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MOLECULAR MECHANISMS INVOLVED IN THE *IN VITRO* PROTECTIVE EFFECTS OF ANTHOCYANINS AGAINST INTESTINAL INFLAMMATION

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ABBREVIATIONS

ADME *Absorption Distribution Metabolism and Excretion*

AJ *Adherent Junctions*

AP *Apical*

AP-1 *Activator Protein 1*

ARE *Antioxidant Response Element*

BBE *Bilberry and Blackcurrant Extract*

BBE-IP *Bilberry and Blackcurrant Extract- Intestinal Phase*

BL *Basolateral*

BMI *Body Mass Index*

CD *Crohn Disease*

COX-2 *Cyclooxygenase-2*

Cul3 *Cullin3*

ERK *Extracellular Signal-Regulated Kinase*

FAD *Flavin Adenine Dinucleotide*

FFA *Free Fatty Acids*

FRAP *Ferric Reducing Antioxidant Power*

GI *Gastrointestinal*

GLUT *Glucose Transporter*

HO-1 *Heme Oxygenase 1*

IBD *Inflammatory Bowel Disease*

IEC *Intestinal Epithelial Cells*

IFN-γ *Interferon gamma*

IKK *IkB Kinase*

IκB *Inhibitor of kappa B*

IL-6 *Interleukin-6*

IL-8 *Interleukin-8*

JNK *c-Jun N-terminal Kinase*

Keap1 *Kelch-like ECH-associated protein 1*

LPS *Lipopolysaccharide*

MAPK *Mitogen Activated Protein Kinase*

NADPH *Nicotinamide Adenine Dinucleotide Phosphate*

NF-κB *Nuclear Factor-κB*

NQO1 *NADPH Quinone Oxidoreductase 1*

NRF2 *Nuclear Factor Erythroid 2-Related Factor-2*

PA *Palmitic Acid*

PKC *Protein Kinase C*
PKR *Protein Kinase RNA-activated*
RNS *Reactive Nitrogen Species*
ROS *Reactive Oxygen Species*
RI *Recovery Index*
SAT *Subcutaneous Adipose Tissue*
SCFA *Short Chain Fatty Acid*
SFA *Saturated Fatty acid*
SGLUT *Sodium-Glucose Transport*
TAA *Total Antioxidant Activity*
TJs *Tight Junctions*
TLR *Toll-Like Receptor*
TNF-α *Tumor Necrosis Factor alfa*
UC *Ulcerative Colitis*
VAT *Visceral Adipose Tissue*
VEGF *Vascular Endothelial Growth Factors*
ZO-1 *Zonula Occludens 1*

Abbreviation of anthocyanins and metabolites

Cya-3-Ara *Cyanidin-3-Arabinoside*
Cya 3-Gal *Cyanidin-3-Galactoside*
C3G / Cya-3-Glu *Cyanidin-3-Glucoside*
Cya-3-Rut *Cyanidin-3-Rutinoside*
Del-3-Ara *Delphinidin-3-Arabinoside*
Del-3 Gal *Delphinidin-3-Galactoside*
Del 3-Glu *Delphinidin-3-Glucoside*
Del-3-Rut *Delphinidin-3-Rutinoside*
Mal-3-Ara *Malvidin-3-Arabinoside*
Mal-3-Gal *Malvidin-3-Galactoside*
Mal-3-Glu *Malvidin-3-Glucoside*
Peo-3-Ara *Peonidin-3-Arabinoside*
Peo-3-Gal *Peonidin-3-Galactoside*
Peo-3-Glu *Peonidin-3-Glucoside*
Pet-3-Ara *Petunidin-3-Arabinoside*
Pet-3-Gal *Petunidin-3-Galactoside*
Pet-3-Glu *Petunidin-3-Glucoside*
PCA *Protocatechuic Acid*
PGA *Phloroglucinaldehyde*

Abstract

The inflammatory bowel disease (IBD) is a group of multifactorial pathologies with an unknown etiology, characterized by an alternation of an acute and a remission phase of the intestinal epithelium inflammation. In the last years there was a worldwide increased incidence of IBD, especially in the industrialized and growing countries. In addition, epidemiological studies reported a positive correlation between gut inflammation and obesity.

Recent *in vivo* and *in vitro* studies have supported the beneficial effects of anthocyanins, a class of flavonoid compounds widely distributed in Mediterranean diet, in various chronic inflammatory diseases, such as IBD, since they possess anti-inflammatory and antioxidant activity.

In the first part of this study, we aimed to evaluate the molecular mechanisms involved in the modulation of intestinal epithelial inflammation by using an *in vitro* model consisting of Caco-2 cells exposed to high concentrations of palmitic acid (PA), and the protective effects exerted by cyanidin-3-O-glucoside (C3G) pre-treatment. For all the experiments, fully differentiated Caco-2 were pretreated for 24h with different concentration of C3G (10 and 20µM), added on the apical side, and then exposed to PA 100µM, added on the basolateral chamber, for 6h.

The data obtained demonstrated the C3G anti-inflammatory activity through the modulation of NF-κB pathway induced by PA. In addition, C3G was able to improve the intracellular redox status through the activation of the adaptive cellular response modulated by Nrf2 pathway. Furthermore, it ameliorates the intestinal barrier by reducing the intestinal paracellular activity altered by PA.

Since the bioavailability of the anthocyanins seems to be very low mainly due to poor stability during gastrointestinal digestion, in the second part of this study an *in vitro* simulated gastrointestinal digestion of a purified and standardized bilberry and blackcurrant extract (BBE), rich in anthocyanins, was performed. We further studied the bioactivity of the BBE, after the static simulated digestion, on an *in vitro* model of intestinal inflammation by using differentiated Caco-2 cells exposed to TNF-α.

The outcomes confirmed the high instability of the anthocyanins in mild alkaline environment of the small intestine reporting a 13% of recovery index. However, although the high loss of anthocyanins, the digested BBE maintained part of its bioactivity, proved by the inhibition of the of NF-κB pathway induced by TNF-α, and by the activation of Nrf2 pathway.

These data hence confirm that anthocyanins, introduced by diet or food supplements, could represent a possible approach for the prevention of IBD.

Keywords: Inflammatory Bowel Disease, anthocyanins, inflammation, free fatty acid, NF-κB, adaptative cellular response, *in vitro* digestion, biostability.

Part 1: Background

Chapter 1

Pathogenesis and molecular aspects of Inflammatory Bowel Disease (IBD)

1.1 Anatomy and physiology of the gut

The intestine represents the last part of the digestive system and is made up of the small intestine and the large intestine. The small intestine is long 6-7 meters and extends from the pyloric sphincter to the ileocecal valve, with the main function of absorbing the products of digestion. Anatomically it is divided into three portions, duodenum, jejunum, and ileum. Whereas, the large intestine extends from the ileal valve to the anal orifice, surrounding the small intestine on three sides. With a length of about 1.5 meters and a diameter of 10 cm at the beginning, and 7 cm at the level of the rectal ampulla, it represents 1/5 of the entire gastrointestinal tract. Anatomically it is divided mainly into 3 portions: caecum, colon, and rectum. The cecum is the first part of the large intestine with a blind-bottomed sack shape and is suspended below the ileocecal valve. The colon in turn is divided into ascending colon, transverse colon, and descending colon. Its main function is to absorb water and electrolytes, to synthesize some vitamins, and to transform the undigested material (kilo) into feces that will be then eliminated through defecation (Drake *et al.*, 2010).

1.1.1 Histology

The large intestine wall is made by 4 concentric layers: mucosa, submucosa, muscular, –and serous. The inner one (mucosa) consists of a layer of simple columnar epithelium which folds inwards forming invaginations called Lieberkühn crypts or intestinal glands. The intestinal epithelium consists of: a) enterocytes, cylindrical cells with a brush border in the apical portion that absorb water, electrolytes and vitamins produced by the bacterial flora, b) globet cells that secrete mucus, which facilitates the transport of feces and protects the intestine from the gases and acids produced by intestinal bacteria, and c) Paneth cells, which are mainly found at the base of the crypt and secrete antibacterial molecules (Clevers, 2013). The submucosa instead contains many blood and lymphatic vessels and the nervous plexus of Meissner. The muscular layer has a circular inner and an external and longitudinal layer divided into 3 bands called taeniae, these are shorter than the colon thus causing the formation of haustra, intestinal gibbosities. Moreover, in the muscular there is also the plexus of Auerbach, which together with the Meissner one form the enteric nervous system (Deakin *et al.*, 2006).

1.1.2 The Intestinal barrier

The intestine represents one of the largest part of the organism that is in contact with the external environment, so in addition to the function to absorb the substance needed, it must prevent the penetration of harmful microorganisms. This last function is explicated by the intestinal barrier. The intestinal barrier is made by physical, biochemical, and immunological elements; from external to inner layer the barrier is constituted by the mucus layer, the intestinal epithelial cells (IEC), and the adaptative and innate immune cells of the lamina propria (**Fig. 1**). The mucus layer covers the entire surface hydrating and protecting the mucosa from the pathogens, through antimicrobial peptides (AMPs) and secretory IgA molecules (sIgA). Small intestine presents only one mucous gel layer while the colon has two, the inner separates the IEC form the microbiota, depleting of the harmful bacteria, while the outer one permits the colonization of the microbiota (Johansson *et al.,* 2016). The intestinal epithelium, instead, represents the real and strongest part of the barrier. As mentioned above it is a polarized monolayer made up by enterocyte, globet cells, Paneth cells, and enteroendocrine cells, which physically separate the lumen from the lamina propria. The selective permeability is regulated by the presence of the junctional complexes; adherens junctions (AJ), tight junctions (TJ), and desmosomes (Galipeau *et al.,* 2016). The tight junctions (TJ) consist of approximately 50 different membrane proteins, located in the apical part and in the lateral region of the IEC. They include: a) integral membrane proteins such as the adhesion molecules claudin and occludin, which extend into the intracellular space acting as a bridge between two cells, b) cytoskeletal linkers such as the proteins of the zonula occludens (ZO-1; ZO-2; ZO-3) and cingulin, c) signal proteins able to act as transcription factors, regulators of the cell cycle, and to activate various downstream cascade reactions (Cereijido *et al.,* 2007). Below the TJs are present the adherens junctions which promote the initiation and stabilization of the cell-cell adhesion and the regulation of actin cytoskeleton. They are mostly composed by cadherins (transmembrane proteins) and catenins (Bischoff *et al.,* 2014)

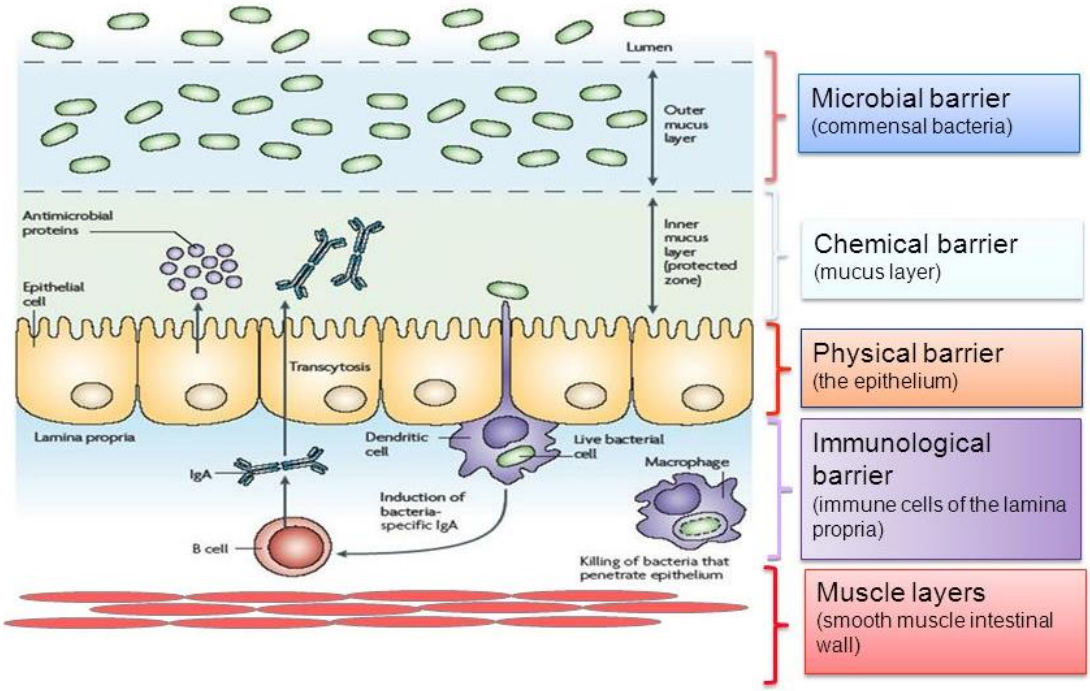


Fig. 1: Intestinal barrier composition (image from http://www.WeiLab.com**)**

1.1.3 Microbiota

The intestine is also the site of the largest bacterial ecosystem in humans. In fact, the intestinal lumen hosts more than 700 different species of non-pathogenic microorganisms. The composition and density of the microbiota varies widely from individual to individual, even of the same species, due to the diet, environmental and genetic factors. In general, the microbiota consists mainly of bacteria but also fungi and viruses are present. The 90% of the total bacteria present in the large intestine derives from 30-40 species belonging to the genera *Bacteroides*, *Bifidobacterium*, *Clostridium*, *Faecalibacterium*, and *Eubacterium* (Guarner *et al.*, 2003; Beaugerie *et al.*, 2004). The relationship between intestinal flora and man cannot be defined as a simple commensal relationship but more as a mutualistic relationship (Sears *et al.*, 2005). In fact, while man provides the ideal environment for bacterial growth, these in turn perform different important duties for humans, such as facilitating chemical digestion and absorption, synthesizing vitamins and saturated short chain fatty acids, preventing the growth of pathogenic microorganisms, “training” the immune system and regulating bowel development (Sherwood *et al.*, 2013). Without microbiota the human organism would not be

able to digest some food components, in fact unlike man some bacteria possess enzymes capable of digesting some polysaccharides, such as fibres, some types of starches, and oligosaccharides and sugars. They metabolize these saccharides into short chain fatty acids (SCFA) such as propionic acid, butyric acid, and acetic acid. These fatty acids can be used by man as an energy source (Gibson *et al.*, 2004). They also increase the production of some cells of innate immunity such as exinophiles, basinophils and neutrophils (Levy *et al.*, 2016). The intestinal microbiota also produces a large amount of vitamins, especially vitamin K, B12, therefore making a significant contribution especially when the dietary intake is very low. Finally, the flora also metabolizes some amino acids such as tryptophan and consequently produces substances (3-indolopropyl acid, 3-indolealdehyde, and indole) which exert a neuro-protective effect and help maintaining homeostasis and reactivity of the intestinal barrier (Zhang *et al*., 2015; Wikkoff *et al.*, 2009). In healthy conditions the flora is well separated from the intestinal tissue thanks to a thick layer of mucus produced by IEC. A damage of the flora and the barrier leads to the infiltration of harmful microorganisms and consequently activation of different inflammatory pathways and diseases, such as the inflammatory bowel disease.

1.2 Inflammatory bowel diseases

Inflammatory bowel diseases (IBD) are a group of pathologies characterized by an alternation of an acute and a remission phase of the intestinal epithelium inflammation (Melgar *et al.*, 2010). The two main forms of IBD are ulcerative colitis (UC) and Crohn Disease (CD), which share similar symptoms, such as diarrhea, abdominal pain, and bleeding, while they differ in the location and intensity of the inflammation. In fact, ulcerative colitis is characterized by a diffuse and interrupted inflammation of the colon with the ulcer that occurs very rarely and only in the most severe conditions of this pathology. On the contrary, ulceration is typical of Crohn's disease, which can penetrate and become transmural affecting the entire digestive tract (from the mouth to the intestine) (Melgar *et al.*, 2010).

1.2.1 Etiopathogenesis of IBD

1.2.1.1 Genetic Factors

The etiopathogenesis is still not clear but it is believed it can be mainly due to: genetic susceptibility, alteration of the immune system, and environmental factors (**Fig. 2**). Several studies have in fact shown that IBD are the result of an anomalous and disproportionate response of the immune system to environmental factors, including bacterial flora, specific antigens, and food-derived agents in genetically susceptible individuals (Kucharzik *et al.,* 2006). Recent genome-wide association studies (GWAS) have shown that more than 40 gene loci belonging to adaptive immunity, intestinal barrier function, and autophagic pathways are related to the development of IBD. Among the specific CD genes, particular importance is given to the NOD2/CARD15. In two different genetic studies three polymorphisms have been identified on the above-mentioned gene on chromosome 16, which increase the probability of developing CD by 20-40% (Hugot *et al.,* 2001; Ogura *et al.,* 2001; Cuthbert *et al.,* 2002). The NOD1 and NOD2 genes recognize a distinct motif present in peptidoglycans and play an essential role in the signalling pathway of innate immunity response. In fact, their activation results in the activation of the caspase signalling pathway and the κB nuclear transcription factor (NF-κB) with a consequent increase in the production of pro-inflammatory cytokines. In the specific, the NOD2 gene encodes an intracellular protein, NOD2, also known as CARD15, which is expressed in Paneth cells and in antigen-presenting cells, and it can be induced in intestinal epithelial cells (Guitierrez *et al.,* 2002; Lala *et al.,* 2003). Once activated by its muramyl dipeptide ligand (MDP), CARD15 interacts with Rick/Rip2 and subsequently induces the activation of NF-κB and the mitogen activated protein kinase pathway (MAPK) (Guitierrez *et al.,*2002; Inohara *et al.,* 2000; Lala *et al.,* 2003). Furthermore, it is considered that NOD2 can have an effect also on the activation of the caspase cascade, however this mechanism has not been completely clarified, in fact the mutations seen in CD patients do not involve the CARD domain considered important for caspase 1 activation. NOD also regulates the expression of α-difensin in Paneth cells and therefore is believed to inhibit the invasion of bacteria present in the intestinal lumen (Kobayashi *et al.,* 2005). Therefore, mutations at the level of this gene result in a protein that is no longer able to correctly interact with the MDP with the consequent inability of the

intestinal mucosa to fight bacterial infection and the initiation of the systemic inflammatory response that leads to an uncontrolled inflammation. Genes associated to colitis instead, include genes coding for matrix proteins (ECM1), interleukins (IL-2, IL-10), E-cadherins (CDH1), suggesting thus that, in contrast to CD, in the pathogenesis of UC there is a loss of intestinal barrier function (Franke *et al.*, 2008; Barret *et al.*, 2009; Fisher *et al.*, 2008; Festen *et al.*, 2009; Silverberg *et al.*, 2009). Finally, genes related to both IBD pathologies belong to the interleukin 23 (IL-23) pathway, to the transducer and activator of transcription 3 (STAT 3), and to the Multi Drug Resistance 1 (MDR-1) (Duerr al., 2006; Franke *et al.*, 2008; Stoll *et al.*, 2004; Kaser *et al.*, 2008)

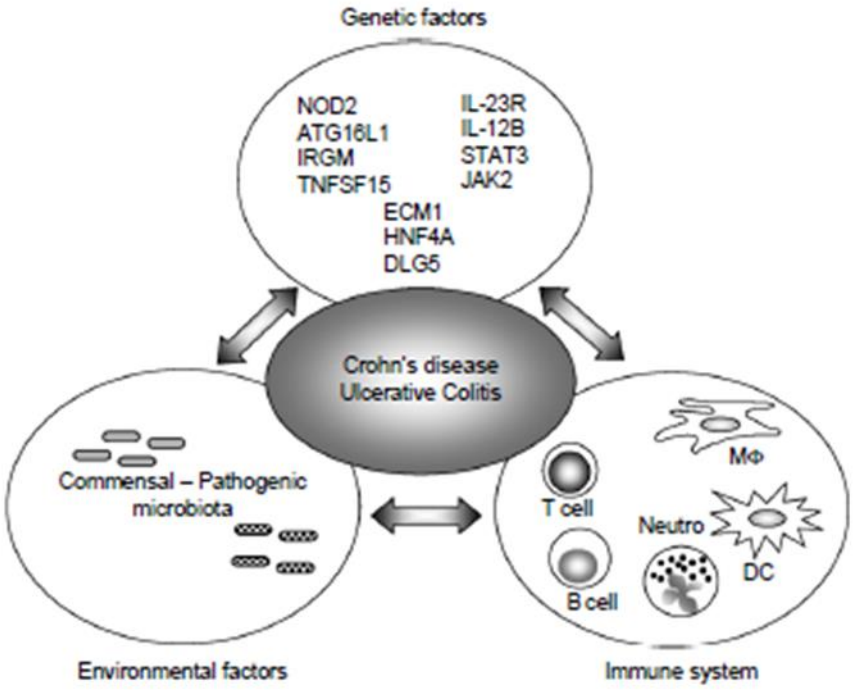


Fig. 2: **Main factors involved in the pathogenesis of IBD** (from Melgar *et al.*, 2010)

1.2.1.2 Immunological factors

The immune system constitutes a defensive barrier between microorganisms located within the lumen and the intestinal epithelium. In physiological conditions the intestinal mucosa is characterized by the abundant presence of lymphocytes both at intraepithelial and subepithelial level. Several studies have shown that a malfunction of the innate and adaptive immune system can contribute to the aberrant intestinal inflammatory response in patients with IBD. In fact, the damage at the intestinal mucosa in IBD patients is related to a high increase in effector immune cells, such as $CD4^+$ and $CD8^+$, intraepithelial cytolytic lymphocytes, perforins and granzymes containing T cells (Singh *et al.,* 2001). It has been shown that the accumulation of $CD4^+$ plays a fundamental role in the development and exacerbation of IBD, and this is probably due to the increased activation of T lymphocytes and to their increased infiltration in the intestinal wall as a response to the increase of adhesion and chemoattractant molecules in the inflamed mucosa and a concomitant decrease in the apoptotic process (Kaser *et al.*, 2010; Ina *et al.*, 1999; Stefulj *et al.,* 2001). Following their activation, $CD4^+$ and $CD8^+$ can differentiate into the various T helper lymphocytes Th1, Th2, Th17 (Neurath *et al.*, 2006). Crohn's disease is associated with a Th1 cell-mediated response characterized by enhanced production of interleukins (IL-1, IL-2, IL-6, IL-12, IL-18), tumor necrosis factor alpha (TNF-α), and interferon gamma (IFN- γ) (Lotz *et al.,* 2006). TNF-α exerts its anti-inflammatory effect by stimulating the production of IL-6 and IL-1β by intestinal macrophages (Baumann *et al.,* 1994). Furthermore, patients with intestinal Crohn's disease have a high number of Th17 lymphocytes and other subgroups of Th cells secreting various interleukins (IL-17A, IL-17F, IL-22, etc). IL-22 is a pro-inflammatory cytokine upregulated both at the serum and tissue levels of CD patients (Schmechel *et al.,* 2008). In contrast, in ulcerative colitis the immune response is regulated by Th2-type cytokines, such as IL-4, IL-5 and IL-10 (Camoglio *et al.,* 1998; Sawa *et al.,* 2003). Activation of the IL-10 secreting $CD4^+$ T lymphocytes at the intestinal level of patients with ulcerative colitis has been proposed to be due to the consequence of elevated IL-10 and IL-8 levels (Melgar *et al.,* 2003).

1.2.1.3 Environmental factors

Without any doubt, environmental factors such as smoking, diet, stress and microbiome play a fundamental role in the development of IBD. In recent years, research is increasingly focusing on the study of microbiota and diet.

The bacterial flora in the intestine of mammals is a complex and dynamic system with a steady state, which can be modified by various environmental factors, such as diet, drugs, and lifestyle. As a consequence of the diet changes there is also a variation in the composition of commensal bacteria (Ley *et al.,* 2006; Sonnerburg *et al.,* 2004; De Filippo *et al.,* 2010). The consumption of so-called Western diets, mainly consisting of processed and refined foods, red meats, sweetened beverages, and at the same time accompanied by a low consumption of fibers, fruits and vegetables, has been associated with the development of metabolic diseases associated with a systemic and mild inflammation (Wellen *et al.,* 2005). In physiological conditions the microbiota maintains a symbiotic relationship with the intestinal mucosa offering crucial functions for metabolism, immunity and protection against pathogens. So, the abundance and diversity of the flora plays a fundamental role in providing essential functions such as, clearance of pathogens, resistance to colonization, and symbiosis (Statovci *et al.,* 2017). An *in vivo* study carried out on wild-type mice fed with a diet rich in lipids and sugars showed a decrease in microbiota biodiversity and an increase in opportunistic pathogens with a consequent reduction in the function of the intestinal barrier (Zhang *et al.,* 2012). Epidemiological studies suggested an increased risk of developing IBD related to a high consumption of red and processed meats, and lipids (saturated and unsaturated fatty acids), and low consumption of vitamin D (de Silva *et al.,* 2014; Wiese et al., 2014) (**Fig. 3**). While a recent study by the European Prospective Investigation in Cancer (EPIC) found no correlation between the body mass index and the development of IBD, suggesting therefore that a high-calorie diet is not enough to trigger intestinal inflammation (Chan *et al.,* 2013).

Saturated fatty acids (SFA) occur naturally in derivatives of plant origin, mainly short-chain ones, while long-chain ones, such as palmitic acid, stearic acid and myristic acid, are found in products of animal origin (lard, butter, pork and beef, etc.) (Vannice *et al.,* 2013). *In vitro* studies have shown that SFA, with a mechanism very similar to LPS, can act as mediators of pro-inflammatory

cytokines by binding and activating the toll like receptor 4 (TRL4) and consequently up-regulating the expression of NF-κB (Lee *et al.,* 2003). The last one plays an important role in the activation of many pro-inflammatory mediators, such as COX-2, TNF-α, IL-1β, IL-6, CXCL8, IL-12, and IFN-γ (Lee *et al.,* 2003) Furthermore, it was demonstrated that palmitic acid (PA) and stearic acid can induce the degradation of the factor κB inhibitor (IκB), the phosphorylation of C-Jun N-terminal kinase (MAPK), and the kinase regulated by extracellular signal (ERK) on macrophages (Lyons *et al.,* 2016). A high intake of SFA also induces a modification of the microbiota by increasing the number of Gram-negative bacteria and therefore the natural ligand for TRL4, LPS, inducing an increase in intestinal permeability, thus leading to a state of endotoxemia (Moreira *et al.,* 2012). The intake of SFA increases the formation of low-density lipoprotein (LDL) and decreases their turnover with the consequent formation of oxidized LDL, which are a well-known molecular pattern associated with damage and recognized by TRL4, inducing therefore an inflammatory response (Stewart *et al.,* 2010). In contrast, monounsaturated acids contained in olive oil, avocado, macadamia nuts and lard, have been shown to reduce cholesterol and increase levels of high-density lipoprotein (HDL) (Jekins *et al.,* 2010). Furthermore, the treatment of pro-inflammatory macrophages (M1) with palmitoleic acid induces an increase in M2 anti-inflammatory profile (Chan *et al.,* 2010). In an *in vivo* study it was shown that the dietary intake of oleic acid reduced the risks of developing intestinal inflammation (de Silva *et al.,* 2014). However, the role of monounsaturated acids in the development of IBD is partly unknown. Polyunsaturated fatty acids (PUFAs), since they contain more than a double bond, are more prone to oxidation. They are divided into two groups: omega 3 (alpha linoleic acid [ALA], docoesanoic acid [DHA]) and omega 6 (linoleic acid [LA], arachidonic acid [ARA]). The latter is the main n-6 PUFA found in inflammatory cells and is involved in the synthesis of eicosanoids such as leukotrienes and prostaglandins, which are known inflammatory mediators (Schmitz *et al.,* 2007). While, omega 3 showed an anti-inflammatory activity, in fact, unlike saturated fatty acids, they inhibit the TRL4 signal and consequently the transcription of pro-inflammatory genes. DHA can improve the function of the intestinal barrier by increasing the expression of tight junctions (Liu *et al.,* 2012). Therefore, in order to maintain an intestinal homeostasis it is important that the omega 6/omega 3 ratio is very low.

Unfortunately, in recent years this ratio has increased dramatically due to processed foods, low consumption of dietary fibers and fish products. In line with this, in a recent study a high ARA: eicosapentaenoic acid (EPA) ratio was found at the level of the inflamed intestinal mucosa of patients with UC (Pearl *et al.*, 2013).

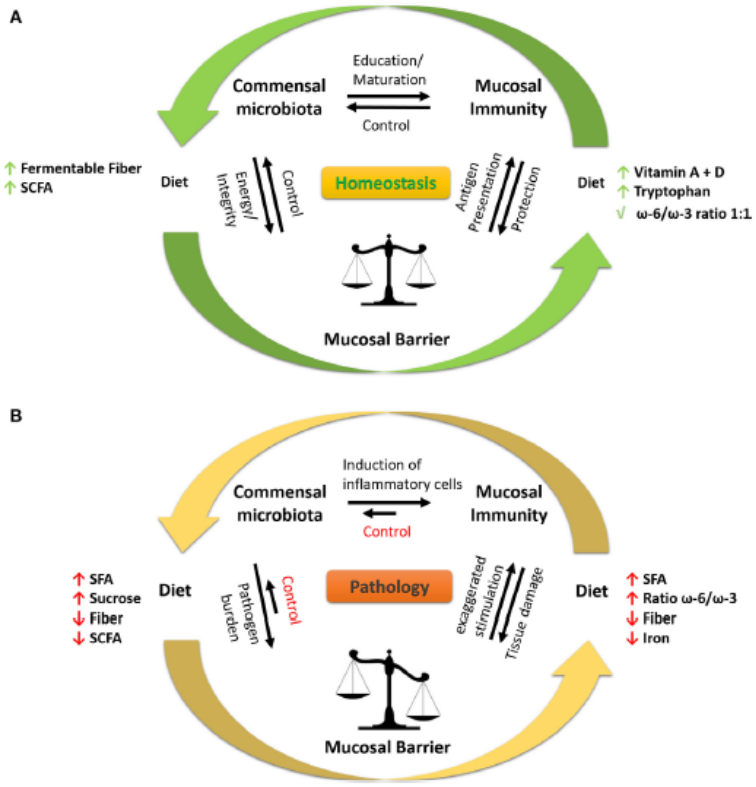


Fig. 3: **Effect of high fatty diet on the intestinal barrier**. A diet rich in fibres and vitamin D helps in the homeostasis maintenance **(A)**. In fact, the fibres introduced by the diet are converted by microbiota into SCFA which increase the mucosal barrier integrity. Furthermore, microbiota induces the maturation and education of immune system cells, this in turn control the microbial activity preventing intestinal damage. On the contrary a high fatty diet **(B)** and low in fibres promotes the pathogenic proliferation and the intestinal barrier disruption. In addition, a microbiota made mainly by non-beneficial bacteria induces the activation of inflammatory cytokines, leading to further activation of inflammatory process and tissue damage. The tissue damage in turn is not anymore able to control the microbiota composition, creating thus a negative and dangerous loop. (from Statovci *et al.*, 2017)

1.3 Obesity and IBD

In recent years the percentage of overweight people has increased by 28% in developed countries and by around 60% in developing countries, and no country has reported a decrease (Ng *et al.*, 2014). In the United States more than a third of the adult population is obese. In parallel with this increase, the incidence of developing IBD is also increasing globally. In an epidemiological study conducted in Scotland on 489 patients with IBD, 18% were obese and 38% were overweight, whereas only 3% of patients with DC and 0.5% of those with UC were underweight (Steed *et al.*, 2009). Similar results were found in cohort studies of infant patients, in fact 9-10% of children with Crohn's disease and 20-34% of children with ulcerative colitis had a body mass index (BMI) for age over 85 percentiles (Kugathasan *et al.*, 2007). Despite the high prevalence of obesity in patients with IBD, mild obesity has been associated with a risk of developing Crohn's disease but not ulcerative colitis (Singh *et al.*, 2017). Furthermore, the effect of obesity on the development of IBD may be age-dependent, in fact, the condition of obesity in juvenile and adolescent age is associated with a greater risk compared to obesity in old age (Singh *et al.*, 2017). The condition of obesity in addition to be a risk factor for CD etiopathogenesis can also alter the course and outcome of the disease. Indeed, it has been shown that patients with Crohn's disease and overweight are more prone to a more active inflammation and also show an inflammation of the anorectal tract, therefore the rate of hospitalization for these patients is greater than in normal weight subjects (Mendall *et al.*, 2011; Blain *et al.*, 2002; Nascimento *et al.*, 2012; Hass *et al.*, 2006). The response to therapy also appears to be different between obese/overweight and normal weight patients. Bhalme *et al.* (2013) observed an increased risk of loss of response to adalimumab, a recombinant anti-TNF-α monoclonal antibody, but not of infliximab, another monoclonal anti-TNF-α antibody, in obese patients. This effect could be due to the increased amount of body fat that modifies the pharmacokinetic properties of the drugs. In addition to this also the high concentration of circulating pro-inflammatory mediators alter the pharmacological effect (Bhalme *et al.*, 2013; Kredel and Siegmund, 2014)

1.3.1 Adipose tissue as an endocrine organ

Adipose tissue has always been considered as a passive form of connective tissue with the only function of storing energy in the form of triglycerides and releasing energy in the form of free fatty acids (FFA). However, due to the wide range of hormones, proteins, cytokines, and enzymes secreted by adipocytes, it is now considered an endocrine organ that performs different functions (Kredel and Siegmund, 2014). It consists of a group of adipocytes, preadipocytes, macrophages, endothelial cells and fibroblasts, and is subdivided into visceral and subcutaneous adipose tissue (Rocha *et al.,* 2007; Gregor *et al.,* 2011). The two types of tissue show a very different immunological and metabolic profile. Subcutaneous tissue (SAT) is approximately 80% of body fat and has a greater number of preadipocytes than visceral tissue. The adipocytes of the SAT have a high sensitivity to insulin and have a greater ability to accumulate FFA and triglycerides. In contrast, visceral adipose tissue (VAT) accounts for 10-20% in men and 5-10% in women of total body fat. It is highly vascularized and has a high content of M1 pro-inflammatory macrophages which mainly secrete TNF-α and IL-1. Furthermore, VAT is characterized by a high expression of pro-inflammatory cytokines, such as interleukins (IL-6, IL-8) and chemokines (MCP-1), adiponectin and cells of innate immunity. Additionally, an increase in visceral adipose tissue is associated with a condition of insulin resistance and metabolic syndrome (Kredel and Siegmund, 2014). In physiological conditions, adipokines regulate lipid metabolism, glucose metabolism, and the effect of insulin through a set of interactions with other cytokines (Balistieri *et al.,* 2010). The condition of obesity is instead characterized by an increase in the entire adipose tissue with different modifications of the humoral, cellular, and stromal components (Deiuliis *et al.,* 2011; Fain *et al.,* 2004; Kintscher *et al.,* 2008). *In vivo* studies carried out on animals and obese human patients have shown how in these subjects there is an increase of cytokines, which modify the expression of different pro-inflammatory mediators and can activate an innate immune response, thus triggering a mild chronic inflammation typical of obesity (Olefsky *et al.,* 2010). Chronic inflammation leads to the development of secondary diseases and further can affect the progress of other diseases, such as for example IBD (Barbarroja *et al.,* 2010; Scaffler *et al.,* 2006). Visceral fat has been recognized to be the most metabolically

active fraction of all body fat compartments and therefore could be a more indicative and specific factor for the risk of developing IBD than the total obesity determined by BMI (Kredel and Siegmund, 2014; Uko *et al.*, 2014). Fat accumulation can also be locally limited, in fact patients suffering from Crohn's disease show a peculiar hyperplasia, independent of body mass, of mesenteric VAT, the so-called “creeping fat”. Creeping fat (CF) is an extension of visceral tissue that extends from the roots of the mesentery and envelops the inflamed part of the large intestine, covering more than 50% of its surface, and consequently acting as a bridge between the accumulation of fat and inflammatory activity (Peyrin-Biroulet *et al.,* 2007; Sheehan *et al*., 1992; Fink *et al.,* 2012). While in a condition of obesity tissue enlargement is generally due to a cellular hypertrophy and very rarely to a hyperplasia, the creeping fat instead is characterized by a hyperplasia of adipose tissue. In fact, the adipocytes of creeping fat are significantly smaller and their number is about 4 times greater than in the normal mesenteric adipose tissue (Peyrin-Biroulet *et al.,* 2007). In the adipose tissue of obese patients there are up-regulated especially genes with pro-inflammatory activity, in CF instead there is an increase in gene expression of both the pro and anti-inflammatory cytokines (Zulian *et al.,* 2010). Due to their small size, the adipocytes of creeping fat produce less pro-inflammatory cytokines and are less responsive to stimulation (Skurk *et al.*, 2007; Kopp *et al.,* 2010). However, despite this, they are the major active producers of different leptin mediators, adiponectin, and resistin (Paul *et al.,* 2006; Batra *et al.,* 2009) (**Fig. 4**). It is worthwhile to be aware that while creeping fat is a distinctive feature of Crohn's disease, it is generally absent in individuals with ulcerative colitis. However, there are some observations of edematous adipose tissue in patients with UC (Edling *et al.,* 1963; Eklof *et al.,* 1970). Furthermore, due to the different molecular profile of adipocytes and tissue morphology, the visceral fat of patients with UC has inflammatory characteristics very different from those of subjects with CD. However, also UC is associated with an obesity condition with an increase in adipose tissue mass (John *et al.,* 2006; Levy *et al.,* 2005).

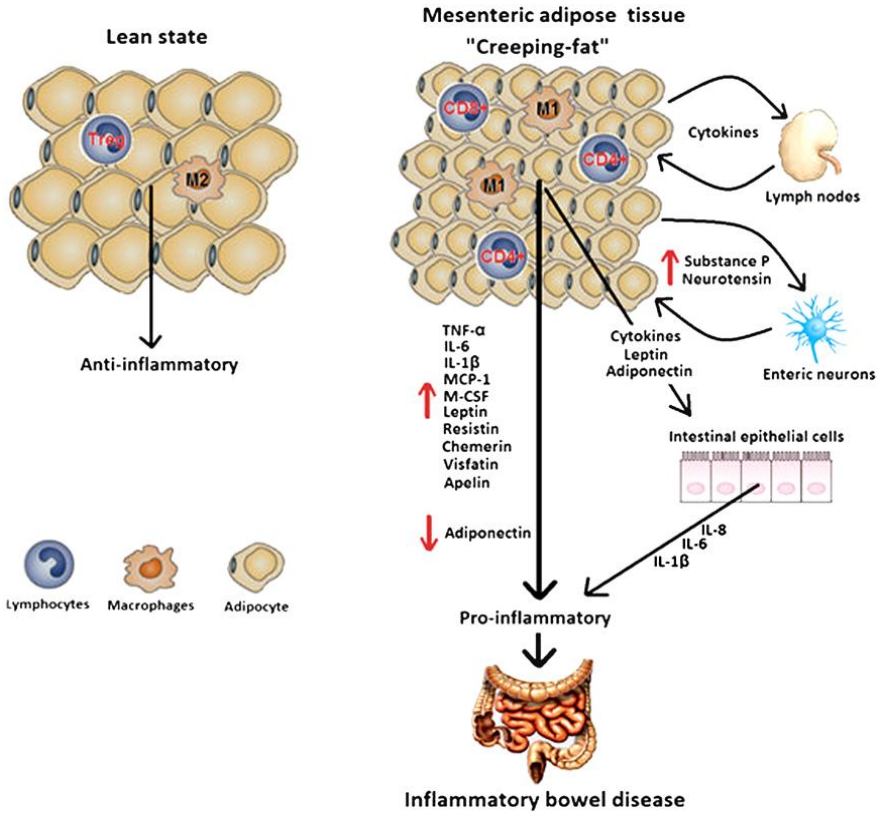


Fig. 4: **Morphological and functional differences between the adipose tissue in healthy state and in patient with CD**. In healthy condition the adipose tissue is characterized by "normal size" adipocytes secreting mainly anti-inflammatory mediators. The creeping fat, instead, is characterized by smaller adipocytes and a high population of macrophages secreting different pro-inflammatory cytokines (TNF-α, IL-6), adipokines (adiponectin, leptin) that induce intestinal inflammation (from Goncalves *et al.*, 2015).

1.3.2 Crosstalk between adipose tissue and bowel in IBD

- ***adipokines***

In the last years, in order to better understand the mechanism underlying this crosstalk between adipose tissue and intestinal inflammation, the research was focused on the role of molecules secreted by adipocytes on the initiation and progression of IBD. It has been reported that adipocytes and preadipocytes secrete more than 50 adipokines (Trayhurm *et al.*, 2004). As mentioned above the adipocytes secrete several pro-inflammatory cytokines, chemokines and adipokines, showing thus to possess a strong pro-inflammatory action and to induce an innate immune response.

VAT can be considered a crucial source of cytokines, produced both by adipocytes as well as by macrophages and lymphocytes, liable for the inflammatory process of

IBD (Bertin *et al.,* 2010; Desreumaux *et al.,* 1999; Karmiris *et al.,* 2006; Fantuzzi *et al.,* 2008; Schsffler *et al.,* 2008). In patients with IBD and in patients with mild obesity was observed a high plasma concentration, directly proportional to body mass, of TNF-α and IL-6 (Bertin *et al.,* 2010). Moreover, it has been reported that the adipose tissue of patients with IBD secrete a higher amount of TNF-α compared to the one of healthy patients, and in addition high levels of this cytokine were also found in the bloodstream, mucosa and faeces, suggesting the involvement of a systemic effect (Gambero *et al.,* 2007; Murch *et al.,* 1991; Breese *et al.,* 1994; MacDonald *et al.,* 1990). Another cytokine that is over-expressed in VAT of people with IBD is IL-6, and several studies have suggested that around 30% of the circulating IL-6 derives from visceral adipose tissue (Park *et al.,* 2005). In addition, since Crohn's disease is associated with the Th1 mediated cell response, which is characterized by an increased production of IL-6, IFN-γ and TNF-α, it can be concluded that creeping fat contributes to the typical Th1 response of UC (Zulian *et al.,* 2012; Goncalves *et al*., 2015). Furthermore, macrophages and T lymphocytes of mesenteric adipose tissue in active CD patients release more cytokines (IL-6, IL-4, and IL-13) than those of non-active and healthy patients. Therefore, the aberrant expression of cytokines in the creeping fat of CD subjects is due in part to macrophages and T lymphocytes of adipose tissue (Jung *et al.,* 2013). The mesenteric adipose tissue of patients with IBD, in addition to an over-expression of cytokines, is characterized also by an overproduction of adipokines, such as adiponectin, resistin, and leptin (Maconi *et al.,* 2008; Barbier *et al.,* 2003). The latter was initially thought to perform only the activity of appetite suppressant, but now several studies have shown its involvement in inflammatory processes. Higher levels of leptin have been found in the intestinal lumen of patients with IBD compared to healthy one, and both its gene and protein expression are overexpressed in patients with Crohn's disease (Bertin *et al.,* 2010; Desreumaux *et al.,* 1999; Sitaraman *et al.,* 2004; Paul *et al.,* 2006). Leptin stimulates and promotes the proliferation of mononuclear cells and plays a crucial role in the inflammatory response by increasing the secretion of inflammatory cytokines using the STAT3 pathway (Matarese *et al.,* 2002; Procaccini *et al.,* 2012; La Cava *et al.,* 2004; Williams *et al.,* 2004). It also stimulates differentiation of T cells in the Th1 phenotype rather than in the Th2 (Lord *et al.,* 2002).

Resistin is mainly secreted by macrophages, mononuclear cells, and stem cells (Karmiris *et al.,* 2008). It has been associated with inflammatory processes since its expression in adipose tissue is induced by pro-inflammatory cytokines, such as IL-1, IL-6, and TNF -α (Kaser *et al.,* 2003). In addition, *in vitro* studies on murine and human macrophages cell lines have reported that the bacterial lipopolysaccharide activates the expression of resistin through a pro-inflammatory cytokine cascade (Lehrke *et al.,* 2004). In turn, resistin once activated induces the activation of IL-12, TNF-α and adhesion molecules in mononuclear cells, human endothelial cells and macrophages (Bokarewa *et al.,* 2005; Pang *et al.,* 2006). In fact, it has been shown that in human mononuclear cells resistin induces, and is also induced by, IL-6 and TNF-α through the NF-κB pathway (Bokarewa *et al.,* 2005; Pang *et al.,* 2006).

Whereas the role of adiponectin in the pathogenesis of IBD is still partially unknown (Bertin *et al.,* 2010). Adiponectin, secreted mainly by mature adipocytes, has anti-inflammatory effects (Karmiris *et al.,* 2008). In fact, *in vitro* studies on different cell lines have shown that it is able to negatively modulate inflammatory pathways through the inhibition of NF-κB, and to interfere with the function of macrophages (Ouchi *et al.,* 1999; Yokota *et al.,* 2000). In addition, adiponectin suppresses TNF-α production by activating the MAPK signalling pathway in murine macrophages, and it further blocks the TRL-induced NF-κB activation in human macrophages (Wolf *et al.,* 2004; Yamaguchi *et al.,* 2005; Zhao *et al.,* 2005). It up-regulates expression of anti-inflammatory cytokines, such as IL-10 and IL-1Ra in dendritic cells, monocytes and macrophages (Wolf *et al*., 2004), suggesting thus that its anti-inflammatory effect is mediated principally by the cells of the immune system. Low levels of adiponectin have been reported in patients with active CD and its expression in adipose tissue is inversely proportional to the degree of intestinal inflammation (Rodrigues *et al.,* 2012). However, an increase in both the levels of gene expression and proteins in VAT have also been reported in subjects with Chron's disease (Yakamoto *et al.,* 2005). Also with regard to the serum levels of this adipokine, conflicting results have been reported (Zhao *et al.,* 2005; Ohashi *et al.,* 2010), suggesting thus that the physiological role of adiponectin in the formation and progression of IBD still remains a question mark.

In the last period the possible involvement of neuropeptides in the pathogenesis of IBD has been demonstrated. Enteric neurons are characterized by a high amount of substance P (SP), neurotensin (NT) and vasoactive intestinal polypeptides (Shepard *et al.*, 1987; Bishop *et al.*, 1980). *In vitro* studies on pre-adipocytes showed an increase in IL-6 and IL-8 expression, via the NF-κB pathway, when treated with NT and SP, respectively. This pro-inflammatory effect could create a cascade reaction that leads to the recruitment of immune cells and the formation of creeping fat (Karagiannides *et al.*, 2008). Also Gross and co-workers have shown that substance P can influence the size of fat depots by acting on the replication and apoptosis of pre-adipocytes. Specifically, the treatment of adipocytes with SP induces an increase in the proliferative activity and at the same time reduces apoptosis (Gross *et al.*, 2009). Therefore, the neuropeptides could act as a bridge between the adipose tissue and the intestinal response during the IBD condition acting on two different fronts: the formation of the creeping fat and the induction of an inflammatory response at the level of the adipocytes (Fink *et al.*, 2013).

1.4 Lipotoxicity

The lipids introduced through the diet are certainly essential in promoting inflammation. Lipids are a heterogeneous class of molecules including fatty acids, sterols, phospholipids and triglycerides. In addition, to be an efficient source of energy, they also act as important components of the cell membrane and molecules that regulate metabolic homeostasis. However, a wrong lifestyle, and genetic and epigenetic factors, can alter the balance between their metabolism and their composition becoming thus harmful to the health with consequent organelle dysfunction, cell death, dysfunction in energy metabolism, and chronic inflammation (Ertnunc and Hotamisligil, 2016). All these negative effects together induce a pathological condition which is called lipotoxicity. A condition of lipotoxicity in addition to causing an alteration of lipid metabolism, converges with the immune and stress response, contributing so to the development of various diseases (Fu *et al.*, 2012). A diet rich in lipids induces an increase in free fatty acids, which enter the mitochondria where they are oxidized or esterified into triglycerides through the β-oxidation process (Pessayre *et al.*, 2001). During the

process of β-oxidation of fatty acids, which depends on the redox reactions of the co-factors NAD^+/NADH and FAD/FADH2, electrons are released and transferred to the mitochondrial electron transport chain, here they can be bound to oxygen forming radicals superoxide anions and other reactive oxygen species (ROS). In turn, ROS and other radical species oxidize the polyunsaturated lipids of fat deposits, thus triggering the process of lipid peroxidation. The intermediates of this metabolic process also react with oxygen to form different ROS, causing so an imbalance in the redox state with the consequent formation of a condition of oxidative stress (Pessaye *et al.,* 2002; Matsuzawa-Nagata *et al.,* 2008). Furthermore, lipid peroxidation and ROS reduce the levels of endogenous antioxidants and vitamins, leading to a further increase in intracellular ROS levels with a consequent increase in ROS-dependent cell damage (Pessaye *et al.,* 2002). The ROS-mediated state of inflammation induces the activation of different inflammatory signal pathways including the NF-κB pathway, one of the main transcription factors related to inflammatory processes, and consequently of other downstream pro-inflammatory mediators of NF-κB such as TNF-α, IFN-γ, and inducible synthetase of nitric oxide (iNOS) (Weisberg *et al.,* 2008). Furthermore, lipids influence the cellular function by activating membrane receptors, in fact, palmitic acid directly activates the inflammatory pathways by increasing the expression of TRL4 on cell membranes or by stimulating the protein kinase R (PKR) (Nakamura *et al.,* 2010). Following its activation by harmful lipids, such as palmitic acid or oxidized cholesterol, PKR induces the activation of the JNK pathway. JNK downstream mediators include genes involved in the inflammatory processes, apoptosis, modulation of inflammasome and of the transcription factor activating protein 1 (AP-1) (Takada *et al.,* 2010; Kang *et al.,* 2012; Peng *et al.,* 2015) (**Fig. 5**). Some of the principal pro-inflammatory mediators involved in the initiation and progression of IBD induced by a condition of lipotoxicity will be described in more detail below.

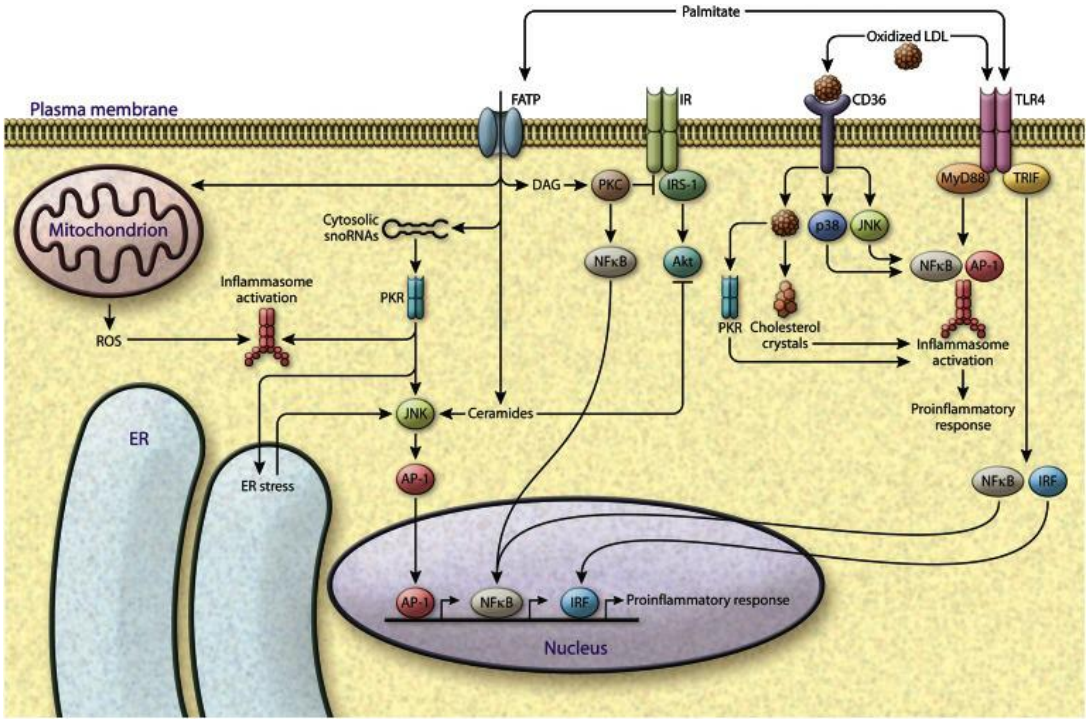


Fig. 5: **FFA-induced lipotoxicity**. Increased amount of toxic lipids causes failure of metabolic regulation, converging on inflammatory and stress pathways. Palmitate can activate TLR4 signalling, leading to activation of inflammasomes and induction of inflammatory gene transcription factors such as NF-κB, AP-1 and interferon regulatory factor (IRF). Palmitic acid directly contributes to the synthesis of diacylglycerols and ceramides, which activate stress kinases, PKCs, the NF-κB and JNK pathways. It further can be transported inside the cell by the FATPs, and through PKR can induce ER stress. Additionally, lipotoxicity can also induce the ROS production from mitochondria, which is linked to inflammasome activation (from Ertnunc and Hotamisligil, 2016).

1.5 MOLECULAR MECHANISMS INVOLVED IN IBD

1.5.1 Reactive oxygen species (ROS)

Chronic intestinal inflammations are multifactorial pathologies characterized by a massive infiltration of granulocytes and macrophages in the intestine, which in addition to inducing the activation of a broad spectrum of pro-inflammatory cytokines also produce a huge quantity of ROS and reactive nitrogen species (NOS) (Biasi *et al.*, 2013). ROS are small molecules including oxygen radicals [superoxy (O_2*), hydroxyl radical (*OH)], non-radical species [oxygen singlet (O_2) and hydrogen peroxide (H_2O_2)] (Speciale *et al.*, 2019). These molecules are highly reactive and are able to cause damage to proteins, lipids and nucleic acids, generating hence oxidized macromolecules which are considered as triggers for

various pathologies (Tian *et al.,* 2017). Although they have always been considered as harmful substances, in reality at low and moderate concentrations ROS are essential for cellular homeostasis and are beneficial for many biological processes. In fact, at the level of the intestinal epithelium, they are involved in the defence mechanism against pathogens by inducing the respiratory burst in the phagocytes (Biasi *et al.,* 2013). In physiological conditions the intracellular levels of ROS are kept stable and balanced by different cellular processes. Endogenous ROS are produced in intracellular organelles such as endoplasmic reticulum, mitochondria, peroxisomes, nucleus, cytosol, and extracellular matrix. Among these, mitochondria are considered the main organelles in which ROS production takes place, they are in fact the site of the electron transport chain, the main source of ROS, as well the site of the catabolism of fatty acids (Novak and Mollen 2015; Poyton *et al.,* 2009). At the same time these organelles are also the most damaged and the primary targets of oxidative stress (Tian *et al.,* 2017). A high ROS production, in fact, is able to suppress the electron transport chain with a consequent decrease in ATP production and damage at the mitochondrial DNA level. When this situation continues over time, the mitochondrial homeostasis is affected and subsequently cell death pathway is activated (Chen *et al.,* 2008; Scherz-Shouval *et al.,* 2007). Several enzymes, such as lipoxygenases, cyclooxygenases, (involved in lipid metabolism), oxidase, peroxidase, through the catalysis of chemical reactions, are considered responsible for the production of endogenous ROS (Kulkarni *et al.,* 2007; Swindle *et al.,* 2007). In addition, external and environmental factors, such as smoking, alcohol, drug use and chemotherapy also participate in the production of ROS (Goyette *et al.,* 2007). Under physiological conditions, eukaryotic cells in order to counteract and avoid over-expression of intracellular ROS, induce the expression of genes coding for antioxidant proteins, thus regulating the intracellular redox state. However, the excessive production of ROS associated with a decrease in antioxidant activity is related to an increase in intestinal permeability, impaired immune response, damage at the DNA level, lipid peroxidation, oxidation of the amino acid lateral chains, apoptosis, and carcinogenesis (Ridnour *et al.,* 2005; Valko *et al.,* 2001; Valko *et al.,* 2007). This condition is called oxidative stress and is due to an imbalance between antioxidants and radical species.

The endogenous defence system against oxidizing substances consists mainly of a) intracellular enzymatic antioxidants: superoxide dismutase (SOD), glutathione peroxidase (GPX), and catalase (CAT), b) non-enzymatic intracellular antioxidants: glutathione (GSH), and c) extracellular antioxidants, uric acid, vitamins, minerals (Tian *et al.*, 2017). The first endogenous antioxidant system evaluated in patients with IBD was GSH/GSSG/GPX. GSH is a water-soluble antioxidant molecule containing a thiol group derived from cysteine and expressed in cytosol, nucleus, and mitochondria. The maintenance of its homeostasis is provided by synthesis starting from cysteine or from the regeneration of oxidized glutathione (GSSG) and uptake of GSH by dependent sodium transport (Vučetić *et al.*, 2017). Together with enzymes glutathione S-transferase (GST) glutathione reductase (GSR) and GPX, it forms an important antioxidant barrier in the intestinal mucosa. The GPX enzyme allows the reduction of hydrogen peroxide in water or of lipidic hydroperoxides (ROOH) in stable alcohols, catalysing the oxidation of GSH in disulphide oxidized glutathione. Currently 8 GPX isoforms have been detected, among these the isoform 2 (GPX2) is specifically expressed in the gastro-intestinal epithelium (Dayer *et al.*, 2008). *In vivo* studies on CD and UC mouse models have shown a pivotal role of GPX2 in the defence against oxidative stress and inflammation in the intestinal mucosa (Dayer *et al.*, 2008; Te Velde *et al.*, 2008). It has also been shown to be induced under pathogenic conditions such as IBD and gastric cancer. One of the mechanisms by which the condition of oxidative stress starts and propagates intestinal inflammation is mediated by the NF-κB signalling pathway, which is responsible for the regulation of several pro-inflammatory mediators.

1.5.2 NF-κB pathway

NF-κB is an inducible redox-sensitive nuclear transcription factor responsible for several biological processes, such as inflammation, immune response, cell growth, and apoptosis (Hayden *et al.*, 2012). Its activation in fact induces the transcription of several pro-inflammatory mediators. It consists of homodimers and heterodimers of proteins of the NF-κB family, which includes 5 monomers, RelA

(p65), RelB, cRel, NF-κB 1(p50), and NF-κB 2. All these proteins share a N-terminal region, the Rel Homology Region (RHR) responsible for dimerization, nuclear translocation, DNA binding, and binding to the κB (IκB) inhibitor (Baldwin, 2001). Furthermore the RelA, RelB, cRel subunits also contain an essential domain for the transcriptional activity, the transcriptional activation domain (TAD), while p50 and p52 are lacking of it, so they act as inhibitors through a mechanism of competition with the TAD-containing dimers for κB site binding (Huxford *et al.*, 2009; Franzoso *et al.*, 1992). In most cells NF-κB is a heterodimer composed of the RelA (p65) and NF-κB (p50) subunits, which also represents the most active and stable form of this family (Speciale *et al.*, 2019). In healthy conditions NF-κB is located in the cytosol bound to the κB inhibitor, which by binding to the RHR domain of the dimer masks the nuclear localization signal, preventing thus its translocation to the nucleus (Huxford *et al.*, 1998). Following NF-κB activation, it translocates to the nucleus where it binds to specific DNA sites and regulates the expression of many genes involved in inflammatory processes, innate immunity, cell proliferation, and apoptosis (Speciale *et al.*, 2019). The activation and therefore the translocation of this factor can occur through two different pathways (**Fig. 6**): the classical or canonical pathway and the non-canonical pathway. Activation by the canonical pathway involves the IκB kinase (IKK) consisting of two subunits (IKKα and IKKβ) and the regulatory protein NEMO (NF-κB essential modulator). External stimuli from pro-inflammatory mediators (TNF-α), associated pathogenic molecular pattern (PAMPs), and associated damage molecular patterns (DAMPs), induce IKK phosphorylation which in turn phosphorylases IκB causing its dependent proteasome degradation and allowing the nuclear translocation of NF-κB (Bonizzi *et al.*, 2004). While stimuli involved in cell differentiation and development, such as the beta lympho-toxin receptor (LTβR), CD40L, TNF receptors etc., activate instead the non-canonical pathway. This pathway is mediated by the NF-κB inducing kinase (NIK) and by IKKα. NIK activation leads to phosphorylation and subsequent proteasomal processing of the NF-κB2 (p100) precursor in p52 through an IKK1/IKKα-dependent manner. The formed p52 mature subunit is free to dimerize with the RelB subunit and consequently to translocate to the nucleus and activate the gene transcription of different mediators (Sun, 2012). At intestinal level it is involved in IEC homeostasis and in the permeability modulation of the intestinal barrier

(Pasparakis *et al.,* 2008). In fact, an abnormal activation of this factor is a typical feature of IBD (Neurath *et al.,* 1996; Rogler *et al.,* 1998). Administration of NEMO-binding drugs that block the link between the NEMO and IKK subunits has been shown to reduce the degree of inflammation in different murine models of colitis and to inhibit IKK activation (Shimozawa *et al.,* 2004). Furthermore, high levels of the p65 subunit in fibroblasts, endothelial cells, and macrophages of patients with IBD have been reported (Gelbmann *et al.,* 2003; Rogler *et al.,* 1998). Excessive NF-κB activation is thought to be responsible for the induction of transcription of several genes having a central role in the pathogenesis of IBD which can be divided into. These genes play a fundamental role in inducing damage to the intestinal barrier by stimulating the production of other NF-κB-dependent cytokines (Seidelin *et al.,* 2005; Clevers *et al.,* 2011)

- genes regulating cell proliferation and apoptosis: cyclic D1 and D3, proteins of the Bcl family, endothelial growth factor (EGF) (Biasi *et al.,* 2013)

- genes regulating intestinal permeability and angiogenesis: claudin, myosin light chain kinase (MLCK), vascular endothelial growth factor (VEGF). For example, TNF-α induces a partial increase in intestinal permeability by activating MLCK in a dependent NF-κB manner (Ye and Ma, 2008)

- metalloproteinases which degrade the extracellular matrix and the cells of the intestinal mucosa (Ravi *et al.,* 2007)

- enzymes inducing eicosanoid, ROS and RNS: COX-2, lipooxygenases, iNOS, which participating in ROS metabolism can induce NF-κB activation and therefore increase cell permeability (Andersen *et al.,* 2005).

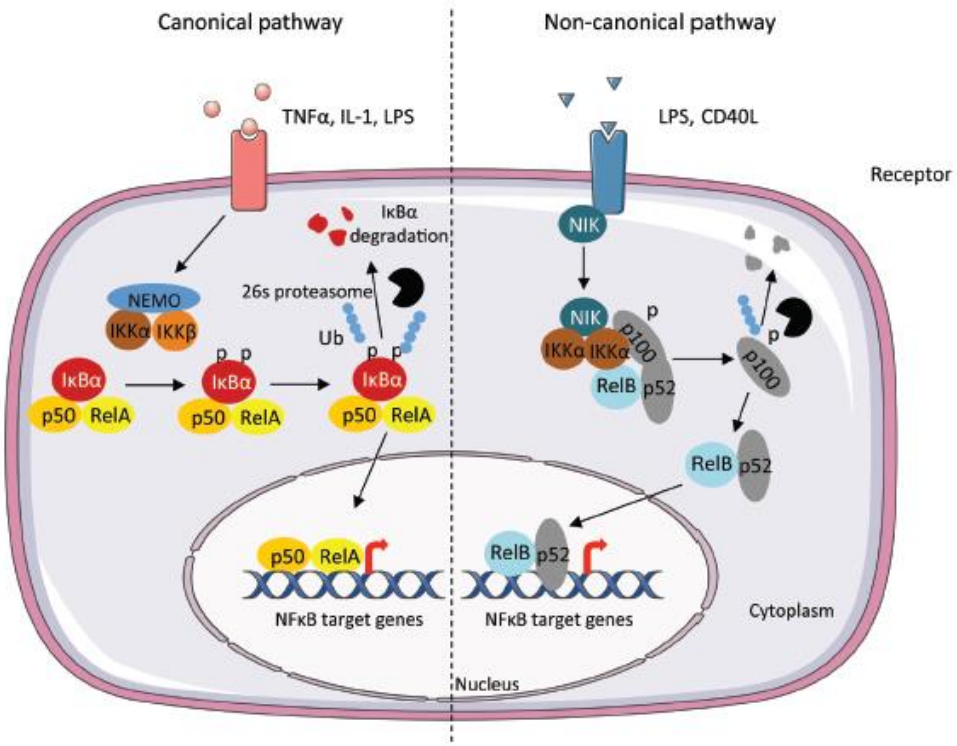


Fig. 6: Canonical and non-canonical pathway leading to the activation of NF-κB. The canonical pathway is triggered by cytokines such as TNFα and Interleukin-1 (IL-1). These induce the phosphorylation of IκBα by the IKK complex, leading to its degradation by the 26S proteasome. The RelA/p50 complex is free to translocate to the nucleus and to activate the transcription of target genes. In the non-canonical pathway TNF cytokine family induce the activation of IKKα through the NF-κB-inducing kinase (NIK) leading to the phosphorylation of the p100 subunit and processing in p52. P52 can dimerize with RelB and translocate to the nucleus (from Viennois *et al.*, 2013).

1.5.3 Nrf2 pathway

The Nuclear factor-erythroid 2 related factor 2 (Nrf-2), is a key transcription factor in the maintenance of redox homeostasis at the level of the intestinal mucosa. In fact, this factor regulates the expression of detoxifying and antioxidant enzymes presenting the Antioxidant Responsive Element (ARE) sequence on their promoter. It is expressed at the level of different tissues such as liver, kidneys (organs involved in the detoxification processes), as well as at the level of the gastrointestinal tract, skin, and lungs (organs more in contact with external agents) (Speciale *et al.*, 2013).

Under physiological conditions, Nrf2 is bound to the cytosolic proteins Keap-1 and Cullin 3 (Cul3), with the last one inhibiting the transcriptional activity of Nrf2 by ubiquitination and proteasomal degradation, while Keap-1 is a substrate that facilitates this reaction. A condition of oxidative stress or other oxidative stimuli induces a burst of the Keap- Cull-3 inhibition system allowing Nrf2 to translocate to the nucleus where it will bind to the ARE sequence and may induce the transcription of genes coding for antioxidant enzymes (Cullian *et al.*, 2004; Kobayashi *et al.*, 2004; Zhang *et al.* 2004) (**Fig. 7**).

Nrf2 modulates the transcription of about 250 genes that can be divided into:

- antioxidant enzymes: heme-oxygenase 1 (HO-1), glutathione peroxidase 2 (GPX2), glutamate-cysteine ligase catalytic subunit (GCLC), glutamate-cysteine ligase-modifying subunit (GCLM), sulfiredoxin 1 (SRXN1) and tioredoxin (TRX)

- enzymes of phase 1 metabolism: NAD(P)H quinone oxidoredutase (NQO1), enzyme responsible for the reduction of quinones, highly reactive species which can cause stress.

- phase 2 detoxifying enzymes: glutathione S microsomal transferase 1 (GTST1) (Hayes *et al.*, 2000; Speciale *et al.*, 2019)

Nrf2 plays an essential role in the pathogenesis of IBD. *In vivo* studies on a Dextran sulfate sodium (DSS)-induced colitis model on Nrf-2 knock out (KO) and wild type (WT) mice showed that KO mice had more severe symptoms associated with a greater expression of pro-inflammatory genes and a decrease in antioxidant genes compared to WT ones (Khor *et al.*, 2006; Khor *et al.*, 2008).

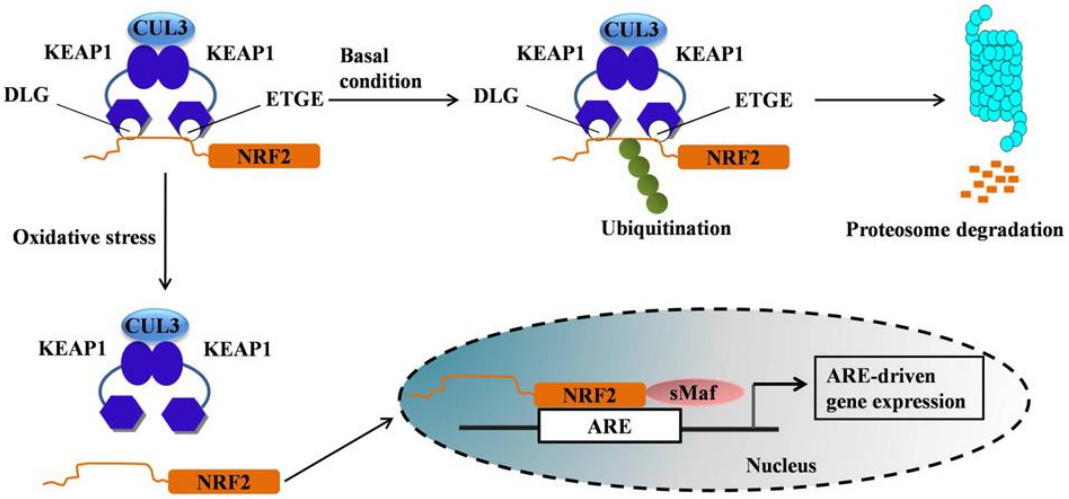


Fig. 7: **Schematic mechanism of Nrf2 activation**. Under basal conditions, Nrf2 binds to its repressors Keap1 and Cul3 which lead to ubiquitination followed by proteasome degradation. Oxidative stress stimuli induce a burst of the Keap-1/Cul-3 inhibition system, and Nrf2 is free to translocate to the nucleus where it binds to ARE genes and can induce transcription of several gene coding for antioxidant and detoxifying enzymes (from Ahmed *et al.*, 2017).

1.5.4 Crosstalk between Nrf2 and NF-κB

In the last few years a possible crosstalk between Nrf2 and NF-κB has been suggested, where the two pathways reciprocally inhibit the transcription and/or activity of downstream proteins (**Fig. 8**). This assumption is based on the fact that different activation and inhibition mechanisms have been demonstrated between the two pathways. In fact, it has been shown that the activation of Nrf2 and HO-1, following the exposure to antioxidants, is able to inhibit the nuclear translocation of NF-κB in prostate cancer cells (Bellezza *et al.*, 2012) In addition, NF-κB nuclear levels obtained from the lungs of Nrf2 KO mice exposed to LPS were much higher than those from WT mice. In line with this, Lee and coworkers (2009) reported that Keap1 is able to induce IKKβ degradation by ubiquitation, consequently inhibiting p65 nuclear translocation.

On the other hand, NF-κB can in turn directly inhibit Nrf2 activity. In fact, since both factors bind to the same region of the transcription co-activator CREB binding protein (CBP), an increase in p65 levels prevents the binding of Nrf2 with CBP, repressing hence its activity (Liu *et al.*, 2008). Furthermore, Yu *et al*. (2011)

demonstrated that p65 inhibits Nrf2 pathway through the nuclear translocation of Keap1.

It is believed that the levels of oxidative stress can move the balance needle towards the activation of one pathway or to the other one. A low degree of oxidative stress induces the activation of the Nrf2 pathway, and therefore an antioxidant response, while an intermediate level activates the NF-κB pathway and the inflammatory response. High levels are instead responsible for the induction of cell death processes (Gloire *et al.*, 2006)

However, many aspects of this crosstalk are still unknown, so further studies are necessary in order to better understand this interaction.

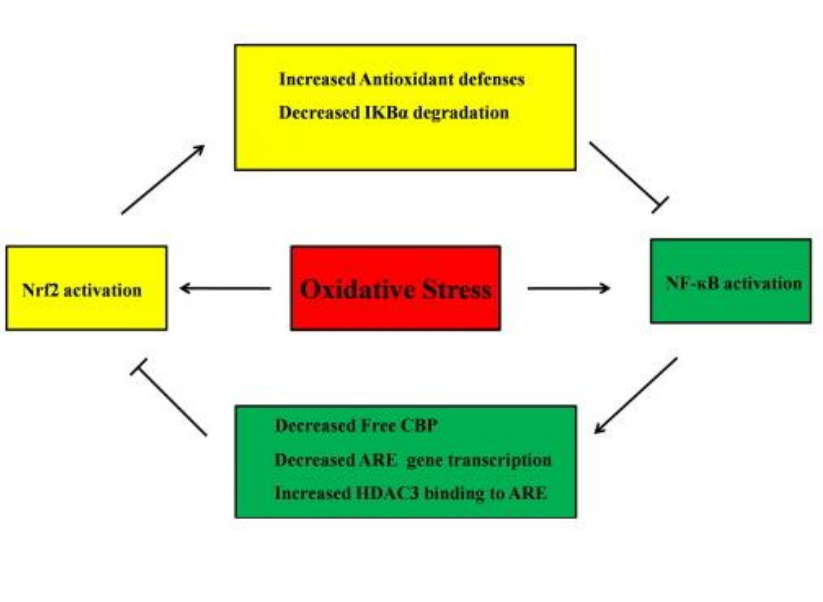


Fig. 8: **Crosstalk between Nrf2 and NF-κB pathways** (modified from Ahmed *et al.*, 2017).

1.6 Oxidative stress and intestinal permeability

Intestinal epithelial cells (IEC) form a barrier over the entire GI tract, preventing the entry of pathogenic microorganisms and toxic molecules, thus maintaining intestinal homeostasis. They are the first cells that come into contact with the external environment and therefore can be considered as a first defence mechanism against possible harmful stimuli. The IECs can recognize pathogenic organisms, and secrete cytokines and chemokines activating thus the immune response

(Pastorelli *et al.,* 2013). Intestinal permeability is regulated by tight junctions, adherent junctions and desmosomes, which firmly bind epithelial cells and regulate molecular flow through the intracellular space. Several studies have shown the connection between malfunctioning TJs and intestinal inflammation. Cani *et al.* (2008) have reported that a diet rich in lipids, through the modulation of the microbiota, induces an increase in intestinal permeability by reducing the gene expression of occludin and ZO-1 (**Fig. 9**). According to this, other groups have seen a down-regulation of claudin-1 and occludin in mice fed for 8 weeks with a diet rich in lipids (Alvarez-Suarez *et al.*, 2014).

The recognition of pathogens is mediated by recognition pattern receptors (PRRs) such as toll like receptor (TRL) and NOD-like receptors (NLRs) which can distinguish commensal bacteria from harmful ones (Pastorelli *et al.,* 2013; Fukata *et al.,* 2013). In particular, TRL-2 mainly recognizes lipoproteins, TRL-3 double-stranded viral RNA, TRL-4 lipopolysaccharides (LPS), TRL-5 flagellin, and finally NOD1 and 2 bacterial peptidoglycans (Takeda and Akira, 2003). As a result of excessive production of ROS at the intestinal level, the resulting oxidative stress induces lipid peroxidation and modifies the function of proteins and thus accelerates cell damage (Anderson *et al.,* 2004). The ROS overexpression induces damage to the cytoskeleton proteins regulating the tight junctions and intestinal permeability, causing thus a malfunction of the intestinal barrier (Rao *et al.,* 2008). Due to barrier damage, pathogenic bacteria can more easily penetrate the intestinal epithelium and activate PRR signals, on the endothelium, by increasing the expression of TRLs and NOD on endothelial cells. Additionally, endothelial cells present the proteins CD40 and CD40L, which belong to the superfamily of tumor necrosis factor receptors (TNFR) and therefore can co-stimulate immune cells during intestinal inflammation, suggesting a role of the endothelium as a second barrier (Pastorelli *et al.*, 2013).

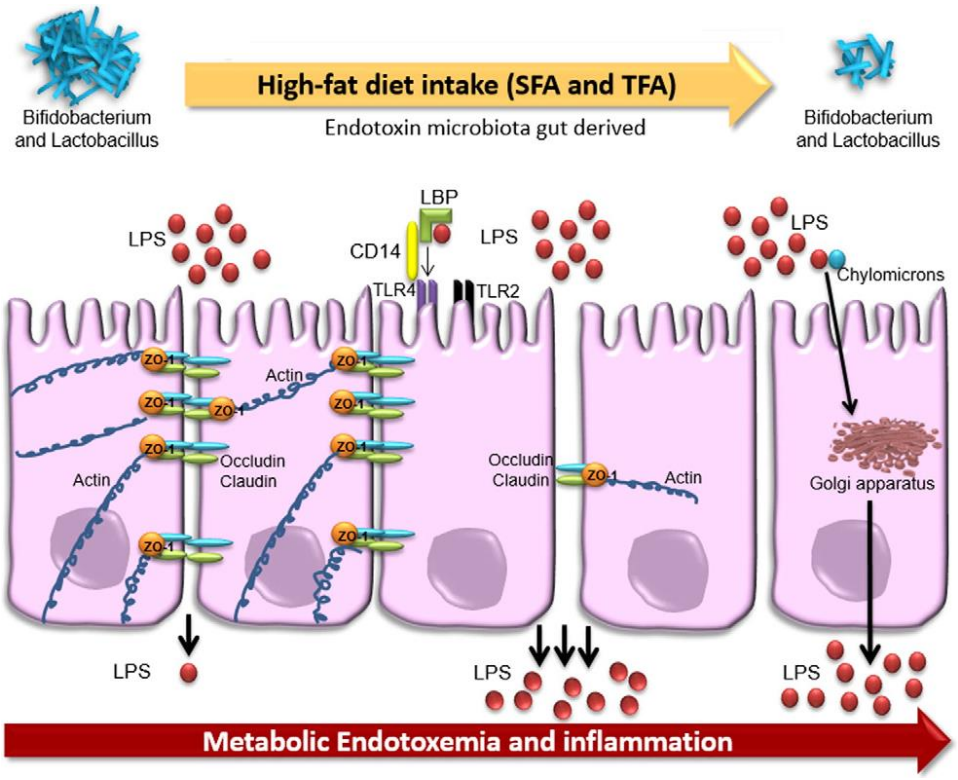


Fig. 9: **Effect of a diet rich in lipid on intestinal barrier**. A high intake of FFA can disrupt TJs and AJs acting on different levels, leading thus to an increased intestinal permeability. Furthermore, an excess of lipids negatively modulates the amount of the good bacteria (such as *Bifidobacterium* and *Lactobacillus*), increasing the number of Gram-negative ones and leading to a state of endotoxemia and inflammation (modified from Morais *et al.*, 2016).

1.7 Therapy

It is widely accepted that intestinal damage is caused by an altered immune response to environmental factors within the intestinal lumen. Therefore, the main purpose of IBD therapies is to reduce inflammation and avoid relapse (Biasi *et al.*, 2013). Conventional therapies involve the use of anti-inflammatory agents, such as 5-aminosalicylates (sulfasalazine, mesalazine) and corticosteroids (prednisolone, dexamethasone, budesonide), which induce a significant suppression of inflammation and a rapid disappearance of symptoms. However, intestinal inflammation is a pathology that requires long-term therapies, and often the use of these drugs leads to side effects, such as anemia, hypersensitivity, and drug intolerance (Biasi *et al.*, 2013). Furthermore, these anti-inflammatory drugs do not prevent the formation of streaks and intestinal fibrosis, requiring hence surgery (Collingwood *et al.*, 2007). A valid alternative of using anti-inflammatory agents is

represented by immunomodulators, which act through an immunosuppression mechanism. Thiopurine, cyclosporin A, and methotrexate are widely used in the treatment of IBD, however they possess cytotoxic effects which can increase other pathologies and infections, therefore their use requires constant monitoring and personalized dose (Chouchana *et al.,* 2012; Khan *et al.,* 2011). Due to the increased incidence and severity of IBD, in recent years research has focused on the study of new revolutionary therapies. Among these, biological therapy, which focuses on the inflammatory response antagonizing pro-inflammatory molecules, is getting more and more pharmacological interest. Nowadays the biologic drugs available on the market are anti-TNF-α monoclonal antibodies (Infliximab, Adalimumab, Certolizumab and Golimumab), anti-α4 integrin receptor (Natalizumab and Vedolizumab) and anti-IL12 and IL23 (Ustekinumab) (Biasi *et al.,* 2013; Reinglas *et al.,* 2018). They are used as a second-line therapy for the treatment of moderate and advanced Crohn's disease (**Fig.10**). Although their use is widely discouraged, even in co-administration with antibiotic drugs, due to the very high risk of developing even serious infections. Thanks to multiple studies it is now known that an over-expression of ROS and an exaggerated activation of the NF-κB pathway are at the basis of the initiation and progression of IBD. Therefore modulating the NF-κB signalling pathway or acting directly on ROS production sites, using both natural and synthetic antioxidants, could represent a new efficient and alternative therapeutic method (Yanai *et al.,* 2016; Kannan *et al.,* 2013). Drugs having antioxidant activity include COX-2 inhibitors, angiotensin 2 inhibitors, inhibitors of hydroxymethylglutaryl CoA reductase, and N-acetyl cysteine (NAC). Clinical studies have reported a decrease in the levels of endogenous ROS in patients with IBD following the intake of COX-2 inhibitors (Marnett *et al.,* 2009). In addition, Telmisartan, an angiotensin 2 inhibitor, has both antioxidant and anti-inflammatory effects, reducing ROS levels by downregulation of NF-κB and COX-2 (Arab *et al.,* 2014). In murine models of IBD, following an oral administration of NAC for a month and a half, a reduction in lipid and protein oxidation was shown with a concomitant increase in GSH and CAT activity with a consequent reduction of oxidative stress (Siddiqui *et al.,* 2006; Guijarro *et al.,* 2008). Finally, recently several clinical tests have shown the positive effect of a continuous diet rich in polyphenols and vitamins A, C and E in decreasing and preventing inflammation. Polyphenols, very abundant molecules in fruits and

vegetables, possess antioxidant, anti-inflammatory, anticancerogenic, and immunomodulatory activity. In fact, they inhibit the production of pro-inflammatory cytokines by modulating the NF-κB, MAPKs, Nrf-2 pathways; they are also powerful free radical scavengers, reducing the condition of oxidative stress and inhibiting the sensitive redox pathways. In a randomized, placebo-controlled clinical trial conducted on CD patients, it was shown that a 6-months treatment with sulfasalazine co-administered with curcumin was able to significantly reduce the incidence of relapse compared to the treatment with the anti-inflammatory alone. For the following 6 months all patients were treated with only the anti-inflammatory; the data shown that the number of relapse cases was similar between the two groups (Hanai *et al.*, 2006). Additionally, the polyphenols positively regulate the intestinal barrier acting on the TJs, and further increase the production of SCFA and the amount of healthy commensal flora (Kaulmann *et al.*, 2016)

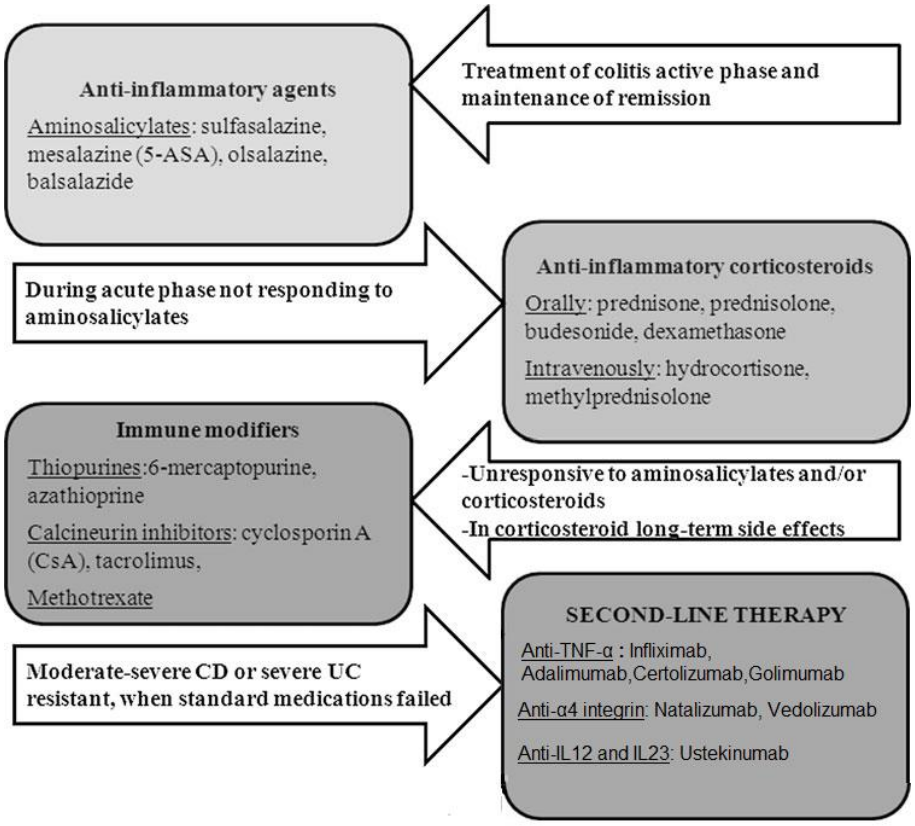


Fig. 10: Conventional therapeutic approaches used for the IBD treatment (modified from Biasi *et al.*, 2013).

Chapter 2

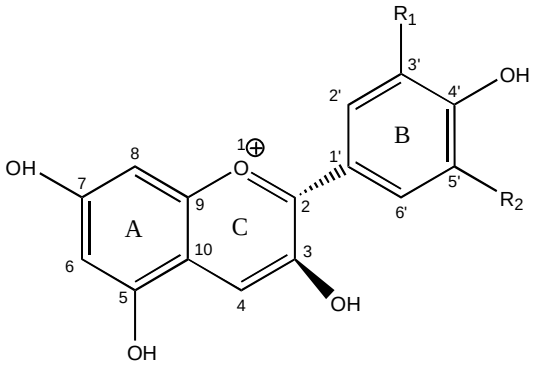
Anthocyanins: bioavailability and molecular activities

2.1 Introduction

The anthocyanins (from the Greek *anthos* meaning flower and *kyanos* meaning blue) are water-soluble pigments responsible for the red, blue and violet colour of fruits, vegetables and flowers (Mazza *et al.,* 2004; He *et al.,* 2010). They belong to a large group of compounds known as flavonoids, which in turn are a class of polyphenols (McGhie *et al.,* 2007). Anthocyanins are found in nature as glycosides of the flavylium salt (2- phenylbenzopyryl), and differ from each other through the many structural variations, such as the number of hydroxyl groups, their degree of methylation, the number and nature of the sugar moiety attached to the molecule, the location, the nature and number of aliphatic or aromatic acids attached to sugar (Faria *et al.,* 2014; Ramawat, *et al.,* 2013)**.**

2.2 Chemistry of anthocyanins

Anthocyanins are present as glycosides of their respective aglycones, anthocyanidins. These consist of an aromatic ring (A) bound to a heterocyclic ring containing an oxygen atom (C) which in turn is bound by a carbon-carbon bond to another aromatic ring (B). So far 17 anthocyanidins have been identified, but only 7 of these are widely distributed in nature: cyanidin (Cya), delphinidin (Del), malvidin (Mal), pelagonidin (Pel), peonidin (Peo) and petunidin (Pet) (**Fig. 11**). Although the anthocyanidins are only 17, the anthocyanins present in the plant kingdom are more than 600. The most common carbohydrates normally bound to the aglycon, in the form of mono, di and trisaccharides, are: glucose (Glu), galactose (Gal), arabinose (Ara), rhamnose (Rham), rutinose (Rut) and xylose (Xyl). The glycosylation, which give greater stability and higher water solubility, occurs mainly on the C3 of the C ring and on C5 and C7 of the A ring, in fact the 3-glucosidic derivatives are 2.5 times more frequent than the other derivatives, and among these prevails the cyanidin 3-O-glucoside (C3G) (Ignat *et al.,* 2011; Wu *et al.,* 2006; Fang 2014; Castaneda-Ovando, *et al.,* 2009).



	R_1	**R_2**
Pelargonidin (Pel)	H	H
Cyanidin (Cya)	OH	H
Delphinidin (Del)	OH	OH
Peonidin (Peo)	OCH_3	H
Petunidin (Pet)	OCH_3	OH
Malvidin (Mal)	OCH_3	OCH_3

Fig. 11: Chemical structure of the anthocyanins.

Anthocyanins are highly unstable compounds and very susceptible to degradation due to various factors such as: temperature, light, pH, oxygen, and enzymes (Fernandez *et al.,* 2014) Their colour can deeply change depending on the substituents present on the B ring. In general the colour saturation increases with the increase of the number of the hydroxyl groups and decreases with the addition of methyl groups (Tsuda *et al.,* 2012). In aqueous solutions the anthocyanins undergo structural changes in response to the change in pH in 4 molecular structures: quinoidal base (blue), flavylium cation (red), carbinol (decoloured), and chalcone (yellowish). In general, anthocyanins are stable in acidic solutions (pH 1-3) where they exist as flavylium cation. At a pH higher than 4 they instead adopt the forms of carbinol and chalcone, and additionally the latter may undergo further modifications producing phenolic acids (Fang 2014) (**Fig. 12**).

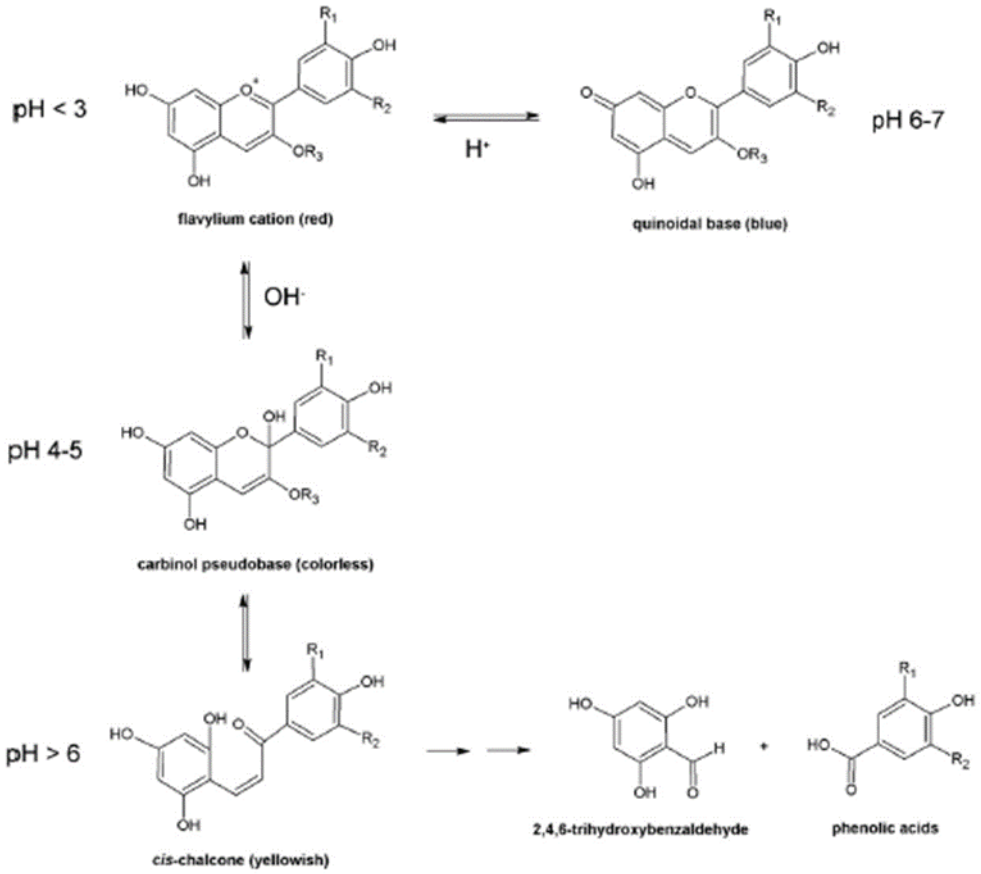


Fig. 12: **pH-dependent anthocyanins structure modification** (from Fang 2014).

2.3 Sources of anthocyanins

Anthocyanins are widely present in the Mediterranean diet, as they are abundantly found in a broad variety of fruits and vegetables. They are especially abundant in dark-coloured fruit such as wild berries, cherries, peaches, grapes, apples, plums, but also in vegetables such as red onions, red turnips, aubergines, red cabbage, legumes (black beans) (Wu *et al.*, 2006; Koponen *et al.*, 2007). In addition to the fruits and vegetables mentioned above, some cereals (black rice, red sorghum, purple corn), tubers, flowers, leaves and red wine are rich in anthocyanins (Williams *et al.*, 2004). The relative abundance and composition of anthocyanins may vary due to intrinsic and extrinsic factors, such as genetics, agronomic variation, light intensity, temperature, harvest time and conservation (Guo *et al.*, 2015). For example, in red grapes (*Vitis vinifera*), depending on the harvest period, the anthocyanins content varies significantly with values reaching the maximum peak at 1.87mg/g of fresh grapes, while in red wine their concentration varies from

411 to 728 mg/L, this depends not only on the degree of maturity of the grape but also on the vinification, fermentation and concentration of the sugar (Bindon *et al.*, 2013).

The daily consumption of anthocyanins varies widely in different regions, seasons and individuals with different socio-cultural backgrounds, in fact their daily intake has been estimated to have a wide range from some milligrams to thousands of milligrams (Wu *et al.,* 2006). In a study conducted in the USA on adults by Wu and colleagues (2006) it was estimated that the daily intake in the USA was 12.5 mg/d, while according to the European Prospective Investigation into Cancer and Nutrition (EPIC) study, conducted in 10 European countries, the average daily intake for each country was between 19.8 and 64.9 mg/d in the male population, while in women the range was between 18.4 and 44.1 mg (Zamora-Ros *et al.*, 2011). This study also shows that the country with the highest consumption of these pigments is Italy, this is most likely due to the Mediterranean diet, rich in red/blue fruits and vegetables and wine. Indeed, according to Wu and colleagues, a glass of red wine is able to provide about 20-35 mg of anthocyanins (Wu 2006). In China the average is 27.6 mg/d, very similar to that in some northern European countries (Germany 35.1 mg/d, England 26.1 mg/d, Denmark 28.2 mg/d, Netherlands 21.4 mg/d). In the Fiji Islands, on other hand, where the diet is mainly made up of animal sources, anthocyanin consumption is equal to 0.04 mg/d (Lako *et al.*, 2006).

2.4 Bioavailability

The bioavailability is the percentage of active ingredients or therapeutic portions contained in the drugs that are absorbed and therefore becomes available at the action site (Jakobek, 2015). The part of the polyphenolic compounds which is released from the food matrix, following digestion, is defined as bio-accessible and is therefore potentially available to be absorbed (Bohn, 2014).

Pharmacokinetic studies of anthocyanins have shown that these substances have a very low bioavailability. In fact, if compared to the non-ingested and undigested dose the amount of total anthocyanins absorbed and excreted through the urine is very low (McGhie and Walton, 2007). However, on the other hand, recent studies

conducted on men to evaluate bioavailability, using ^{13}C3G, have reported a huge metabolism and an absorption of 12.4% (Czank *et al.*, 2013), suggesting thus that their bioavailability could be much higher of what was said previously thanks to the identification of some metabolites. For this reason it is fundamental to have a deeper and deeper knowledge of the fate of anthocyanins following their intake, studying their processes of absorption, distribution, metabolism and excretion (ADME)

Following the intake of anthocyanins through diet or food supplements, they are rapidly absorbed, extensively metabolized and eliminated through urine and faeces. Metabolism includes both phase 1 (oxidation, reduction, and hydrolysis) as well phase 2 (conjugation reactions such as methylation, glucuronidation, sulfation, etc.) (Lila *et al.*, 2016; Hribar *et al.*, 2014). In addition, before the conjugation reactions, the aglycon can be degraded into phenolic acids and aldehydes by the enzymes present in the epithelial cells and in the intestinal lumen and by the microbiota. (**Fig.13**) All ADME processes will be then described during the various stages of digestion.

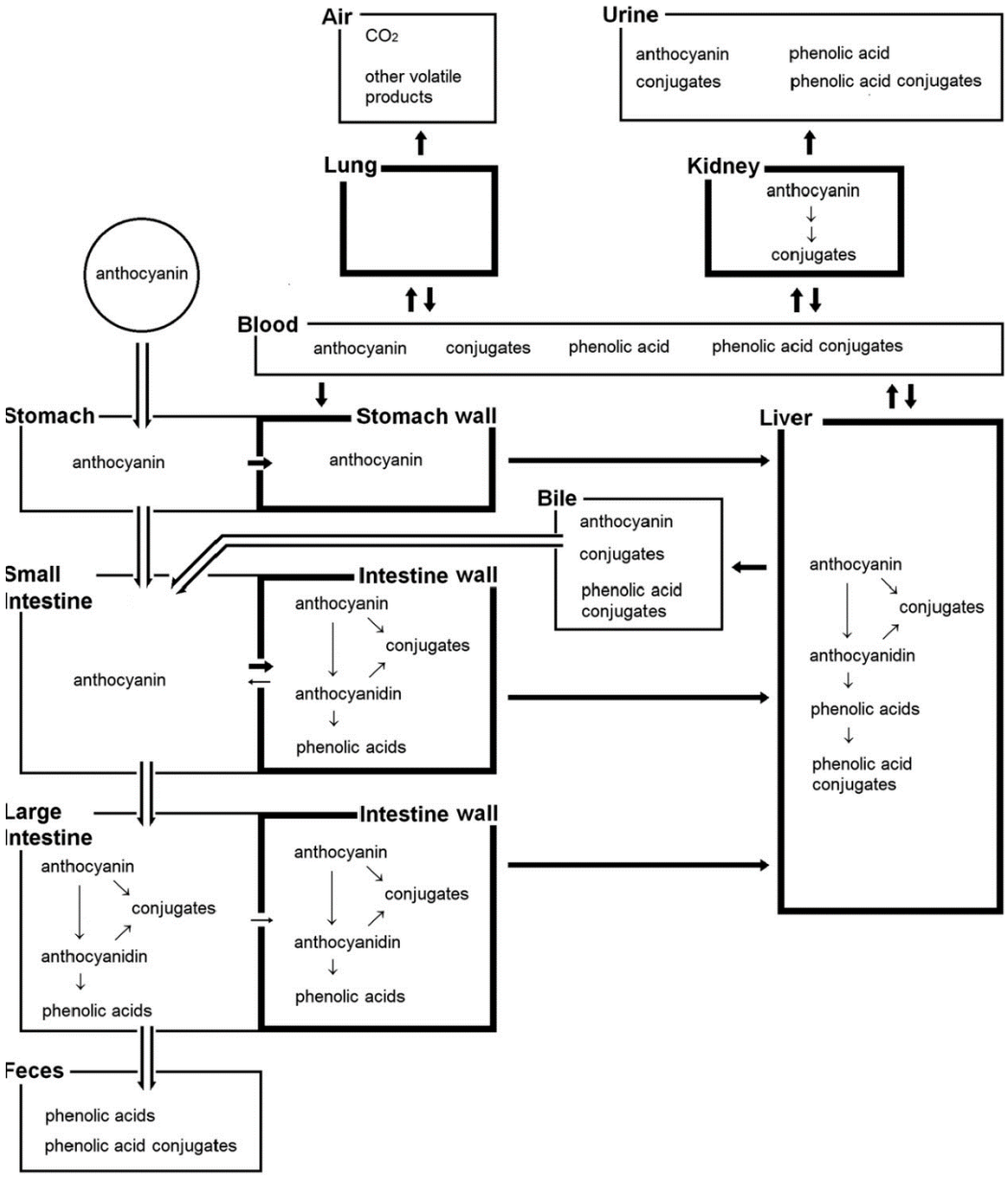


Fig. 13: Schematic representation of the ADME processes of anthocyanins (Fang 2014)

The physiological process of digestion can be divided into 4 phases: oral digestion, gastric digestion, intestinal digestion (small intestine) and fermentation (colon).
The first step of the digestion of anthocyanins passes through the oral cavity where the food matrix containing the anthocyanins is ground and mixed with saliva and other food components. The saliva has a pH 7 and contains the enzyme salivary amylase responsible for the breaking of starches into simple carbohydrates. The neutral pH of the oral cavity causes the formation of a hemiketal and consequently

the opening of the C ring (Olivas-Aguirre *et al.,* 2016). In this step the composition of the food, the rheology, the structure are essential for the formation of the food bolus (Chen *et al.,* 2014). The anthocyanins can also be metabolized during the oral phase; in fact it has been shown that they are deglycosylated by salivary β-glycosylases in protocatechuic acid (PCA) and in cyanidin glucuronide (Mallery *et al.*, 2011). However, oral metabolism is closely related to the time of exposure to oral enzymes (Kamonpatana *et al.*, 2012).

Subsequently the oral bolus formed arrives in the stomach where the acidic pH (1-2) provides excellent conditions for the stability of the anthocyanins allowing them to exist in the form of glycoside (Kop-Bolanz *et al.,* 2014; Oliveira *et al.,* 2015). The presence of glycosides in the bloodstream after a few minutes from the intake suggests that anthocyanins can be absorbed intact also at the stomach level. (Fang 2014; Hribar *et al.,* 2014) Since anthocyanins are hydrophilic substances, they cannot pass the cell membrane through passive transport but need a transport system. It is suggested that a membrane carrier called bilitranslocase may be involved in this transport mechanism, as well as in the gastric mucosa it is also expressed in the vascular endothelium, kidneys and liver. (Fang 2014; Passamonti *et al.,* 2002). It has also been suggested that glucose transporters (GLUT1 and GLUT 3), and the monocarboxylate transporter 1 (MCT1) may also be involved in the gastric absorption of anthocyanins (Oliveira *et al.,* 2015; Del Bo '*et al.*, 2012). The transport mechanism within gastric epithelial cells appears to be depend on the conformation of the B ring and the sugar moiety (Zou *et al.*, 2014)

The next step of digestion takes place at the level of the small intestine where, contrary to what happens in the stomach, the mild alkaline environment and the presence of various enzymes cause a loss of bioavailability of about 40-60%. (Olivas- Aguirre *et al.,* 2016). The small intestine represents their main site of absorption they can be transported inside the enterocytes both through active and passive transport (**Fig. 14**). In the first case, the anthocyanins are transported intact in the form of glycosides by the glucose sodium dependent transport systems (SGLUT-1) and GLUT2. However, if the concentrations are high (above 40 μM) a saturation effect can be observed. (Lila *et al.,* 2016; Hribar *et al.,* 2014; Zou *et al.,* 2014). Other studies have shown, instead, that the absorption of anthocyanins does not depend only on SGLUT-1 but also on a transport system for flavonoid

molecules, as demonstrated by the inhibition of C3G absorption by quercitin-3-glucoside (Walton *et al.*, 2015). Active transport is the main mechanism of absorption of anthocyanins in the intestine. This is confirmed by the numerous studies on Caco2 as *in vitro* intestinal absorption models, which have shown that anthocyanins are widely transported intact inside the enterocytes (Kamiloglu *et al.*, 2015). Whilst in the second case the anthocyanins are initially deglycosylated by the lactase-floridizin hydrolase, at the level of the brush border of the intestinal epithelial cells, with consequent production of lipophilic aglycones which can therefore penetrate the epithelial cells through passive transport. Once inside the epithelial cells the aglycones can either pass through the basolateral membrane to reach the bloodstream or firstly be metabolized and then cross the basolateral membrane (Fang 2014)

However, the high concentration of anthocyanins within the intestinal tissue is in contrast with the low bioavailability reported in the literature, thus suggesting that these molecules may undergo a vast first passage metabolism (Fang 2014). Inside the enterocytes, anthocyanins are transformed into different phenolic acids and conjugated by phase 1 and 2 enzymes. In general, the microbiota and entero-hepatic metabolism are responsible for phase 1 metabolism while other enzymes, such as catechol O-methyltransferase (COMT), phenyl sulfotransferase (PST), are responsible for the biotransformation in more hydrophilic substances that facilitate their elimination via the kidney (Fang 2014; Felgines *et al.*, 2010; Felgines *et al.*, 2006).

The most important anthocyanin degradation products are PCA and PGA (Xie et al, 2016; Miyazawa *et al.*, 1999). The PGA derives from the cleavage of the ring A, since it is a lipophilic molecule it can be absorbed through a passive mechanism reaching thus the plasma within the first two hours of intake (Olivas-Aguirre *et al.*, 2016). On the contrary the PCA deriving from the opening of the ring C undergoes various modifications by first phase enzymes obtaining different metabolites, such as for example hippuric acid, vanillic acid, which in turn are further metabolized by the phase 2 enzymes (de Ferrars *et al.*, 2014)

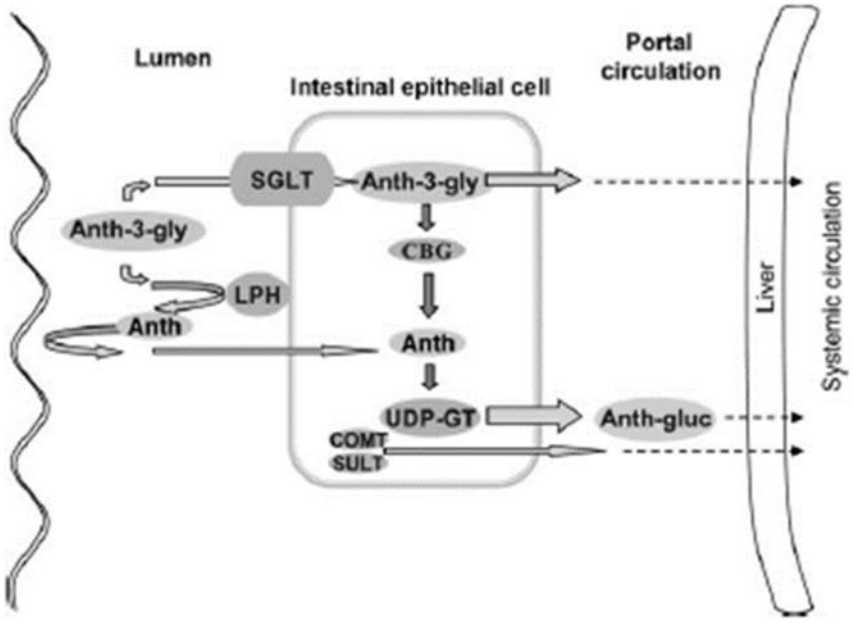


Fig. 14: Mechanism of intestinal absorption of anthocyanins. The anthocyanins are mainly absorbed as intact glycoside by the SGLT and, once inside the cell, they can either directly cross the basolateral membrane into the portal circulation, or be hydrolysed by cytosolic β-glucosidase, metabolized and transported as glucuronide derivates into the systemic circulation. Further the anthocyanin glycosides could be hydrolysed at the mucosal brush-border membrane by lactate phlorizin hydrolase and then be absorbed into the IECs, here they will follow one of the routes mentioned above. Abbreviations; SGLT, sodium–glucose co-transporter; Anth-3-gly, anthocyanin 3-glycoside; CBG, cytosolic b-glucosidase; LPH, lactate phlorizin hydrolase; UDP-GT, UDP-glucuronosyltransferase; Anth-gluc, anthocyanin glucuronide; COMT, catechol-O-methyltransferase; SULT, sulfotransferase. (from Kay 2006)

The anthocyanins not absorbed at the level of the small intestine reach the last section of the digestive tract, the colon, where they further undergo into processes of deglycosylation, metabolism phase 1 and 2. The non-absorbed and metabolized anthocyanins are excreted in the faeces, and it has been shown that less than 0.005% of the ingested dose is excreted intact (de Ferrars *et al.,* 2014). Also in this case, the slightly basic pH favours the formation of the chalcone and the opening of the rings A and C with consequent formation of further phenolic acids. Furthermore, the presence of the microbiota deeply influences the bioavailability of anthocyanins. In fact, in the colon they are exposed to more than 300-500 different bacterial species, among which the *Bifidobacterium*, *Bacteroides*,

Eubacterium and *Clostridium* stand out the most. The bacterial flora releases many deglycosylation enzymes which split the sugar portion and release the aglycon which in turn produces different phenol acids such as protocathecuic acid, vanilla acid, ferulic acid or aldehydes (Hribar *et al.*, 2014; Faria *et al.*, 2014).

Moreover, Hanske and colleagues, in an *in vivo* study conducted on germ free mice and not, demonstrated how the products of metabolism of both the phase 1 and 2 were excreted 2-3 times more in mice presenting the bacterial flora compared to germ free mice (Hanske *et al.*, 2013)

In conclusion, therefore, the amount of anthocyanins decreases during the passage in the gastrointestinal tract, while the quantity of phenolic acids increases. These can be absorbed in the intestine by epithelial monocarboxylic acid transporters and then further metabolized in the kidneys or liver (Faria *et al.*, 2014; de Ferrars *et al.*, 2014).

2.4.1 Systemic bioavailability

Pharmacokinetic studies have suggested that the anthocyanins and their conjugated metabolites reach the bloodstream very quickly, with a Cmax ranging from to 596 nM after about 2 h, while the urinary concentration is between 0.03 and 4% of the ingested dose with a time of half-life of 1-3 h. (Czank *et al.*, 2013). In addition to anthocyanins and their phase 2 metabolites, have also been reported elevated plasma levels of degradation products of anthocyanins, in particular PCA. de Ferrars and colleagues studied the pharmacokinetic profile of C3G, isotopically marked on ring A and B, administered daily to 8 male subjects. The reported data showed several phenolic acids and their phase 1 and 2 metabolites in the bloodstream. Phenolic acids exhibited a very different pharmacokinetic profile compared to their origin products; in fact, while the anthocyanins reached Cmax (141nM) after about 2h and were eliminated after 6h, the metabolite peaks appeared with a concentration range between 0.2 and 2 µM, after 10 h and remained in circulation up to 48 h after intake. In addition to this, the metabolites showed a biphasic profile with an initial and slight appearance between 1 and 5 h from intake and a second more evident peak between 6 and 48 h later, suggesting thus an extensive metabolism at the level of the colon (de Ferrars *et al.*, 2014).

Finally, their bioavailability depends significantly on the chemical structure, indeed it can be influenced by the structure of the aglycon and the sugar portion. For example, in both human and rats, the urinary concentration of anthocyanins derived from delphinidin is much lower than those based on malvidin. This is due to the difference in hydrophobia, in fact malvidin has fewer free hydroxyl groups and therefore is less hydrophilic and can be easily transported into tissues. (McGhie *et al.,* 2003). These data are also supported by perfusion *in situ* on rat study in which the amount of malvidin 3-O-glucoside and cyanidin 3-O-glucoside was 10.7% and 22.4% respectively. (Talavèra *et al.,* 2004). In addition, the sugar portion also has a great influence on bioavailability: for example galactose is more absorbed than arabinose, with a consequent greater plasma concentration for galactose (McGhie *et al.,* 2003). At the intestinal level the anthocyanin glycosides are extensively hydrolysed by the microbiota in about 20 – 120 min depending on the carbohydrate structure (Keppler and Humpf 2005).

2.4.2 Effect of food matrix on the bioavailability

The beneficial effect and the bioavailability of the phenolic compounds do not depend exclusively on food sources but also, as previously mentioned, by their stability which in turn may depend on the raw material processing method, the matrix in which it is incorporated, and endogenous factors such as digestive enzymes and intestinal flora.

The heat treatment of foods, such as pasteurization, ensures greater microbiological stability and durability of food, but at the same time causes a degradation of the bioactive compounds present, such as polyphenols, vitamins etc (Marszalek *et al.,* 2015). Furthermore, the composition of the matrix and the components of other foods significantly influence the bio-accessibility, absorption and metabolism of anthocyanins. In the food matrix, generally, the anthocyanins are mixed with other macromolecules such as proteins, carbohydrates and lipids. Polyphenols, with large numbers of hydroxyl groups, have a great affinity with proteins, forming complexes that tend to reduce their absorption (Bohn, 2014). Some studies have shown that the interaction between polyphenols and milk proteins, in particular casein, is able to reduce the antioxidant capacity of coffee,

tea and chocolate. (Serafini *et al.,* 2003). However, other studies disagree with this by showing that the addition of milk proteins does not change the polyphenolic concentration in plasma (Keogh *et al.,* 2007). Probably the inhibitory effect of milk on the absorption of anthocyanins is linked to the concentration of these molecules. Milk proteins reduce absorption of polyphenols when there is a low concentration instead it seems to have no impact in the presence of a high concentration (Jakobek *et al.,* 2015). High molecular weight polyphenols are generally linked with covalent bonds to the fibres and their bioavailability depends on the release from this complex, which in turn depends on the structure of the polyphenols, the complexity of the carbohydrate-polyphenol structure and whether or not the enzyme can reach the carbohydrate (Jakobek *et al.,* 2015). According to Ortega and colleagues in a study of an *in vitro* digestion model, soluble dietary fibres increase the stability of polyphenolic compounds during the duodenal phase, since the fibres form an entrapment matrix that prevents the enzymes to reach polyphenols contained in them (Palafox-Carlos *et al.,* 2011). Moreover, thanks to their antioxidant power, polyphenols help protect the intestinal lumen from oxidative stress and therefore carbohydrates and polyphenols that have reached the colon can have beneficial effects in the growth of bacterial flora (Mrduljas *et al.,* 2017)

2.5 Biological activity of anthocyanins

Despite their low bioavailability, anthocyanins have shown to play an important role in the prevention and in many diseases, such as diabetes, cardiovascular and immune system diseases, cancer through anti-proliferative, antioxidant, anti-inflammatory, anti-angiogenic activities (Domitrovic *et al.,* 2011).

2.5.1 Anti-inflammatory activity

Inflammation is a complex biological response to physical and chemical agents, infections or injuries and is often associated with the initiation, development and progression of tumours (Germolec *et al.,* 2010). *In vitro* studies have shown that anthocyanidins possess anti-inflammatory activity through the regulation of different inflammatory modulators, such as NF-κB, MAPK, COX-2 in murine macrophages activated with LPS or the inhibition of gene and protein expression of inducible nitric oxide (iNOS) in activated J774s (Hou *et al.,* 2005; Hämäläinen *et al.,* 2007). Furthermore, the treatment of LPS-activated macrophages with anthocyanin extract was able to down-regulate the expression of pro-inflammatory cytokines IL-1β and TNF-α (Lee *et al.,* 2014).

One of the principal enzymes involved in the progression of the inflammatory process is NF-κB. NF-κB is an oxidative stress sensitive nuclear transcription factor and it is ubiquitously expressed in cells. It is mainly activated by pro-inflammatory cytokines, such as TNF-α, IL-6 IL-1 and IFN-γ, hyperglycaemia, ultraviolet radiation, oxidative stress. Once activated NF- κB in turn up-regulates the expression of proinflammatory cytokines, chemokines, adhesion molecules, the inducible form of the enzyme nitric oxide synthase, Cyclooxygenase (COX)-2 (Heininger *et al.,* 2000). Numerous studies have reported that anthocyanins can modulate the NF-κB pathway at different levels. For example black soya bean extract showed a dose dependent inhibition of p65 nuclear translocation in human gastric epithelial cells infected with *H. pylori*. and in human keratinocytes (HaCaT) exposed to UV-B (Kim *et al.,* 2013; Tsoyi *et al.,* 2008). Similarly, C3G extracted from red rice has also been shown to inhibit NF-κB pathway in activated LPS macrophages (Min *et al.,* 2010). Delphinidin attenuates the activation of NF- κB in mouse epidermal cells exposed to UV-B rays and in murine epidermal cells exposed to TNF-α. (Kwon *et al.,* 2009; Hwang *et al.,* 2009). Moreover C3G is able to inhibit the activation of IKK and therefore the nuclear translocation of p65 in Caco2, exposed for 6h to TNF-α (Ferrari *et al.,* 2016). Further the same work group reported the ability of anthocyanins to attenuate PA-induced inflammation in murine adipocytes by inhibiting the NF-κB pathway (Muscarà *et al.,* 2019).

COX are enzymes responsible for converting arachidonic acid into prostaglandins. Antioxidants inhibit COX-2 gene expression by interfering with different cell signalling pathways that modulate its expression, among these the NF- κB, the CCAAT binding protein (C / EBP), the activator protein 1 (AP-1) and the binding protein- CRE (CREB) pathways. C/EBP regulates the production of COX-2, while AP-1 and CREB are essential for both basal and induced transcription of COX-2 (Hou *et al.,* 2005). Anthocyanins have been shown to possess several *in vitro* degrees of inhibition against COX-1 and COX-2 (Wang *et al.,* 1997; Seeram *et al.,* 2001; Seeram *et al.,* 2003; Hou *et al.,* 2004; Munoz-Espada *et al.,* 2006). The inhibitory effect of anthocyanidins on COX activity is influenced by the number and position of hydroxyl groups depending mainly on the free hydroxyl groups. The presence of hydroxyl groups in position 3'and 4' on the ring B confers to cyanidin a more effective inhibitory power against COX, while the trihydroxy substitution in 3,4',5' greatly reduces the inhibitory capacity of delphinidin. However, in another *in vitro* study on macrophages activated with LPS, delphinidin was shown to be a more effective inhibitor of cyanidin, whereas pelargonidin, peonidin and malvidin did not show any activity (Hou *et al.,* 2004).

2.5.2 Antioxidant activity

Oxidative stress is a pathological condition in which there is an imbalance of redox homeostasis, the quantity of ROS (reactive oxygen species) and RNS (reactive nitrogen species) greatly exceeds the amount of endogenous antioxidants, leading to an oxidation of lipids, DNA and proteins. Oxidative stress is considered to be one of the main factors able to induce diseases, such as cardiovascular, cancer, cellular aging (Dai and Mumper 2010). Antioxidants can inhibit or delay the oxidizing action by: a) decreasing the local concentration of oxygen, scavenging the ROS / RNS, b) binding to metal ions in order to prevent the formation of radical species (hydroxyl radicals, ferryl), c) converting peroxides into non-radical products, like alcohols, d) scavenging intermediate radical species preventing the continuous abstraction of hydrogen (Dai and Mumper, 2010; Miguel, 2010). Like the anti-inflammatory activity also the antioxidant one depends on anthocyanins chemical structure and not all of them possess the same activity in scavenging the

ROS and RNS species. In fact the orientation of the ring determines which hydrogen atom can be donated to the free radical and the capacity of the anthocyanins to support the unpaired electron (Kay 2004). Furthermore, the effectiveness of scavenge the different ROS species differs from one anthocyanin to another; for example delphinidin is the most active against superoxide anion followed by cyanidin and pelargonidin while the last one is the most active against hydroxyl radicals (Tsuda *et al.,* 1996; Antal *et al.,* 2003). In general the antioxidant activity is related to the number of free hydroxyl groups around the pyranic ring, higher is the number higher is the activity. For example, anthocyanins with di-hydroxyl groups in position 3'-4' can quickly chelate metal ions to form anthocyanin-stable metal complexes. (Sarma *et al.,* 1997). In acidic environment (pH 2-4) the anthocyanins are found in the form of flavylium cation and are very susceptible to nucleophilic attacks in position 2 and 4, therefore the hydroxylation in these positions increase their chelating capacity. While anthocyanins with hydroxyl groups in ortho position have a high activity against hydroxyl radicals, chelating iron and consequently inhibiting the generation of HO (Bakowska-Barczak 2005; Noda *et al.,* 2000). Further, besides the position of the hydroxyl groups on the ring B also the degree and position of methoxy groups influences the stability and therefore the activity of such molecules; for example malvidin-3-glucoside and petunidin-3-glucoside showed a lower activity than the cyanidin-3-rutinoside and delphinin-3-glucoside (Muselik *et al.,* 2007).

The number and position of the sugar portion can influence the antioxidant capacity of anthocyanins. For example, the number of glycosyl group on C3 is inversely proportional to the activity. In fact, some *in vitro* studies have reported that delphinidin- and cyanidin- 3-rutinoside are less active than their respective monoglucosides (Muselik *et al.,* 2007). Finally, glycosylation also appears to induce a decrease of antioxidant capacity by reducing free hydroxyl groups and chelation sites (Wang and Stoner 2008).

The presence of acyl groups seems to have an influence on the antioxidant activity, however also in this case the activity is dependent on the type of free radical. In fact, some authors have shown that the pyranoanthochyanin of cyanidin, malvidin, petunidin and pelargonidin have a greater ability to scavenge the superoxide anion,

but they are not active against hydroxyl radicals. (García-Alonso *et al.,* 2005), However Muselik and coll. (2007) have shown how the addition of pyruvic acid to delphinidin and malvidin 3-monoglucoside causes a decrease in antioxidant activity in aqueous phase assays.

The possible mechanism by which anthocyanins act includes direct and indirect pathways. In the first case the activity is due to their ability of the flavonoid to donate electrons (Fukumoto and Mazza 2000; Borkowski *et al.,* 2005) which can bind ROS (superoxide, singlet of oxygen, peroxide of hydrogen and hydroxyl radicals). While in the second case they act through different mechanisms such as repair or increase of superoxide dismutase (SOD) activity and glutathione peroxidase, reducing the formation of endogenous ROS through the inhibition of NADPH oxidase and xanthine oxidase or through the modification of mitochondrial respiration and arachidonic metabolism (Steffen *et al.,* 2008).

The activation of these enzymes is modulated by the erythroid nuclear transcription factor -2 (Nrf-2), expressed mainly at the level of the liver and kidneys, organs involved in the detoxification processes, and at the level of the gastrointestinal tract, skin, lungs (most organs exposed with external agents) (Speciale *et al.,* 2013). (**Fig. 15**) Numerous studies have suggested that the intake of flavonoids may induce an upregulation of the nuclear transcription factor Nrf2. It has been shown that C3G treatment is able to activate an adaptive response of Nrf2 in HUVEC cells exposed to TNF-α by counteracting the inflammatory process including the activation of the NF-κB pathway and H_2O_2 accumulation (Speciale *et al*., 2010). Furthermore, the same working group demonstrated that the pre-treatment with C3G in HUVEC exposed to TNF-α was able to activate the Nrf2 pathway, through the activation of the MAPKs pathway (ERK1/2) (Speciale *et al.,* 2013). In line with this, Fratantonio *et al*. (2015) demonstrated a reduction in the oxidative stress induced by palmitic acid in HUVEC through the activation of Nrf2 and consequently of antioxidant and cytoprotective genes (Fratantonio *et al.,* 2015). In addition, the effect of anthocyanins on the Nrf2 pathway has been further studied also on intestinal epithelial cells. Ferrari *et al*. (2016) evaluated the transcriptional activity of Nrf2 on Caco2 exposed to TNF-α, demonstrating elevated nuclear levels of Nrf2 and an up-regulation of H0-1 and NQO-1. All these data confirm the role of Nrf2 in a cellular adaptation response induced by anthocyanins.

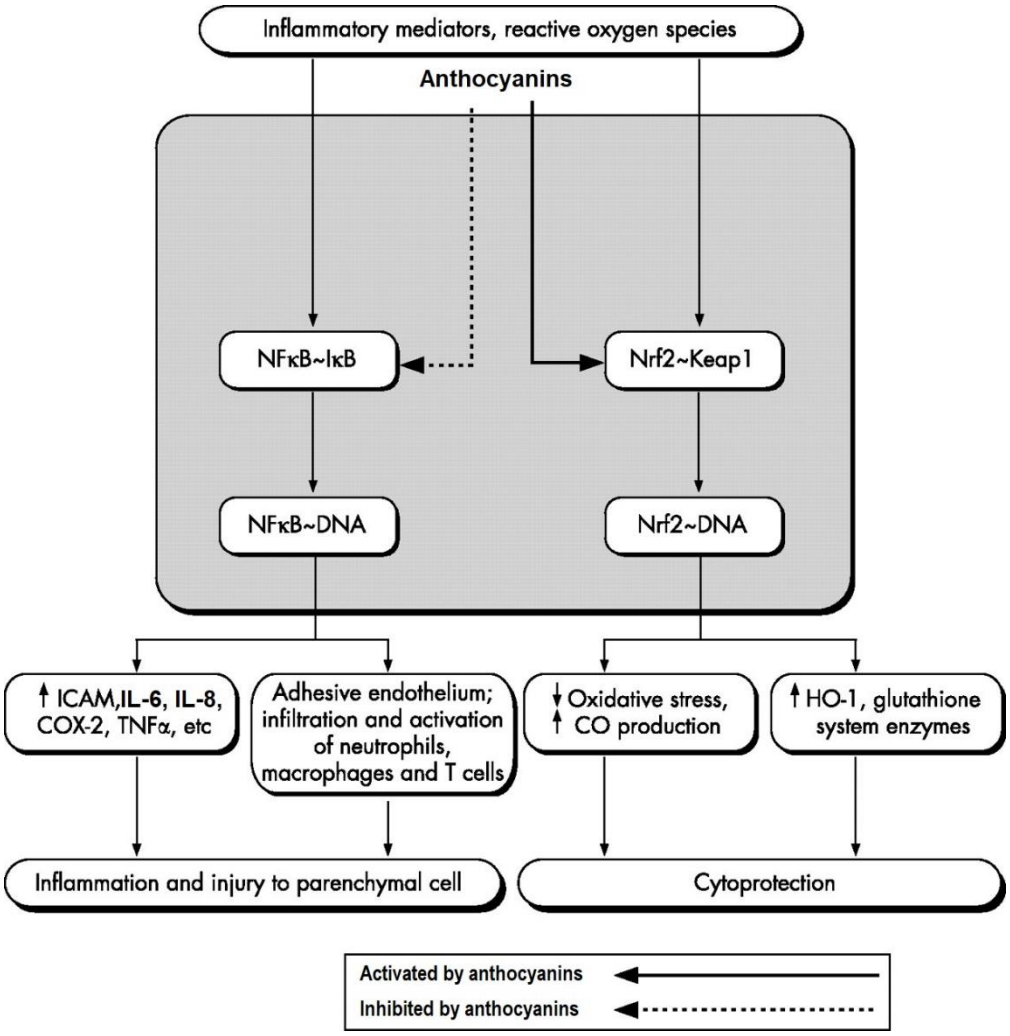


Fig. 15: Anthocyanins attenuate inflammation down-regulating inflammatory genes and up-regulating cytoprotective ones. COX-2, cyclooxygenase-2; HO-1, heme-oxygenase-1; IkB, inhibitor of kB; ICAM, intercellular adhesion molecule; iNOS, inducible nitric oxide synthase; NFkB, nuclear factor k B; Nrf2, nuclear factor E2-related factor; TNFα, tumour necrosis factor-α. (modified from Shapiro et al., 2007)

2.6 Conclusions

In conclusion the literature data showed the ability of the anthocyanins to interfere with different molecular pathways, preventing different pathological conditions. Regarding the gut barrier, epidemiological studies have reported that daily intake of anthocyanins, through the diet or food supplement, reduce the risk to develop IBD (Tian *et al.*, 2017). Additionally, it has been reported their ability to positively modulate the microbiota improving the intestinal permeability (Kaulmann *et al.*, 2016). However, the molecular mechanisms underlying this effect are still not fully established, so further and extensive studies are needed to completely understand their role in IBD. Moreover, it worthy to be aware that the majority of literature data reporting the multiple pharmacological activity of the anthocyanins were carried out on *in vitro* and *in vivo* experiments, using often not realistic and physiologic concentration of anthocyanins. So it must be taken in consideration the extensive metabolism of anthocyanins and their low bioavailability. Hence, more *in vivo* and *in vivo* studies investigating the effect of anthocyanins and their metabolites on the gene and protein expressions are necessary to fully comprehend the role of these compounds in IBD prevention.

Part 2: Experimental

INTRODUCTION

Inflammatory bowel diseases (IBD) are a group of multifactorial pathologies, including Crohn disease and ulcerative colitis, that affect the gastrointestinal tract. They are characterized by intermittent phases of acute inflammation and remission (Melgar *et al.,* 2010). The chronic inflammation on IBD leads to a massive infiltration of granulocytes and macrophages, which cause an high production of pro-inflammatory mediators and ROS with consequently aberrant immune system response and alteration of redox homeostasis (Biasi *et al.,* 2013) In the last decade there has been a worldwide increase in incidence and prevalence of IBD especially in the industrialized and developing countries. In fact, it was reported more than 2 million people in Europe and 1.5 million in USA suffer from IBD and more than 100 thousand new cases are reported in developing countries (Molodecky *et al* 2011).

Nonetheless, in the last period, the research has made great discovery, even if the etiopathogenesis is still in part unknow. However, it is supposed that is IBD is due to an interaction of three different element: genetic susceptibility, immune system and environment factors (Kucharzik *et al.,* 2006).

Interestingly it has been reported a parallelism between the increase of the obesity and IBD (Steed *et al.,* 2009). In line with this cohort studies, it has been demonstrated that 18% of patient with IBD are obese and 38% are overweight. These evidences taken together with the fact that the highest incidence of IBD is reported in western and in developing countries (Molodecky *et al* 2011), which are adopting a westernized lifestyle and diet, suggest that the environment factors plays a key role in the initiation and progression of IBD. In particular diet has a huge impact on IBD pathogenesis. Indeed a westernized diet rich in processed meat, sugary beverage, lipid, and poor in dietary fiber is associated to a low grade of inflammation, condition typically of obesity, diabetes and metabolic disorder (Cani *et al.,*2007; Wellen *et al.,*2005). In line with this, several epidemiological studies showed that an increased risk in developing IBD is correlated with a high consumption of processed food and dietary fat especially n–6 polyunsaturated acid and saturated acid (e.g. palmitic acid, PA) (de Silva *et al.,* 2014; Wiese *et al.,* 2016). *In vitro* studies evidenced that saturated fatty acids, in particular PA, induce the proteasomal degradation of IκBα and thereby activate the nuclear

transcriptional factor NF-κB, which in turn up-regulate several pro-inflammatory mediators (Lyons *et al.*, 2016; Lee *et al.,* 2003). Similarly other saturated fatty acid (such as lauric acid) induce the activation of TRL4, increasing thus the expression of NF-κB, and disrupt the composition of the commensal microbiota increasing thus the risk of the colonization of pathogens (Moreira *et al.,* 2012). Additionally, a high intake of lipid can increase the plasmatic levels of free fatty acids (FFA) and thereby, through the β-oxidation, induce an overproduction of ROS. The overexpression of ROS leads to an oxidative stress that in turn activate the NF-κB, and others proinflammatory cytokines inducing so an inflammatory response (Tian *et al.,* 2017).

It was also reported that an oxidative stress can induce an impairment of the intestinal barrier, through a damage on the cytoskeleton of protein regulating the tight junctions, affecting the responsiveness to the pathogenic microorganisms (Rao 2008). The intestinal barrier provides separation between lumen and the rest of the body, blocking the passage of harmful microorganism, acting thus as a first line defense against pathogens. When it is damaged, a high number of harmful microorganisms can penetrate the epithelium and to further induce and inflammatory response (Pastorelli *et al.,* 2013). In addition, the information on the molecular mechanism of FFA on IBD are few and more data needed to elucidate the role of lipids on the IBD pathogenesis.

In the last years there has been growing interest in the study of polyphenols, widely present in dietary fruits and vegetables, as preventive and alternative therapy against different disease (cardiovascular, diabetes, cancer, and chronic inflammatory disease). Particularly dietary anthocyanins, a subclass of polyphenols, have shown several benefits for the human health. Indeed different studies have demonstrated their antioxidant activity, mostly through the scavenging of free radicals (Dai and Mumper, 2010), and antiinflammatory properties, thanks to the inhibition of NF-κB pathway (Romier-Crouzet *et al.,*2009) or activation of the adaptive response Nrf2 mediated (Speciale *et al.,* 2011). *In vivo* studies have reported that a diet rich in polyphenols is associated with a lower risk of IBD (Tian *et al.,*2017). In line with this, other studies demonstrated that the anthocyanins can positively modulate the tight junctions and thus reduce the intestinal permeability, moreover they can increase the ratio of the healthy microorganism on the bowel vs unhealthy (Kaulmann *et al.,* 2016).

The aim of this work was to evaluate the in vitro beneficial effects of anthocyanins in counteracting the intestinal inflammation. At this aim we used the Caco-2 cells, human epithelial cells derived from colorectal adenocarcinoma with the unique ability to differentiate spontaneously in enterocyte like cells. Thanks to this reason Caco-2 represent a highly validate in vitro model of intestinal epithelium. Caco-2 cells were exposed to proinflammatory agents such as palmitic acid (PA) or TNF-a.

The first part of this research was focused principally on the effects of C3G and PA on:

- one of the main nuclear transcription factors involved in the inflammation, NF-κB and its downstream proinflammatory mediators (IL-6; IL-8; COX-2).
- oxidative stress and adaptive cellular response, assessing ROS levels and Nrf2 pathway.
- intestinal barrier function, evaluating the paracellular permeability.

However, the reported beneficial effect of the anthocyanins is mostly based on *in vitro* studies and on animal models and the concentration used in these studies are much higher than the normally arrives *in situ* after the ingestion with diet or food supplements. It has been in fact reported that the bioavailability of anthocyanins is very poor, ranging from 0.5-1% of the ingested part (McGhie and Walton, 2007). During the passage through the gastrointestinal tract the anthocyanins undergo to different chemical processes that produce to several phenolic metabolites (Fang 2014)

Hence knowing the fate and the stability of anthocyanins after the digestion is important for a more realistic evaluation of the beneficial effect of the anthocyanins. Therefore, with the aim to have a more realistic knowledge about the potential protective effect of anthocyanins, in the second part of this research, an *in vitro* simulated gastrointestinal digestion of a purified and standardized bilberry and blackcurrant extract rich in anthocyanins was performed. *In vitro* gastro-intestinal digestion models are very advantageous system for assessing the fate of a specific food or substance and the impact of the food matrix and food component on their stability and bio activity.

Our research focused on the change of anthocyanin composition and antioxidant activity after the simulated digestion. We further studied the *in-situ* effect of the intestinal phase of the simulated digestion on an *in vitro* model of intestinal inflammation by using differentiated Caco-2 cells exposed to TNF-α. In particular we assessed NF-κB proinflammatory pathway and the activation of an antioxidant and detoxifying response modulated by Nrf2 pathway.

3. *MATERIALS AND METHODS*

3.1 Materials

The dimethylsulfoxide sterile hybri-max (DMSO), Dulbecco's Modified Eagles Medium (DMEM), Dulbecco's phosphate-buffered saline solution (DPBS), Fetal Bovine Serum (FBS), L-Glutamine, penicillin/streptomycin solution, non-essential amino acid (MEM), tripsin-EDTA, sodium salicylate, sodium chloride, Bradford reagent, bovine serum albumin (BSA fraction V), ponceau S, Coomassie® Brillant Blue, Tween 20, dithiothreitol (DTT), leupeptin, benzamidine, aprotinin, sodium fluoride, hydrochloric acid, sodium hydroxide, igepal, ammonium persulphate, TEMED, trizma base, trizma HCl, sodium dodecylsulphate (SDS), Ethylenediaminetetraacetic acid (EDTA), ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), potassium persulphate,, sodium salt fluorescein, 2′,7′-Dichlorodihydrofluorescein diacetate (DCFH-DA), SYBR green JumpStart Taq Ready Mix and all other reagents, if not differently specified, were purchased from Sigma Aldrich (Milan, Italy).

Cyanidin-3-O-glucoside (C3G) was obtained from Polyphenols AS, Sandnes, Norway, and was of HPLC grade. The bilberry and blackcurrant extract was purchased from Medpalett AS, Sandnes, Norway.

Methanol and ethanol were obtained from Carlo Erba Reagent (Milan, Italy) in their highest commercially available purity grade.

Palmitic acid (PA) was supplied by Cheyman Chemicals.

Acrylamide/Bis-Acrylamide was bought from Fisher BioReagents.

The nitrocellulose membrane Hybond-P PVDF, the blocking agent (non-fat milk powder) and the ECL plus detection kit system were bought from Amersham Biosciences (Milan, Italy).

The primary antibodies anti-NF-κB p65, anti-Nrf2 were bought from Santa Cruz Biotechnology. The antibodies anti- pIKK α/β, anti-COX-anti-β-Actin, anti-Lamin

B and secondary antibodies HRP-labeled goat anti-rabbit Ig were all purchased from Cell Signaling Technology.

The E.Z.N.A. Total RNA Kit was bought from OMEGA bio-tek (VWR). The Quanti-IT RNA assay and the Tumor Necrosis Factor-α (TNF-α) were purchased from Invitrogen GIBCO (Milan, Italy).

Porcine α- amylase, Porcine pepsin, porcine pancreatin, porcine bile, potassium chloride, potassium monobasic phosphate, sodium bicarbonate, magnesium chloride, ammonium carbonate, Folin-Ciocalteau reagent, ammonium acetate, sodium carbonate were all purchased from Sigma Aldrich (UK)

3.2 Cell culture and treatment

3.2.1 Cell culture

Caco-2 is a human epithelial continuous cell line derived from a colorectal adenocarcinoma, developed for the first time in 1977 by Fogh at Sloan-Ketterig Institute (USA) (Fogh et al. 1977). Thanks to their peculiar and unique ability to differentiate spontaneously in enterocyte-like cells they are extensively used as a valid *in vitro* model of intestinal barrier. Indeed, although they have cancerogenic origin, after reaching confluence they undergo spontaneous differentiation, in normal culture conditions, and present all the characteristics of mature enterocytes (such as brush border, polarized morphology, and tight junctions). 5-20 days after confluence there is a gradual reduction of the cell size, a gradual formation of tight junctions between adjacent cells, and an increase of microvilli density and length (Sambuy *et al.*, 2004).

The Caco-2, obtained from the American Tissue Culture Collection (ATCC), were grown in DMEM supplemented with 10% heat-inactivated FBS, 4 mM L-glutamine, 100 U/mL penicillin/streptomycin solution, 1% not essential aminoacidic. The cells were maintained at 37°C in a humidified atmosphere with 5% CO_2, and subcultured twice a week after reaching 80% ca. of confluence. The growth medium was changed every two days. Caco-2 reach confluence 4-5 days after seeding and differentiate in 18-21 days post-confluence.

For all the experiments, in order to better reproduce the *in vivo* intestinal condition, Caco-2 were cultured on permeable filter support (ThinCerts - Greiner Bio) (**Fig. 1**). These polycarbonate filter divides each well in two different compartments, the apical (AP) side, corresponding to the intestinal lumen, and the basolateral (BL) side, corresponding to the bloodstream, recreating thus the gut environment. Furthermore, these filter support presents a high density of pores that allows the passage of ions and nutrients across the two sides of the monolayer.

To obtain the polarized monolayer, Caco-2 were seeded on polycarbonate membranes with a pore size of 0.4 μm at density of $4*10^4$ cells/insert (12 well plate) or $10*10^4$ cells/insert (6 well plates). The cells were cultured for 21 days post confluence with growth medium changes every two or three days (**Fig.1)**

All experiments were carried on fully differentiated cells.

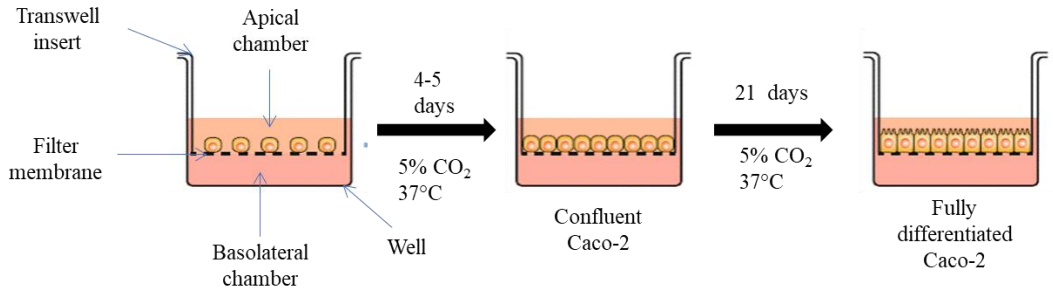


Fig. 1: Schematic model of the different stage of Caco-2 differentiation.

3.2.2 Preparation of the PA - bovine serum albumin complex

Lipid-containing media was prepared by conjugation of PA to bovine serum albumin (BSA) according to the method described by Fratantonio *et al*. (2015). Briefly, PA was dissolved in ethanol at 200 mM as stock solution and then diluted, before use, to 4 mM in medium with 10% FFA-free bovine serum albumin (BSA) at 60°C. The mixture was gently agitated to dissolve the fatty acid and pH was finally adjusted to 7.4 with 1N NaOH. The fatty acid-albumin molar ratio was kept at < 3 to ensure that the fatty acid was bound to albumin.

3.2.3 Pretreatment with C3G and exposure to PA

Fully differentiated Caco-2 were pretreated on the apical side with different concentrations of C3G (10-20 µM) and incubated at 37°C for 24h. At the end of the incubation time cells were rinsed twice with DPBS containing calcium and magnesium, in both the compartments, and exposed to PA 100 µM for 6h added on the basolateral side. PA concentration and time point were selected based on our previous screening data (**Fig. 2**)

For all the experiment C3G was freshly solubilized in DMSO. The final concentration of DMSO in the growth medium was always 0.05% (v/v). The cells treated with DMSO alone were considered as controls.

C3G concentrations and exposure time used were consistent with that employed successfully in our previous studies as well as in others (Ferrari et al., 2016; Fratantonio et al., 2015; Serra et al., 2013)

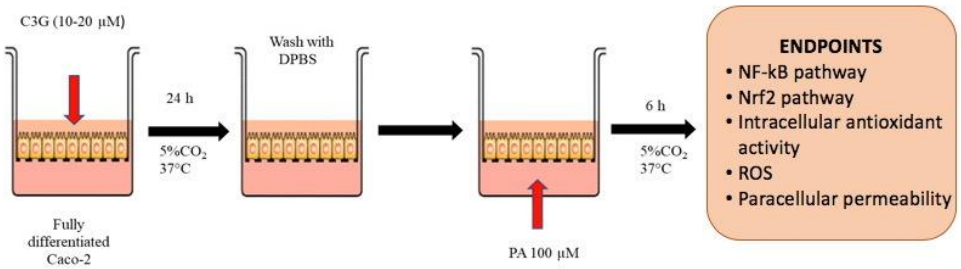


Fig. 2: Schematic workflow of the *in vitro* model of inflammation on human intestinal Caco-2 cells.

3.3 Methods

3.3.1 Western blot analysis

3.3.1.1 Nuclear and cytoplasmic proteins extraction

After the appropriate treatments the cells were rinsed with DPBS, harvested with trypsin-EDTA, resuspended and lysed in a hypotonic buffer (Hepes 10 mM, $MgCl_2$ 1.5 mM, KCl 10 mM, and glycerol 5%) containing a cocktail of protease inhibitors (aprotinin 2 µg/mL, leupeptin 1 µg/mL, and benzamidine 1 mM) and DTT 1 mM, kept in ice for 15 min and then Igepal 10% was added. The lysed cells

were then centrifuged and the supernatant containing the cytoplasmic protein were isolated and stored at -20°C. While the pellet containing the nuclei was lysed in a hypertonic buffer (Hepes 20 mM, $MgCl_2$ 1 mM, NaCl 400 mM, EGTA 1mM, EDTA 0.1 mM and glycerol 10%) containing the protease inhibitor (aprotinin 2 µg/mL, leupeptine 1 µg/mL, and benzamidine 1 mM) and DTT 1mM. After centrifugation the nuclear fraction was collected and stored at -20°C until use.

3.3.1.2 Total proteins extraction

At the end of the treatments, Caco-2 were rinsed with DPBS, harvested with Trypsin-EDTA, and resuspended in a lysis buffer containing Tris HCl 10 mM, NaCl 150 mM, Triton X-100 1% and $EDTANa_2$ 5mM, a cocktail of protease inhibitors (aprotinin 2 µg/mL, leupeptin 1 µg/mL, and benzamidine 1 mM) and DTT 1 mM. following the centrifugation, the supernatant containing the proteins was collected and stored at -20°C.

3.3.1.3 Determination of protein content

The protein content was quantified by the Bradford colorimetric assay (Bradford, 1976) using BSA as a standard. The Bradford assay exploits the change of the absorption peak of the Coomassie Brilliant Blue dye when it binds to arginine and to the hydrophobic amino acid residues present in proteins. In the anionic form, the linked dye is blue and has an absorbance maximum at 595 nm. In its cationic form, the unbound dye is green and red. Briefly, 5 min after adding the dye to the samples, absorbance was red at 595 nm. The increase in absorbance is proportional to the amount of dye bound to protein. All analyses were performed in triplicate.

3.3.1.4 Immunoblotting

For the immunoblotting analysis, 40 µg of protein were denatured in 4X SDS-PAGE reducing sample buffer (Tris-HCl 260 mM pH 8, glycerol 40% (v/v), SDS 9.2% (w/v), bromophenol blue 0,04% and as a reducing agent the 2-mercaptoethanol) and subjected to the SDS-PAGE on 12%

acrylamide/bisacrylamide gels. Following the separation, the proteins were transferred on a nitrocellulose membrane (Hybond-P PDVF, Amersham Bioscience). Residual binding sites were blocked, through 2h incubation with 5% of lyophilized non fat milk solubilized in TBS-T (TRIS base 10 mM, NaCl 100 mM, Tween 20 0.1%). The membrane when then probed with specific primary antibodies: rabbit anti-NF-κB p65 monoclonal antibody (Santa Cruz Biotechnology) (1:1000); rabbit anti-Phospho-IKK α/β (Cell Signaling Technology) (1:1000); rabbit anti- Nrf2 policlonal antibody (Santa Cruz Biotechnology) (1:500); rabbit anti- COX-2 monoclonal antibody (Cell Signaling Technology) (1:1000); rabbit anti-β-Actin monoclonal antibody (Cell Signaling Technology) (1:6000), rabbit anti-Lamin-B monoclonal antibody (Cell Signaling Technology) (1:1500), followed by a 2h incubation with peroxidase-conjugated secondary antibody HRP labeled goat anti-rabbit Ig (Cell Signaling Technology) (1:6000) and visualized with an ECL plus detection system (Amersham Biosciences). Quantitative analysis was performed by densitometry.

3.3.2 Evaluation of gene expression

3.3.2.1 RNA extraction

RNA was extracted using the E.Z.N.A. Total RNA Kit I (OMEGA bio-tek VWR) following the manufacturer's instructions. At the end of the extraction RNA was solubilized in an appropriate volume of nuclease free water preheated at 70°C.

3.3.2.2 RNA integrity

After the extraction, RNA concentration was measured using the Quant-iT RNA assay kit with the Qubit fluorimeter (Invitrogen, Milano). The quality of RNA was evaluated by verifying its integrity by electrophoresis on denaturant agarose gel with SYBR® Gold. The integrity of RNA was evaluated on ribosomal double strand RNA (28S and 18S rRNA). Briefly, 3 μg of total RNA was taken and diluted with Loading Buffer 2X (consisting of deionized formamide, MOPS, formaldehyde, glycerol, bromophenol blue, and water) in a 1:1 ratio. The sample was heated to 65°C for 10 min and then allowed to cool on ice for 5 min. Then

RNA samples were loaded in a 1% agarose gel containing 0.66 M formaldehyde and run at 100 V for about 1 h. At the end of the run the RNA was verified through a transilluminator to highlight the 28S and 18S rRNA bands.

3.3.2.3 Preparation of cDNA

After verifying RNA integrity, to obtain the corresponding cDNA, reverse transcription was carried out following the manufacturer's instructions (Sigma, Product Code M1302).

At this aim, the following components were added in a nuclease free tube:

- 1 µL oligo$(dT)_{23}$ primer (final concentration 70 µM)
- mRNA template
- 1 µL 10 mM dNTP mix
- Nuclease-free water up to 10 µL of total volume

Then the tube was centrifuged to collect all the components on the bottom. The sample was heated to 70°C for 10 min to denature the RNA and then allowed to cool on ice for 5 min. At this point the remaining components were added:

- 2 µL 10X M-MLV reverse transcriptase buffer
- 1 µL M-MLV reverse transcriptase
- 0,5 µL RNase inhibitor (40 units/ml)
- 6.5 µL nuclease-free water

_____

- 20 µL of final volume

The tube was incubated at 37°C for 50 min to activate the enzyme. Then the reaction tube was heated at 85°C for 10 min to denature the M-MLV reverse transcriptase.

3.3.2.4 Quantitative RT-PCR

mRNA levels were determined by Real-Time qPCR (Applied Biosystems 7300 Real-Time PGR System, GA, USA) with SYBR green chemistry (SYBR green JumpStart Taq Ready Mix - Sigma). Each cDNA sample (1 μL) was subjected to amplification using primer pairs specific for individual genes (**Table 1**). To check the possible contamination of the reagents, a sample containing all the reagents except the cDNA was included (NTC - No Template Control). All Real-Time PCR reactions were performed in triplicate and normalized with ROX. The ROX dye is used as a reference. Normal pipetting errors do not affect the determination of the Ct values in real-time PCR instrument using a reference dye. A constant concentration of the reference dye along the plate provides a signal proportional to the real volume in the wells. The instrument software normalizes the differences in volume of the wells by normalizing the signals of the SYBR Green dye with the reference.

In the PCR tubes the following reagents were added:

- 12.5 μL SYBR Green JumpStart Taq ReadyMix
- 1X ROX
- Forward Primer 0.2 μM
- Reverse Primer 0.2 μM
- H_2O
- 1 μL of cDNA sample previously obtained by reverse transcription

Then the tubes were vortexed and finally centrifuged. The following parameters were used for amplification. 40 cycles each consisting of the following steps:

- Denaturation: 94 ° C for 15 seconds
- Annealing and extension: 60 ° C for 1 minute

Before carrying out the 40 cycles a step of 2 minutes at 94°C to activate the TAQ polymerase is required.

Finally, at the end of the amplification cycles, a step of dissociation has been carried out to assess the presence of other amplified products and to determine the

melting temperature (Tm) of our amplified product. The Tm was compared with that calculated using the DNA Star program (Madison, USA). The melting temperature is specific for each amplified product and corresponds to the temperature at which 50% of the DNA is denatured and depends also on the

percentage of G and C bases present.

Primer			Amplificon		References
			Length	Tm	
18S rRNA	Fwd	5'-GTAACCCGTTGAACCCCATT-3'	153bp	84°C	Ferrari *et al.*, 2017
	Rev	5'-CCATCCAATCGGTAGTAGCG-3'			
IL-8	Fwd	5'-ACTGAGAGTGATTGAGAGTGGAC-3'	112 bp	82°C	Primer Bank (ID 10834978a 2) Wang and Seed, 2003
	Rev	5'-AACCCTCTGCACCCAGTTTTC-3'			
IL-6	Fwd	5'-ACTCACCTCTTCAGAACGAATTG-3'	149bp	82°C	Primer Bank (ID 10834978a 2) Wang and Seed, 2003
	Rev	5'-CCATCTTTGGAAGGTTCAGGTTG-3'			
NQO-1	Fwd	5'-AAGAGCACTGATCGTACTGG-3'	172bp	84.5°C	Cimino *et al.*, 2013
	Rev	5'-CTTCAGTTTACCTGTGATGTCC-3'			

Tab. 1: Forward and reverse primer sequences used for Real-Time qPCR.

3.3.2.5 Post-analysis elaboration

The data were processed using the SDS 1.3.1 software (Apphed Biosystems, Foster City, CA, USA) and expressed as threshold cycle (Ct), which indicates the cycle at which the detected fluorescence exceeds the threshold line. The Ct values for each target gene and for the reference gene were obtained, and then the difference (ΔCt) vas calculated. The efficiency of the primers for the genes tested was

corresponding to that of the primers for 18S rRNA (reference gene). The purpose of the reference gene is to normalize the PCR for RNA added in the reactions of reverse transcription. For a valid calculation of ΔΔCt, the amplification efficiency of the target and the reference must be approximately equal. The fold increase in mRNA expression compared with the control cells not treated and not exposed to PA was determined using the $2^{-\Delta\Delta Ct}$ method (Livak *et al.*,2008). The $2^{-\Delta\Delta Ct}$ method is a convenient method for analyzing the results of gene expression obtained with qPCR experiments. Data analyses of gene expression that used the $2^{-\Delta\Delta Ct}$ method have been reported in the literature.

3.3.3 Determination of ROS

The oxidative stress condition was evaluated through the determination of ROS levels. The intracellular ROS levels were quantified using the DCFHDA, a fluorogenic and lipophilic substance. Thanks to its hydrophobic nature, it can easily penetrate into the cells, where the cellular esterase deacetylate it, and consequently trapped inside the cell. Then, it is oxidized by the intracellular ROS in 2',7'dichlorodihydrofluorescein (DCF), which is highly fluorescent. Briefly, at the end of 6h exposure to PA, cells were washed twice with DPBS at both the compartment, and afterward DCFH-DA 50 µM were added in the apical side, followed by incubation at 37°C for 30 min. Then, fluorescence was determined at 485nm and 530 nm (excitation and emission respectively) using a fluorimeter. The fluorescence intensity is directly proportional to the ROS amount. Results are expressed as fluorescence intensity per mg of proteins against control.

3.3.4 Total Antioxidant Activity

Following the appropriate treatment cells were rinsed harvest and then separated through centrifugation at 10000 rpm for 10 min. Then the pellet was solubilized and homogenized in Triton X100 0.05% and subsequently incubated for 30 min at 4°C. The samples were centrifugated at 14000 rpm for 15 min and the supernatant was used for the determination of the total antioxidant activity. The antioxidant activity was determined using ABTS as described by Cimino *et al.* (2013). Briefly

a 1.7 mM solution of 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) was mixed with a 4.3 mM solution of potassium persulfate and incubated for 12-16h in the dark. Afterward samples were diluted in the ABTS/$K_2S_2O_8$ mixture and incubated for 6 min in the dark. Subsequently the absorbance was read at 734 nm, using a UV /vis spectrophotometer (Shimadzu, Japan). The sample concentration was calculated from Trolox standard curve equation, and the results were expressed as mg Trolox equivalent (TE)/mg of protein.

3.3.5 Fluorescein permeability

The paracellular permeability was evaluated by the transport of fluorescein from the apical to the basolateral side. At the end of the experiments, Caco-2 monolayer was washed with DPBS and an aliquot of DPBS was added on the basolateral side while Fluorescein (100 μM in DPBS) was used on the apical side. After 30 min, the basolateral liquid was withdrawn, and the fluorescence was measured using a multiplate reader (FluoStar ® Omega BMG Labtech) with excitation and emission respectively at 492 nm and 518 nm. The values are expressed as fold change against the control.

3.4 *IN VITRO* GASTROINTESTINAL DIGESTION MODEL

3.4.1 Simulated gastrointestinal digestion of an anthocyanin extract

The blackcurrant and bilberry extract (BBE) used in the present study is an anthocyanin enriched dietary supplement (Medpalett AS, Sandnes, Norway), commercially available, and consists mainly of 17 purified ACNs (all glycosides of cyanidin, peonidin, delphinidin, petunidin, and malvidin) isolated from wild Scandinavian bilberries (*Vaccinium myrtillus*) and blackcurrant (*Ribes nigrum*) from New Zealand (Hassellund *et al.*, 2012*;* Qin *et al.*, 2009).

The experiments concerning *in vitro* gastrointestinal digestion of BBE were carried out in the laboratories at Quadram Institute Bioscience (Norwich Research Park, Norwich, UK, under the supervision of Prof. Pete Wilde) according to the method described by Minekus *et al*. (2014). The digestion consists in three different phases: oral, gastric, and intestinal (small intestine) phase. Instead of withdrawing aliquots from the reaction tubes at the end of the oral, gastric or intestinal digestion, individual digestions were carried out for each phase of digestion.

For all the digestions, the BBE was solubilized in water at concentration of 320 µg/mL of anthocyanins expressed as C3G equivalents (as evaluated by the pH differential method as described below).

The **oral digestion** was performed incubating one mL of BBE solution with salivary simulated fluid (SSF) (mix ratio 1:1) [KCl 15.1mM, KH_2PO_4 3.7mM, $NaHCO_3$ 13.6 mM, $MgCl_2(H_2O)_6$ 150 µM, $(NH_4)_2CO_3$ 60 µM] pH=7 and salivary α-amylase (final conc. 75 U/mL) for 2 min in a continuous agitation (130 rpm), using a shaking waterbath (New Brunswick CO. INC New Jersey USA) at 37°C.

The remaining oral digesta were submitted to the **gastric digestion**: 2 mL were diluted in gastric simulated fluid (GSF) (mix ratio 1:1) [KCl 6.9mM, KH_2PO_4 900 µM, $NaHCO_3$ 25 mM, $MgCl_2(H_2O)_6$ 100 µM, $(NH_4)_2CO_3$ 500 µM, NaCl 47.2 mM, ultra-pure water and $CaCl_2$ 75 µM (final conc.). The pH was adjusted to 3 with HCl 1M and then incubated with porcine pepsin (final conc. 2000 U/ mL), freshly prepared in ultra-pure water, in a shaking waterbath (130 rpm) at 37°C for 2h.

Finally, the **intestinal digestion** was performed by mixing the resulting gastric chyme (4 mL) with the intestinal simulated fluid (SIF) (mix ratio 1:1) [KCl 6.8 mM, KH_2PO_4 800 µM, $NaHCO_3$ 85 mM, $MgCl_2(H_2O)_6$ 330 µM, NaCl 38.4 mM], pure water and $CaCl_2$ 300 µM (final conc.) and the pH neutralized (pH=7) with the addition of NaOH 1M. Immediately the mixture was incubated with bile (10 mM) and porcine pancreatin (final conc. 100 U/mL) in shaking waterbath for 2 h at 37°C.

The **sham digested** extract was prepared by eight-fold dilution of 1mL of the initial solution of BBE in water in order to reach a similar total volume of intestinal phase. Similarly, at the end of oral and gastric digestion, the final volume of the samples was adjusted to 8mL (corresponding to the final volume of intestinal digesta). In addition, pH of all the samples were adjusted to pH 2 and kept on ice in order to stop the enzymatic activity and later centrifuged at 5000 rpm for 10 min. The supernatant was collected and stored at -80 °C for additional assays or HPLC analysis **(Fig. 3).**

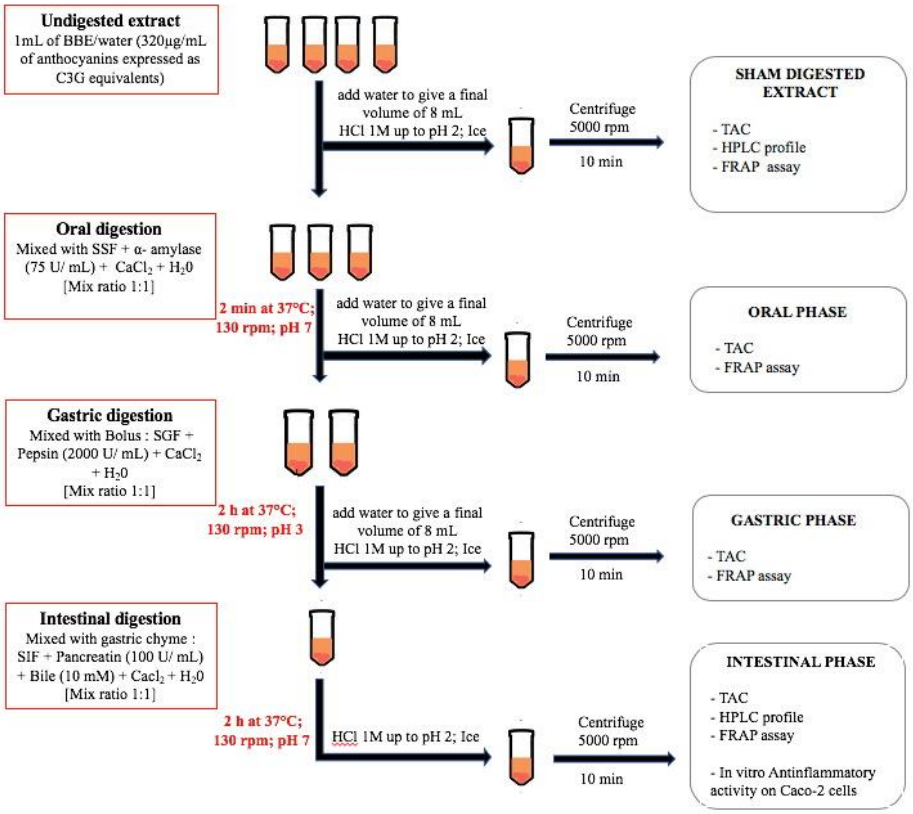


Fig. 3: Schematic workflow of the *in vitro* digestion method.

3.4.2 Total anthocyanins content

The total anthocyanins amount was evaluated through the pH differential method (AOAC, 2005). The method is based on the capability of anthocyanin pigments to reversibly change color with a change in pH; the colored flavylium cation form exists at pH 1.0, and the colorless hemiketal form predominates at pH 4.5. Each sample was diluted with 0.025 M potassium chloride buffer (pH 1) and 0.4 M sodium acetate buffer (pH 4.5) and incubated in dark for 15 min. The absorbance of each dilution was measured in a UV/vis spectrophotometer at 520nm (wavelength at which there is the maximum absorbance peak of monomeric anthocyanins) and 700 nm (no absorbance of anthocyanins), against distilled water. The difference in the absorption of the pigments at 520 nm is proportional to the pigment concentration. To eliminate the possible haze, the absorbance of each sample was read also at 700 nm. The monomeric anthocyanin pigment concentrations were expressed as C3G equivalents/mL (Giusti *et al.*, 2001). The mg/mL of monomeric anthocyanins were calculated using the following equation:

$$(mg/ml) = (A * MW * DF)/\varepsilon * l$$

where: $A = (A_{520} - A_{700})_{pH1} - (A_{520} - A_{700})_{pH4.5}$; MW= 449.2; l = 1 cm, ε = 26 900 l/mol cm; DF = dilution factor

With the aim to analyze the fate of the anthocyanins after the digestion process, the recovery index (RI) of these was calculated according to the method described by Ortega et al. (2011). The RI allows to estimate the quantity of the anthocyanins recuperated after each phase of the *in vitro* digestion.

% Recovery Index (RI) = (TAC digesta / TAC sham digested BBE) *100

3.4.3 HPLC profile of anthocyanins

Anthocyanins profiles of undigested and GI digested samples were determined by High Performance Liquid Chromatography-diode array detector (HPLC-DAD) (Agilent 1200, Agilent Technologies) according to Czank and coworkers (2013). Briefly 20 µl of sham digested extract or intestinal extract were injected into Kinetex XB-C18 column (100 x 4.6 mm, 2.6 µm particle size) and analyzed using a gradient elution with a flow rate of 1 mL/min. The mobile phases were composed by formic acid in water (5:95, v/v) as solvent A and formic acid in acetonitrile (5:95, v/v) as solvent B. The solvent gradient consisted of 2.5% B at 0 min, 25% B at 15 min, 45% B at 20 min, and 100% B at 25−30 min at a flow rate of 1 mL/min (Czank *et al.*, 2013).

To identify the anthocyanins present in each sample, the spectra from 200 to 600 nm were monitored for all peaks, and the chromatograms were recorded at 520, 280, 330 and 370 nm. Anthocyanins show a characteristic and specific absorption peak at 520 nm deriving from the absorption of the benzopyran ring (A and C ring), and at 280 nm (coming from the absorption of B ring) which is common for all the subclasses of polyphenols. The identification of anthocyanins compounds was carried out by comparing UV absorption spectra and retention times of each compound with those of previously published data and if available of undigested pure stand standards (Fischer *et al*., 2011). For a preliminary anthocyanin quantification peak area was recorded at 520 nm.

3.4.4 Ferric reducing antioxidant power (FRAP) assay

The antioxidant activity of sham digested or digested BBE was determined using the FRAP assay according to the method described by Boussahel *et al*. (2015). The FRAP reagent consists in a uncolored complex Fe^{3+}-TPTZ, which in acidic condition is reduced by non-enzymatic antioxidant, such as polyphenol compounds in blue complex Fe^{2+}-TPTZ. Briefly, the samples were diluted in the FRAP reagent (made up freshly) and incubated for 4 min a 20°C, afterward the absorbance was measured at 593 nm. The samples concentration was assessed using the standard curve ($FeSO_4$) equation and the values were expressed as µmol $FeSO_4$ equivalent /ml of digesta or sham digested extract.

3.4.5 Caco-2 cells pretreatment with digested BBE and exposure to TNF-α

The anti-inflammatory activity of intestinal digested BBE was evaluated in an *in vitro* model of intestinal inflammation using human Caco-2 cells.

Since *in vitro* studies addressing the protective effects of anthocyanins have been evaluated a concentration between 10 and 100 µM, which seem to be largely too high to be achieved in the target site under physiological conditions, we aimed to study the effects of physiological intestinal concentrations of these compounds.

In our experiments, the highest intestinal digested extract concentration used was 1.5µg/ml (calculated with pH differential method and expressed as C3G), since this corresponds (taking in account a 16.9% of intestinal recovery) almost to a starting undigested concentration of 20µM of C3G, commonly used for *in vitro* studying anthocyanin anti-inflammatory effects (Ding *et al., 2006;* Ma *et al., 2018;* Ferrari *et al., 2016;* Speciale *et al.*, 2014). Then, the differentiated Caco-2 cells were pre-treated for 24h with different concentrations of the BBE intestinal phase (BBE-IP) (1.5, 0.75, 0.37 and 0.18 µg/ml expressed as C3G). At the end of the incubation time, cells were rinsed twice with DPBS with Calcium and Magnesium, at both the compartments and were exposed to TNF-α 50 ng/mL for 6h, added on the basolateral side (**Fig. 4**).

Inflammatory pathway and adaptive cellular response were studied in Caco-2 cells evaluating NF-κB and Nrf2 pathways

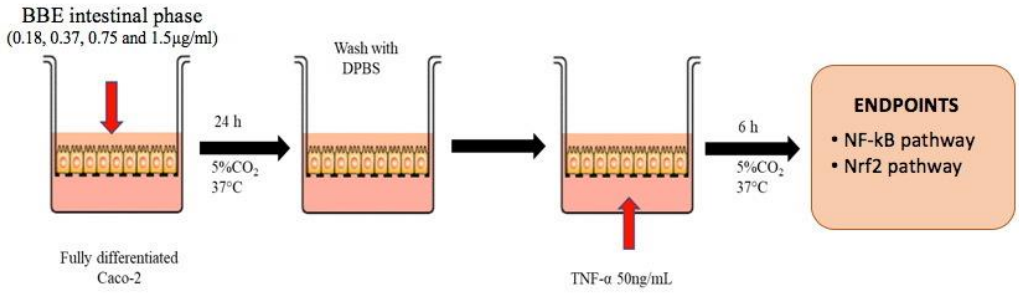


Fig. 4: Schematic representation of the *in vitro* BBE-IP pretreatment and exposure of Caco-2 cells.

3.5 Statistical analysis

All the experiments were carried out in triplicate and repeated three times. Results are expressed as mean ± SD from three independent experiments and statistically analyzed by a one-way ANOVA test, followed by Tukey's HSD, using the statistically ezANOVA (http://www.sph.sc.edu/comd/rorden/ezanova/home.html), differences between groups and treatments were considered significant for $p < 0.05$. Correlation analysis was performed between total anthocyanins content and antioxidant activity of extract using Pearson correlation analysis.

4. *RESULTS AND DISCUSSIONS*

4.1 STUDIES ON AN *IN VITRO* MODEL OF PA-INDUCED INTESTINAL INFLAMMATION

4.1.1 Effect of C3G on NF-κB pathway

Several scientific papers reported that PA acts as a pro-inflammatory mediator inducing the activation of the transcription factor NF-κB, MAPKs, and JNK pathway (Lyons *et al.,* 2016). In line with this, other studied showed an up-regulation of NF-κB in intestinal tissue of patients with IBD (Biasi *et al.* 2013). NF-κB is a redox sensible nuclear factor that plays a pivotal role in the regulation of different pro-inflammatory mediators, such as interleukins (IL-6, IL-8, IL-12, IL-1 β), TNF-α, IFN-γ, and myosin light chain kinase (MLCK) (Biasi *et al.* 2013). In physiological conditions it is found in an inactive form in the cytoplasm complexed with the inhibitor of κB, IκB. External inflammatory stimuli activate the kinase of IκB (IKK) which in turn phosphorylate and induce the proteasomal degradation of IκB. NF-κB/p65 thus is free to translocate into the nucleus and to induce the transcription of several pro-inflammatory mediators (Bernotti *et al.,* 2003).

Hence, with the aim to assess the beneficial effect of C3G on PA induced intestinal inflammation we evaluated the nuclear translocation of p65 and the activation of IKK.

Firstly, in order to determine the appropriate exposure time to PA, a kinetic of NF-κB activation was performed exposing the differentiated monolayer to PA 100 μM for 6 or 24 h. The results demonstrate that after 6h there is an increase of NF-κB levels that remains persistent till 24h, suggesting thus that 6h are enough to induce a cellular response (**Fig. 5**). Hence, based on this outcome and on previous published data (He *et al.,* 2012), 6h of PA exposure was selected.

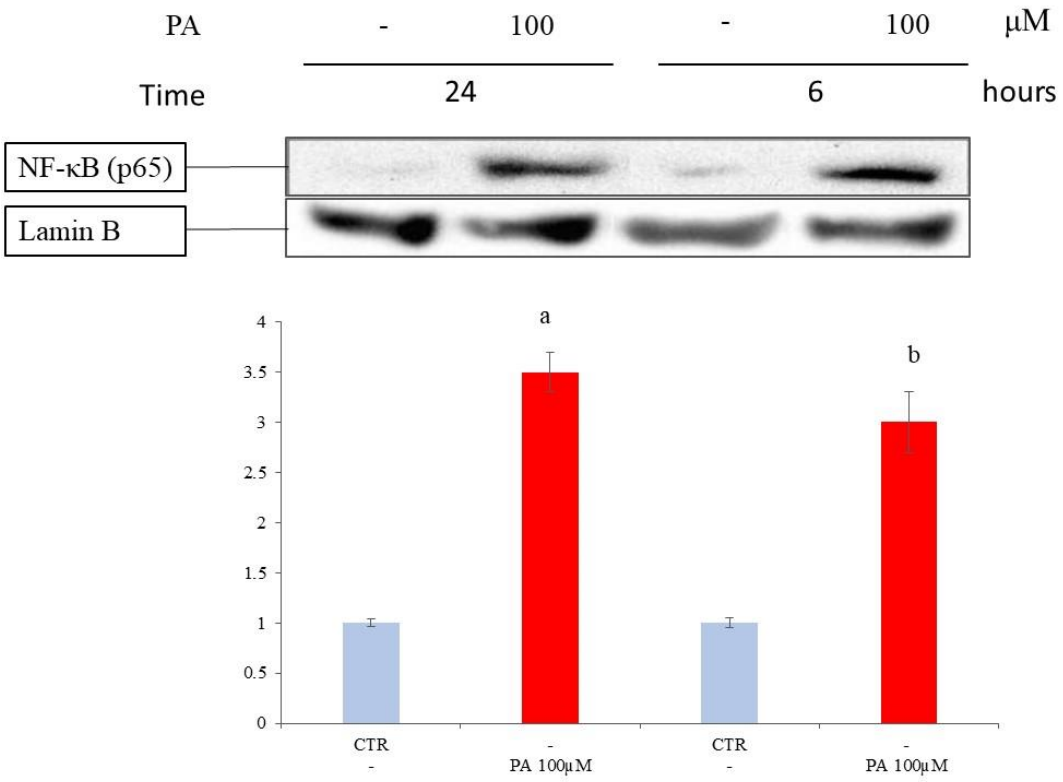


Fig. 5: Nuclear translocation of NF-κB (p65). Caco-2 cells were exposed to PA 100 μM for 6 and 24 hour. Cultures treated with the vehicle alone (0.05% DMSO) were used as controls (CTR). Results are reported as fold change against control and expressed as mean ± SD of three independent experiments. NF-κB values were normalized to the corresponding Lamin B value. $^{a}p < 0.05$ vs CTR 24h; $^{b}p < 0.05$ vs CTR 6h.

Hereafter we evaluated C3G effects on PA induced damage (He *et al*.,2009). The results shown in **fig. 6** reported an increase of p65/NF-κB nuclear levels in the sample exposed to PA 100 µM for 6h. On the contrary its nuclear translocation was inhibited by the pretreatment with C3G (10 and 20 µM) in a dose-dependent way. C3G 20µM was able to restore the nuclear level of p65 to the control value. These results demonstrate the inhibition of PA-induced NF-κB pathway exerted by C3G.

Furthermore, p65/NF-κB nuclear levels result lower in C3G 20µM pretreated cells when compared to controls, irrespective to the presence of PA. This effect is probably due to the modulation of redox status exerted by natural polyphenols as reported in previous studies (Fratantonio *et al*., 2015).

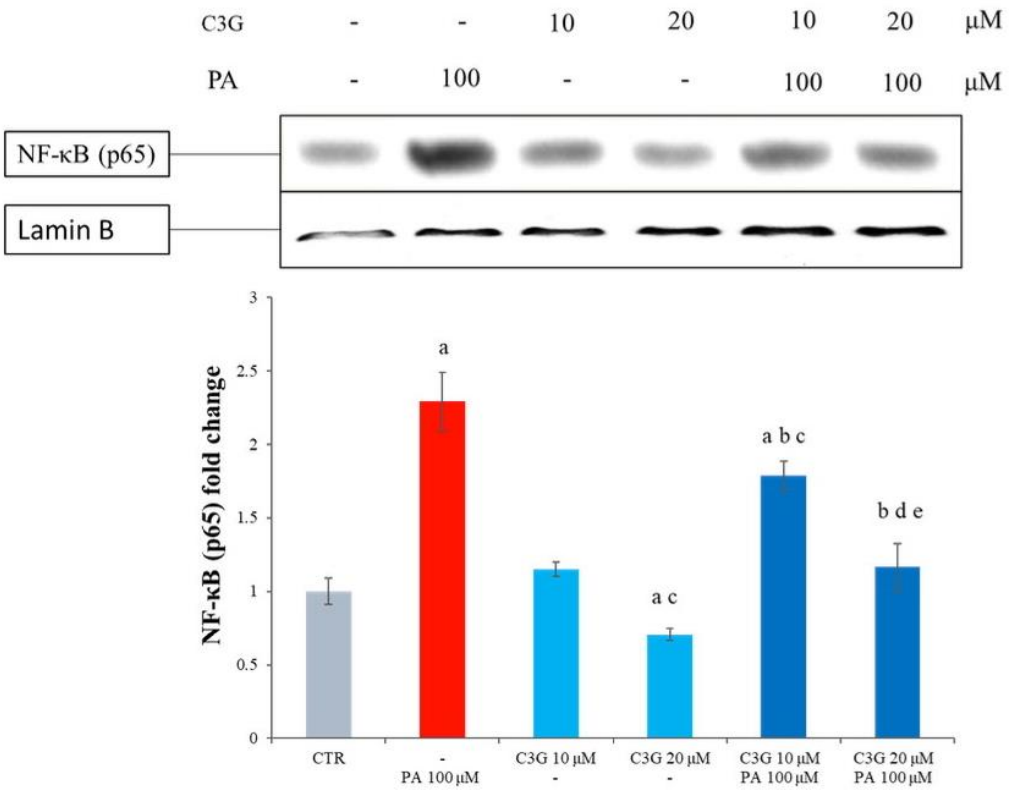


Fig. 6: Nuclear translocation of NF-κB (p65). Differentiated Caco-2 were pretreated or not for 24 hours with C3G (10 and 20μM) and subsequently exposed to PA 100 μM for 6 hours. Cultures treated with the vehicle alone (0.05% DMSO) were used as controls (CTR). Results are reported as fold change against control and expressed as mean ± SD of three independent experiments. NF-κB values were normalized to the corresponding Lamin B value. [a]$p < 0.05$ vs CTR; [b]$p < 0.05$ vs PA 100 µM; [c]$p < 0.05$ vs C3G 10 μM; [d]$p < 0.05$ vs C3G 20 μM; [e]p <0.05 vs C3G 10 µM+ PA 100 µM.

Furthermore, in order to better elucidate the mechanism by which C3G exerts its effects on NF-κB nuclear translocation, we evaluated the cytoplasmic levels of IKK α/β. The data shown in **fig. 7** demonstrate that C3G, at both the concentrations tested, is able to reduce, in a dose dependent manner, the activation of IKK induced by PA 100 µM, and consequently to inhibit p65 nuclear translocation.

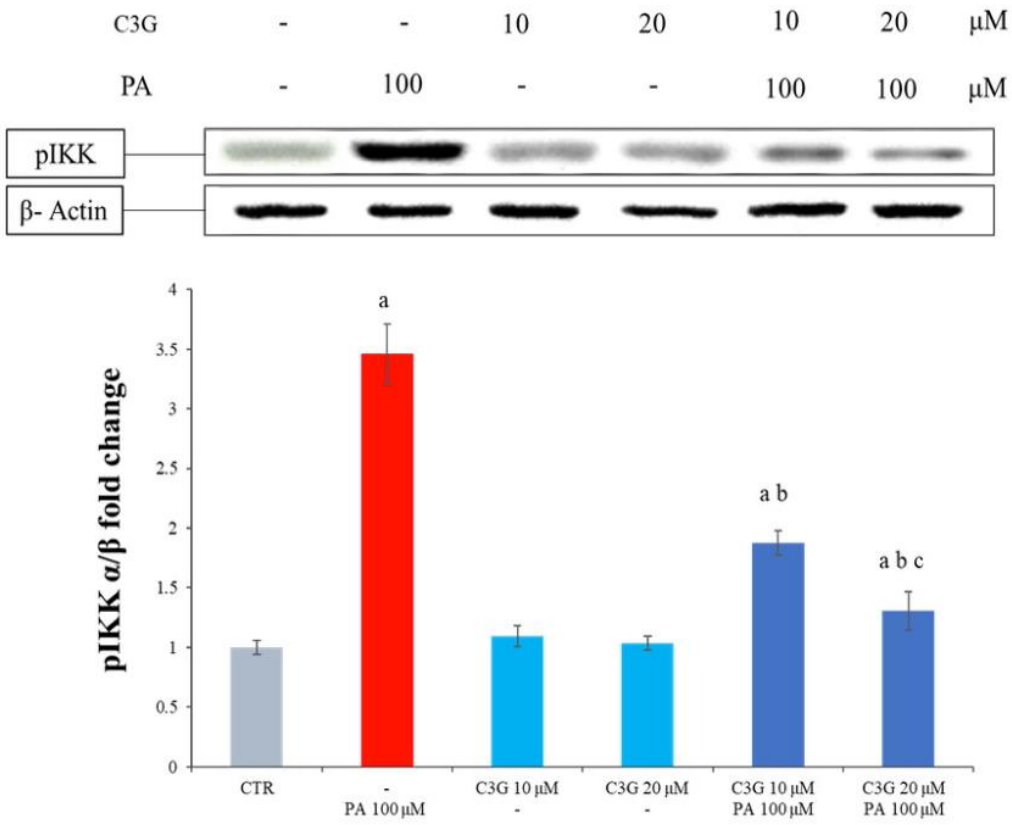


Fig. 7: Cytoplasmic levels of p-IKK α/β. The Caco-2 monolayer was pretreated or not for 24 hours with C3G (10 and 20μM) and subsequently exposed to PA 100 μM for 6 hours. Cultures treated with the vehicle alone (0.05% DMSO) were used as controls (CTR). Results are reported as fold change against control and expressed as mean ± SD of three independent experiments. p-IKK values were normalized to the corresponding β-Actin value. [a]$p < 0.05$ vs CTR; [b]$p < 0.05$ vs PA 100 µM; [c]p <0.05 vs C3G 10 µM+ PA 100 µM.

4.1.2 Effect of C3G on NF-κB transcriptional activity

As mentioned before, NF-κB regulates the expression of several genes, encoding pro-inflammatory mediators such as interleukin IL-6, IL-8, COX-2 etc, having on their promoter a κB binding region. Hence, to confirm the transcriptional activity of NF-κB and the effects of C3G, gene expression of IL-6 and IL-8 was evaluated.
IL-6 is a pleiotropic cytokine involved in the innate and adaptive immune system. In fact, it controls the differentiation of T cells, balancing the levels of pro- and anti-inflammatory cytokines (Waldner *et al.*, 2014). Recent scientific evidences have suggested a pivotal role of IL-6 in different inflammatory diseases (rheumatoid arthritis, systemic juvenile idiopathic arthritis, various types of cancer,

and IBD) (Nishimoto *et al.*, 2006). Clinical studies have shown that the serum and tissue levels of IL6 were increased in patients with IBD compared to healthy ones. Furthermore, IL-6 levels were higher in patients with active Crohn disease respect to patients with an inactive disease, suggesting that high levels of IL-6 are correlated with the severity of IBD (Takac *et al.*, 2014).

The data, shown in **fig. 8**, demonstrate a statistically significant up-regulation of IL-6 mRNA after incubation with PA for 6h, confirming the PA induces the inflammatory response. This upregulation was significantly inhibited by C3G pretreatment, at both the tested concentrations, restoring gene expression levels to control value.

Furthermore, also in this case, the presence of C3G was associated to a decrease of IL-6 gene expression, irrespective to the presence of PA. As observed for NF-κB, it is possible to speculate that the modulation of redox status, exerted by C3G, reduce NF-κB transcriptional activity.

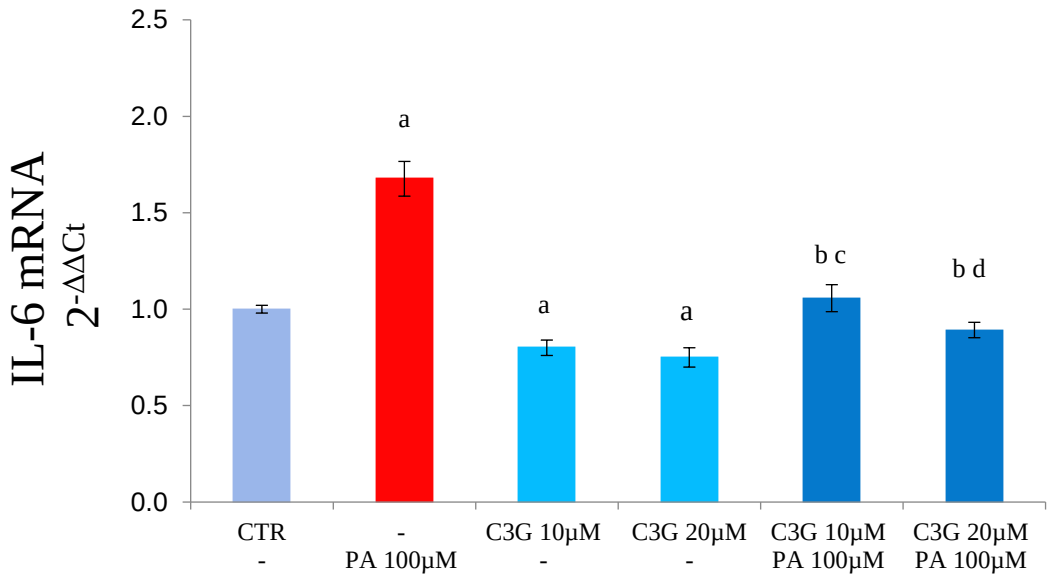


Fig. 8: IL-6 gene expression. Differentiated Caco-2 cells were pretreated or not with C3G for 24 h, and subsequently exposed to PA 100 µM for 6h. Cells treated with the vehicle alone (0.05% DMSO v/v) were used as controls (CTR). Results, from three independent experiments, are expressed as $2^{-\Delta\Delta Ct}$ (mean ± SD) and normalized to CTR. 18S rRNA was used as housekeeping gene. $^{a}p < 0.05$ vs CTR; $^{b}p < 0.05$ vs PA 100 µM; $^{c}p < 0.05$ vs C3G 10 µM; $^{d}p < 0.05$ vs C3G 20µM.

IL-8 is a chemokine involved in the recruitment of neutrophils from the peripherical blood to the inflamed tissue. In addition, IL-8 is found to be overexpressed in patients with ulcerative colitis respect to healthy patients (Mahida *et al.*, 1992)

In our experimental model, the data confirmed the PA-induced transcriptional activity of p65, as suggested by the upregulation of IL-8 mRNA. Whilst, the pretreatment with C3G showed a high and significant reduction of the IL-8 gene expression. Interestingly, the Caco-2 treatment with only C3G reduced basal mRNA levels of IL-8, as previously observed for IL-6 (**Fig. 9**).

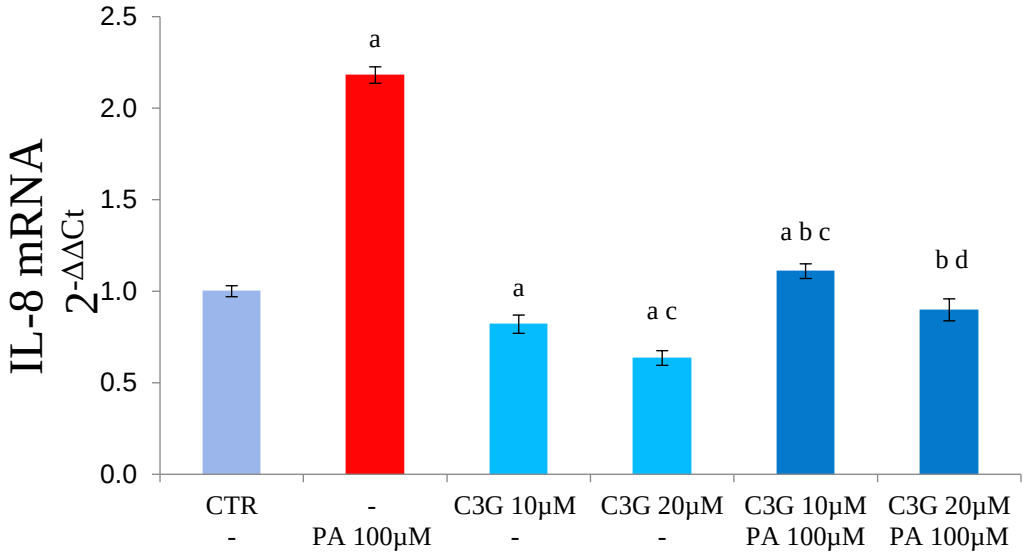


Fig. 9: IL-8 gene expression. Differentiated Caco-2 cells were pretreated or not with C3G for 24 h, and subsequently exposed to PA 100 µM for 6h. Cells treated with the vehicle alone (0.05% DMSO v/v) were used as controls (CTR). Results, from three independent experiments, are expressed as $2^{-\Delta\Delta Ct}$ (mean ± SD) and normalized to CTR. 18S rRNA was used as housekeeping gene. $^{a}p < 0.05$ vs CTR; $^{b}p < 0.05$ vs PA 100 µM; $^{c}p < 0.05$ vs C3G 10 µM; $^{d}p < 0.05$ vs C3G 20µM.

4.1.3 Effect of C3G on COX-2 activation

The anti-inflammatory effect of C3G was further evaluated taking into account another NF-κB down-stream target, COX-2. COX-2 is the inducible form of the cyclooxygenase, the enzyme regulating the synthesis of prostaglandins and leukotrienes. COX-2 represents an early and immediate inflammatory response protein. It is activated by pro-inflammatory cytokines, TNF-α, IL-1, IFN-γ etc, via nuclear transcriptional factors NF-κB and AP-1 (Wang *et al.*, 2005; Dubois *et al.*, 1998).

The results obtained show that the exposure to PA for 6 h induces an increase of COX-2 levels in Caco-2 cells. On the contrary C3G, at both tested concentrations, reduced its activation, confirming once more the protective effect of C3G on PA-induced intestinal inflammation (**Fig. 10**). Also in this case, the presence of C3G in the culture medium was associated to reduced COX-2 levels in comparison to control cells and cells exposed to PA.

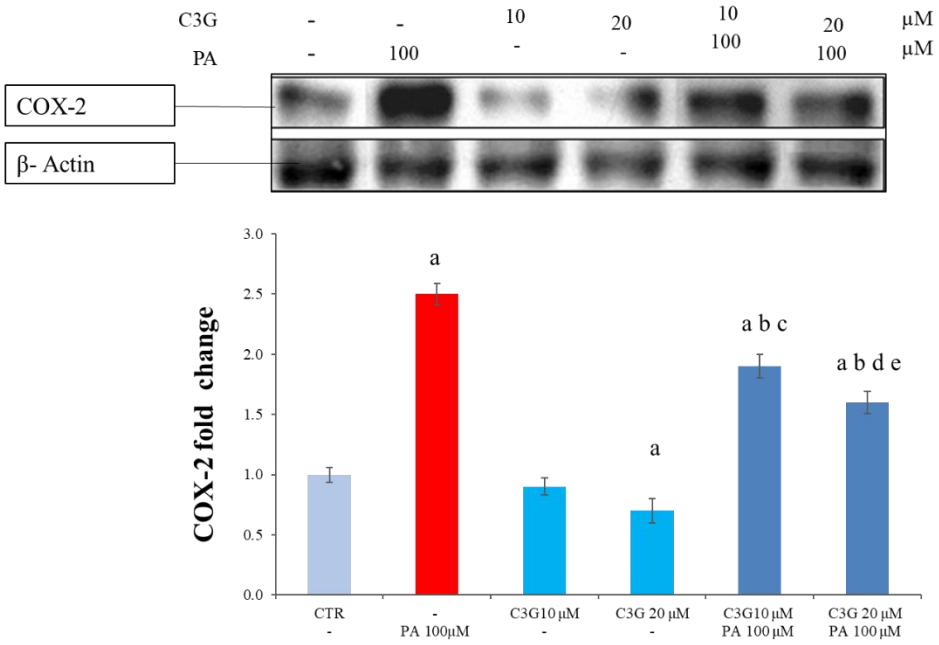


Fig. 10: COX-2 protein expression. Differentiated Caco-2 were pretreated for 24 hours with C3G (10 or 20μM), and subsequently exposed to PA 100 μM for 6 hours. Cultures treated with the vehicle alone (0.05% DMSO) were used as controls (CTR). Results are reported as fold change against control and expressed as mean ± SD of three independent experiments. COX-2 values were normalized to the corresponding β-actin value. [a]$p < 0.05$ vs CTR; [b]$p < 0.05$ vs PA; [c]$p < 0.05$ vs C3G 10 μM; [d]$p < 0.05$ vs C3G 20 μM; [e]$p < 0.05$ vs C3G 10 μM + PA 100 µM.

4.1.4 Effect of C3G on oxidative stress

Plasma concentrations of FFAs are increased in metabolic syndrome, and the increased fatty acids may cause cellular damage via the induction of oxidative stress [Furukawa *et al*., 2004]. Once FFAs enter into the cell, they are metabolized toward the β-oxidation, with production of ROS. FFA overload induces an overproduction of ROS that in turn causes an impairment of the redox balance and consequently oxidative stress (Pessaye *et al.,* 2002; Matsuzawa-Nagata *et al.,* 2008). In addition, a high fat diet, through ROS involvement, may induce the activation of NF-κB, and thus of its down-stream pro-inflammatory cytokines (Weisberg *et al.,* 2008; Kesh *et al.,* 2016).

Hence, in order to assess the role of PA on oxidative stress and the effects of C3G, we evaluated intracellular levels of ROS. Data showed higher level of ROS in Caco-2 exposed to PA when compared to control. The pretreatment with C3G reduced the intracellular levels of ROS at both the concentrations used. All these data suggest that palmitate induces an inflammatory response in intestinal epithelium, as evidenced by the activation of NF-κB pathway, through oxidative stress, and that C3G probably exerts its anti-inflammatory activity via ROS inhibition (**Fig. 11**). Interestingly, Caco-2 cells treated only with C3G exhibited lower ROS levels compared to control. This effect has been reported also for interleukins and COX-2, so supporting the involvement of an antioxidant response probably modulated by the Nrf2 pathway (Speciale *et al.,* 2014; Ferrari *et al.,* 2016).

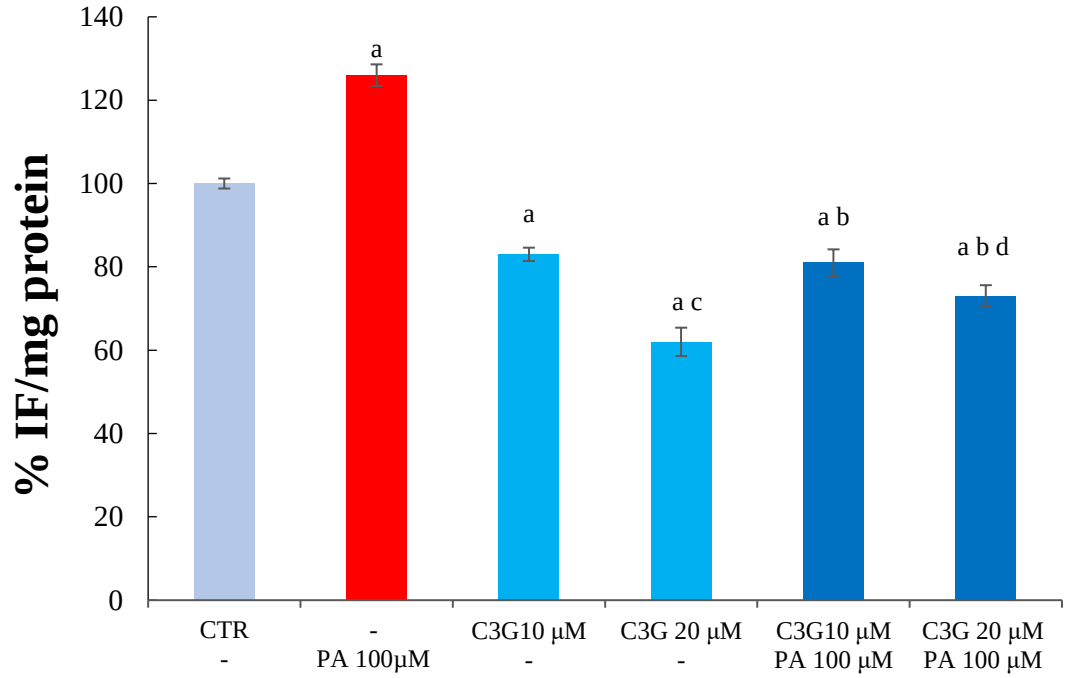


Fig. 11: ROS intracellular levels. Caco-2 were pretreated for 24 hours with C3G (10 and 20μM), and subsequently exposed to PA 100 μM for 6 h. Cultures treated with the vehicle alone (0.05% DMSO) were used as controls (CTR). The results are reported as % change of fluorescence intensity/mg of proteins against control and expressed as mean ± SD of three independent experiments. [a]$p < 0.05$ vs CTR; [b]$p < 0.05$ vs PA100 µM; [c]$p < 0.05$ vs C3G 10 μM; [d]p <0.05 vs C3G 20 µM.

It is well known that anthocyanins improve intracellular redox status also through the scavenging of free radical and so contrasting the formation of ROS (Speciale *et al.*, 2014). Thus, in order to confirm the cellular redox status altered by PA, the intracellular total antioxidant activity (TAA) was evaluated. In our experimental conditions PA reduced the TAA, confirming further the ability of PA to impair the redox balance. Interestingly, we observed a statistically significant and dose dependent increase of antioxidant activity, expressed as mmol of Trolox equivalents, in cells treated with C3G, and this activity was still evidenced after PA exposure. Taken together these results confirm the capacity of C3G to induce an antioxidant response so restoring and maintaining redox homeostasis (**Fig. 12**).

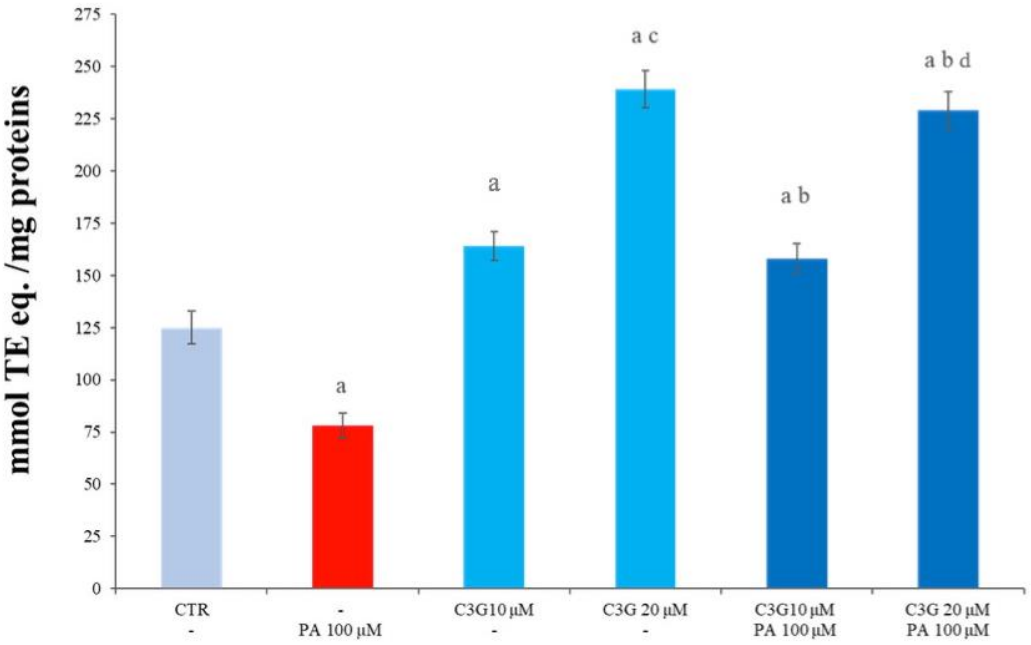


Fig. 12: Total Antioxidant Activity. Caco-2 cells were pretreated for 24 hours with C3G (10 and 20μM) and subsequently exposed to PA 100 μM for 6 h. Cells treated with DMSO alone (0.05%) were used as controls (CTR). The results are reported as nmol TE/mg protein and expressed as mean ± SD of three independent experiments. [a]$p < 0.05$ vs CTR; [b]$p < 0.05$ vs PA 100 µM; [c]$p < 0.05$ vs C3G 10 μM; [d]$p < 0.05$ vs C3G 10 μM + PA 100 µM

4.1.5 Effect of C3G on Nrf2/Keap1 pathway

Mammalian cells, in order to counteract ROS overproduction and oxidative stress, induce the expression of genes encoding for antioxidant proteins through the activation of Nrf2/Keap1 pathway (Valko *et al.*, 2007). Under basal conditions Nrf2 is found in the cytoplasm bonded with its inhibitor Keap1. Following oxidative stimuli, Nrf2 is activated and released from Keap1, and subsequently it is free to translocate and accumulate into the nucleus (Zhang *et al.*, 2003). Once in the nucleus, it recognizes and bind the antioxidant response element (ARE) motif, so activating the transcription of genes encoding for antioxidant and detoxifying proteins (Toki *et al.*, 1997). Therefore, with the aim to study the mechanism underlying the C3G antioxidant effects, we focused our studies on Nrf2/Keap1 pathway, evaluating nuclear levels of Nrf2 and its transcriptional activity.

Our data demonstrates that PA affects Nrf2 transcription factor by inhibiting nuclear accumulation (**Fig. 13**). On the contrary, Caco-2 cells treatment with C3G

(20µM) for 24h induced an increase of the Nrf2 nuclear levels and these effects were evident also in presence of PA. These data suggest the ability of C3G to induce an adaptive cellular response, and thus counteracting oxidative stress through the activation of Nrf2 pathway. This effect was evidenced also in other experimental models (Ferrari *et al.*,2016; Fratantonio *et al.*, 2015; Cimino *et al.*, 2013; Speciale *et al.*, 2013) demonstrating the main rule of this transcription factor in the protective effects of anthocyanins.

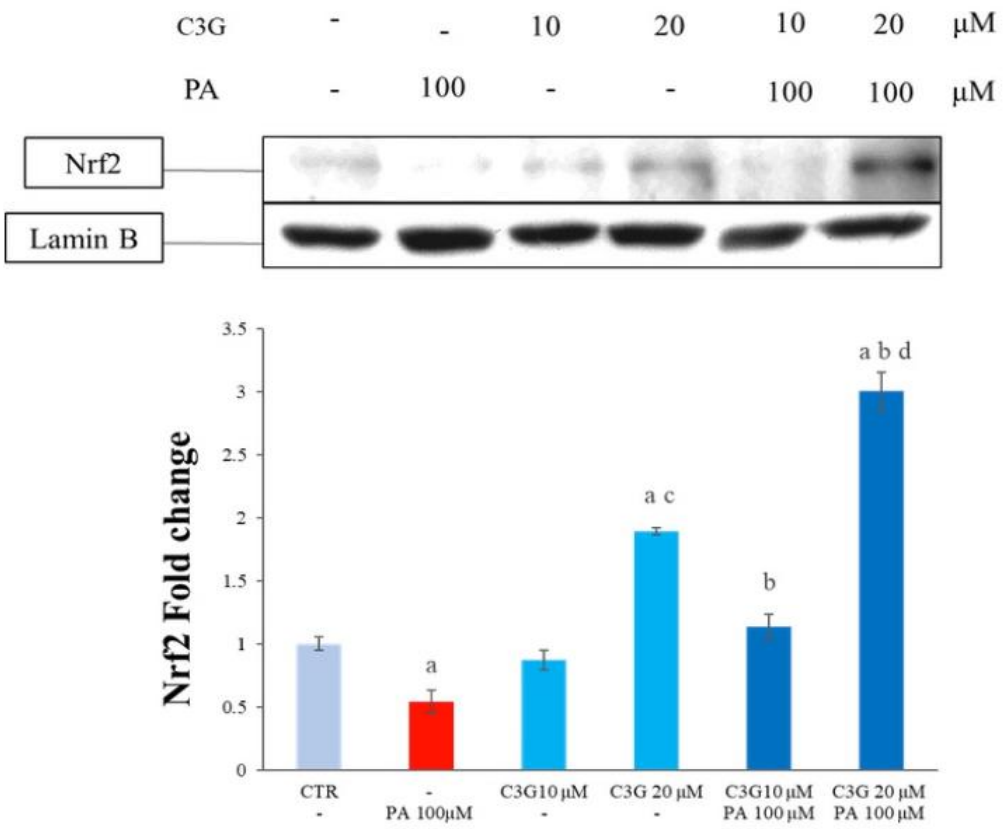


Fig. 13: Nuclear level of Nrf2. Differentiated Caco-2 were pretreated or not for 24 hours with C3G (10 and 20μM) and subsequently exposed to PA 100 μM for 6 hours. Cultures treated with the vehicle alone (0.05% DMSO) were used as controls (CTR). Results are reported as fold change against control and expressed as mean ± SD of three independent experiments. Nrf2 values were normalized to the corresponding Lamin B value. $^{a}p < 0.05$ vs CTR; $^{b}p < 0.05$ vs PA 100 µM; $^{c}p < 0.05$ vs C3G 10 μM; $^{d}p < 0.05$ C3G 10 µM+ PA 100 µM.

To confirm Nrf2 transcriptional activity, the expression of NQO-1 gene, bearing an ARE sequence, was evaluated. NQO-1 is a FAD-dependent flavoprotein able to reduce quinones to hydroquinones, preventing the electron reduction and consequently the production of free radicals (Dinkova-Kostova and Talalay, 2010). External and internal stimuli both induce its transcription through the activation of Nrf2/Keap1 pathway (Dinkova-Kostova *et al.*, 2012).

Data obtained showed a downregulation of NQO-1 after Caco-2 cells exposure to PA. Interestingly, an overexpression of NQO-1 mRNA in cells treated with the highest C3G concentration, irrespective to the presence of PA, was observed. This further confirms the activation of Nrf2 as an important mechanism involved in the protective effect of C3G on gut inflammation (**Fig. 14**).

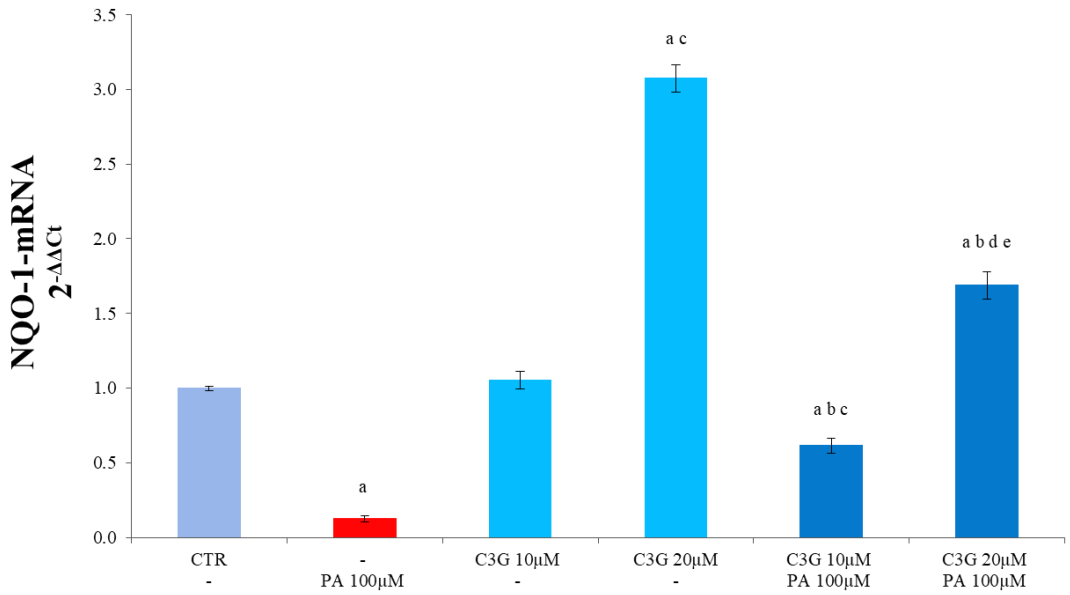


Fig. 14: **NQO-1 gene expression**. Caco-2 monolayer was pretreated or not with C3G for 24 h and subsequently exposed PA 100 µM for 6h. Cells treated with the vehicle alone (0.05% DMSO v/v) were used as controls (CTR). Results, deriving from three independent experiments, are expressed as $2^{-\Delta\Delta Ct}$ (mean ± SD) and normalized to CTR. 18S rRNA was used as housekeeping gene. $^{a}p < 0.05$ vs CTR; $^{b}p < 0.05$ vs PA 100 µM; $^{c}p < 0.05$ vs C3G 10 µM; $^{d}p < 0.05$ vs C3G 20 µM, $^{e}p < 0.05$ vs C3G 10 µM+ PA 100 µM.

4.1.6 Effect of C3G on PA-induced impaired intestinal permeability

The intestinal epithelial cells (IEC) form a first line defense against potentially harmful microorganisms. In fact, IEC recognize pathogens, secerns cytokines and chemokines, activating the immune response. In addition, IEC efficiently control intestinal permeability allowing only the passage of nutrients and blocking the passage of pathogens. Intestinal permeability is highly regulated by tight junctions (TJ) (Pastorelli *et al.*, 2013). It was widely demonstrated that oxidative stress and inflammation induce a damage on the cytoskeleton affecting proteins regulating the TJ (Rao 2008). Hence, in line with our data described above, the next step of our research was to assess the protective role of C3G on the intestinal epithelial permeability.

The permeability was evaluated by the fluorescein transport assay. Our results showed an increase of the transport of fluorescein from apical to the basolateral side after exposure to PA, suggesting the impairment of the barrier. Whereas, C3G pretreatment reduced, in a dose-dependent way, the quantity of fluorescein on the basolateral side, indicating the protective effect of C3G in restoring the permeability altered by PA (**Fig. 15**)

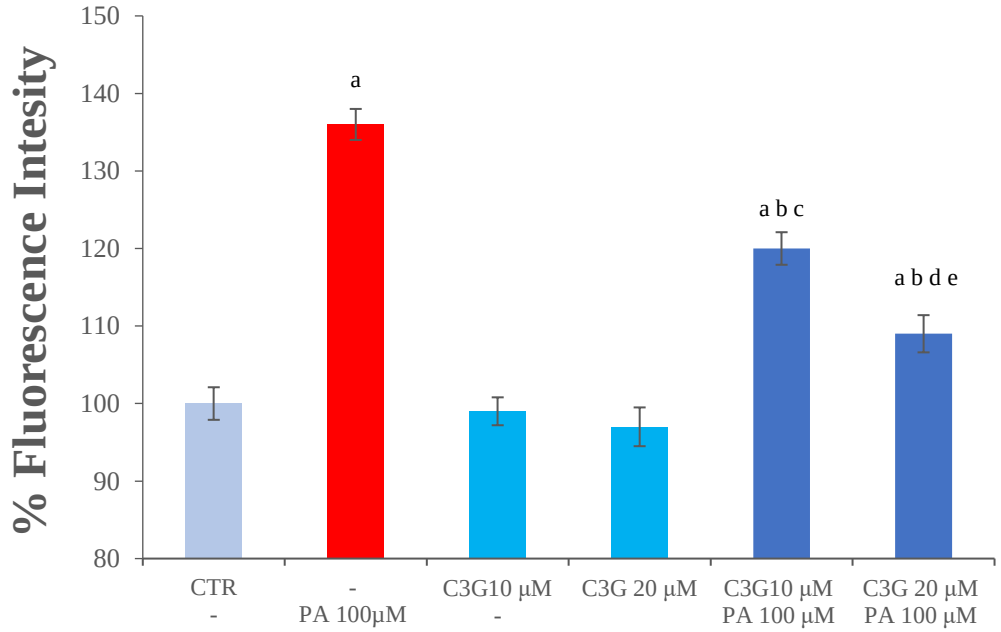


Fig. 15: **Intestinal epithelium permeability**. Caco-2 cells were pretreated for 24 hours with C3G (10 and 20μM) and/or subsequently exposed to PA 100 μM for 6 h. Cells treated with DMSO alone (0.05%) were used as controls (CTR). The results are reported as % fluorescence intensity change vs control and as mean (± SD) of three independent experiments. $^{a}p < 0.05$ vs CTR; $^{b}p < 0.05$ vs PA 100 µM; $^{c}p < 0.05$ vs C3G 10 µM; $^{d}p < 0.05$ vs C3G 20 µM, $^{e}p < 0.05$ vs C3G 10 µM+ PA 100 µM.

4.2 STUDIES ON *IN VITRO* SIMULATED GASTROINTESTINAL DIGESTION

4.2.1 Effect of gastrointestinal digestion on anthocyanins recovery

In vivo and *in vitro* studies reported several health benefits of anthocyanins since they possess antioxidant, anti-inflammatory, and anti-angiogenic activity (Domitrovic, 2011). Nonetheless anthocyanins are extremely instable and susceptible to degradation due to pH, temperature and enzymes. In acidic conditions (pH 1-3) they exist as the more stable form, the flavylium cation, while at pH higher than 4 they are present as chalcone and carbinol, which undergo easily in chemical degradation producing phenolic compounds (Fang 2014). For this reason, anthocyanins would be affected by the gastrointestinal tract before they are absorbed. The gastric and intestinal digestions have different effects on the qualitative/quantitative phenolic profiles of plants products, resulting in changes in their antioxidant activity (Gonzales *et al.,* 2015). In last decade the *in vitro* simulated digestion has been widely used to assess the stability, the bioavailability and the bioactivity of different phytochemicals (Correa-Betanzo *et al.,* 2004; Lucas-Gonzalez *et al.,* 2016; Burgos-Edwards *et al.,* 2017; Bouayed *et al.,* 2011; Thomas-Valdés *et al.,* 2018).

Therefore, with the aim to evaluate the stability of the anthocyanins after gastrointestinal digestion, a static *in vitro* digestion of a purified and standardized bilberry and blackcurrant extract (BBE) was performed. After each step we analyzed the total amount of anthocyanins by the differential pH method (AOAC, 2005) and a Recovery Index (RI) of digesta *vs* sham digested sample was evaluated (Ortega *et al.,* 2011) (**Fig. 16**). Our results showed that after the oral digestion there was a loss of the anthocyanins amount (RI 64.2 %) respect to the BBE sham digested extract, probably due to the neutral environment (pH 7) and to their transformation into the colorless and instable form, carbinol. In addition, gastric simulated digestion with pepsin under acidic condition, although in a non-statistically significant way, affects the anthocyanins content respect to the oral phase, still remaining lower than BBE sham digested extract (RI 50.4 %). A strong and significant reduction was observed after the intestinal digestion. In fact, the incubation with pancreatin under neutral environment deeply affected the recovery

of this subclass of polyphenols with a RI of 16.9% (**Fig. 16**). This effect was probably due to the anthocyanins transformation into carbinol and/or especially to their degradation into metabolites (Fang 2014). These results are in accordance with other studies in which a decrease of berries anthocyanins after gastrointestinal digestion was reported (Marhuenda *et al.*, 2016; Correa-Betanzo *et al.*, 2004).

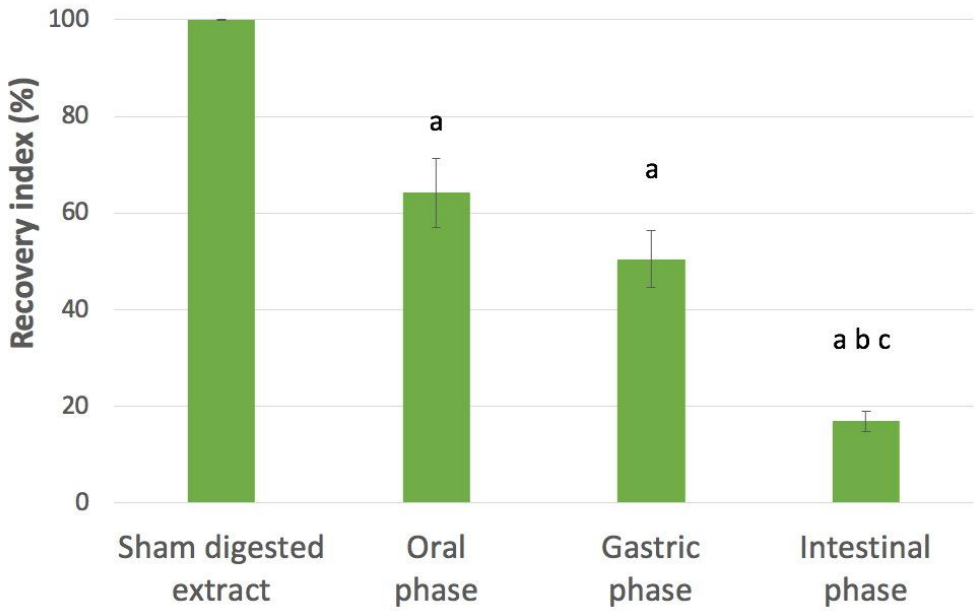


Fig. 16: Recovery Index (RI) of anthocyanins. The anthocyanins content in sham digested and BBE digested was analyzed by the pH differential method, expressed as C3G equivalents and finally RI was calculated. The sham digested extract was used as reference for the RI calculation (see material and methods). Results are expressed as mean ± SD calculated from three independent experiments. [a]p <0.05 vs sham digested; [b] p <0.05 vs oral phase [c] p <0.05 vs gastric phase.

4.2.2 Stability of anthocyanins after simulated gastrointestinal digestion

To further investigate the fate of the single anthocyanins after the gastrointestinal digestion, HPLC-UV/Vis analysis was performed. The data from the BBE sham digested extract (**Tab. 2**) confirmed the presence of 17 anthocyanins. The most abundant anthocyanins were delphinidin and cyanidin derivates. In particular, cyanidin-3-rutinoside and delphinidin-3-rutinoside were the two most present, followed by cyanidin-3-glucoside and delphinidin-3-glucoside. In addition, small amounts of glycosides of peonidin, petunidin and malvidin were revealed.

Stability of anthocyanins during gastrointestinal digestion is presented in **fig. 18** and **table 2.**

The intestinal digestion reduced the BBE anthocyanins content, as previously demonstrated (**Fig. 16**). Different rates of decrease were observed for different anthocyanins. In fact, from the initial 17 anthocyanins, after the simulated intestinal digestion, only nine of them were detected and the order of the decreases among the remaining anthocyanins were as follows: cyanidin-3-galactoside (29.97 %) > cyanidin-3-rutoside (29.43%) > malvidin-3-glucoside (26.53%) > cyanidin-3-arabinoside + petunidin-3-galactoside (25.83%) > cyanidin-3-glucoside (21.83%) > peonidin-3-glucoside (16.72%) > malvidin-3-galactoside (16.71%) > petunidin-3-glucoside (4.60%) (**Fig. 17**). The rate of decrease of anthocyanins also reflects their stability, with the higher rates of decrease for delphinidin and derivatives indicating their lower stability.

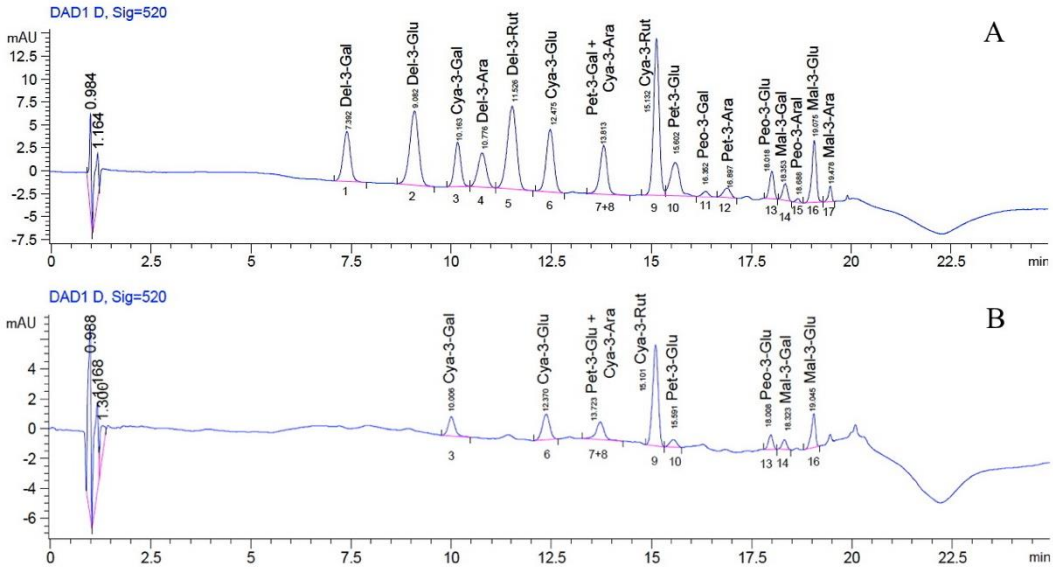


Fig. 17: Anthocyanin profile of undigested extract (sham digested) (**A**) and intestinal digested phase (**B**). The BBE was dissolved in water so to obtain a solution containing 320µg/ml of anthocyanins (determined through colorimetric assay and expressed as C3G equivalent) and 8-fold diluted before injection (sham digested extract). The chromatograms were extracted at 520nm. Peaks: **1** – *Delphinidin-3-Galactoside*; **2** – *Delphinidin-3-Glucoside*; **3** – *Cyanidin-3-Galactoside;* **4** – *Delphinidin-3-Arabinoside*; **5** – *Delphinidin-3-Rutinoside*; **6** – *Cyanidin-3-Glucoside*; **7+8** *Cyanidin-3-Arabinoside + Petunidin-3-Galactoside*; **9** – *Cyanidin-3-Rutinoside;* **10** – *Petunidin-3-Glucoside*; **11** – *Peonidin-3-Galactoside;* **12** – *Petunidin-3-Arabinoside*; **13** – *Peonidin-3-Glucoside*; **14**- *Malvidin-3-Galactoside*; **15** – *Peonidin-3-Arabinoside;* **16** – *Malvidin-3-Glucoside*; **17**- *Malvidin-3-Arabinoside*.

Anthocyanins			Sham digested extract	Intestinal phase	
peak number		**Retention Time min**	**mAU*s**	**mAU*s**	***RI (%)***
1	Del-3 Gal	7.382	68.61 ± 4.85	ND	-
2	Del 3-Glu	9.082	125.74 ± 8.89	ND	-
3	Cya 3-Gal	10.163	52.30 ± 3.69	15.67 ± 1.11 [a]	*29.97*
4	Del-3-Ara	10.776	54.36 ± 3.84	ND	-
5	Del-3-Rut	11.526	146.71 ± 10.37	ND	-
6	Cya-3-Glu	12.475	91.37 ± 6.46	19.95 ± 1.41 [a]	*21.83*
7 + 8	Pet-3-Gal + Cya-3-Ara	13.813	66.11 ± 4.67	17.08 ± 1.21 [a]	*25.82*
9	Cya-3-Rut	15.132	170.49 ± 12.05	50.18 ± 3.55 [a]	*29.42*
10	Pet-3-Glu	15.602	59.73 ± 4.22	2.75 ± 0.19 [a]	*4.60*
11	Peo-3-Gal	16.352	12.86 ± 0.90	ND	-
12	Pet-3-Ara	16.897	10.75 ± 0.76	ND	-
13	Peo-3-Glu	18.018	23.56 ± 1.66	3.94 ± 0.28 [a]	*16.72*
14	Mal-3-Gal	18.353	15.56 ± 1.10	2.60 ± 0.18 [a]	*16.71*
15	Peo-3-Ara	18.688	9.25 ± 0.65	ND	-
16	Mal-3-Glu	19.075	54.20 ± 3.83	14.38 ± 1.02 [a]	*26.53*
17	Mal-3-Ara	19.478	9.59 ± 0.68	ND	-
	TOTAL		**971.24 ± 68.68**	**126.55 ± 8.98 [b]**	***13.03***

Table 2: Stability of BBE anthocyanins before and after *in vitro* intestinal digestion. Data represent peak area (mAU*s) at 520 nm. RI: % is the recovery index of each anthocyanin *vs* the initial content in sham digested extract. Results are expressed as ± SD of three different experiments [a]p <0.05 vs respective anthocyanin from sham digested extract, [b]p <0.05 *vs* sham digested extract.

It is worthwhile to be aware that the anthocyanins present, after the intestinal digestion, were mostly cyanidin derivates, suggesting that probably this outcome is due to their chemical structure and higher initial content in BBE. Scientific literature reported that the presence of a higher number of substituent on B ring, especially hydroxyl groups (Cy < Dp), negatively influences anthocyanins stability during GI digestion, while the presence of alpha-methoxy group, like in malvidin, peonidin and petunidin derivatives, may protect them from degradation (Woodward *et al.*, 2009; Kamonpatana *et al.*, 2014). In addition, our results are in agreement with other studies which reported the detection of only two cyanidin derivates after the intestinal digestion of maqui berry containing cyanidin and delphinidin derivates (Lucas-Gonzalez *et al.*, 2016). At the same extend, Perez-Vicente and coworker reported a general decrease of all the individual anthocyanins after in vitro intestinal digestion of a sweet pomegranate juice, more marked for delphinidin glycosides respect to cyanidin derivates (Perez-Vicente *et al.*, 2002).

4.2.3 Effect of simulated digestion on the antioxidant activity of BBE

According to many studies, anthocyanins have very strong antioxidant proprieties (Dai and Mumper, 2010), but due to the chemical changes to which they undergo during the GI digestion this antioxidant capacity may change (Fang 2014). The antioxidant capacity of the digested and crude extracts was determinate by the Ferric Reducing Antioxidant Power (FRAP). This test is based on the ability to reduce Fe^{3+} in Fe^{2+} in acidic condition. The obtained data (**Fig. 18**) show, after the oral phase, a significant decrease (32 %) in the reducing power of the digested extract compared to the sham digested one, followed by a further 10 % decrease after the gastric digestion. In the final step of the simulated digestion, the reducing capability was significantly diminished (59 %) with respect to that of the undigested value. Our data agree with other *in vitro* studies which report similar results for currant, strawberry, and apple subjected to simulated GI digestion (Burgos-Edwards *et al.*, 2017; Bouayed *et al.*, 2011; Thomas-Valdés *et al.*, 2018).

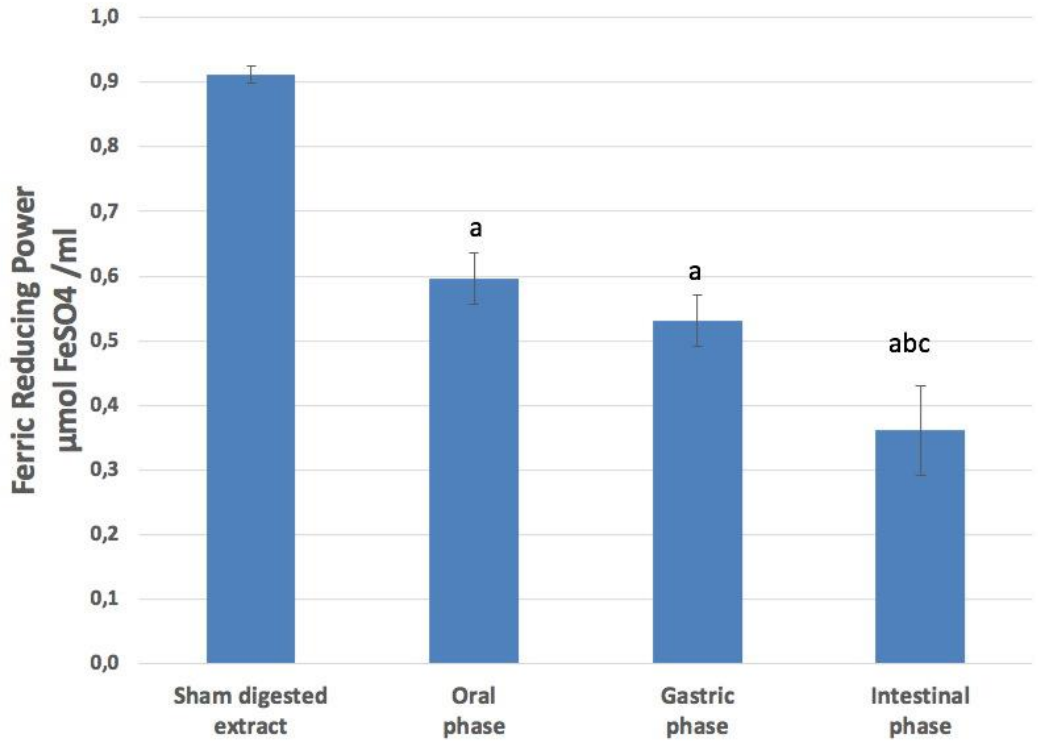


Fig. 18: FRAP Assay before and after *in vitro* digestion of BBE. Results are reported as µmol $FeSO_4$/ml of digesta or sham digested extract and expressed as mean ±SD of three independent experiments. [a] $p < 0.05$ vs sham digested extract; [b] $p < 0.05$ vs oral phase; [c] $p < 0.05$ vs gastric phase.

4.2.4 Pearson's correlation test

Recent studies have suggested that bioactivity of dietary polyphenols is hard to relate to only one specific class of compounds, due to their variability and complexity, so that the activity can be due to a synergic effect between the matrix, and the major and minor compounds present (Genskowsky *et al.*, 2016; Lucas-gonzales *et al.*, 2016). Thus, with the aim to clarify the relationship between the anthocyanins content of each sample and the correspondent antioxidant activity, the correlation coefficient was evaluated. The Pearson's correlation coefficient (**Tab. 3**) showed a strong and significant correlation between the anthocyanins content and the FRAP assay for the undigested extract and also for the extracts undergone oral and gastric digestion, suggesting that the anthocyanins are the major responsible for the antioxidant capacity of bilberry and blackcurrant extract. However, in the intestinal phase, results demonstrated a weak correlation,

suggesting that different degradation products, such as the phenolic compounds produced from anthocyanins during the simulated GI digestion, partly contribute to the antioxidant activity. In conclusion our outcomes support the fact that the lost in the anthocyanin concentration, due to the GI digestion, may be balanced by the formation of new antioxidant metabolites.

Antioxidant assay	**Sham digested**	**Oral phase**	**Gastric phase**	**Intestinal phase**
	TAC	*TAC*	*TAC*	*TAC*
FRAP	0.997	1.000	0.882	0.516

Tab. 3: Pearson's correlation coefficient between the total anthocyanins content (TAC), measured by the pH differential method, and the FRAP of BBE following sham digestion, oral, gastric and intestinal digestions.

4.2.5 Effect of digested BBE on TNF-α induced intestinal inflammation

The bioactivity of anthocyanins after GI digestion was evaluated on an *in vitro* model of inflamed intestinal epithelium, using the Caco-2 cell line and TNF-α as inflammation inductor agent. This is a well-established *in vitro* model for studying intestinal inflammatory mechanisms. In addition, we have previously demonstrated the protective effects of C3G (20 and 40μM) in the same *in vitro* experimental model (Ferrari *et al.*, 2016; 2017). However, the majority of available *in vitro* studies addressing the understanding of anthocyanin mechanisms of action have considered concentrations between 10 and 100 μM and these concentrations seem to be too high to be achieved in the target site under physiological conditions.

For this reason, we aimed to test if an anthocyanin-rich extract, such as BBE, can be effective in reducing *in vitro* intestinal inflammation also after (simulated) GI digestion. In our experiments, the highest intestinal digested extract concentration used was 1.5μg/ml (calculated with the pH differential method and expressed as C3G) since it corresponds to a starting sham digested concentration of around 20μM of C3G. Thus, the differentiated Caco-2 cells were pre-treated for 24h with different concentrations of the BBE intestinal phase (BBE-IP) (1.5, 0.75, 0.37 and 0.18 μg/ml expressed as C3G). After 24h, the cells were exposed to TNF-α for 6h.

It was demonstrated that stimulation of cells with various proinflammatory agents induces a rapid transcription of genes that modulate inflammation, mainly through the activation of the signaling pathways regulated by the redox sensitive transcription factor NF-κB. As shown in **Fig. 20**, the translocation of NF-κB into the nucleus was evidenced by the increased nuclear levels of the p65 NF-κB in cells exposed to TNF-α. BBE-IP pretreatment dose-dependently prevented TNF-α-induced nuclear translocation of p65, and the highest concentration tested was able to restore it to levels similar to those found in control cells. Treatment with BBE-IP alone didn't affect the nuclear translocation of NF-κB.

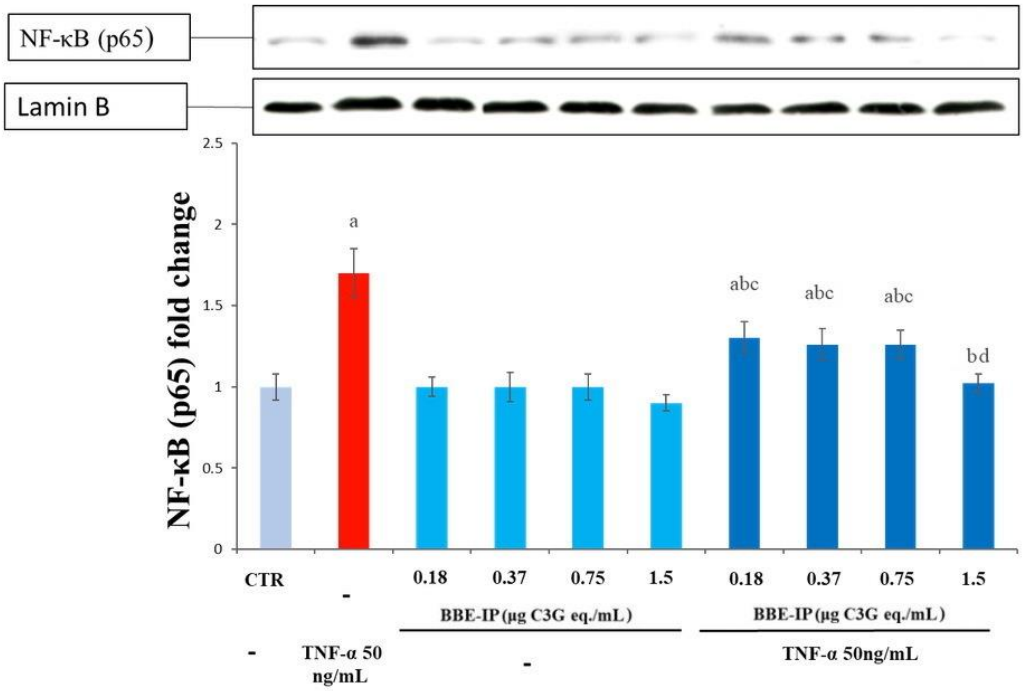


Fig. 19: Effect of intestinal digested BBE on p65 nuclear translocation. Differentiated Caco-2 cells were pretreated or not for 24h with different concentrations of the BBE-IP (1.5, 0.75, 0.37 and 0.18 µg/ml expressed as C3G). Cells were subsequently exposed to 50ng/mL TNF-α for 6 h. Cells treated with the growth medium and vehicles were used as controls (CTR). Results are reported as fold change against control and expressed as mean ± SD of three independent experiments. p65 values were normalized to the corresponding Lamin B value. $^{a}p < 0.05$ vs CTR; $^{b}p < 0.05$ vs TNF - α; $^{c}p < 0.05$ vs all BBE-IP unexposed to TNF-α; $^{d}p < 0.05$ vs all the lower BBE-IP exposed to TNF-α.

The binding sites of NF-κB are localized in the promoter regions of many genes codifying for proinflammatory cytokines and immunoregulatory mediators, such as IL-8 and IL-6. Our data demonstrated that TNF-α is able to induce IL-8 gene expression in a statically significant manner. Pre-treatment with the intestinal phase reduced significantly the level of the cytokine gene expression in a dose-dependent way. Interestingly, the highest concentration used was able to restore levels to those observed in control cells. Treatment with BBE-IP alone did not affect mRNA levels (**Fig. 20**).

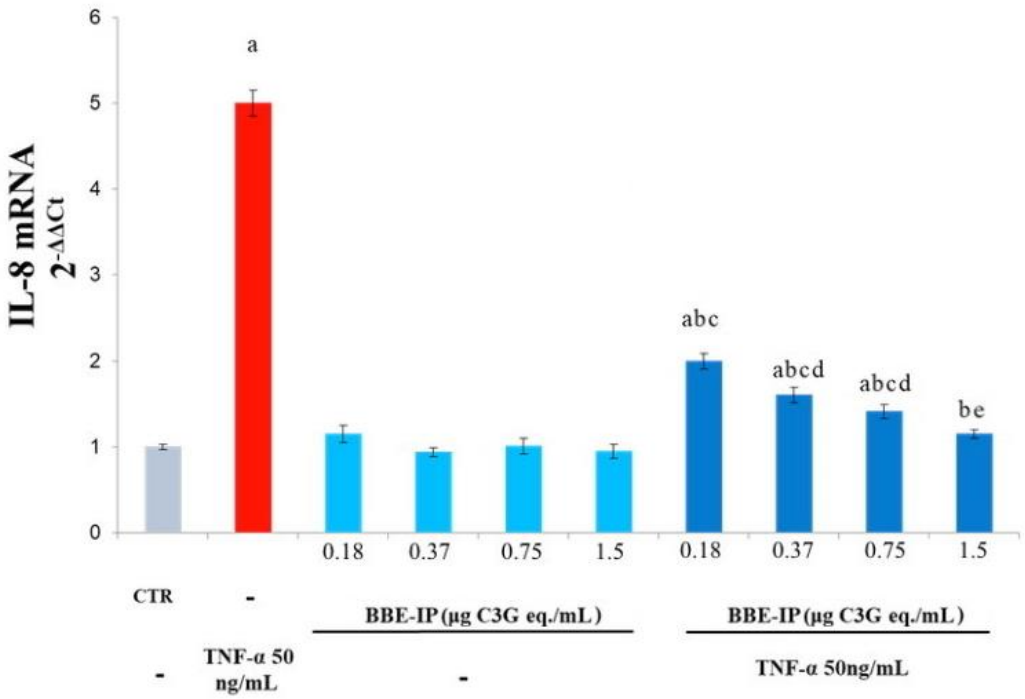


Fig. 20: Effect of intestinal digested BBE on TNF-α induced IL-8 gene expression. Differentiated Caco-2 cells were pretreated or not for 24h with different concentrations of the BBE-IP (1.5, 0.75, 0.37 and 0.18 µg/ml expressed as C3G). Cells were subsequently exposed to 50ng/mL TNF-α for 6 h. Cells treated with the growth medium and vehicles were used as controls (CTR). IL-8 mRNA expression was analyzed by real time PCR and data are expressed as $2^{-\Delta\Delta Ct}$ and normalized to CTR. 18S rRNA was used as housekeeping gene. [a]p <0.05 vs CTR; [b]p < 0.05 vs TNF -α; [c]p <0.05 vs all BBE-IP concentrations not exposed to TNF-α; [d]p <0.05 vs BBE-IP 0.18µg + TNF-α 50 ng/mL; [e]p <0.05 vs all the lower BBE-IP concentrations exposed to TNF-α.

The in vitro anti-inflammatory effect of the intestinal digestion was additionally evaluated through the mRNA level of another cytokine involved in the chronical intestinal inflammation, IL-6. This cytokine is reported to be highly present in the serum of patients with IBDs, and actively contributing to the intestinal inflammatory process (Seegert *et al.*, 2001).

The data shown in **fig. 21** confirmed the ability of TNF-α to induce the inflammatory process, as suggested by the increase of IL-6 mRNA levels. This increase was significantly reduced by the pre-treatment with the digested BBE at all the concentrations tested and in a dose-dependent way. In addition, the two highest concentrations used (1.5 and 0.75 µg/ml) reduced mRNA levels to those of control.

These data, taken together, confirm the capacity of the digested BBE to reduce and prevent the inflammation induced by TNF-α. Interestingly, this positive effect was seen even with very low concentrations (0.18 µg C3G eq./mL), suggesting that even after the drastic reduction in the anthocyanin concentration, caused by the GI digestion, BBE is able to exert its beneficial effect. However, we can speculate that the effect is probably due to a synergistic effect of the remaining anthocyanins and their degradation products.

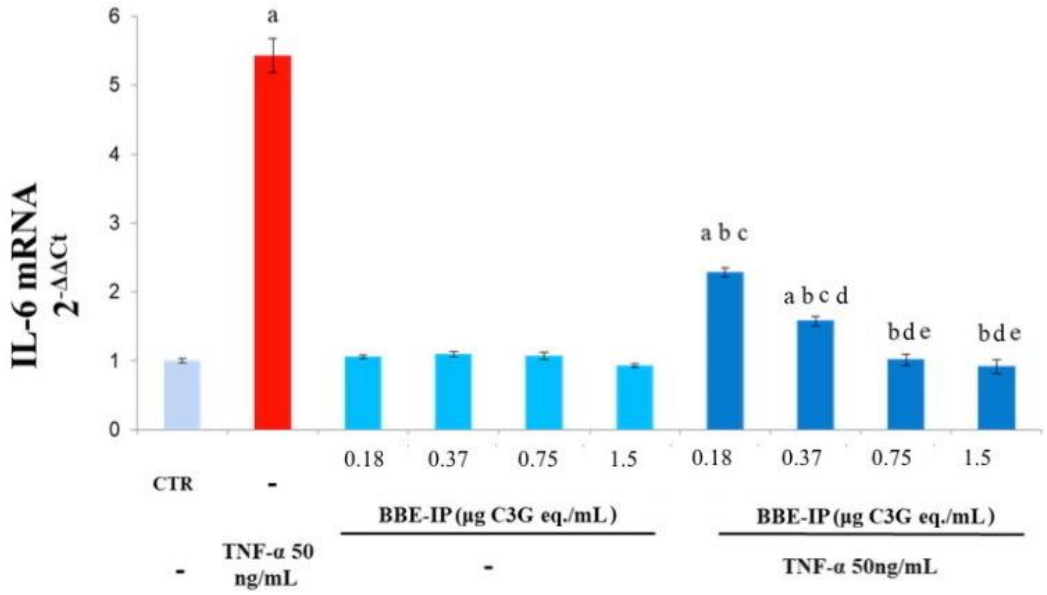


Fig. 21: Effect of intestinal digested BBE on TNF-α induced IL-6 gene expression. Differentiated Caco-2 cells were pretreated or not for 24h with different concentrations of the BBE-IP (1.5, 0.75, 0.37 and 0.18 µg/ml expressed as C3G). Cells were subsequently exposed to 50ng/mL TNF-α for 6 h. Cells treated with the growth medium and vehicles were used as controls (CTR). IL-6 mRNA expression was analyzed by real time PCR and data are expressed as 2-ΔΔCt and normalized to CTR. 18S rRNA was used as housekeeping gene; $^{a}p < 0.05$ vs CTR; $^{b}p < 0.05$ vs TNF -α; $^{c}p < 0.05$ vs all BBE-IP concentrations not exposed to TNF-α; $^{d}p < 0.05$ vs BBE-IP 0.18µg + TNF-α; $^{e}p < 0.05$ vs BBE-IP 0.37µg + TNF-α.

4.2.6 Effect of digested BBE on Nrf2/Keap1 pathway

Dietary anthocyanins have been demonstrated to exert indirect antioxidant activity through the induction of Keap1/Nrf2 system-mediated antioxidant and detoxification enzymes (Speciale *et al.*, 2018). In order to study the potential mechanisms underlying BBE-IP protective effects, we evaluated, in the same

experimental conditions, the nuclear localization of Nrf2 and its transcriptional activity, through the expression of NQO-1 gene.

Our results demonstrated that TNF-α didn't affect the activation of Nrf2 pathway as previously demonstrated (Ferrari *et al.*, 2016). Conversely, treatment with BBE-IP at 0.75 and 1.5µg/ml for 24 h was able to increase Nrf2 translocation, in a dose-dependent manner, in TNF-α-exposed Caco-2 cells. In addition, BBE-IP alone was able to induce Nrf2 pathway at 1.5µg/ml (**Fig. 22**).

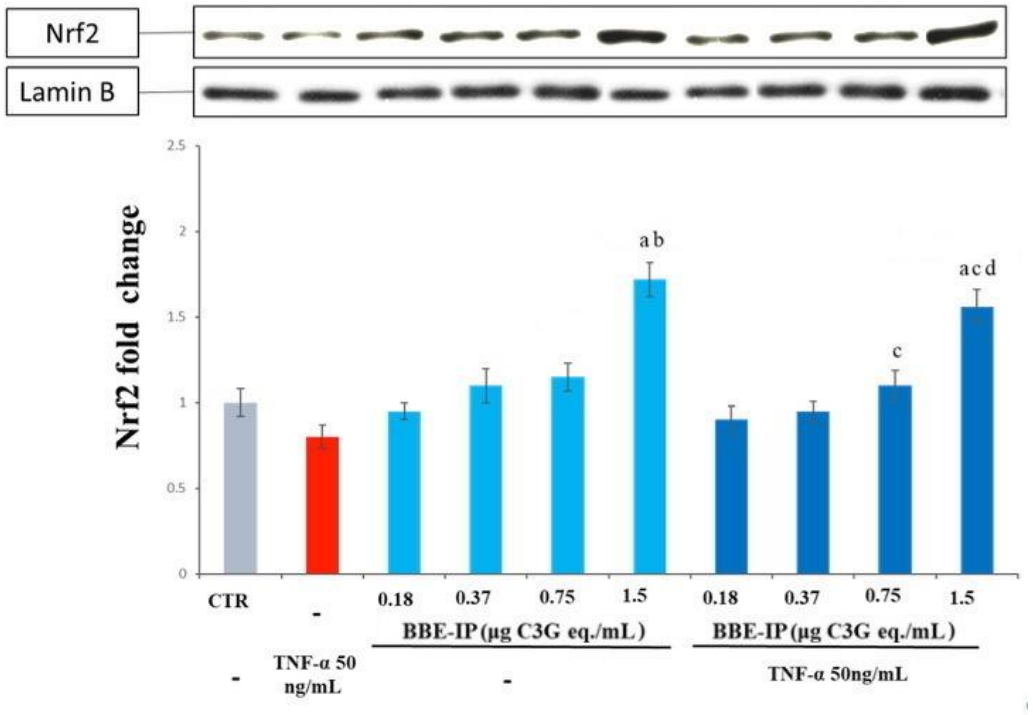


Fig. 22: Effect of intestinal digested BBE on nuclear Nrf2 translocation. Differentiated Caco-2 cells were pretreated or not for 24h with different concentrations of the BBE-IP (1.5, 0.75, 0.37 and 0.18 µg/ml expressed as C3G). Cells were subsequently exposed to 50ng/mL TNF-α for 6 h. Cells treated with the growth medium and vehicles were used as controls (CTR). Results are reported as fold change against control and expressed as mean ± SD of three independent experiments. Nrf2 values were normalized to the corresponding Lamin B value. $^{a}p < 0.05$ vs CTR; $^{b}p < 0.05$ vs all the lower BBE-IP concentrations not exposed to TNF-α; $^{c}p < 0.05$ vs TNF-α; $^{d}p < 0.05$ vs all the lower BBE-IP concentrations exposed to TNF-α.

In order to confirm BBE-IP ability to activate an antioxidant adaptive response, NQO-1 gene expression, representative of Nrf2 transcriptional activity, was evaluated. Data obtained (**Fig. 23**) showed that TNF-α did not affect mRNA levels of NQO-1, as elsewhere reported (Ferrari *et al.*, 2016). Cells pretreatment with BBE-IP was able to induce NQO-1 gene expression at concentrations starting from 0.75 µg C3G eq/mL and this effect was still evident even in the cells exposed to TNF-α. Taken together, these results support the hypothesis that Nrf2 signaling activation is involved in BBE-IP protective effect on epithelial inflammation induced by TNF-α.

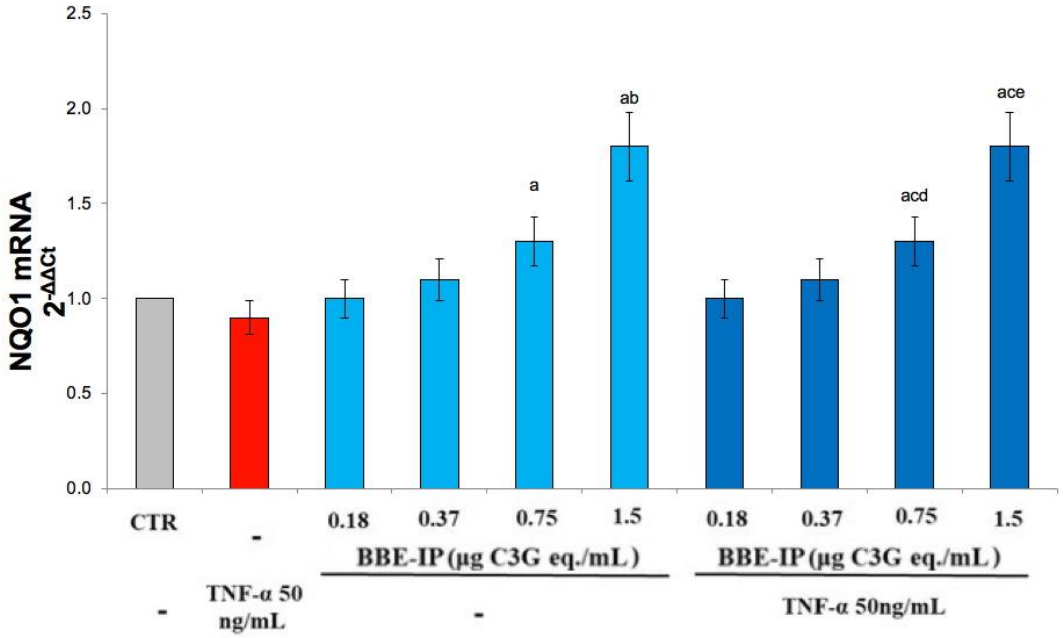


Fig. 23: Effect of intestinal digested BBE NQO-1 gene expression. Differentiated Caco-2 cells were pretreated or not for 24h with different concentrations of the BBE-IP (1.5, 0.75, 0.37 and 0.18 µg/ml expressed as C3G). Cells were subsequently exposed to 50ng/mL TNF-α for 6 h. Cells treated with the growth medium and vehicles were used as controls (CTR). NQO-1 mRNA expression was analyzed by real time PCR and data are expressed as 2-ΔΔCt and normalized to CTR. 18S rRNA was used as housekeeping gene. [a]p <0.05 vs CTR; [b]p < 0.05 vs all BBE-IP concentrations not exposed to TNF-α; [c]p <0.05 vs TNF-α; [d]p < 0.05 vs BBE-IP 0.18 µg + TNF-α; [e]p <0.05 vs all the lower BBE-IP concentrations exposed to TNF-α.

5. *CONCLUSIONS*

IBD represents a group of chronic diseases characterized by an altered immune response and a persistent inflammatory state. The molecular mechanisms underlying IBD are still partly unknow. Since IBD is mainly found in industrialized areas of the world, it suggests that environmental factors, like sedentary lifestyle and diet, together with genetic risk factors can influence disease development and progression (Kreuter *et al.*, 2019). In addition, a correlation between overweight or obesity and an increased risk of IBD has been reported (Singh *et al.*, 2017). In fact, circulating FFAs are associated with enhanced pro-inflammatory cytokines production by a number of cells including macrophages, endothelial cells, and intestinal epithelial cells (Suganami *et al.*, 2005, Mendall and Gunasekera, 2011). Several *in vitro* and *in vivo* studies have shown that higher levels of intracellular FFAs determine greater expression of inflammatory mediators with a consequent activation of inflammatory signalling pathways, such as NF-κB, and cause overproduction of ROS, leading to an oxidative stress condition (Tian *et al.*, 2017).

In the last decades, there has been increasing interest on micronutrients and natural substances, present in fruits and vegetables, as a source of therapeutic agents. Several epidemiological studies reported a beneficial role of anthocyanins, a group of plant polyphenols, in numerous diseases including IBD (Farzaei *et al.*, 2018) and their beneficial effects are associated principally to the antioxidant and anti-inflammatory proprieties. In particular, anthocyanins exert antioxidant activity directly, scavenging reactive oxygen and/or nitrogen species, and indirectly through the modulation of ARE/Nrf2 pathway and then the transcription of enzymes involved in the activation of the adaptive cellular response (Speciale *et al.*, 2013)

In the first part of this study we demonstrated the protective effect of C3G on PA-induced intestinal inflammation and the molecular mechanisms underlying this effect. From the data obtained, it is evident that C3G is able to inhibit the proinflammatory NF-κB pathway induced by PA. Indeed PA-induced NF-κB and IKKα/β, which regulate NF-κB activation, are significantly lower in Caco-2 cells pre-treated with C3G. Furthermore, the inhibitory effects of C3G on NF-κB pathway was confirmed by the evaluation of some NF-κB downstream

inflammatory modulators. In fact, our results showed an upregulation of the interleukins (IL-6 and IL-8) in cells exposed to PA for 6 h, while the pre-treatment with the C3G significantly reduced, in a dose-dependent way, the mRNA levels of both the interleukins. Additionally, the COX-2 protein levels were evaluated, and the outcome supports, once again, the anti-inflammatory activity of C3G against PA-induced damage.

In our experimental model, the exposure of Caco-2 to PA for 6 h induced an overproduction of intracellular ROS, thus leading to an oxidative stress condition. In addition, PA diminished the levels of TAA (and index of the intracellular redox status) if compared to the control cells. The pre-treatment with C3G for 24 h significantly reduced the ROS levels and improved intracellular antioxidant markers, so demonstrating a shifting of the redox balance from an oxidative to a reduced state.

However, it is important to note that the pre-treatment with C3G alone showed a decrease of ROS levels and, at same time, increased intracellular antioxidant markers than control, even in absence of external stimuli, so supporting that, also in normal gut epithelium cells, C3G is able to induce an adaptive antioxidant response.

Our data confirmed the activation of the cellular antioxidant adaptive response modulated by the Nrf2 transcription factor in cells pre-treated with C3G. In fact, C3G increased Nrf2 nuclear levels and the mRNA levels of its downstream gene NQO-1, irrespective to the presence of PA. These effects were reported also in other experimental models, so demonstrating an indirect antioxidant mechanism for anthocyanin through the upregulation of cytoprotective and antioxidant proteins (Aboonabi and Singh, 2015; Ferrari *et al.*, 2016, Speciale *et al.*, 2013). This seems to be relevant since it has been proposed that anthocyanins should be able to contribute to the quenching of NF-κB activation as a downstream effect, at least in part, of the increased Nrf2 response, so supporting the existence of a crosstalk between these two transcription factors.

Lastly, PA induces a damage on the intestinal barrier as suggested by the increased paracellular permeability in Caco-2 monolayer cell exposed to PA for 6 h. On the contrary, the pre-treatment with C3G counteract this effect stabilizing the intestinal barrier.

The findings of the first part of this study all demonstrate the protective effect of C3G against the activation of the inflammatory process resulting from the induction of NF-κB pathway by PA, and these beneficial effects appear to be due to its ability to activate cellular protective responses modulated by Nrf2.

However, several studies have reported that bioavailability of anthocyanins is very poor, mostly due to the acidic pH and the biotransformation during the gastrointestinal digestion (Fang 2014). Until now, the majority of available studies on anthocyanins activity used purified molecules at high concentrations which could be not reached following the GI digestion (Day *et al.*, 2014).

For these reasons, in the second part of this study, we investigated the stability, as well as the changes in antioxidant activity, of an anthocyanins-enriched extract, using an *in vitro* simulated GI digestion of a standardized extract obtained from bilberry and blackcurrant (BBE).

Our data demonstrates that anthocyanins are very unstable under simulated *in vitro* GI digestion, as suggested by the very low recovery index in comparison to the undigested extract. HPLC analysis further confirmed the high instability of the anthocyanins in intestinal environment reporting a 13% of recovery index. Additionally, from the initial 17 anthocyanins, only 9 of them were detected after the GI digestion, mostly cyanidin derivates suggesting how the chemical structure possesses a high relevance on their stability. The results also confirm that fewer free hydroxyl groups and more methoxy groups in the B-ring improve anthocyanin stability.

The elevated loss in anthocyanins during GI digestion is related to a decrease of BBE reducing power as suggested by the FRAP assay. However, the Pearson's correlation test evidences, after the intestinal phase, a weak correlation between the FRAP assay and the total anthocyanins content of the GI digested BBE, suggesting thus that the antioxidant power is not only due to the anthocyanins content but probably to a synergic effect between the present compounds and newly formed metabolites.

In a previous study we have demonstrated the protective effect of C3G (20 and 40 μM) on TNF-α induced intestinal inflammation (Ferrari *et al.*, 2016). Since these

concentrations should be too high to be reached in the target site, we tested if the anthocyanin rich extract BBE can be also effective in reducing *in vitro* intestinal inflammation after simulated GI digestion. Our data confirmed the inhibitory effect of digested BBE on the activation and transcriptional activity of NF-κB induced by TNF-α in Caco-2 cells. In fact, we observed a significant reduction of the nuclear levels of p65/NF-κB and interleukins (IL-6, IL-8) gene expression in cell pre-treated with the BBE intestinal phase. The research continued with the study of the Nrf2 pathway. Also, in this case the results allowed us to confirm the ability of digested BBE to activate the adaptive antioxidant cellular response even at very low concentration (0.75 µg/mL expressed as C3G eq./ml). Indeed, in Caco-2 pre-treated with BBE intestinal phase for 24 h there is a significant increase of the Nrf2 nuclear levels as well as of NQO-1 gene expression. Furthermore, these data support again the hypothesis of functional interactions between the Nrf2 and NF-κB pathways in the protective effects exerted by anthocyanins.

In conclusion, our research contributes to clarify the molecular mechanisms underlying the protective effects of anthocyanins on intestinal inflammation and suggests the possible protective role of these compounds in the prevention of IBD. In addition, even if anthocyanins are very unstable under GI conditions, our result confirm the anti-inflammatory and antioxidant activities of digested anthocyanins-based products following GI digestion. We can speculate that the loss in the anthocyanin concentration, exerted by the GI digestion, may be balanced by the formation of new antioxidant metabolites

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