells as compared to the control group. Histogram show average number of TUNEL positive cells per field (Ctrl n = 13, Promotion and Initiation n = 11) B) Sections from distal colon of AOM-DSS treated mice following different feeding protocols stained with Ki67 antibody for cell proliferation analysis. EPA-FFA significantly reduces Ki-67 nuclear staining in both promotion and initiation arms as compared to the control. Histogram show average number of Ki-67 positive nuclei/total nuclei (Ctrl n = 13, Promotion and Initiation n = 11) C) β-catenin nuclear staining in Control and EPA-FFA feeding groups of AOM-DSS treated mice. EPA-FFA arms showed significant less nuclear β-catenin translocation as compared to control. Histogram show average number of β-catenin positive nuclei/crypt (Ctrl n = 13, Promotion and Initiation n = 11) Data represent means ± SEM $P < 0.05$; ** $P < 0.001$; *** $p < 0.0001$.

4.4 EPA-FFA modulate colonic fatty acid composition in AOM-DSS-treated mice

To assess the success of the feeding protocol, and in order to establish the effect of EPA-FFA diets on fatty acids composition, the incorporation of most representative fatty acids was analyzed in nonpolypoid colonic tissues of AOM-DSS treated mice using GC-MS. Mucosal EPA content significantly increased in the promotion and initiation groups compared to the control arm ($p < 0.0001$), indicating that supplemented EPA-FFA was efficiently incorporated in colonic tissues of mice treated with AOM-DSS (Figure 14). However, we didn't found differences of EPA incorporation between the promotion and initiation arms, although the latter started EPA-FFA diet 40 days before.

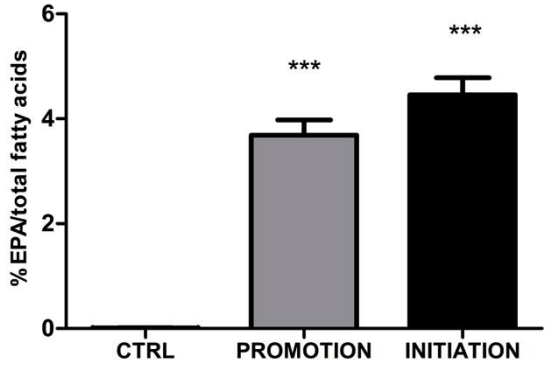

Figure 14 Incorporation of dietary EPA-FFA in colonic tissues EPA colonic content was measured by GC-MS in AOM-DSS treated animals at the end of the end of the experimental protocol. Control group fed a Control diet and induced colon cancer by AOM and DSS (n = 13); Promotion and Initiation arms fed a diet containing 1% EPA-FFA and induced colon cancer by AOM and DSS (n = 11 mice per group).
Promotion and initiation arms showed significant increased percentage content of colonic EPA-FFA as compared to the Ctrl arm.
EPA levels were expressed as relative percentages of total fatty acids. Data are shown as means ± SEM. $P < 0.05$; ** $P < 0.001$; *** $p < 0.0001$; One-way ANOVA followed by Turkey's for multiple comparison.

As regards the effects of EPA-FFA diets on total fatty acid composition, no variations were observed in the percentage content of palmitic and stearic acid among the three different groups. Otherwise, a lower percentage of oleic, linoleic and arachidonic acid was found in the colonic tissues of EPA-FFA treated mice respect to the Control group associated with a modest increase of linolenic acid content. Importantly, we found that, compared to the Control group, in the colonic tissues of mice belonging to the promotion and initiation groups, EPA, DPA and DHA percentages, significantly increased in response to dietary EPA-FFA indicating the ability of the mice to efficiently incorporate EPA-FFA and to promote the conversion to DPA and DHA. The differences in fatty acids content between Control and EPA-FFA-supplemented groups were summarized in Table 4.

Table 4 Effects of experimental diets on colonic fatty acids composition

Fatty acids	Control	Promotion	Initiation	value of p (ANOVA)
C16:0 (Palmitic)	26,29 ± 0,67	27,28 ± 1,22	25,60 ± 1,19	ns
C18:0 (Stearic)	9,85± 1,74	14,50 ± 2,33	16,00 ± 2,37	ns
C18:1 n-9 (oleic)	36,14 ± 2,13	28,08 ± 2,38	26,80 ± 2,23	0.0051
C18:2 n-6 (linoleic)	17,97 ± 1,48	13,49 ± 1,61	13,68 ± 1,56	0.0419
C20:4n-6 (arachidonic)	8,12 ± 1,77	5,62 ± 1,05	5,58 ± 0,78	0.0116
C18:3 n-3 (linolenic)	0,37 ± 0,08	0,42 ± 0,07	0,54 ± 0,09	0.0071
C20:6n-3 (docosahexaenoic)	0,90 ± 0,26	4,17 ± 0,64	4,29 ± 0,47	0.0371
C22:5 n-3 (docosapentaenoic acid)	0,32 ± 0,06	2,76 ± 0,46	3,11± 0,46	0.0025
C20:5n-3 (eicosapentaenoic acid)	0,01± 0,00	3,69 ± 0,28	4,46 ± 0,32	< 0.0001

Data were expressed as percentage of fatty acid methyl esters ± SEM

A higher ω-6/ω-3 PUFAs ratio is found in the western diet and resulted associated with a greater incidence of inflammatory related diseases including IBDs (Hou et al., 2011). Given the importance of an optimal ω-6/ω-3 ratio for health benefits, rather than the absolute percentage of individual PUFAs, we decide to focus our attention on the effects of EPA-FFA diet on the overall ω-6/ω-3 content. As shown in Figure 15, in EPA-FFA treated mice compared to the Control group, the combination of EPA, DPA and DHA, which represents ω-3 PUFAs, resulted significantly increased ($P < 0.0001$ for

each group vs. Control) (ANOVA $P < 0.0001$) at the expenses of the combined percentage content of mucosal ω-6 PUFAs arachidonic and linoleic acids which was significantly decreased in the same groups ($P < 0.0001$ for each group s. Control) (Figure 15). These results indicate that EPA-FFA dietary intake not only enriched the EPA content in the cellular membrane, but also increased the total (n-3) polyunsaturated fatty acid content reducing the ω-6/ ω-3 ratio.

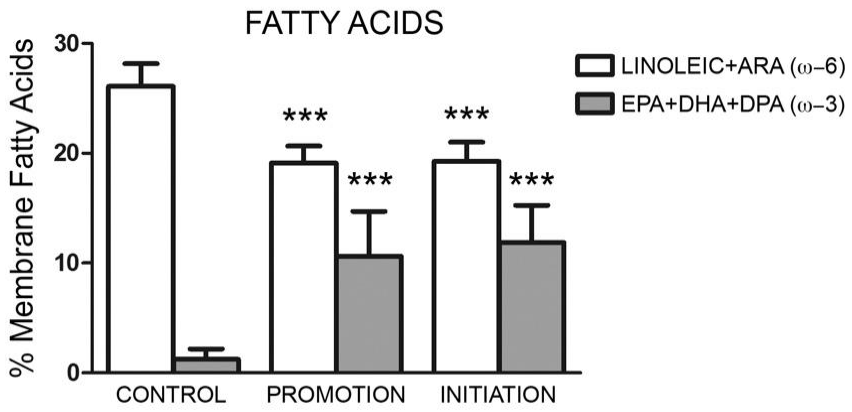

Figure 15 EPA-FFA modulates colonic fatty acids incorporation Colonic fatty acids composition was evaluated using GC-MS. A significant switch toward ω-3 PUFAs at the expenses of ω-6 PUFAs was observed in both promotion and initiation arms as compared to the Control group. Fatty acids levels were expressed as relative percentages of total fatty acids and analyzed using one-way ANOVA followed by Turkey's for multiple comparison (Ctrl n = 13, Promotion and Initiation n = 11). * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$

4.5 EPA-FFA incorporation reduces urinary PGE-M excretion

To determine if changes in fatty acids composition associated with EPA-FFA consumption could be related to a modulation of PGE-2 production, we investigated the effect of EPA-FFA diet on PGE-2 biosynthesis *in vivo* by measuring urinary levels of its major metabolite PGE-M, by LC-MS/MS. We found that EPA-FFA dietary intake led to an important and statistically significant decrease of urinary levels of PGE-M in both promotion and initiation arms, indicating a lower systemic PGE-2 production respect to the Control arm. Interestingly, the percentage of PGE-M reduction appear to be dependent to the exposure time of the diet being of 20.5% in the promotion group and 50.1% in the initiation group ($P < 0.001$ initiation *vs.* Control; $P < 0.05$ promotion *vs.* Control) (ANOVA $P = 0.009$) (Figure 16).

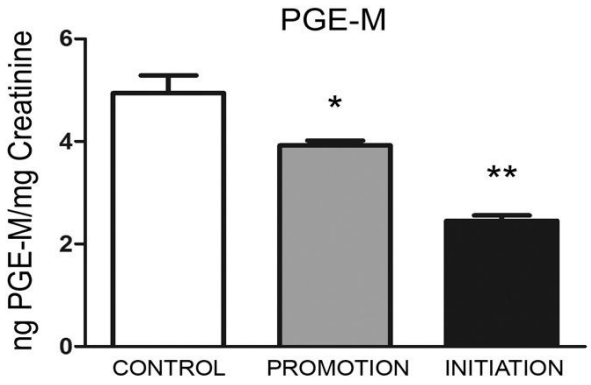

Figure 16 EPA-FFA reduces urinary PGE-M excretion. Pooled urinary samples deriving from each animal in a given treatment were analyzed using a LC-MS/MS system. EPA-FFA diet, compared to the Control group, resulted in decreased levels of PGE-M excretion in particular in the initiation arm. Data were expressed as nanogram PGE-M per mg creatinine and reported as means ± SEM. Analysis was performed using one-way ANOVA followed by Turkey's for multiple comparison (Ctrl n = 13, Promotion and Initiation n = 11). * $P < 0.05$; ** $P < 0.001$; *** $P < 0.0001$.

4.6 EPA-FFA reduced systemic but not colonic inflammation in AOM-DSS mouse model

The exposition to the carcinogen AOM in combination with repeated DSS administrations led to a chronic inflammatory state in the colon and trigger a systemic inflammatory response, which contribute to promote colon tumorigenesis. For this reason, in this study, we investigated the influence of EPA-FFA dietary supplementation on systemic and colonic inflammation. Our results showed that the amount of pro-inflammatory cytokine IL-1β was significantly lower in the promotion group compared to the Control arm ($P < 0.05$ for promotion *vs.* Control). In addition, IFN-γ serum levels were lower in both EPA-FFA treated arms respect to the Control arm, while reaching statistical differences only in the initiation group ($P < 0.0001$ initiation *vs.* Control). Otherwise, the content of all the other monitored cytokines (IL-6, IL-10, IL-17 and TNF-α) was comparable in all treated groups, suggesting that EPA-FFA did not affect the circulating levels of these mediators (Figure 17).

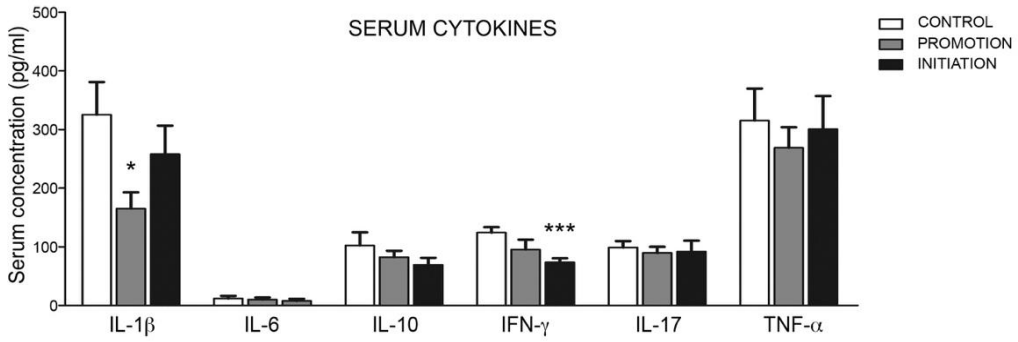

Figure 17 Effects of EPA-FFA diets on circulating cytokines levels Serum levels of indicated cytokines were measured with a multiplex bead-based sandwich immunoassay (Bio-Plex) in AOM-DSS treated mice feeding the Control diet or EPA-FFA diet. Data were reported as means ± SEM. Analysis was performed using one-way ANOVA followed by Turkey's for multiple comparison. Significant changes in circulating cytokines were observed for IL-1β (promotion) and IFN-γ (initiation). * $P < 0.05$, ** $P < 0.001$, *** $P < 0.0001$ *vs*. control. (Ctrl n = 13, Promotion and Initiation n = 11

In order to investigate the effect of EPA-FFA on colonic inflammation, colon lenght was used as macroscopically indication of colonic inflammation, while microscopic inflammatory alterations were estimated by using a histological scoring system. Despite the modest changes found on systemic inflammation, no significant differences were appreciated among the three different groups for colon lenght (Figure 18A) and colonic inflammatory score (Figure 18B).

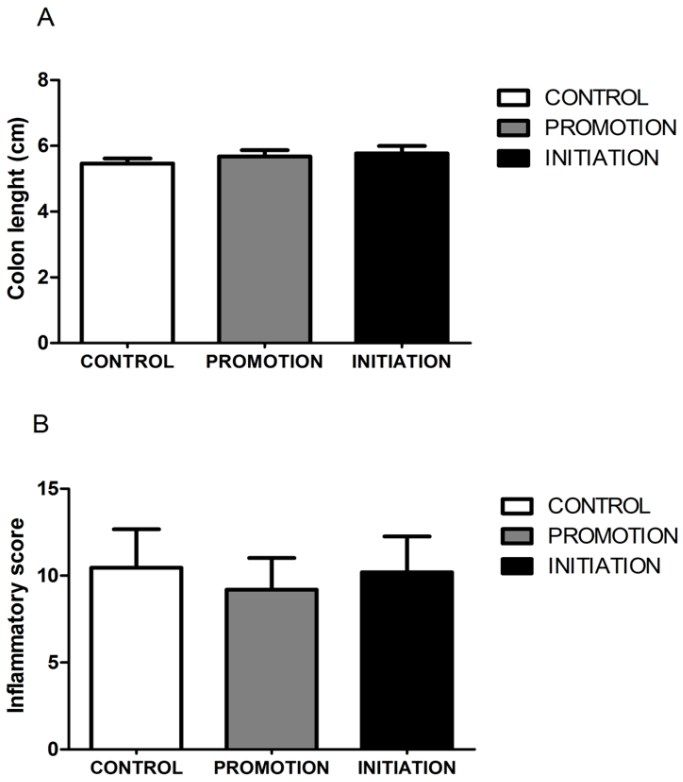

Figure 18 Effect of EPA-FFA on colon length and inflammatory score. Colon length was measured at the sacrifice. Inflammatory score was obtained using histological criteria indicated in the materials & methods. No differences were found in term of colon length (A) and inflammatory score (B) among the Control arm and EPA-FFA treated groups. Data were represented as mean ± SEM (Ctrl n = 13, Promotion and Initiation n = 11).

The intestinal inflammation was also evaluated analyzing the gene expression profile of pro-inflammatory mediators in colonic tissues. We observed an induction of IFN-γ, IL-6 and IL-1β mRNA levels in the AOM-DSS Control arm compared to the healthy mice,

while no differences were observed for TNF-α (Figure 17). However, according to the histological evaluation, we found no significant differences in the transcription levels of inflammatory mediators (IFN-γ, IL-6, IL-1β and TNF-α) among the Control group and EPA-FFA treated groups with an increasing trend in IFN-γ, IL-1β and TNF-α mRNA levels in the initiation group (Figure 19). These results indicate that while having a strong chemopreventive effect, the diets containing EPA-FFA had no effects on intestinal inflammation.

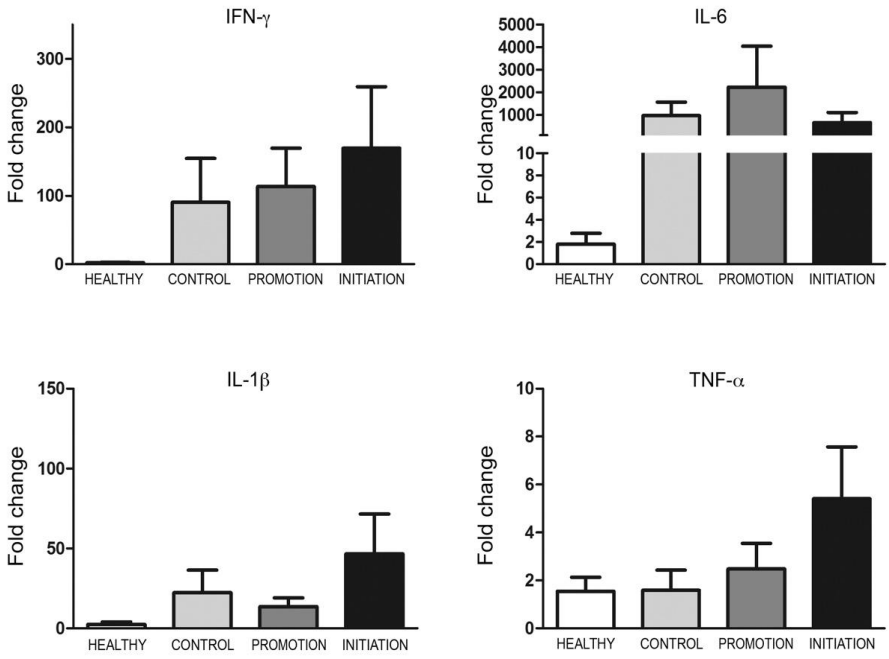

Figure 19 Expression profile of colonic inflammatory cytokines Analysis by qRT-PCR of mRNA expression levels for indicated proinflammatory cytokines in colon from healthy mice and AOM-DSS treated mice fed with the Control diet or EPA-FFA diet. No significant changes were observed in the gene expression profile of proinflammatory cytokines IFN-γ, IL-1β, TNF-α and IL-6 among the different arms. Data were normalized to GAPDH mRNA and expressed as means ± SEM (Healthy n = 8, Ctrl n = 13, Promotion and Initiation n = 11).

4.7 EPA-FFA induces Notch1 signaling activation in AOM-DSS mouse model

Recent evidences support the hypothesis that deregulation of Notch signaling could play a crucial role in the evolution of CAC (Garg et al., 2011). In order to establish whether the chemopreventive effects of EPA-FFA in AOM-DSS mouse model could be connected to a modulation in Notch signaling pathway, and to establish whether EPA-FFA treatment transcriptionally affect Notch signaling in this model, we measured changes in RNA levels of Notch1 downstream target genes Hes1, Nrarp and Atoh1. In addition, we evaluate the expression profile of the Notch1 ligand Jagged1 in this context. Gene expression analysis revealed a suppression of Notch signaling induced by AOM-DSS treatment compared to healthy mice as demonstrated by a marked reduction of Hes1, Nrarp and Jagged1 and reciprocal increase of Atoh1 (Figure 20). Importantly, EPA-FFA treatment has proven to be able to counteract the Notch1 repression caused by AOM-DSS exposition resulting in a significant upregulation of Nrarp in the initiation group ($P < 0.001$ for initiation *vs.* Control; P = n.s. for promotion *vs.* Control) (ANOVA $P = 0.0032$), an increasing trend for Jagged1 and a reciprocal decreasing trend for Atoh1. Moreover, despite the abolition of Hes1 expression observed in the Control group respect to the healthy mice, EPA-FFA treatment was also associated with a modest increase of Hes1, in particular in the initiation arm.

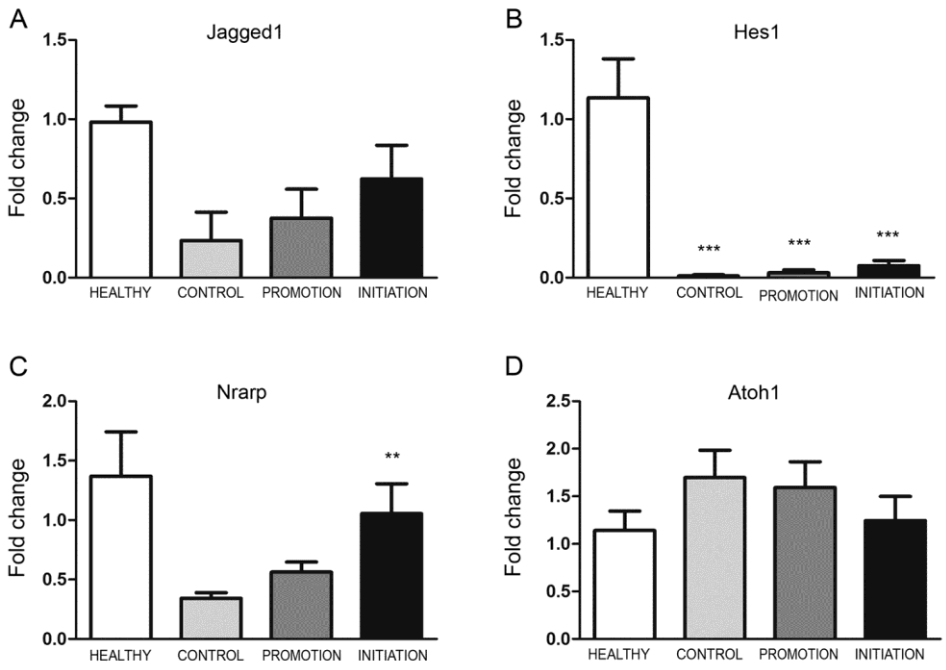

Figure 20 EPA-FFA affect Notch1 signaling pathway mRNA expression levels of Notch1 ligand Jagged 1 and downstream target genes of Notch signaling by q-RT-PCR in healthy mice and AOM-DSS treated mice fed with the Control diet or EPA-FFA diet. Compared to healthy mice, AOM-DSS treatment was associated to a downregulation of (A) Jagged1, (B) Hes1 and (C) Nrarp with a reciprocal increase of Atoh1 (D). Of note, EPA-FFA counteract the effects induced by AOM-DSS. Data were normalized to GAPDH mRNA and expressed as means ± SEM (.Healthy n = 8, Ctrl n = 13, Promotion and Initiation n = 11).

4.8 EPA-FFA treatment modifies the gut microbiota composition in CAC

Growing recent evidences suggest that the gut microbiota composition importantly contributes to the aetiology of CRC and CAC (Irrazábal et al., 2014). To test the hypothesis that EPA-FFA may exert its protective activity inducing specific changes in the microbial community structure associated with inflammation-driven CRC, we evaluated the gut microbiota composition using stool samples from AOM-DSS treated mice feeding the different diets. As a baseline control, we used the faecal samples taken from healthy mice. We observed that while the microbiota structure was mainly represented by Bacteriodetes, which are typical of the mouse gut microbiota, the AOM-DSS treatment resulted to be associated to the appearance of *Akkermansia sp*., a mucin-degrading species, belonging to the Verrucomicrobia phylum compared to the healthy mice (Figure 21). Importantly, EPA-FFA diet induced a significant enrichment of *Lactobacillus sp*. compared to the Control group ($P = 0.003$), which appeared to be more relevant in the promotion group. Interestingly, also a decreasing trend in *Akkermansia* population was noticed in both arms feeding EPA-FFA diet, while not reaching statistical differences. All data for each arm were reported in histograms describing the relative abundance of the microbial groups in each arm (Figure 21)

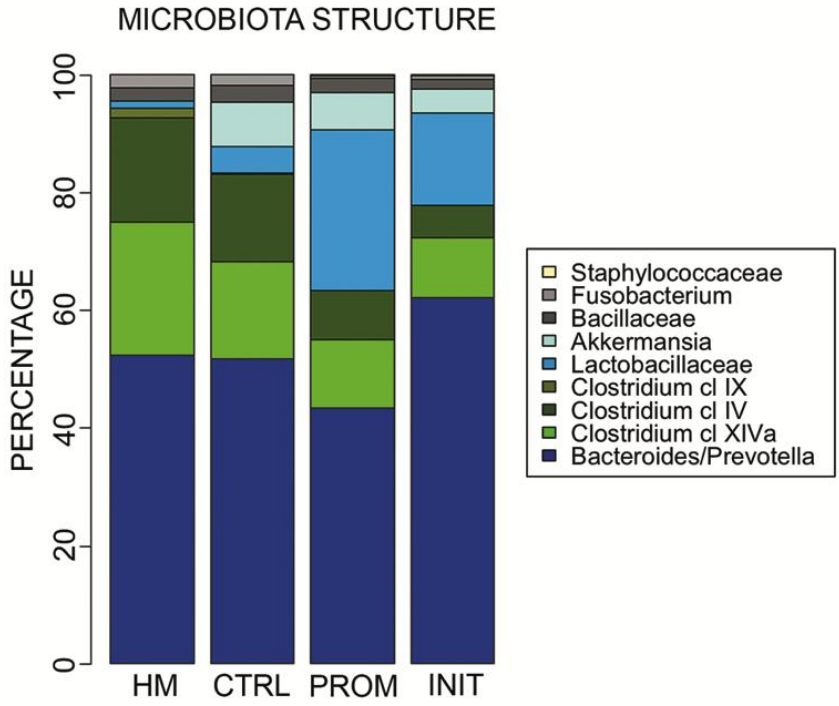

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Figure 21 EPA-FFA induces changes in gut microbiota composition Composition of intestinal microbiota was evaluated by pooling faecal samples deriving from each animal in a given treatment using HTF. Microby Array. EPA-FFA led to significant increase of Lactobacillaceae (P = 0.003 Promotion and Initiation *vs.* Control). Data were reported as relative percentage of the indicated microbial groups in each arm (Healthy n = 8, Ctrl n = 13, Promotion and Initiation n = 11).

4.9 Expression of miR34-a in AOM-DSS treated mice and the influence of EPA-FFA

Due to the known tumor soppressor function of miR-34a in CRC (Gao et al., 2014), we decided to explore the ability of EPA-FFA to exert its anti-cancer effects through a modulation of miR-34a in our model. In this thesis we evaluated the expression of this microRNA in normal colonic mucosa of AOM-DSS treated mice at the end of the experimental protocol. The results revealed an AOM-DSS-driven downregulation of miR-34a in normal colonic mucosa of mice feeding the Control diet compared with healthy mice. Interestingly, as shown in Figure 22, EPA-FFA dietary supplementation resulted in a progressive induction of miR-34a expression, in both arms treated with EPA-FFA. In particular, in the initiation group miR34-a levels reached values similar to the healthy group, while not reaching statistical significant differences.

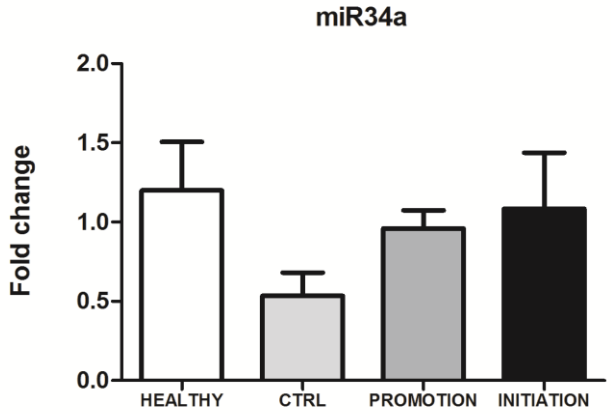

Figure 22 Influence of EPA-FFA on miR34a expression qRT-PCR analysis of miR-34a on normal colonic mucosa of healthy mice and AOM-DSS treated mice fed with the Control diet or EPA-FFA diet. An upregulation of miR34a was observed in both arms fed with EPA-FFA diet compared to the Control group. No significant variations were observed among the experimental groups. Data were reported as relative percentage of the indicated microbial groups in each arm. (Healthy n = 8, Ctrl n = 13, Promotion and Initiation n = 11).

5. DISCUSSION

The primary end point of this study was to investigate the potential chemopreventive effect of EPA-FFA on both initiation and promotion of CAC. We found that both feeding protocols containing EPA-FFA appeared equally efficient in counteracting the tumor incidence and multiplicity induced by the AOM-DSS treatment. Importantly, tumors size resulted significantly reduced in both arms fed with EPA-FFA diet compared to the Control group. Surprisingly, we didn't find significant differences in terms of outcomes between the promotion and initiation protocols, although the latter was designed to start EPA-FFA diet 4 weeks prior to the AOM injection. We hypothesized that the lack of differences between the two experimental protocols could be due to the rapid incorporation and reaching a steady-state level by EPA-FFA. Indeed, the free-fatty acid formulation (FFA) doesn't require the hydrolysis by pancreatic lipase and is therefore more efficiently absorbed and highly bioavailable. Supporting this hypothesis, no significant differences were observed in terms of ω-3 PUFAs incorporation between the promotion and initiation arms although, as compared to the control animals, they showed a significant increase. In addition, the possibility to use an FFA formulation allowed us to use a very low dosage of EPA in the diet (1%) approaching to a condition more suitable in humans. The impact of dietary fish oil (FO) in the context of a chemically-induced inflammatory-driven cancer model, was previously investigated only in another work performed by Monk et al which evaluated the effect of dietary ω-3 PUFAs in mice WT or harboring a deletion of PPARδ within the intestinal epithelium (PPARδ$^{\Delta IEpC}$) (Monk et al., 2012) . Interestingly, while conditional PPARδ knock-out appeared to have no effect on tumor incidence, they observed an exacerbation of colitis in PPARδ$^{\Delta IEpC}$ mice and found a reduction of colon injury and tumor incidence in mice feeding a diet enriched in FO compared to the group consuming a Corn oil (CO) diet (Monk et al., 2012) . However, differently from our study, in which the genetic background of the animals is the same in all arms of treatment, they employed a more generic formulation of FO at a higher percentage in the diet (4% of FO *vs.* 1% EPA-FFA) and showed a protective effect only on CAC initiation. Importantly, our data show a strong chemopreventive effect of EPA-FFA not only in the initiation of CAC, but also in the promotion group which simulate the clinical scenario observed in patients with long-standing IBD. Different cellular and

molecular mechanisms have been suggested in order to explain the anti-cancer effects of ω-3 PUFAs including EPA, although most of them have emerged from *in vitro* studies and need to be further characterized and confirmed *in vivo*. One of the first proposed anti-cancer behaviour of EPA is linked to the ability of this natural compound to be incorporated into the cellular membranes and function as substrate for oxygenases such as COX-2 and cytochrome P450 mono-oxygenases. In particular, COX-2 dependent synthesis of PGs in colorectal mucosa, especially through the PGE_2 production, represents a critical event in the pathogenesis of CRC (Hull et al., 2004). In this context, the interesting aspect of this potential EPA-driven mechanism is the capability to redirect the synthesis pathway of eicosanoids toward the production of anti-inflammatory and anti-tumorigenic PGE_3 at the expenses of pro-inflammatory and protumorigenic PGE_2 series, deriving from the arachidonic acid metabolism (Cockbain et al., 2012b). In this study, we found that EPA-FFA introduced with the diet was efficiently incorporated into the cellular membrane inducing important changes in the cellular fatty acids composition. Noteworthy, in both arms treated with EPA-FFA, we observed a significant increase of the overall ω-3 PUFAs content including EPA, DPA and DHA at the expenses of the percentage content of ω-6 PUFAs (arachidonic + linoleic acids). Interestingly, strengthening the previously mentioned hypothesis, this increased content of ω-3 PUFAs into the cellular membrane resulted associated with a decreasing trend of circulating levels of PGE_2 in both arms feeding the EPA-FFA diet compared to the Control group, with a stronger effect evident in the initiation group. Immunohistochemical analysis of colon sections harvested from this study also revealed that EPA-FFA diet inhibited tumor growth slowing down the intestinal cell proliferation as highlighted by the reduced reactivity of Ki-67 observed for both the promotion and initiation protocols at the same rate. In addition, there was also an increased number of TUNEL positive cells, a marker for apoptosis induction, in both EPA-FFA feeding arms with an higher rate of apoptotic cells detectable in the initiation group. It is also important to underlie that DHA exposure derived from fish oil increased colonocytes apoptosis in a study conducted by Ng Y et al. (Ng et al., 2005) and the chemopreventive properties of DHA against CRC were also extensively reported (Cockbain et al., 2012b). In this study we observed an efficient conversion of dietary EPA-FFA to DHA and DPA in both arms feeding EPA-FFA. Given the higher overall content of ω-3

PUFAs found in the promotion and initiation arms compared to the Control group, the impact of EPA-FFA on apoptosis and cell proliferation could be enhanced through a synergistic effect of the combined ω-3 PUFAs. Importantly, in this work we found for the first time that EPA-FFA is able to modulate Notch1 signaling *in vivo*. Notch signaling has been recognized as a crucial molecular pathway for regulating self-renewal of stem cell population in the normal intestinal mucosa, as well as for the determination of cell fate and terminal differentiation of proliferating cells (Guilmeau, 2012). The canonical Notch signaling activation relies on the ability of a specific ligand (Jagged or DLL) to bind the extracellular portion of Notch receptors resulting in the intramembrane receptor proteolysis sequentially mediated by ADAM and γ-secretase and subsequent cleavage and release of the Notch intracellular domain (NICD). This released active Notch fragment travels into the nucleus where, through the association with DNA binding protein CSL and the recruitment of the transcriptional coactivator Mastermind (MAM), assemble a transcription machinery that activates downstream target genes (Kopan and Ilagan, 2009). The critical involvement of Notch signaling in both CRC and CAC has been recently demonstrated in different studies (van Es et al., 2005)(Garg et al., 2010). Interestingly, a complex interplay between Wnt and Notch signaling was reported in models of sporadic or FAP-related CRCs. In particular, the study conducted by Rodilla et al. described the Notch1 activation as a downstream event of Wnt signaling activation through beta-catenin-mediated transcriptional induction of the Notch-ligand Jagged1 and showed that deletion of one allele of Jagged1 in mice $Jag1^{+/\Delta}$ crossed with APC $^{Min/+}$ mice is sufficient to reduce the tumor size in APC mutant background indicating that activation of Notch1 by Jagged1 confers an advantage to β-catenin dependent tumors (Rodilla et al., 2009). Differently, in a later study performed by Kim et al. it was revealed a suppression of Wnt/ β-catenin target genes in Apc $^{Min/+}$ mouse model upon activation of Notch signaling. Importantly, this Notch-mediated negative effect on CRC development, appeared to be particularly evident during the promotion, rather than the initiation of CRC (Kim et al., 2012b). In this study, we observed a suppression of Notch1 activation in the normal colonic mucosa of AOM-DSS treated mice feeding the Control diet compared with healthy mice, as revealed by a marked downregulation of Jagged1, Nrarp and Hes1 mRNA levels, associated with a reciprocal increasing trend for Atoh1. Importantly, EPA-FFA

treatment was able to rescue the suppression of Notch1 activation induced by AOM-DSS in both arms of treatment, but in particular in the initiation arm. Noteworthy, in the same experimental protocols a significant increase of apoptosis rate and a considerable reduction of tumor incidence was observed indicating that Notch1 works as a tumor suppressor in our model and more importantly Notch signaling constitutes a new *in vivo* target of EPA-FFA. Interestingly, in this study we also confirmed the existence of a negative correlation between the Notch1 activation and the β-catenin induction. Indeed, in AOM-DSS control arm, in which Notch1 signaling appeared to be off, we observed high levels of nuclear β-catenin as revealed by immunohistochemical analysis. Noteworthy, a significant reduction of nuclear β-catenin translocation was detected in both arms feeding EPA-FFA compared to the Control arm. Overall, our results were in line with a previous study by Garg et al. which proposed a tumor suppressor function of MMP-9 in CAC which occur directly modulating Notch activation leading to a concomitant suppression of β-catenin expression. The exact mechanism through which EPA-FFA can modulate Notch1 signaling is unknown. However, it has been demonstrated that ω-3 may exert anticancer effects inducing changes in lipid raft organization and function or altering caveolae lipid microenvironment (Yaqoob and Shaikh, 2010), (Ma et al., 2004). Importantly, caveolin-1, the principal component of caveolae, has been shown to critically modulate Notch1/NICD and Hes1 expressions in the brain regulating astroglial differentiation (Li et al., 2011). In addition, multiple mechanisms, including microRNAs, regulate Notch1 or Jagged 1 expression. For instance, miR-21 which resulted upregulated in AOM-DSS mouse model (Gao et al., 2013), has been shown to repress Jagged1 (Hashimi et al., 2009). Interestingly, miR-21 is targeted by ω-3 PUFAs in breast cancer cells (Mandal et al., 2012). The importance of alterations in gut microbiota structure in CRC occurring on inflammatory background was recently elucidated by Arthur et al in AOM-treated *Il10*$^{-/-}$ mice which revealed the crucial role of bacterial species with genotoxic properties during CAC development (Arthur et al., 2012). They proposed a model in which inflammation promotes CAC onset and progression acting on both the host and the microbiota, leading to the expansion of microorganisms with genotoxic and mucin-degrading capabilities (Arthur et al., 2012). Of note, in this study we provide new evidences that support the implication of the gut microbiota composition in CAC development showing relevant

changes in the gut microbiota structure among healthy mice, AOM-DSS control and EPA-FFA fed mice. As expected, we found that the mouse intestinal microbiota of healthy animals which are fed with a control diet containing 1% of CO, is dominated by *Bacteroides-Prevotella spp*. from the Bacteroidetes phylum together with the specific populations of clostridial cluster XIVa. Noteworthy, in the AOM-DSS control group respect to the healthy mice we appreciated the appearance of *Akkermansia sp.*, whose major representative *A. muciniphila*, a mucin-degrading species, resulted to be 4-fold higher in patients with CRC (Weir et al., 2013). In addition, according to our results, the increased abundance of *Akkermansia sp.* was also found by Berry D. et al in DSS-treated mice which proposed the putative mucin degraders *Akkermansia* and *Mucispirillum* as indicator phylotypes for DSS treatment (Berry et al., 2012). These observations were supported by the evidence that one of the features that marks the inflammatory bowel disease UC is the reduction of goblet cells and a progressive loss of mucus secretion (Kim and Ho, 2010). Strikingly, when AOM-DSS treated mice were fed with EPA-FFA diet, a decreased content of *Akkermansia sp* was progressively observed and more importantly, an enrichment of *Lactobacillus sp*. was found in both promotion and initiation arms compared with the AOM-DSS group fed with the control diet. The beneficial and protective properties of probiotic microorganisms against CRC development , including strains belonging to the Lactobacillus and Bifidobacterium genera, were reported in different studies (Khazaie et al., 2012), (Wollowski et al., 2001). Moreover, Gosh et al recently showed that a dietary supplementation with ω-3 PUFAs in a mouse model of infection-induced colitis, was able to induce the growth of beneficial bacterial strains such as *Lactobacillus sp*. and *Bifidobacteria sp*., as opposed to the effect induced by ω-6 PUFA rich diets (Ghosh et al., 2013). Our findings are in accordance with Gosh et al. suggesting that *Lactobacillus sp.*, which is known to counteract the growth of potential pathogens, may have an important role in the protective effects of EPA-FFA in the context of CAC. Thus, we demonstrated that EPA-FFA diet was able to ameliorate the intestinal dysbiosis induced by the DSS treatment, promoting the recovery of an healthy gut microbial ecosystem.

Several studies have shown important regulatory functions of miRNAs in different cancer-related biological processes including cell proliferation, apoptosis, invasion and metastasis (Lujambio and Lowe, 2012). Among the miRNAs with tumor suppressor functions, miR-34a is a typical one whose role was reported in different types of cancer, including both CRC and CAC (Gao et al., 2014), (Rokavec et al., 2014). Importantly, it has been recently reported that mice deficient for miR34-a (Mir34a–/–) are more susceptible to tumor development in AOM-DSS mouse model promoting tumor invasion (Rokavec et al., 2014). In our study, AOM-DSS treatment resulted in decreased expression of miR-34a in colonic mucosa of mice feeding the Control diet compared with healthy mice. Interestingly, EPA-FFA appeared to be able to reverse this phenomenon restoring miR-34a expression notably upon the initiation arm in which miR-34a levels reached values close to the healthy mice. Given the ability of EPA-FFA to induce miR-34a in AOM-DSS mouse model, we can speculate that this effect may represent a further chemopreventive mechanism mediated by EPA-FFA which need to be further investigated. Interestingly, in this study, despite the strong effect found in terms of chemoprevention of CAC, EPA-FFA treatment did not improve the colonic intestinal inflammation in mice treated AOM-DSS, although in the latter modest changes in systemic inflammation were found. Although the potential anti-inflammatory properties of ω-3 PUFAs were highlighted in different studies, recent literature data suggested that a dietary supplementation with fish oil or other compounds performed during DSS-induced colitis could exacerbate the intestinal inflammation and increasing the mortality rate impairing the beneficial effects of these substances (Matsunaga et al., 2008), (Jia et al., 2011). Since these effects were mainly observed following an acute inflammatory insult induced by the DSS administration, we hypothesize that this effect may be due to the chemical interaction between chemopreventive substances and DSS. This assumption was sustained by the evidence that *fat-1* transgenic mice, which can catalyze the endogenous conversion of ω-6 PUFA to ω-3 PUFA by introducing a double bond into fatty acyl chain, inducing an enrichment in endogenous ω-3 PUFAs, didn't show exacerbation of colitis during DSS treatment (Matsunaga et al., 2008). Thus, in our work in order to minimize the potential harmful effect of ω-3 PUFAs, we decided to withhold EPA-FFA feeding during DSS cycles by switching to Control diet. Importantly, we didn't find significant differences in

the mortality rates among the three different groups, while intensifying the inflammatory background which initiated and promoted the AOM-induced carcinogenesis performing three short DSS cycles (4 days) at a high concentration (2.5%). Of note, in order to simulate a dosage more suitable in humans, we used a purified formulation of EPA-FFA at a lower concentration (1%) compared with a previous study performed by our research group in Apc $^{Min/+}$ mouse model (Fini et al., 2010). Therefore, we speculate that the controversial behavior of EPA-FFA in the resolution of inflammation, while having a strong effect on carcinogenesis, could be due to the use of a low dosage of EPA-FFA in a context of chemically-induced robust inflammation. Our findings were in accordance with previous evidences showing inconsistency between the levels of colonic inflammation and tumor development in CAC mouse models (Grivennikov et al., 2009),(Arthur et al., 2012). Concluding, we described new potential anticancer effects of EPA which appeared to be independent from the modulation of colonic inflammation in this system and importantly, we demonstrated that EPA-FFA is an effective chemopreventive agent in AOM-DSS mouse model. These evidences suggest that an early EPA-FFA supplementation could represent a good strategy to prevent CRC development in patients with IBD.

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