UNIVERSITÀ DEGLI STUDI DI MILANO

SCUOLA DI DOTTORATO DI RICERCA IN SCIENZE BIOCHIMICHE, NUTRIZIONALI E METABOLICHE

DOTTORATO DI RICERCA IN BIOCHIMICA
CICLO XXVI

TESI DI DOTTORATO DI RICERCA

IMMUNOMODULATORY EFFECTS OF PHENOLIC COMPOUNDS FROM EXTRA-VIRGIN OLIVE OIL ON INTESTINAL EPITHELIAL CELLS

BIO 10

ERI MUTO

Matr. R09100

TUTOR: Dott. DONATELLA CARUSO

COORDINATORE : Prof. FRANCESCO BONOMI

ANNO ACCADEMICO 2012/2013

INDEX

CHAPTER 1

INTRODUCTION

1. 1. 1. The Gut

The gut is traditionally considered as an organ that mediates nutrient digestion, absorption. Besides the gut host the largest collection of microbes and the ability of the immune system to coevolve with the microbiota during postnatal life allow the host and microbiota to coexist in a mutually beneficial relationship [(1)]. As show in Fig 1, the intestinal epithelial cell layers form villi and crypt structures and are composed of different cell lineages. In the intestine there are two cells which have secretory functions, one is goblet cells which secrete mucus, other is paneth cells which is the main secretors of antimicrobial peptides. On the other hand the colon has much higher bacterial load and a markedly different immune cell composition. The colon contains only crypts, no villi. Also, there are no paneth cells, which mean that enterocytes have a much more important contribution to antimicrobial peptide production. However, there is a high prevalence of goblet cells [(2)].

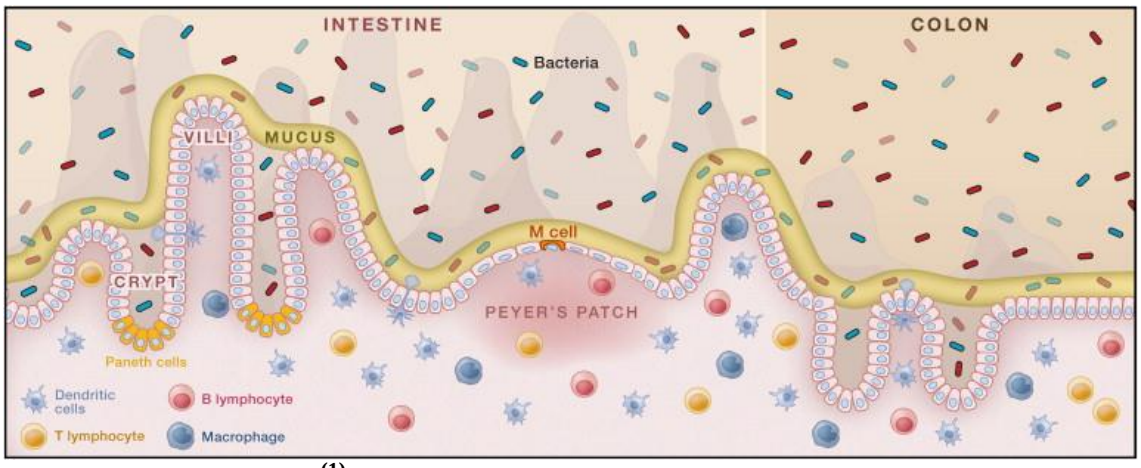


Fig 1 The gut Landscape [(1)]

Furthermore, recent research has been suggesting that gut tract has more roles and contribution on our health. For instance, the duodenum owing to the ability to sense nutrient influx and trigger negative feedback loops to inhibit glucose production and food intake to maintain metabolic homeostasis [3]. Additionally from pathological point of view, Henao-Mejia et al. reported the association of the gut microbiota and exacerbation of hepatic steatosis and inflammation through influx of toll-like receptor 4 (TLR4) and toll-like receptor 9 (TLR9) agonists into the portal circulation, which leads the enhancement of hepatic tumour-necrosis factor α (TNF-α) expression and drives progression of nonalcoholic steatohepatitis (NASH) [4]. As just described one of the example, the intestinal microbiota has been implicated in the pathogenesis of several autoimmune and chronic inflammatory diseases (Fig 2).

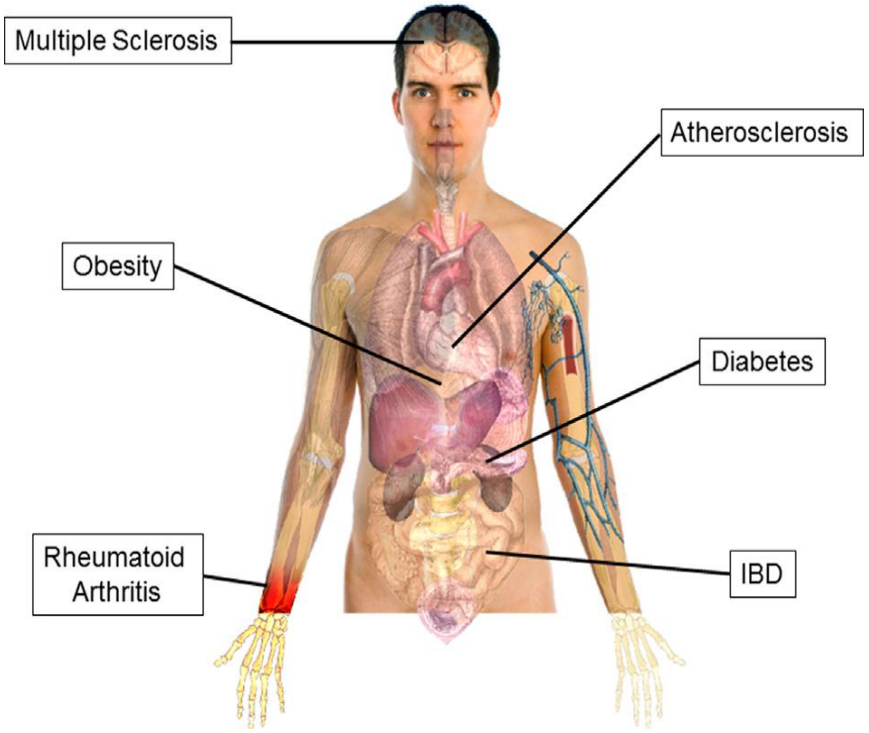


Fig 2 Autoimmune and chronic inflammatory diseases in which the intestinal microbiota has been implicated [5]

1. 1. 2. Inflammation in intestine

As mentioned at 1.1.1. section, the human gut harbors a large collection of commensal bacteria, such as 100 different species of bacteria and around 100 trillion of enterobacteria, the intestines (especially colonic) lumen serves as a reservoir of lipopolysaccharide (LPS: ~50 μg/ml in the colon) [6; 7], which is constantly produced by gram-negative bacteria. But regardless of the huge number of enterobacteria and of the presence of LPS from gram-negative bacteria, intestine usually does not develop inflammation. This is due to the state of immune tolerance to resident intestinal microbes. On the other hand, intestinal inflammation can be also considered as natural and protective process, which is crucial to maintain gut integrity and functioning [8] in the case of food intoxication, stress and infection. Intestinal epithelial cells also respond to various inflammatory mediators secreted by the immune cells, by modulating the epithelial monolayer permeability and secretion. Then, further amplifying the inflammatory process [9].

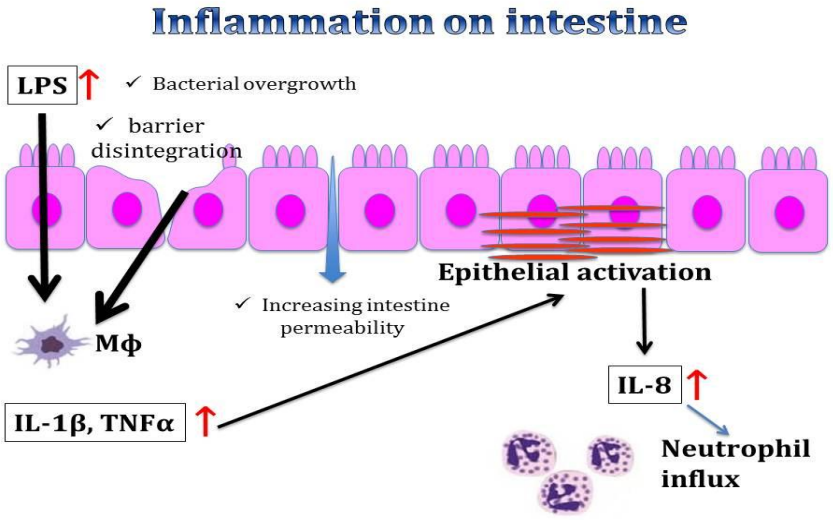


Fig 3 Inflammation on intestine

As shown in Fig 3, one of the initial steps of the inflammatory response could be started from bacterial LPS. The LPS is the major constituents of the outer membrane of gram-negative bacteria and a specific ligand of TLR4. The LPS is contributing to the inflammatory reaction through two processes. First, LPS directly binds to the intestinal epithelial cell surface TLR4 and recruitment of nuclear factor-kappa B (NF-κB) from the cytosol to the nucleus. Then, once NF-κB translocation occurs, transcription of genes encoding pro-inflammatory cytokines, such as interleukin-1beta (IL-1β), and other genes related to inflammation, such as cyclooxygenase 2 (COX2) and inducible nitric oxide synthase (iNOS), would be committed. In the other way, LPS induces the activation of monocytes and macrophages involving the TLR4 and results in the production of key pro-inflammatory cytokines, IL-1, IL-6, IL-8, and TNF-α, which mediate further inflammatory reactions [10; 11].

The cytokine IL-1β is a multifunctional cytokine playing a major role both in the initiation and the amplification of many inflammatory conditions [12] and it has been

found that the inflammatory bowel disease (IBD) patients retain high concentrations of the cytokines in intestine [13]. As mention above, IL-1β is released by various cell types including monocytes–macrophages, neutrophils and endothelial cells and mediates important features of IBD, such as the generation of fever, the reduction of appetite, the release of mediators and the recruitment of leukocytes [8] . The *in vitro* study demonstrated that the effects of IL-1β, TNF-α, interferon- γ (IFN-γ) and LPS effects on intestinal epithelial cells consist of activation of intracellular cascades, leading to an increased transcriptional activity and the secretion of interleukin IL-8 [14].

In addition to its direct immune-activating effects, both LPS and IL-1β could cause an increase in the paracellular permeability through defects in tight junction function or assembly [15; 16]

1. 2. Concept of Mediterranean diet and influence on human health

Mediterranean diet is characterized by high consumption of fresh vegetable (fruit, vegetables, breads, other forms of cereals, potatoes, beans, nuts, and seeds), fresh fruit as the typical daily dessert, olive oil as the principal source of fat, dairy products (principally cheese and yogurt), and fish and poultry are consumed with low to moderate amounts, zero to four eggs are consumed weekly, red meat consumed in low amounts, and wine consumed in low to moderate amounts, normally with meals (Fig 4). This diet is low in saturated fat, with total fat ranging from 25% to 35% of energy throughout the region[17]. This dietary pattern has been typically recognized around Mediterranean area, it was originally represented as the food habits of southern Italy and Greece around the 1970s. Then, Keys et al reported the study which indicated the

association between a Mediterranean diet and a reduction in all-cause mortality, especially a reduction in cardiovascular mortality in the Seven Countries Study [18]. Studies to date, many papers reported the benefit of "Mediterranean diet" on the human health subjects. Recently, Estruch *et al also* demonstrated in the paper which shows the idea and the impact of the Mediterranean diet for human health [19]. This may also be related to the high consumption characteristic of the Mediterranean diet which is rich in olive oil supplies 10–20 mg of phenols per day [20].

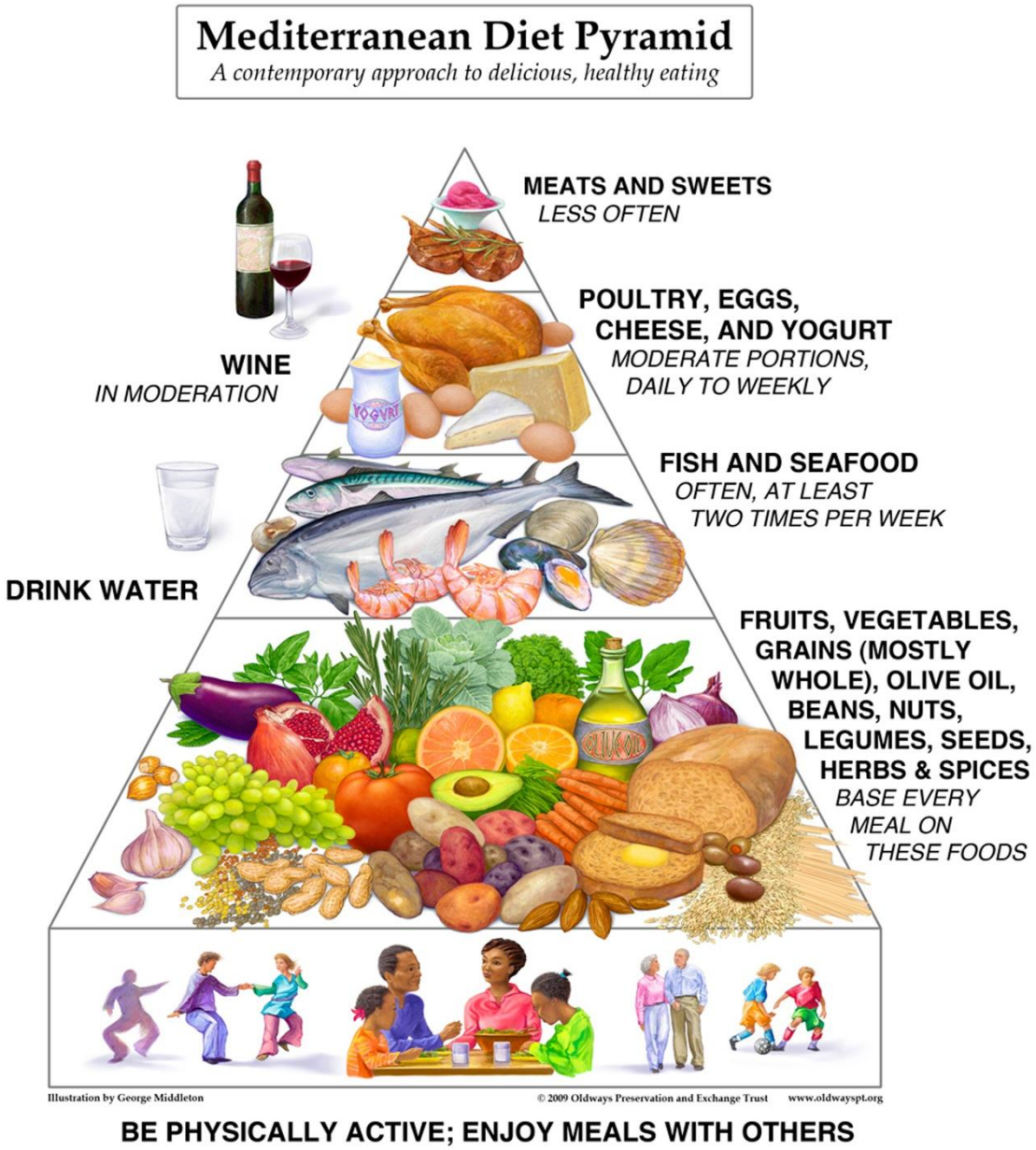


Fig 4 The Mediterranean diet pyramid

Japan is one of the country with long living people. Japanese diets are also characterized by high consumption of rice, fresh fish, beans, fiber- rich foods and fermented foods such as “miso” and “natto”. Recently, Pallauf et al mentioned in his review that the similarity of food consumption habits in Asian and the Mediterranean diets (Fig 5) and the contribution of low incidence of cardiovascular disorders [21]. This

fact indicates that the contribution of quality of foods and the contents of nutrients in what we are consuming during our life time really impact on our future prevalence rate of diseases.

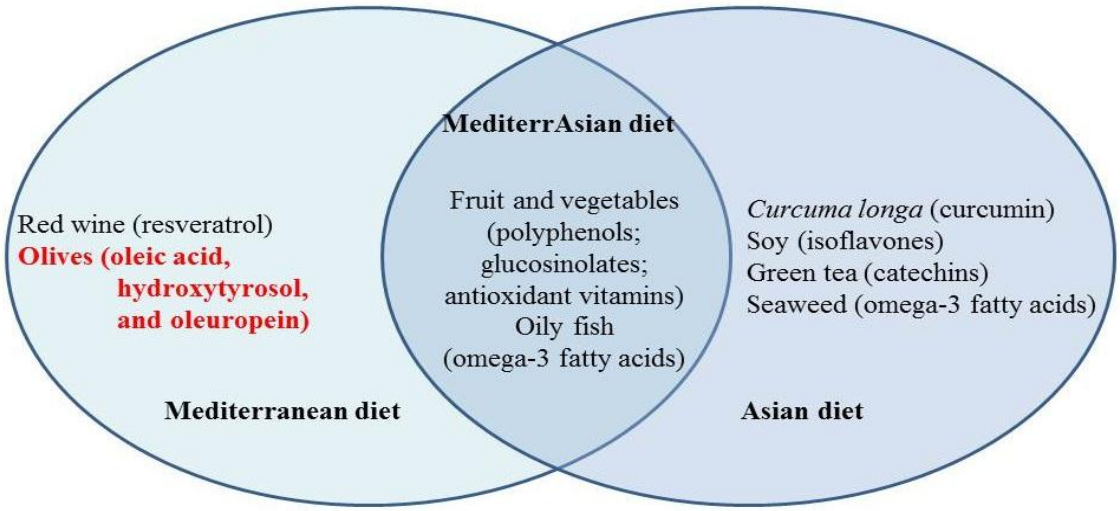


Fig 5 Important food items and their corresponding constituents in the Mediterranean, Asian, and so-called MediterrAsian diets [(21)]

1. 3. Olive oil

As we already know olive oil is the principal fat source of the traditional Mediterranean diet, the range between 25-50ml per day [(22)], a regimen that has been associated with different beneficial effects on human health. Furthermore, the phenolic content and profiles of the considered Italian cultivars greatly depended on place of growing and olive maturity index.

1. 3. 1. Components and composition

Olive oil contains very high level of monounsaturated fatty acids (MUFA) more notably oleic acid, and more than 300 minor components are present. The health

benefits of olive oil, however, may be affected by both the quality and the culinary use of olive oil. The MUFA content does not vary significantly between different qualities of olive oil, but olive oil also contains a "nonsaponifiable" fraction comprising various triterpenes (mostly squalene), phytosterols (mostly b-sitosterol), tocopherols (mostly vitamin E), and phenolic compounds. Among olive oils, extra-virgin olive oil is particularly rich in phenolic compounds. In the Mediterranean area, people put importance on the quality of Olive oil, because in extra virgin olive oil at least 30 phenolic compounds are presents [23] but the ordinary refined variety olive oil poorly contains such beneficial compounds. The phenolic composition of extra extra-virgin olive oil may vary in quantity (50 to 1000 mg/kg) and quality, depending on the olive variety, degree of ripeness, soil composition, climate, processing techniques, and storage [24; 25]. As shown in Fig 6, the major phenolic compounds in olive oil are oleuropein-aglycon, Ligstroside-aglycon, hydroxytyrosol (2-(3,4-dihydroxyphenyl)ethanol) and tyrosol (2-(4-hydroxyphenyl)ethanol).

In vivo, approximately 55–66 mol/100 mol of olive oil phenols were absorbed in the small intestine. Then after, less than 4 mol/100 mol of ingested tyrosol and hydroxytyrosol will reach the colon. However, the amount of ingested oleuropein aglycon and ligstroside aglycon reaching the colon remains to be elucidated [26].

In the present study, we made a characterization of olive oil phenols composition with the above-mentioned 4 major phenolic compounds together with apigenin and luteolin.

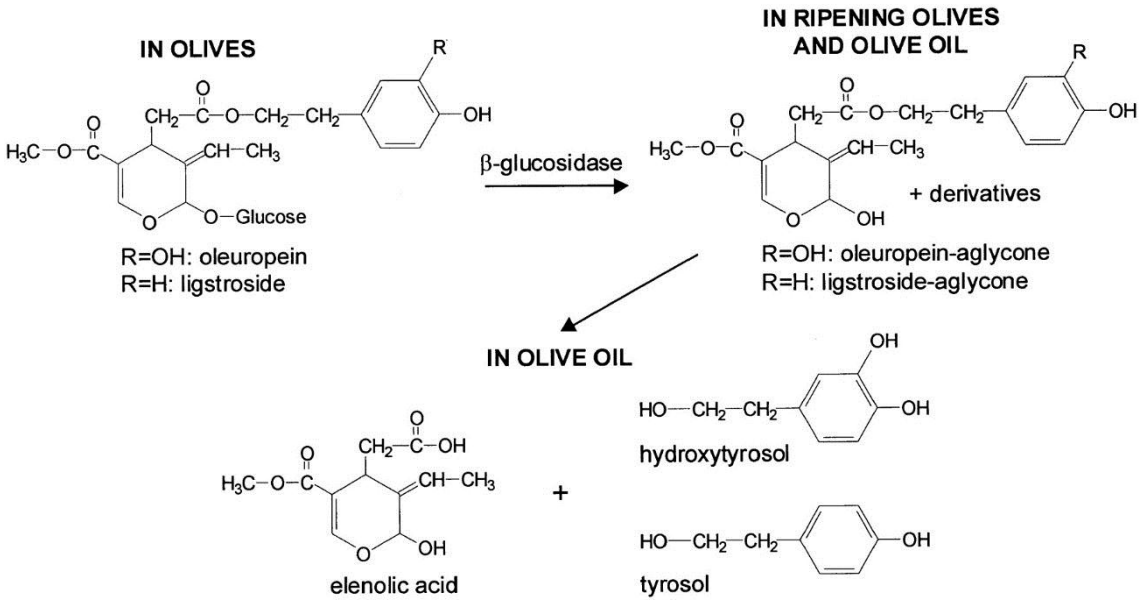


Fig 6 Structures of phenols present in olives and olive oil, their degradation into aglycon during ripening, and hydrolysis of aglycon into tyrosol and hydroxytyrosol[27]

1. 3. 2. Biological activities

In-vitro and *in vivo* studies suggested that the phenolic compounds present in olive oil act as an effective antioxidants and radical scavenger properties. Typically, hydroxytyrosol is a superior antioxidant and radical scavenger than oleuropein and tyrosol [28]. As shown in Fig 7, olive oil phenols have biological activities which may be important in the reduction in risk and severity of certain chronic diseases. So far, many papers have demonstrated the beneficial effects of olive oil for human health and diseases prevention such as, cardiovascular diseases, hypertension, cancer prevention, e.g. colon cancer and breast cancer, and immune response. To see the potential importance of individual phenolic compounds in virgin olive oil include lignans, which are associated with reduced breast cancer risk, [29; 30] hydroxytyrosol, which has the cardio protective and anticancer activity and the anti-inflammatory potential in

experimental systems [31; 32]. Furthermore, recent studies demonstrated that, apigenin has a biological activity as anti-diabetic, anti-Alzheimer's disease and anti-inflammatory properties [33] and luteolin is demonstrated to have the anti-inflammatory properties in gestational tissues by interacting with NF-κB, AP-1 and their downstream targets [34].

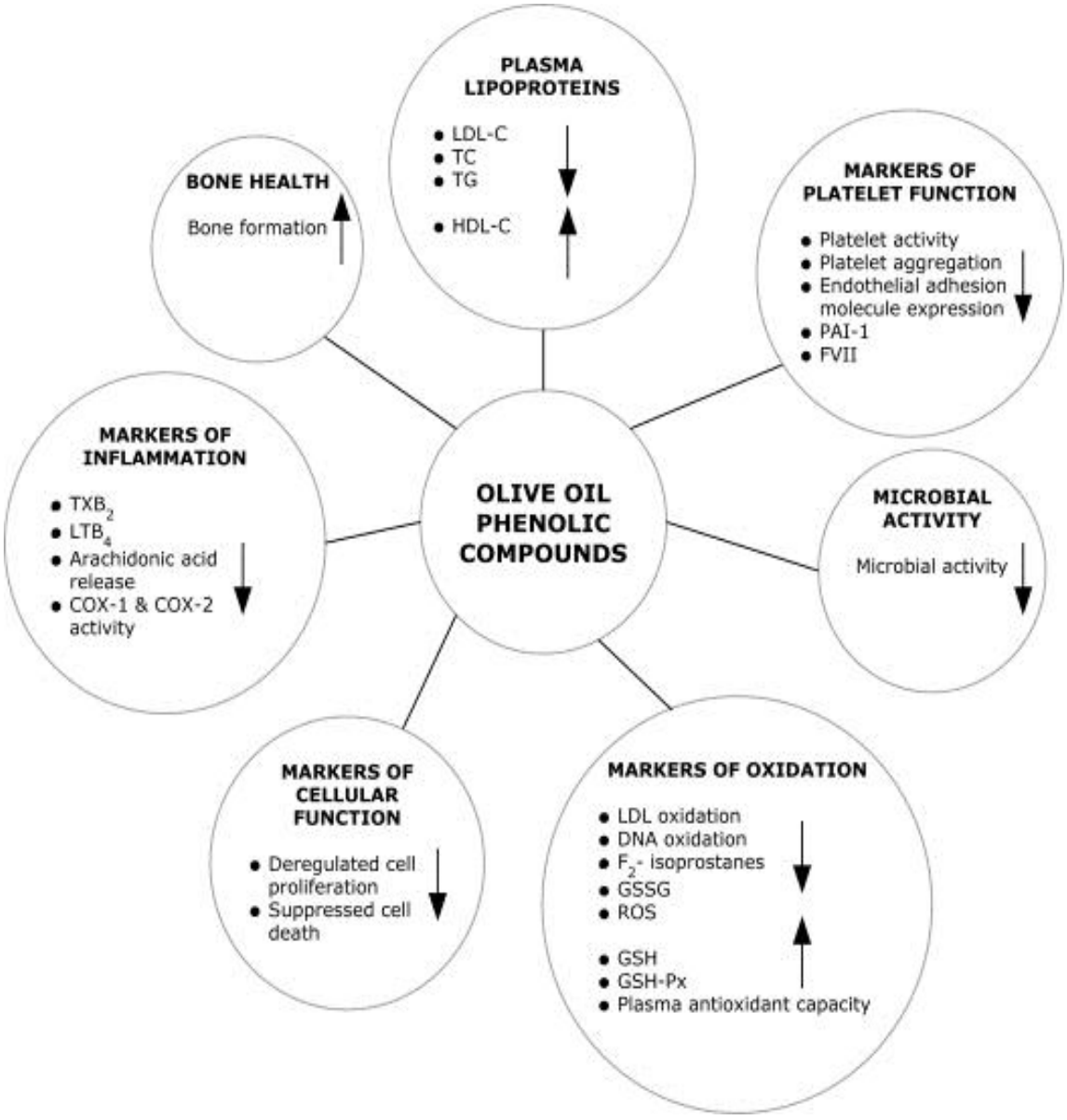


Fig 7 Biological activities of olive oil phenol compounds [35]

1. 3. 2. a. In-vitro studies

Gill et al. demonstrated that a phenol mixture extracted from virgin olive oil is capable of inhibiting, at least *in vitro*, multiple key stages in the colon carcinogenesis pathway including initiation, promotion and metastasis[36]. Moreover, several *in-vitro* experiments have demonstrated the potent antioxidant activity of olive oil phenols on different cellular systems [37; 38; 39; 40; 41]. These data suggest that a significant part of the chemopreventive ability of olive oil on the malignancy could be derived from the minor phenolic compounds that possess these antioxidant properties.

Scoditti et al demonstrated on that how olive oil and red wine polyphenols reduce inflammatory angiogenesis in cultured endothelial cells. They claimed that it is through metalloproteinase-9 (MMP-9) and COX-2 inhibition[42]. The impact of olive oil on the immune system has also been investigated in the isolated oleuropein fraction of the oil. In the isolated mouse macrophages, oleuropein enhanced nitrite production following an LPS challenge concentration-dependently and during endotoxin challenge, oleuropein potentiates the macrophage-mediated response, resulting in higher nitrous oxide production[43] In the freshly isolated human monocytes, hydroxytyrosol increased TNF-α production and considerably reduced the expression of COX2 at both the mRNA and protein level and the reduction of prostaglandin E2 (PGE2) released into the culture medium[44].

1. 3. 2. b. In-vivo studies

In rat models, dietary olive oil prevented the development of aberrant crypt foci and colon carcinomas suggesting that olive oil may have chemo preventive activity in colon carcinogenesis[45]. Sanchez-Fidalgo reported that extra virgin olive oil diet has

protective/preventive effects in the ulcerative colitis-associated colorectal cancer. The mice which DSS administrated and fed an extra virgin olive oil diet presented a lower immunoreactivity of β-catenin, a reduction of pro-inflammatory cytokines levels together with no modification of p53 expression and, a reduction of COX-2 and iNOS protein expression in the colonic tissue, when compared with the sunflower oil diet fed mice[46].

Hydroxytyrosol has been reported to exhibit significant anti-inflammatory actions and attenuate TNF-α and IL-1β expression in an animal model of inflammation, which are pro-inflammatory cytokines often observed in inflammatory disease[47]. However, we have to be aware of the previous paper where the authors demonstrated the administration of hydroxytyrosol after being extracted from its original matrix could be not only non-beneficial but indeed harmful for health[48]

1. 3. 2. c. In clinical studies

In-vivo studies demonstrated that olive oil phenols are well absorbed in human. The analysis of olive oil phenols absorption has demonstrated that both free tyrosol and hydroxytyrosol levels are correlated with their intake[49] and consequently homovanillyl alcohol levels [50] in urine. Heretofore, there are many studies that olive oil has the ability to decrease cardiovascular risk factors. The therapeutic properties of olive oil are often attributed to its high levels of MUFA. Diets rich in olive oil have been shown to be more effective in lowering total cholesterol and low density lipoprotein (LDL) cholesterol than conventional dietary treatments not containing high levels of MUFA[51; 52; 53]. Moreover, there are some evidences that olive oils have abilities to modify gene expression coding for proteins participating in cellular mechanisms involved in

oxidative stress resistance [54], lipid metabolism[55], other atherosclerosis-related traits/pathways [56; 57], and inflammation. Camargo et al. also showed that intake of virgin olive oil based breakfast, which is rich in phenol compounds, is able to repress *in vivo* expression of several pro-inflammatory genes, thereby switching activity of peripheral blood mononuclear cells to a less deleterious inflammatory profile[58]. On other study, Bonani et al. showed that extra virgin olive oil consumption reduces inflammatory markers and increase serum antioxidant capacity at postprandial state [59]. Furthermore, Blanco-Colio et al has showed that a virgin olive oil-rich Mediterranean diet, during postprandial state, reduces inflammatory response of peripheral blood mononuclear cells mediated by transcription factor NF-κB [60; 61]. Also, Lucas et al. mentioned that several clinical trials disclosed that the consumption of olive oil can influence different inflammatory markers such as, IL-7, COX1 and COX2[62] in humans.

1. 3. 3 Our laboratory observation and course

In our laboratory, we have previously reported the protective role of olive oil on early atherogenesis, by down-regulation of the expression of adhesion molecules in endothelial cells and inhibition of proteolytic activity of circulating cells [63]. From the mechanistic point of view, we demonstrated that olive oil polyphenols interfere with NF-*κ*B signalling in monocytes, hence exhibiting anti-inflammatory action [64].

As shown in Fig 8, olive oil phenolic components are mainly absorbed at the intestinal level [27]. Therefore, gastric and intestinal epithelial cells may be exposed to these compounds at high concentrations.

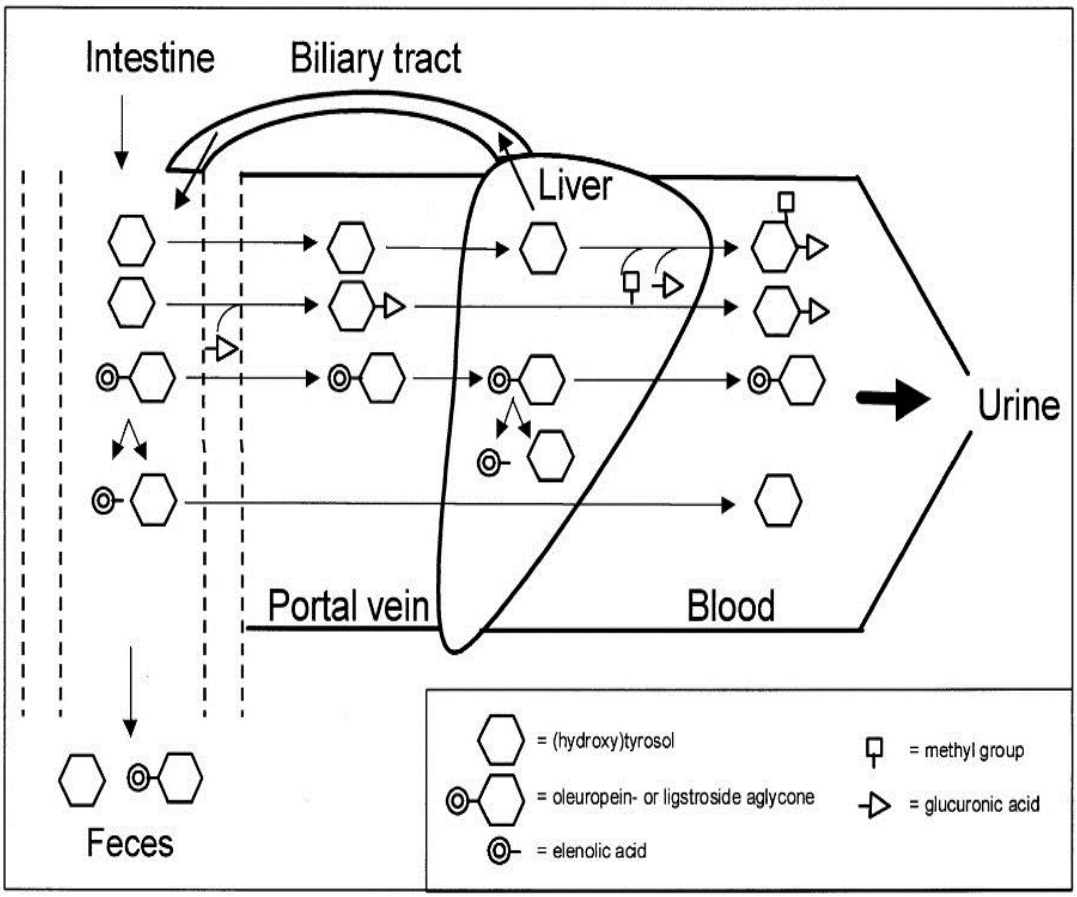


Fig 8 Schematic presentation of the possible metabolism of olive oil phenols [(27)]

Thus, we investigated whether phenolic extracts from extra virgin olive oils exert anti-inflammatory effects on gastric adenocarcinoma cells (AGS). We found that both the phenolic extracts and the individual substances (such as oleuropein aglycon, ligstroside aglycon, hydroxytyrosol and tyrosol) showed inhibitory effects on the NF-κB-driven transcription and its translocation on AGS cells. Therefore, we hypothesized that olive oil could be also effective as a natural anti-inflammatory food to prevent the inflammation in the intestine as already demonstrated in monocytes [(64)] and gastric epithelial cells [(65)]. To clarify whether the olive oil phenolic extracts can display anti-

inflammatory effects to the intestine, we used the human epithelial colorectal adenocarcinoma cell line (Caco-2) as an *in vitro* model.

1. 3. 3. a. Caco-2 cells as a model of intestinal epithelial cells

The Caco-2 cells are widely used as *in vitro* model of intestine to study intestinal absorption and metabolism of various compounds and on the transport of these substances [66; 67]. One of the reasons for this is due to the difficulty of isolation of primary intestinal cells. Other reason is that differentiated Caco-2 cells can be used for the examination of the transport, biotransformation, and barrier function of small intestinal enterocytes [68]. These include how to develop brush border microvilli, measure the enzymatic activity of hydrolase (ex, sucrase-isomaltase, intestinal alkaline phosphatase) and the development of the tight junctions for a polarization of cell monolayer [69].

The Caco-2 cells are originally isolated from 72 years old male, the patient with colorectal adenocarcinoma and were established as a human colonic adenocarcinoma cell line. These cells have the outstanding features such as spontaneous differentiation. The differentiation process was conducted in order to mimic the existing conditions in the intestine *in vivo*. The Caco-2 cells can express the morphological, biochemical characteristic of colonocytes in undifferentiated state, but after reaching the confluence, cells begin the differentiation process and show the transport, biotransformation and barrier function such as those we can see on small intestinal enterocytes [68; 70]. Turck et al. demonstrated that 60 different protein profiles were observed between

undifferentiated and differentiated Caco-2 cells by the proteomic analysis. [71]. Engle et al. demonstrated that from day 3 to day 18 after the confluence, the Caco-2 cells express increased enterocytes markers (e.c. α_1-antitrypsin, and alkaline phosphatase activity) and supressed the colonocyte marker (e.c. surfactant protein A and surfactant-like particle) on plastic growing Caco-2 cells [72]. Hence, we used the Caco-2 cells for the experiments, 24 hours after seeding as undifferentiated Caco-2 cells which represent features of colonocytes, and 18 days after seeding as differentiated Caco-2 cells which represent features of enterocyte.

1. 4. Regulation of molecular mechanism in Inflammation

1. 4. 1. NF-κB and inflammation

The Nuclear transcription factor κB (NF-κB) was discovered by David Baltimore in 1986, and is a ubiquitous factor that resides in the cytoplasm. Activation of the NF-κB/Rel transcription family, by nuclear translocation of cytoplasmic complexes, plays a central role in inflammation through its ability to induce transcription of pro-inflammatory genes [73]. NF-κB is activated by free radicals, inflammatory stimuli, carcinogens, tumor promoters, endotoxins, γ-radiation, ultraviolet (UV) light, and X-ray exposures. Upon activation, NF-κB induces the expression of more than 200 genes that have been shown to suppress apoptosis and induce cellular transformation, proliferation, invasion, metastasis, chemo-resistance, radio-resistance, and inflammation. Hence, it is not surprising that NF-κB has been linked to the wide variety of diseases, because most diseases are caused by poorly regulated inflammation processes [74]. As mentioned above, NF-κB is clearly one of the most important regulators of pro-inflammatory gene

expression. Synthesis of cytokines, such as TNF-α, IL-1β, IL-6, and IL-8, is mediated by NF-κB, as is the expression of cyclooxygenase 2 (Cox-2). Therefore, agents that can suppress NF-κB activation, in principle, have the potential to prevent, delay the onset of, or treat inflammatory diseases.

As shown in Fig 9, activation of NF-κB occurs by releasing from the IκB molecules or by cleaving of the inhibitory ankyrin repeat domains of p100 and p105. These events are achieved by proteasomal degradation of the inhibitors or by partial degradation of the precursors [75].

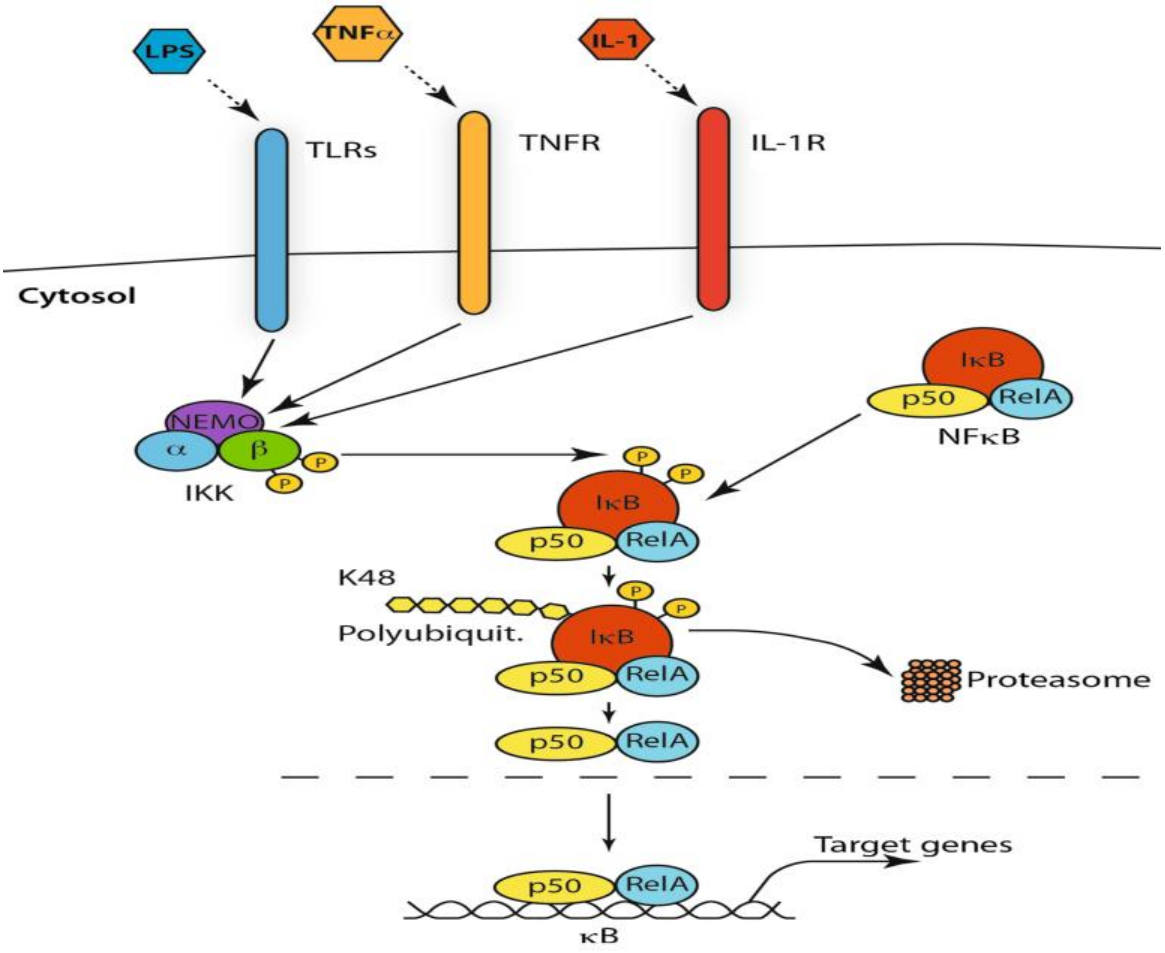


Fig 9 The canonical NF-κB signalling pathway [75]

1. 4. 2. MAPK and inflammation

Mitogen-activated protein kinases (MAPKs) are the group of serine/threonine protein kinases that regulate the transcription of inflammatory cytokines, including interleukin IL-8, in response to various extracellular stimuli through a cascade of protein phosphorylation, leading to the activation of transcription factors [(76)]. Some other studies have shown that olive oil phenol interacts with p38 and is involved in cAMP response element binding protein (CREB) phosphorylation and exert a strong inhibitory effects on cancer cell proliferation [(77)] Furthermore, there have been many reports that IL-8 production and secretion are regulated by the activity of MAPKs [(78; 79)]. The signalling pathways of MAPKs can be mainly divided into 3 pathways, extracellular signal-regulated kinase 1/2 (ERK1/2), p38 MAPK, and c-Jun N-terminal kinase (JNK). Heretofore, most studies have discussed the activation of the MAPKs in relation to cell growth and stress response. In vertebrates, the three major MAPK pathways are represented by kinase cascades leading to activation of ERK, JNK and p38 MAPKs [(80)]. In normal condition, ERK modulates the responses to cellular differentiation, whereas JNK and p38MAPK are activated by stress-associated stimuli, such as heat shock, inflammation, ultraviolet light and irradiation.

1. 4. 3. IL-8 regulation

The Interleukin-8 (IL-8), which is rapidly induced in response to pro-inflammatory cytokines, bacterial and cellular stresses, and is involved in the acute phase of gastrointestinal inflammation by attracting and activating neutrophils [(79)]. The synthesis of IL-8 is controlled by the cooperation of at least three different signalling

pathways. As show in Fig 10, in the first, derepression of the gene promoter; in the second, transcriptional activation of the genes by NF-κB, JNK and ERK pathways; and in the third, stabilization of the mRNA by p38 MAPK pathway [78].

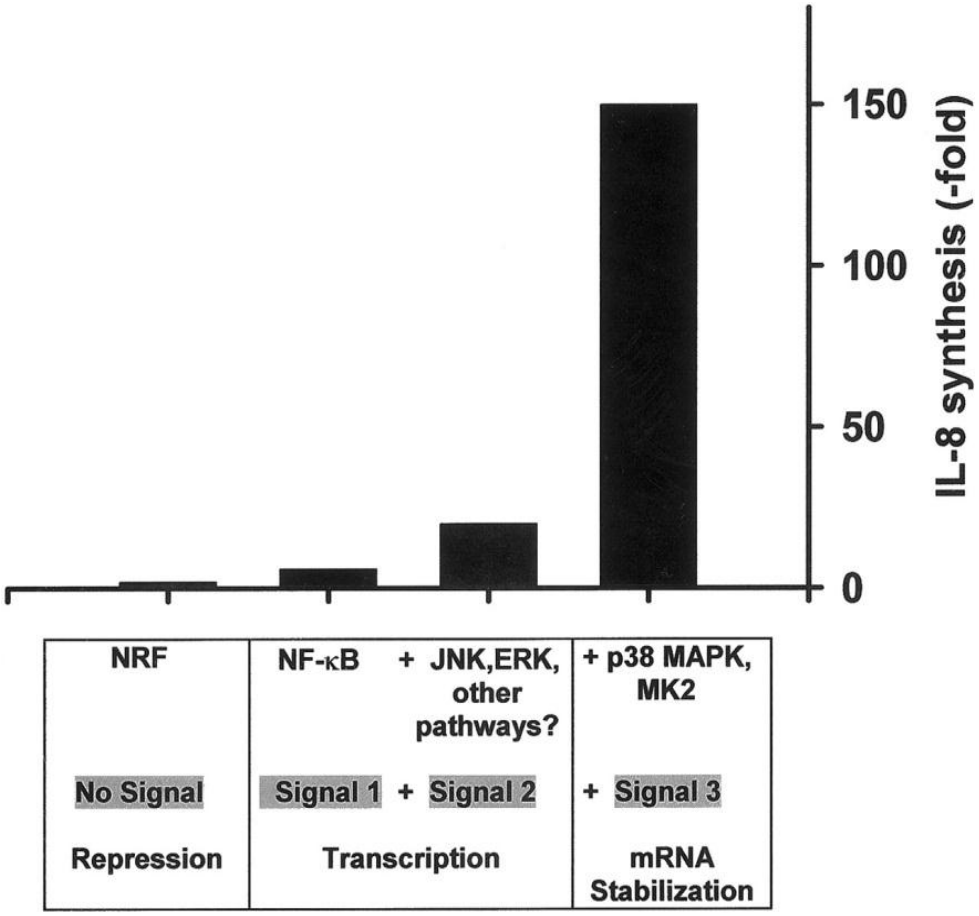


Fig 10 Quantitative control of IL-8 synthesis by cooperation of at least three signalling pathways. [79]

Additionally, Zhang et al. reported that the up-regulation of the nuclear factor (erythroid-derived 2)-like 2 (Nrf2) expression significantly increased IL-8 mRNA levels and protein secretion. Nrf2 caused only a weak induction of IL-8 transcription, but significantly increased the half-life of IL-8 mRNA. Considering the evidence that Nrf2 activation is mainly cytoprotective, these observations raise the possibility that under

certain circumstances, IL-8 may serve as an anti-inflammatory role and thereby contribute to the resolution of tissue injury [81].

CHAPTER 2

AIM OF THE STUDY

Olive oil is the principal fat source of the traditional Mediterranean diet, a regimen that has been associated with different beneficial effects on human health. *In vitro* and *in-vivo* studies have suggested that olive oil phenols (OPs), main components of the unsaponified fraction of olive oil, act as effective antioxidants. In our laboratory, we have reported that OPs down-regulate the expression of adhesion molecules in endothelial cells [(63)] and inhibition of expression and activity of metalloproteinase-9 (MMP-9) in human monocytic cells (THP1) [(64)]. Furthermore, from the mechanistic point of view, we demonstrated that OPs interfere with nuclear factor-kappa B (NF-κB) signalling pathway in monocytes, hence exhibiting immunomodulatory effect on these cells.

Regarding the *in vivo* situation, however, we should consider that olive oil components, before reaching other compartments, are absorbed through the gastrointestinal tract. Therefore, gastric and intestinal epithelial cells may be exposed to OPs at high concentrations. In previous studies, we demonstrated that phenolic extracts inhibited NF-κB-driven transcription and its nuclear translocation in human gastric adenocarcinoma cells (AGS), as *in vitro* model [(65)]. This study clearly demonstrated that OPs may have beneficial effects on gastrointestinal inflammatory states, although little is known about the biological activity of OPs on inflamed intestine. Therefore, the present research was aimed to investigate the potential immunomodulatory activities of phenolic extracts on the intestinal epithelial cells.

In general, intestinal epithelial cells are exposed to numerous bacteria and enterobacteria. In spite of the exposure to these bacteria and even the presence of lipopolysaccharide (LPS) from gram-negative bacteria, the intestine does not usually

develop an inflammatory state. This is though to be due to the immune tolerance to resident intestinal microbes. However, when this immune tolerance is perturbed for several reasons, intestinal epithelial cells become responsive to various inflammatory mediators such as interleukin-1 beta (IL-1β) and TNF-α secreted by the immune cells residing in the intestine. In such inflammatory state, intestinal epithelial cell modulates the epithelial monolayer permeability and secretion, then further inflammatory responses will occur [82]. In particular, NF-κB is one of nuclear transcription factors which are involved in transcription of many cytokines and chemokines. In the presence of cytokines such as IL-1β or TNF-α and of the endotoxin LPS, NF-κB is recruited from the cytosol to the nucleus. Once NF-κB translocates into the nucleus, transcription of genes encoding pro-inflammatory cytokines, such as IL-1β, interleukin-6 (IL-6), TNF-α, and chemokine interleukin-8 (IL-8) occurs. Herein, we focused our attention on IL-8, because it is rapidly induced in response to pro-inflammatory cytokines, bacterial and cellular stresses, and is involved in the acute phase of inflammatory events of gastrointestinal cells by attracting and activating neutrophils [79]. Furthermore, IL-8 is deeply involved in several inflammatory diseases, such as inflammatory bowel disease (IBD); for this reason IL-8 could be considered one of the important markers of intestinal homeostasis and inflammation [83; 84; 85]. Previous studies have shown that the synthesis of IL-8 is controlled by the cooperation of at least three signalling pathways; in the first, derepression of the gene promoter; secondly, transcriptional activation of the gene by NF-κB, JNK and ERK pathways; and in the third, stabilization of the mRNA by p38 MAPK pathway [78].

In this study, we first confirmed the effects of olive oil phenolic extracts on gastric epithelial AGS cells as reported before. In the published paper, we used

commercially available extra-virgin olive oil to extract OPs, while in these experiments we decide to use an extra-virgin olive oil, produced at a low scale following artisanal procedures, thus it is characterized by better organoleptic quality, compared to olive oils from industrial sources. Based on these considerations, we decided to repeat the experiments on AGS cells to assess the biological activity of this particular olive oil.

We further investigated the effects of OPs on the inflamed intestinal epithelial Caco-2 cells. In order to induce the first step of inflammation state, we used bacterial LPS, then to mimic the next step of inflammation state we used cytokine IL-1β, which is secreted by activated macrophages. We examined NF-κB promoter activity, its nuclear translocation and IL-8 native promoter activity in Caco-2 cell line, continuous cells of heterogeneous human epithelial colorectal adenocarcinoma cells. To study the molecular mechanisms by which the olive oil phenolic extracts affect the gut inflammation states, we performed the experiments in two different phases of differentiation process; undifferentiated (features of colonocytes) and differentiated (features of enterocytes) Caco-2 cells; in particular, we examined IL-8 mRNA expression and its secretion into the medium. Furthermore, we tried to get the insight of molecular mechanism of how OPs interact on IL-8 expressions. By integrating the approaches mentioned above, we expected to gain further insights on the molecular mechanisms by which OPs modulate the inflammatory response in intestinal cells.

CHAPTER 3

MATERIALS AND METHODS

3.1 Reagents

DMEM-F12 medium, L-glutamine 200mM, 0.25% trypsin-EDTA, penicillin and streptomycin were purchased from Life Technologies (Milan, Italy). DMEM medium, nonessential amino acid, protease inhibitor cocktail, actinomycin D (A94159), the pharmacological inhibitors of MAPK pathways, U0126 (ERK inhibitor), SB203580 (p38 inhibitor), SP600125 (JNK inhibitor), lipopolysaccharide, tumor necrosis factor-α phloretin, tyrosol were purchased from Sigma Aldrich (Milan, Italy), and fetal bovine serum was purchased from Euro clone (Milan, Italy). IL-1β was purchased from Immunotools (Friesoythe, Germany). The apigenin and luteolin were purchased from Extrasynthese (Lyon, France). The oleuropein aglycone and ligstroside aglycone were obtained from their corresponding glucoside (Extrasynthese, Lyon, France) by enzymatic digestion with β-glucosidase; their purity of 99% was confirmed by TLC and electrospray ionization-mass spectrometry (ESI-MS) analysis. The hydroxytyrosol was from Cayman Chemical Co. (Tallinn, Estonia). All compounds used for the analytical determinations and for the biological assays were of HPLC purity grade. The plasmid NF-*κ*B-LUC containing the luciferase gene under the control of 3 *κ*B sites was a gift from Dr. N. Marx (Department of Internal medicine II - Cardiology, University of Ulm, Germany). Native IL-8-LUC promoter was kindly provided from Dr. T. Shimohata and Prof. A. Takahashi (Departments of Preventive Environment and Nutrition, University of Tokushima Graduate School, Japan). AGS cells were obtained from LGC Standard S.r.l., (Milano, Italy) and Caco-2 cells (at 105 passages) were kindly donated by Dr. Y. Sambuy (INRAN - National Research Institute on Food and Nutrition, Italy).

3.2 Cell cultures

The human adenocarcinoma cells (AGS) were grown in Dulbecco's Modified Eagle's Medium (DMEM) / F12 medium supplemented with 100 units penicillin/ml, 100 mg streptomycin/ml, and 10% heat-inactivated fetal calf serum. The human epithelial colorectal adenocarcinoma cells (Caco-2) were cultured in DMEM containing 10% fetal bovine serum, 4mM L-glutamine, 1mM sodium pyruvate, 1% non-essential amino acid and 1% of penicillin streptomycin. Both cell lines were incubated at 37℃ in a humidified atmosphere containing 5% CO_2 and medium was changed 3 times a week.

3.3 Phenolic extractions from extra virgin olive oil

Phenolic extracts were obtained from Extra virgin olive oil "PLANETA" by the method according to Montedoro *et al.* with minor modifications [86]. Briefly, extra virgin olive oil (100g) was delipidated with 110ml of hexane and extracted with a mixture of methanol and water (methanol / water = 80 / 20; v/v; 110ml) three times by shaking the funnel gently. Collected methanol phase was evaporated under nitrogen stream, and remaining water phased was frozen and lyophilized. Obtained phenolic extracts were stored at -20℃ and re-suspended by ethanol before characterization.

3.4 Characterization of phenolic extracts

3.4.1 Total phenol contents

Total phenol concentrations were measured by the Folin-CIOCALTEAU assay [87]. Briefly, phenolic extracts were diluted with ethanol to the appropriate concentration, then water and Folin-CIOCALTEAU reagents were added, respectively. After adding Folin-CIOCALTEAU reagent, reaction was neutralized with sodium carbonate, and resulting blue color absorbance was measured by spectrophotometry at 750 nm by JASCO model V-530. Gallic acid was used to obtain calibration curve.

3.4.2 Individual phenolic compound characterization

Then individual phenolic compound was characterized with gas chromatography-mass spectrometry (GC-MS/MS) and liquid chromatography (LC-MS). The operating parameters were previously described [88]. Concentrations of Ligstroside aglycon, Oleuropein aglycon, apigenin and luteolin were measured by LC-MS. The calibration curve was measured with phloretin (Oleuropein aglycon; 10 μg/sample, others 1μg/sample) as an internal standard and increasing amounts of the authentic phenols (0-20 μg for Ligstroside aglycon, 0-100 μg for Oleuropein aglycon, 0-5μg for apigenin and 0-15μg for luteolin). Concentrations of tyrosol and hydroxytyrosol were quantified by GC-MS/MS with 50 ng/sample of deuterium-labeled hydroxytyrosol as an internal standard, and calibration curve was obtained with pure 0-50ng of tyrosol and hydroxytyrosol.

3.5 Cytotoxicity

The cytotoxicity of phenolic extracts to the cells was assessed by the 3,4,5-dimethylthiazol-2-yl-2-5-diphenyltetrazolium bromide (MTT) assay. MTT assay is based on the reduction of MTT by the mitochondrial dehydrogenase of intact cells to a purple formazan product. Before experiments, each condition was tested by MTT assay to assess whether treatment with inflammatory stimuli and phenolic extracts caused cytotoxicity. After each treatment, culture media were carefully removed by aspiration and 200 ml of 0.1 mg/ml MTT in PBS were added to each well and incubated for 30min. Then, 100 ml of 10% isopropanol + DMSO (dimethyl sulfoxide) solution was added to each well to dissolve the formazan crystals formed. The amount of formazan was determined by measuring the absorbance at 550nm using a microplate reader iMark^{tm}, Bio-rad.

3.6 Transfections

The AGS cells were seeded 1×10^4 cell per well on 24-wells plates, then after 3 days, NF-κB-luc plasmid was transfected by using calcium phosphate method. The Caco-2 cells were plated at the density of 4×10^4 on 24-wells plates when cells reached approximately 70-80% confluency, 500 ng of NF-κB-LUC, native IL-8-LUC plasmids were individually transfected to the cells. To transfect the plasmids into the Caco-2 cells we used Lipofectamine 2000 with 1 : 2 (μl reagent : μg plasmid DNA) ratio according to the mamanufacturer's instructions. The native IL-8-LUC plasmid used in these experiments contains sequences responsive to other transcription factors such as activator protein 1 (AP-1), CCAAT-enhancer-binding protein-β (C/EBPβ), and NF-κB binding elements.

After 18 hours of transfection, cells were pre-treated for 1 hour with OPs at increasing concentrations (AGS; 0.1-2.5μg/ml, Caco-2; 2.5-10μg/ml) then treated cells with individual stimulus (AGS;10ng/ml of TNF-α, Caco-2; 50μg/ml of LPS or 5ng/ml of IL-1β) together with olive oil phenols (OPs) for the next 6 hours.

3.7 NF-κB nuclear translocation

To assess the effects of phenolic extracts on NF-κB (p65) nuclear translocation in inflamed Caco-2 cells, the cells were plated at a density of 1.5×10^6 cells in 10-mm plates. After 48 hours, cells were treated for 1 hour with the inflammatory mediators and the OPs. Nuclear extracts were prepared using Nuclear Extraction Kit from Cayman Chemical Company (Michigan, USA) and stored at -80℃ until assayed. The concentration of nuclear proteins was assessed by Bradford method and the same amount of nuclear extract (10μg/well) was loaded for the analysis. Then, NF-κB nuclear translocation was assessed by using the NF-κB (p65) transcription factor assay kit (Cayman) followed by spectroscopy (signal read 450 nm, 0.1 s).

3.8 RNA extraction and quantitative RT-PCR analysis

Caco-2 cells were seeded on 24-wells plate at high density (6×10^5 cells/ well). The undifferentiated Caco-2 cells were treated 1 day after seeding while the differentiated Caco-2 cells were obtained after growth for 18 days. Both cells were pre-treated for 1 hour by following concentrations of phenolic extracts before inducing inflammation reaction with 50μg/ml LPS or 5ng/ml IL-1β, respectively. After 1 hour of pre-treatment with phenolic extracts, medium were replaced with medium containg

stimulus alone or together with phenolic extracts and cells were incubated for the next 6 hours. After these treatments, total RNA was extracted using the Nucleospin RNA II kit (Macherey–Nagel, Duren, Germany) and the purity and concentration were checked by nano drop (Thermo Fisher Scientific ND-1000). RNA was analysed by TaqMan CFX384 RT-qPCR using the iScript^{TM} one-step RT-PCR kit for probes (Bio-Rad; Milano, Italy). Samples were run in 384 well formats in triplicate as multiplex reactions with a normalized internal control (GAPDH). IL-8 and GAPDH probe and primers were purchased from Eurofins MWG-Operon (Milano, Italy) The sequences are reported blow.

Gene Symbol	RefSeq ID	Sequences (5' to 3')
hIL-8	NM_000584	Forward ATACTCCAAACCTTTCCACCC Reverse TCTGCACCCAGTTTTCCTTG Probe CCACACTGCGCCAACACAGAAA
hGADPDH	NM_001256799	Forward CGGGGCTCTCCAGAACATC Reverse ATGACCTTGCCCACAGCCT Probe CCCTGCCTCTACTGGCGCTGCC

3.9 Intracellular IL-8 contents and secreted IL-8 protein concentration

After treatments, the medium was removed and stored at -20℃ till the assay of IL-8 secretion. To obtain cell lysate, cells were washed twice with cold PBS then lysed with lysis buffer (100 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1mM EDTA, 1% Triton X - 100, 0.5% sodium deoxycholate, and protease Inhibitor Cocktail). Cell lysates were homogenized and then centrifuged at 13,000 rpm for 10 minutes at 4℃. Total protein

concentration was determined by the Bradford method and the lysated were stored at -20℃ till measured. The levels of IL-8 protein released into the culture medium and intracellular contents were assessed by an enzyme-linked immunosorbent assay (ELISA) kit (peprotech, Rocky Hill, NJ, USA), in accordance with the manufacturer's instructions.

3.10 IL-8 mRNA stability

Undifferentiated and differentiated Caco-2 cells were pre-treated with phenolic extracts for 1 hour and then stimulated with 5ng/ml IL-1β for 6 hours as described. After 6 hours of incubation, the medium was replaced with new medium which contains 10 μg/ml actinomycin D to block further transcription. Total RNA was extracted at 0, 30 and 120 minutes after Actinomycin D addition. IL-8 mRNA stability was assessed by real time PCR method as described above. GAPDH was used as a housekeeping gene to normalize the data.

3.11 MAPKs inhibitor experiments

Caco-2 cells were pre-treated with phenolic extracts and/or inhibitors of individual MAPK pathways by following concentrations10μM U0126 (ERK inhibitor), 30μM SB203580 (p38 inhibitor), and10μM SP600125 (JNK inhibitor). After pre-treatment, the cells were exposed to 5ng/ml IL-1β for the next 6 hours. After 6 hours of treatment, cells were collected and IL-8 mRNA expression was analysed as described above.

3.12 Statistical analysis

All data are expressed as mean ± SEM. Data were analyzed by unpaired *t* test, one-way analysis of variance (ANOVA), or two-way analysis of variance (ANOVA) followed by Bonferroni as post-hoc test. Statistical analysis was done using GraphPad Prism 5.02 software (GraphPad Software Inc., San Diego, CA, USA). $p<0.05$ was considered to be statistically significant. IC_{50} was calculated using GraphPad Prism 5.02.

CHAPTER 4

RESULTS

4. 1. Determination of total phenolic content by Folin-CIOCALTEU method

Total phenolic contents in olive oil phenol extracts were assessed by the Folin – CIOCALTEU method as reported in the Materials and Methods section 3.4.1. The total phenolic amount and composition of olive oils are variables which are from 50 to 1000 mg/kg [(25)]. The OPs from "PLANETA" contained 394mg/kg of total phenols, this value was similar to that found previously in other extra-virgin olive oils of our laboratory [(64)]

4. 2. Analytical quantification of individual phenolic compounds

We only measured major individual phenolic compounds which are already known as having biological activity in *in vivo* and *in vitro* studies, in the OPs used in the present experiments. Quantification of secoiridoids and flavonoids content was performed by LC-MS/MS, and phenolic alcohol was measured by GC-MS. As reported in table 1, secoiridoids such as oleuropein aglicone (OleA) and ligstroside aglicone (LigA) were the most abundant compounds and both of them accounted for about 44% of the total phenols. The phenolic alcohols tyrosol (TY) and hydroxytyrosol (HY) were present in the range of 9-10%, and the concentration of flavonoids apigenin (AP) and luteolin (LU) was below 1%.

Secoiridoids	[OleA]	Oleuropein aglycone	28.3%
	[LigA]	Ligstroside aglycone	15.6%
Phenolic alcohols	[TY]	Tyrosol	4.8%
	[HY]	Hydroxytyrosol	4.8%
Flavonoids	[AP]	Apigenin	0.5%
	[LU]	Luteolin	0.2%
		Other	45%

Table 1 Content of individual OPs measured by GC-MS and LC-MS

4. 3. Cytotoxicity

To decide the concentration range of OPs to be used for the biological assays, we evaluated the cxytotoxicity by performing the MTT assay on AGS cells, and in undifferentiated and differentiated Caco-2 cells. Furthermore, MTT assays were performed in all experimental settings (i.e. different cell density, cotreatment, and agents to be completed), as mentioned in the Materials and Methods section 3.5.

4. 4. The effects of phenolic extracts on NF-κB driven transcription in AGS cells stimulated with TNF-α

As thoroughly discussed in the introduction, NF-κB nuclear transcription factor is deeply involved in the transcription of pro-inflammatory genes such as IL-8, IL-6, IL-1β and TNF-α. Previously, our laboratory demonstrated that OPs from other commercial olive oils can interfere with NF-κB promoter activity [65]. To confirm this biological activity of the new phenolic extracts from "PLANETA" olive oil, we assessed its effect on NF-κB promoter activity in AGS cells treated with TNF-α. NF-κB promoter activity was assessed in AGS cells transiently transfected with the NF-κB-LUC reporter system, in which the luciferase gene is under control of 3 κB-sites. Ten ng/ml of TNF-α induced 4-fold increase in the promoter activity compared with that obtained from the cells without stimulation (Fig. 1). To assess the activity of the OPs, transfected cells were pre-treated with olive oil extract at concentrations ranging from 0.1-2.5μg/ml withTNF-α stimulus. In cells treated together with TNF-α and OPs, the NF-κB promoter activity was reduced in a concentration-dependent manner. The calculated half maximal inhibitory concentration (IC50) was 1.38μg/ml. Therefore, the

present results demonstrated that the ability of OPs to inhibit activation of NF-κB pathway induced by TNF-α in AGS cells is independent on the origin and manufacturing procedure of the olive oil.

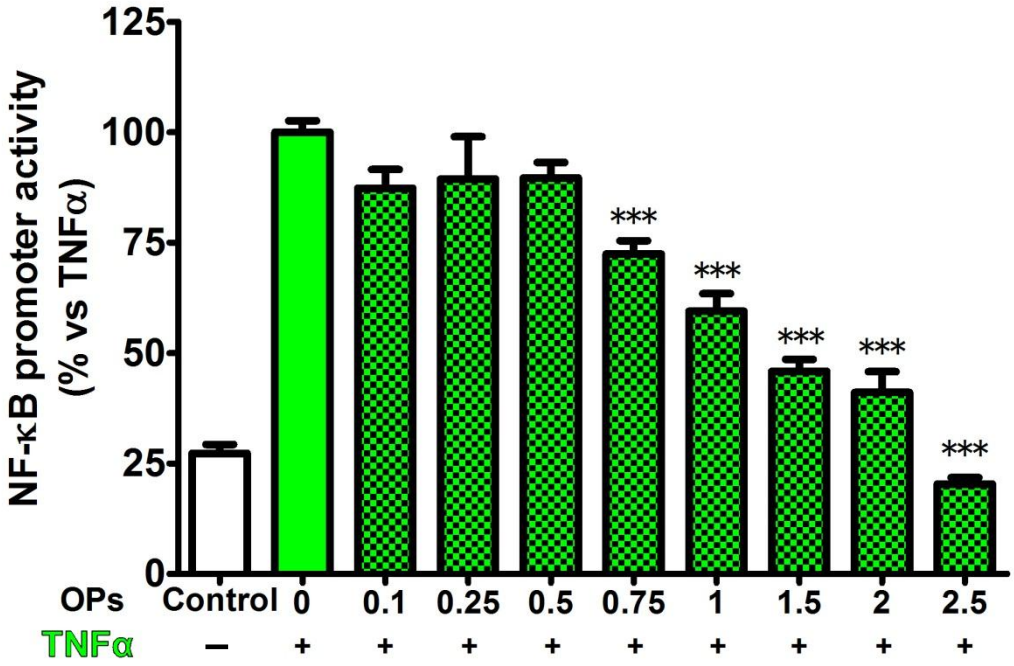


Fig 1 OPs inhibit the NF-κB promoter activity in a concentration-dependent manner in the AGS cells. The AGS cells were transfected with NF-κB-luciferase gene by calcium phosphate methods; after 18 hours of transfection, cells were pre-treated for 1 hour with OPs at different concentrations (0.1-2.5μg/ml). Then, we induced the inflammation states by treating with10ng/ml of TNF-α for next 6 hours. Data are expressed as percentages with respect to luciferase activity measured in cells treated with TNF-α alone. The graph shows the means ± SEM of one representative experiment that was repeated 3 times yielding similar results, n=4 per conditions, ***$p<0.0001$ *vs* stimulus alone. Data were analyzed by one-way analysis of variance (ANOVA), followed by Bonferroni as post-hoc test.

4.5. The influence of OPs on NF-κB driven transcription in Caco-2 cells stimulated with LPS

We moved our attention to human epithelial colorectal adenocarcinoma (Caco-2) cells, because most OPs are absorbed in the intestine [(26)]. As described in introduction part ***1. 3. 3. a.***, Caco-2 cells have two states, an undifferentiated state which is characterized by features typical of colonocytes, and differentiated Caco-2 cells, which more closely resemble enterocytes. All transfection experiments were performed in undifferentiated Caco-2 cells, since differentiated cells are less efficiently transfectable by common commercially available transfection reagents. To induce the acute phase of the inflammation state, we decided to use high concentration of bacterial LPS. To check the involvement of NF-κB promoter activity, we transfected NF-κB-LUC reporter system into undifferentiated Caco-2 cells. LPS did not induce NF-κB promoter activity when compared with the cells without stimulation (Fig 2). As discussed in the Introduction, LPS may activate not only NF-κB but also other signalling pathways, depending on the repertoire of receptors and intracellular effector molecules expressed in target cells. Our results suggest that in undifferentiated Caco-2 cells LPS acts through mechanisms independent of NF-κB. However, treatment with 7.5 μg /ml of OPs reduced the basal activity by about 50%.

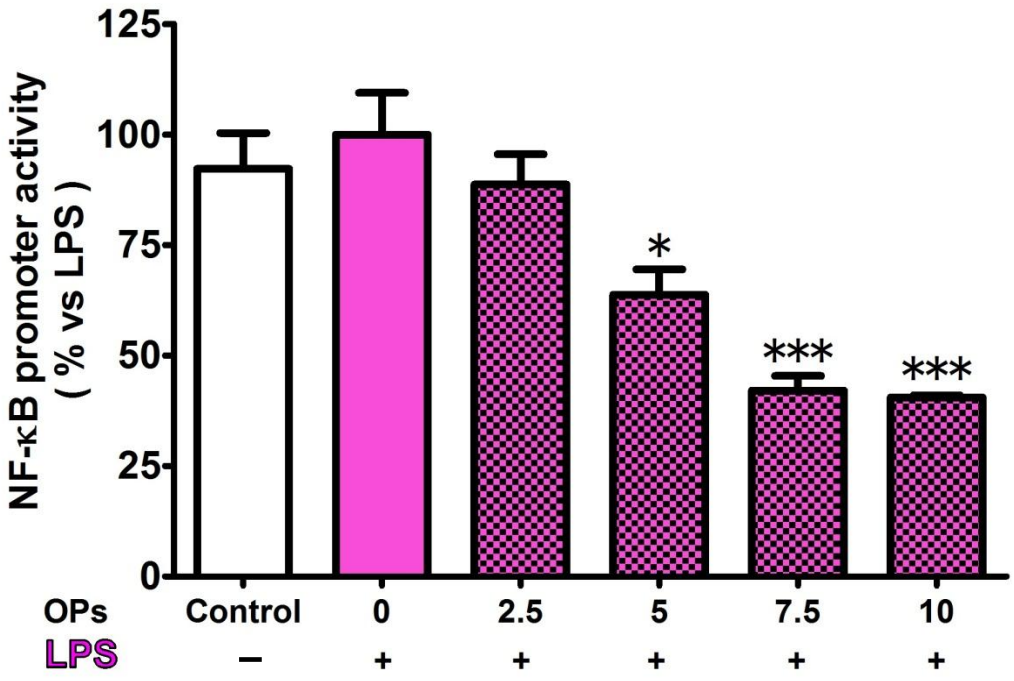
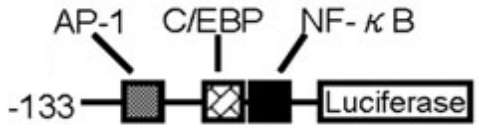


Fig 2 Lipopolysaccharide (LPS) did not induce NF-*κ*B promoter activity. But OPs still suppressed the NF-*κ*B promoter activity in a concentration-dependent manner. The undifferentiated Caco-2 cells were transfected with Lipofectamine 2000, after 18 hours of transfection cells were pre-treated for 1 hour with increasing concentration of OPs (2.5-10µg/ml). Then, we treated cells with 50µg/ml of LPS together with OPs for the next 6 hours. Data are expressed as percentages with respect to luciferase activity measured in cells treated with LPS alone. Data ere expressed as means ± SEM, the graph show a representative experiment that was repeated 3 times yielding similar results, n=4 per conditions, *$p<0.05$, ***$p<0.0001$ *vs* stimuli alone. Data were analyzed by one-way analysis of variance (ANOVA), followed by Bonferroni as post-hoc test.

4.6. Effects of OPs on native IL-8 promoter activity in Caco-2 cells stimulated with LPS

The results shown in fig.2 were obtained using an artificial promoter that contains 3 NF-κB binding sites deriving from the E-selectin gene. To assess the effect of OPs on NF-κB-transcriptional activity in the context of a native promoter, we transfected Caco-2 cells with a plasmid carrying the luciferase gene downstream the interleukin-8 (IL-8) proximal promoter (Fig. 3). Among the numerous genes whose promoters contain NF-κB binding sites and other sequences responsive to inflammatory cues, we selected IL-8 because this chemokine is known to play a key role in the acute phase of intestinal inflammation and immune response as described in introduction ***1. 4. 3***.



Skematic diagram of the IL-8 proximal promoter (Shimohata, et al., 2011)

As shown in Fig. 3, LPS increased the wild-type IL-8 promoter activity by 3-fold compared to that of unstimulated cells, and phenolic extracts suppressed this induction in a concentration-dependent manner. Treatment with 5 μg/ml of OPs reduced the induction of IL-8 promoter activity to the basal level (Fig 3). By using the native promoter of IL-8, which contains several responsive sequences, we demonstrated that, LPS stimulates the transcription of genes related to inflammation regulation. Moreover, the inhibition observed in cells treated with the OPs suggests that these compounds may modulate the transcription of inflammatory genes by multiple mechanisms, in addition to inhibition of NF-κB signalling.

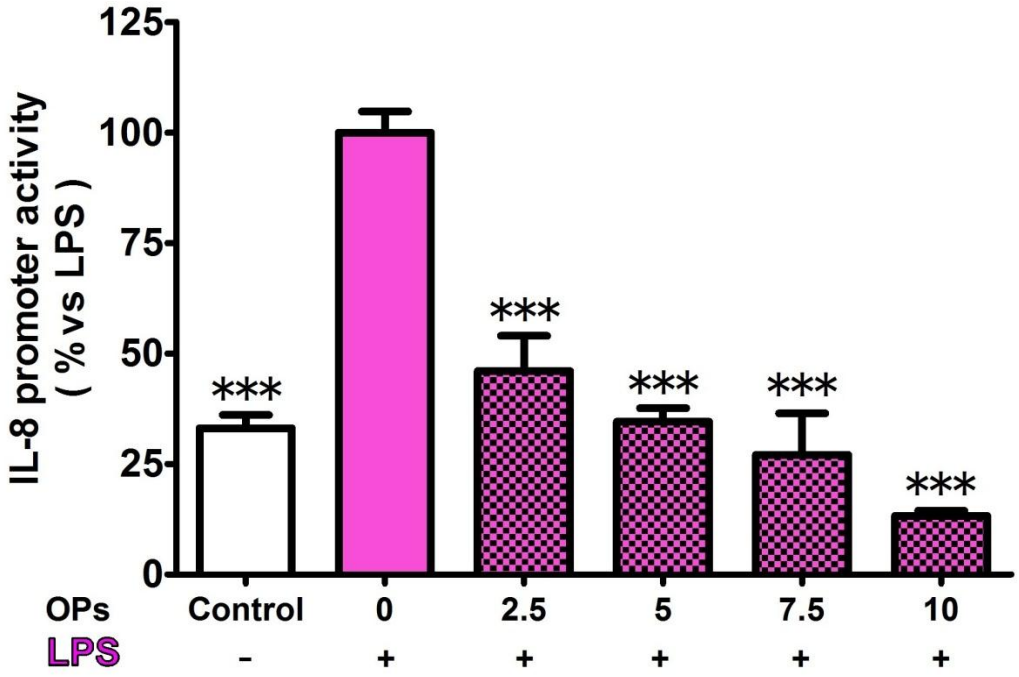


Fig 3 OPs inhibit the IL-8 native promoter activity at in cells treated with LPS. The undifferentiated Caco-2 cells were transfected with the plasmids by use of Lipofectamine 2000, after 18 hours of transfection cells were pre-treated for 1 hour with phenolic extracts at increasing concentrations (2.5-10μg/ml). Then we treated cells with 50μg/ml of LPS together with OPs for the next 6 hours. Data are expressed as percentages with respect to luciferase activity measured in cells treated with LPS alone. The graph shows the means ± SEM of the representative experiment that was repeated 3 times yielding similar results, n=4 per conditions, ***$p<0.0001$ *vs* stimuli alone. Data were analyzed by one-way analysis of variance (ANOVA), followed by Bonferroni as post-hoc test.

4.7. The influence of OPs on NF-κB driven transcription in Caco-2 cells stimulated with IL-1β

IL-1β is one of the cytokines more deeply involved in the acute phase of inflammation; it is secreted by macrophages, after activation with stimuli such as LPS and, by targeting other cells, such as intestinal epithelial cells, it contributes to further amplify the inflammatory/immune response. Treatement with 5 ng/ml of IL-1β induced a 5-fold increase in the promoter activity of the NF-κB reporter system, compared with

that obtained from the cells without stimulation. This finding indicates that, in contrast to LPS, undifferentiated Caco-2 cells are fully responsive to IL-1β. The olive oil phenol suppressed this activity in a concentration-dependent manner. Treatment with 10 μg/ml of olive oil extract suppressed the induction of NF-*κ*B promoter activity to the basal level (Fig 4). These results demonstrate that the phenolic extract inhibits NF-*κ*B activity in undifferentiated Caco-2 cells, similarly to the effects reported in other cell types [64; 65].

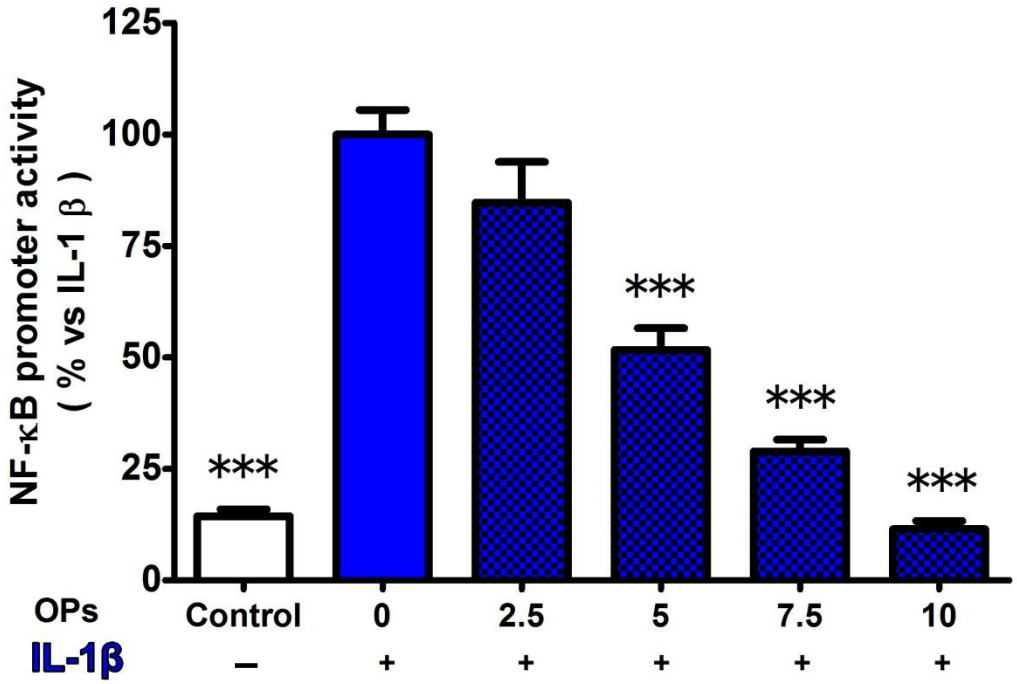


Fig 4 OPs inhibits the NF-*κ*B promoter activity in a concentration-dependent manner in cells treated with IL-1β. The undifferentiated Caco-2 cells were transfected with the plasmids by use of Lipofectamine 2000, after 18 hours of transfection cells were pre-incubated for 1 hour with increasing concentrations (2.5-10μg/ml) of OPs. Then we treated cells with 5ng/ml of IL-1β together with OPs for the next 6 hours. Data are expressed as percentages with respect to luciferase activity measured in cells treated with IL-1β alone. The graph shows the means ± SEM of one representative experiment that was repeated 3 times yielding similar results, n=4 per conditions, ***$p<0.0001$ *vs* stimuli alone. Data were analyzed by one-way analysis of variance (ANOVA), followed by Bonferroni as post-hoc test.

4.8. The contribution of OPs on NF-κB nuclear translocation in undifferentiated Caco-2 cells stimulated with IL-1β

To further investigate the effect of phenolic extracts on NF- κB signalling, we performed NF-κB nuclear translocation experiment. Treatment of undifferentiated Caco-2 cells with 5 ng/ml of IL-1β induced 4-folds increase of nuclear translocation of p65, the transcriptional active subunit of NF-κB, compared with those obtained from unstimulated cells. The OPs suppressed the IL-β-induced nuclear translocation of NF-κB in a concentration-dependent manner (Fig 5), but the effect was less pronounced compared with those observed on NF- κB-driven transcription (Fig 4). At the concentration of 10 μg/ml at which we observed the highest inhibition of the transcriptional activation, it correcponds only 30% inhibition of nuclear translocation (Fig 5). These results suggest that probably the OPs affect NF-κB signalling at distinct levels, such as translocation and transcriptional activity.

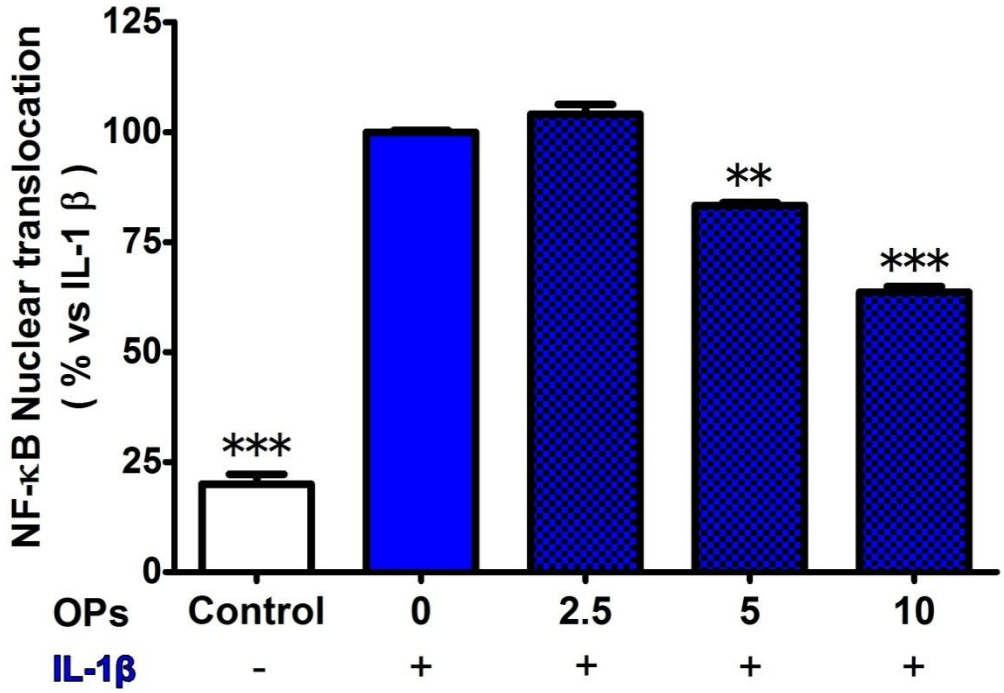


Fig 5 OPs inhibit the NF-*κ*B nuclear translocation in a concentration-dependent manner in cells treated with IL-1β. The Caco-2 cells were seeded on 10cm petri dishes, after 2-3days, when cells reached approximately 80% confluence, they were pre-treated for 1 hour with OPs at increasing concentrations (2.5-10μg/ml). Then,we induced the inflamed state by adding 5ng/ml IL-1β together with the reported concentrations of the OPs for 1 hour. The nuclear content of p65 was measured by ELISA and normalized by protein content. Data are expressed as percentages with respect to cells treated with IL-1β alone. The graph shows the means ± SEM of the representative experiment that was repeated 3 times yielding similar results,**p<0.01 ***p<0.0001 *vs* stimulus alone. Data were analyzed by one-way analysis of variance (ANOVA), followed by Bonferroni as post-hoc test.

4.9. Effect of OPs on IL-8 promoter activity, in Caco-2 cells stimulated with IL-1β

We then evaluated the ability of the OPs to modulate the native IL-8 promoter activity in cells treated with IL-1β. Wild-type IL-8 promoter activity was induced 10-folds by IL-1β and OPs suppressed this induction in a concentration-dependent manner (Fig 6). Ten μg/ml of OPs was able to suppressed the IL-8 promoter activity to the

basal level. The regulation of the native IL-8 promoter activity in cells treated with IL-1β closely resembles the effects observed with the NF-*κ*B reporter system, thus suggesting that this cytokine regulates gene transcription mainly acting through NF-*κ*B. Also, the effect of the phenolic extract on IL-8 promoter activity mirrors the effect observed on the NF-*κ*B-luc construct. It is worth mentioning that these findings in Caco-2 cells are consistent with the results obtained in AGS cells treated with TNF-α (Fig 1).

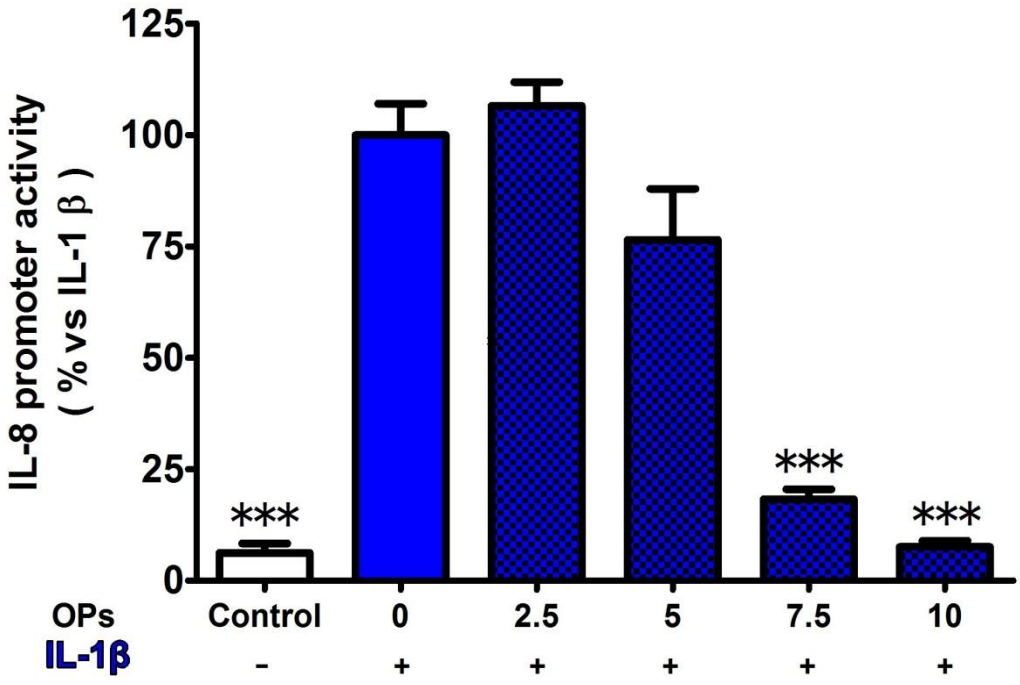


Fig 6 OPs inhibits the IL-8 native promoter activity in cells treated with IL-1β. The undifferentiated Caco-2 cells were transfected with the plasmids by use of Lipofectamine 2000, after 18 hours of transfection cells were pre-treated for 1 hour with OPs at increasing concentrations (2.5-10μg/ml). Then we treated cells with 5ng/ml of IL-1β together with OPs for the next 6 hours. Data are expressed as percentages with respect to luciferase activity measured in cells treated with IL-1β alone. The graph shows the means±SEM of one representative experiment that was repeated 3 times yielding similar results, n=4 per conditions, ***$p<0.0001$ *vs* stimuli alone. Data were analyzed by one-way analysis of variance (ANOVA), followed by Bonferroni as post-hoc test.

4.10. Time course of IL-8 mRNA expression

To assess whether the effects of OPs on promoter activity translate into the modulation of IL-8 expression, we first examined the time course of IL-8 mRNA expression in both undifferentiated and differentiated Caco-2 cells treated with inflammatory stimuli. As we expected, IL-1β rapidly induced IL-8 transcription in both differentiated and undifferentiated conditions that reached a maximum at 1 h. Undifferentiated Caco-2 cells showed strong sensitivity to IL-1β stimulation compared with differentiated Caco-2 cells (Fig 7). We also measured IL-8 secretion into the medium. Upto 2 hours we did not detect any IL-8 protein in the medium by ELISA, but at 4-6 hours IL-8 protein reached a plateau in both undifferentiated and differentiated Caco-2 cells (Data not shown). Based on these results, we decided to treat cells for 6 h with stimuli to assess IL-8 mRNA and protein expression.

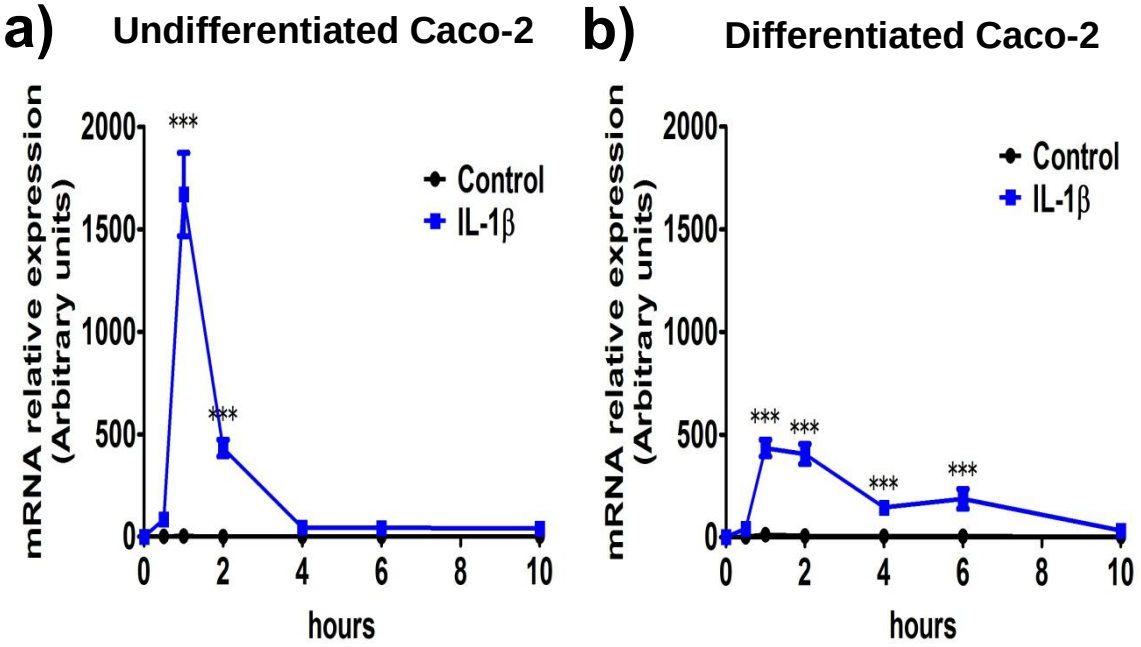


Fig 7 The time course IL-8 mRNA expressions induced by IL-1β The Caco-2 cells were seeded 6×10^5 cells/well on 24 wells plates, after 24 hours (undifferentiated state) or 18 days (differentiated state) cells were treated with 5ng/ml IL-1β for following hours till maximum10 hours. The mRNA levels of IL-8 were measured by RT real time qPCR. IL-8 mRNA levels results were expressed as fold induction, with respect to the control time 0 (without stimulus) set as 1(a, b). Data were expressed as means ± SEM, n=3 per conditions, ***p<0.0001 *vs* Control. Data were analyzed by two-way analysis of variance (ANOVA), followed by Bonferroni as post-hoc test.

4.11. Effect of OPs on IL-8 mRNA expression and protein secretion in undifferentiated and differentiated Caco-2 cells at basal conditions

We assessed whether the OPs modulate the expression of IL-8 at the basal conditions. We also examined the effects of the OPs on NF-κB-LUC and IL-8 native promoter activity at the basal condition in undifferentiated Caco-2 cells. Both transfection assays suggest that OPs significantly suppressed the basal NF-κB-LUC and IL-8 native promoter activities, respectively (data not shown). Either in undifferentiated or differentiated Caco-2 cells, IL-8 mRNA levels significantly respond to OPs at a naive condition (Fig 8 a and c). On the other hand, we did not observe any change of IL-8 secretion level in response to phenolic extract treatment in both cells (Fig 8 b and d). Thus, either in undifferentiated or differentiated cells, OPs induced the IL-8 mRNA expression by about 3 fold but did not affect IL-8 protein secretion. These results were quite unexpected, because the effects on mRNA are not always translated into the protein secretion.

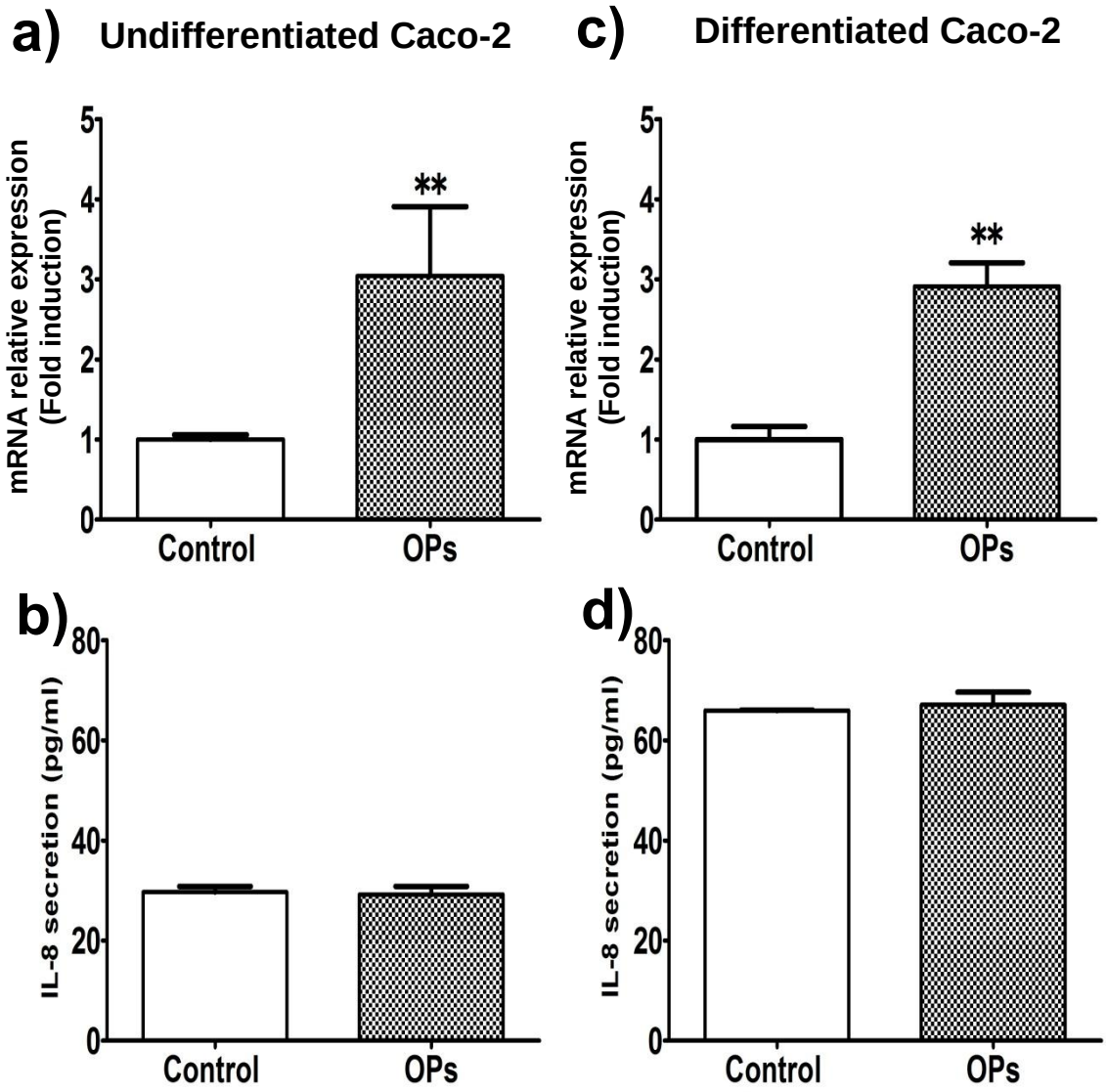


Fig 8 Effect of OPs on basal condition, assessed by IL-8 mRNA expression and IL-8 secretion in both undifferentiated and differentiated Caco-2 cells. The Caco-2 cells were seeded 6×10^5 cells/well on 24 wells plates, after 24 hours (undifferentiated state) or 18 days (differentiated state) cells were pre-treated for 1 hour with 10µg/ml of phenolic extract. IL-8 mRNA levels of IL-8 were measured by RT real time qPCR and are expressed as fold induction, setting at 1 each control (without stimuli) (a, c). Data were expressed by means of 4 times repeated experiments as means ± SEM, n=3 per condition, **p<0.01 vs Control. Data were analyzed by Student's t test.

4.12. The effect of OPs on IL-8 mRNA expression and protein secretion in LPS-stimulated undifferentiated and differentiated Caco-2 cells

To evaluate the effects of OPs in a condition mimicking the intiation of the acute phase inflammation, we treated Caco-2 cells together with bacterial LPS and OPs. As shown in figure 9, LPS induced IL-8 mRNA (a and c) and IL-8 secretion (b and d) significantly compared to control cells. Furthermore, we clearly observed the significant suppression of IL-8 mRNA levels compared with those found in both undifferentiated and differentiated Caco-2 cells treated with LPS alone (Fig 9 a and c). The same tendency was observed on IL-8 secretion (Fig 9 b and d).

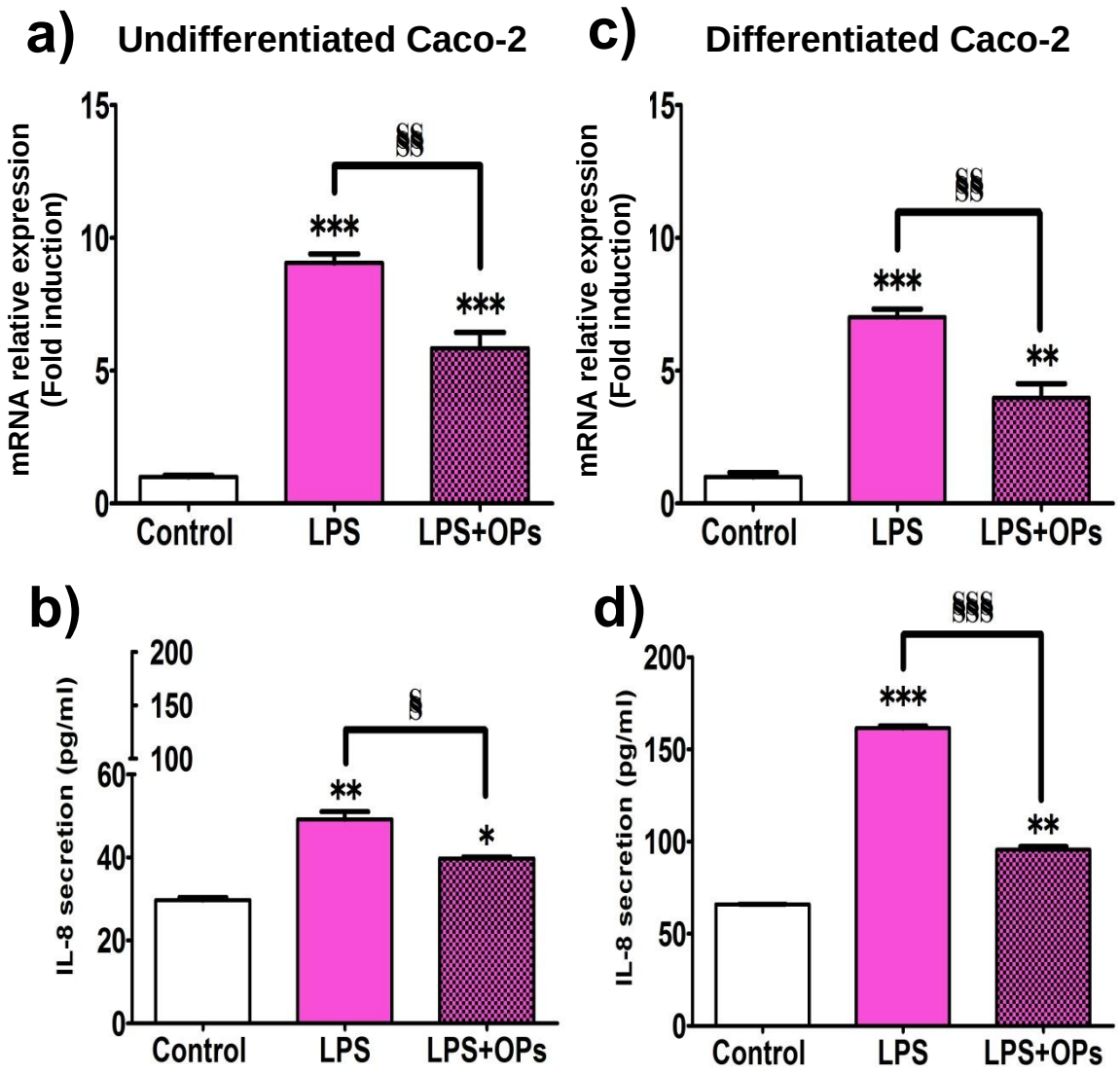


Fig 9 Effect of OPs on LPS-induced IL-8 mRNA expression and IL-8 secretion in both undifferentiated and differentiated Caco-2 cells. The Caco-2 cells were seeded 6×10^5 cells/well on 24 wells plates, after 24 hours (undifferentiated state) or 18 days (differentiated state) cells were pre-treated for 1 hour with 10µg/ml of OPs. Then we induced the inflamed state with 50µg/ml of LPS for the next 6 hours together with OPs. IL-8 mRNA levels were expressed as fold induction, with respect to each control (without stimulus) set as 1(a, c). IL-8 secretion was measured by ELISA as described in material and methods (b, d) Data were expressed as means ± SEM of and shown one representative results from 4 different experiments, n=3 per conditions, *p<0.05, **p<0.01, ***p<0.0001 vs Control, §p<0.05, §§p<0.01, §§§p<0.0001 vs stimulus. Data were analyzed by one-way analysis of variance (ANOVA), followed by Bonferroni as post-hoc test.

4.13. Effect of OPs on IL-8 mRNA expression in IL-1β stimulated undifferentiated and differentiated Caco-2 cells

Then, we also treated Caco-2 cells with cytokine IL-1β. As we expected, IL-1β induced IL-8 mRNA and IL-8 protein secretion strongly compared with bacterial LPS. Moreover, when we treated Caco-2 cells with IL-1β together with OPs, we observed a strong induction of IL-8 mRNA in both undifferentiated Caco-2 cells (6.5 fold increase compared with IL-1β treatment alone; Fig 10 a) and differentiated Caco-2 cells (2.3 fold increase compared with IL-1β treatment alone; Fig 10b). These results seem to be consistent with what we found in transfection assay: OPs inhibit IL-1β- induced NF-κB signalling, but not the mRNA levels of IL-8, a NF-κB regulated gene. Moreover we observed a stronger induction of IL-8 mRNA in the presence of phenolic extract.

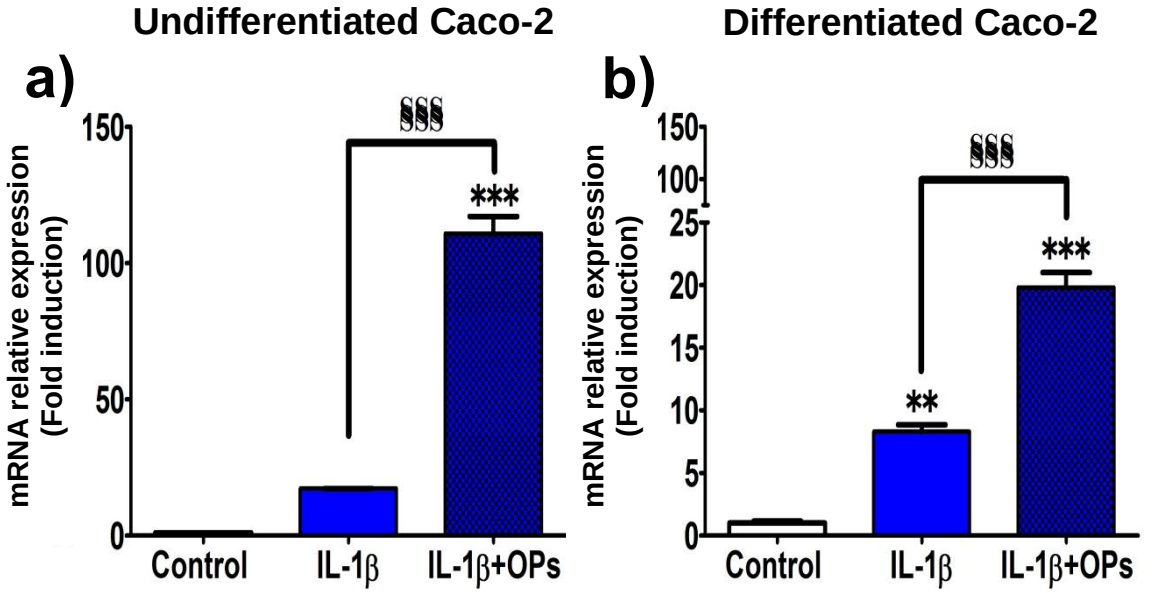


Fig 10 Effect of OPs on IL-1β-induced IL-8 mRNA expression in both undifferentiated and differentiated Caco-2 cells. The Caco-2 cells were seeded 6×10^5 cells/well on 24 wells plates, after 24 hours (undifferentiated state) or 18 days (differentiated state) cells were pre-treated for 1 hour with 10µg/ml of phenolic extracts. Then, we induced the inflamed state by 5ng/ml of IL-1β for the next 6 hours together with OPs. IL-8 mRNA level results were expressed as fold induction, which respected each control (without stimulus) set as 1 (a, c). Data were expressed as means ± SEM of and shown one representative results from 4 different experiments, n=3 per conditions, **p<0.01, ***p<0.0001 vs Control, §§§p<0.0001 vs stimulus. Data were analyzed by one-way analysis of variance (ANOVA), followed by Bonferroni as post-hoc test.

4.14. Effect of OPs on the intracellular IL-8 protein contents and secreted IL-8 protein in IL-1β stimulated undifferentiated and differentiated Caco-2 cells

Under most circumstances, the effects observed on secretion of IL-8 reflect the modulation of its transcription and translation. The results described in the previous section, however, indicate that in the presence of IL-1β the OPs modulates IL-8 transcription and expression in an unexpected way in cells treated with IL-1β, addition of the OPs further induced IL-8 mRNA levels, in contrast to the inhibitory effects observed on promoter activity. To get the better insight of how OPs affect IL-8 protein expressions, we first measured intracellular IL-8 protein contents by ELISA. In both undifferentiated and differentiated Caco-2 cells, intracellular contents of IL-8 protein significantly increased in the presence of both IL-1β and OPs compared with the treatment with IL-1β alone (Fig. 11 a and c). Notably, these results were consistent with the mRNA expression in both undifferentiated and differentiated Caco-2 cells (Fig. 10 a and c). To our surpise, when we focused our attention on secreted IL-8 protein level (Fig 11 b and d), the amount of IL-8 protein released into the medium did not exactly follow the profile of IL-8 mRNA expression (Fig 10 a an b) and intracellular IL-8 contents (Fig 11 a, c). Especially in differentiated Caco-2 cells (fig 11 d), we observed a significant reduction of IL-8 protein secreted into the culture medium, indicating that the observed effects also depend on the differentiation state of Caco-2 cells. Thus, these data suggest that the OPs potentiate the effect of IL-1β on both IL-8 mRNA and protein levels.

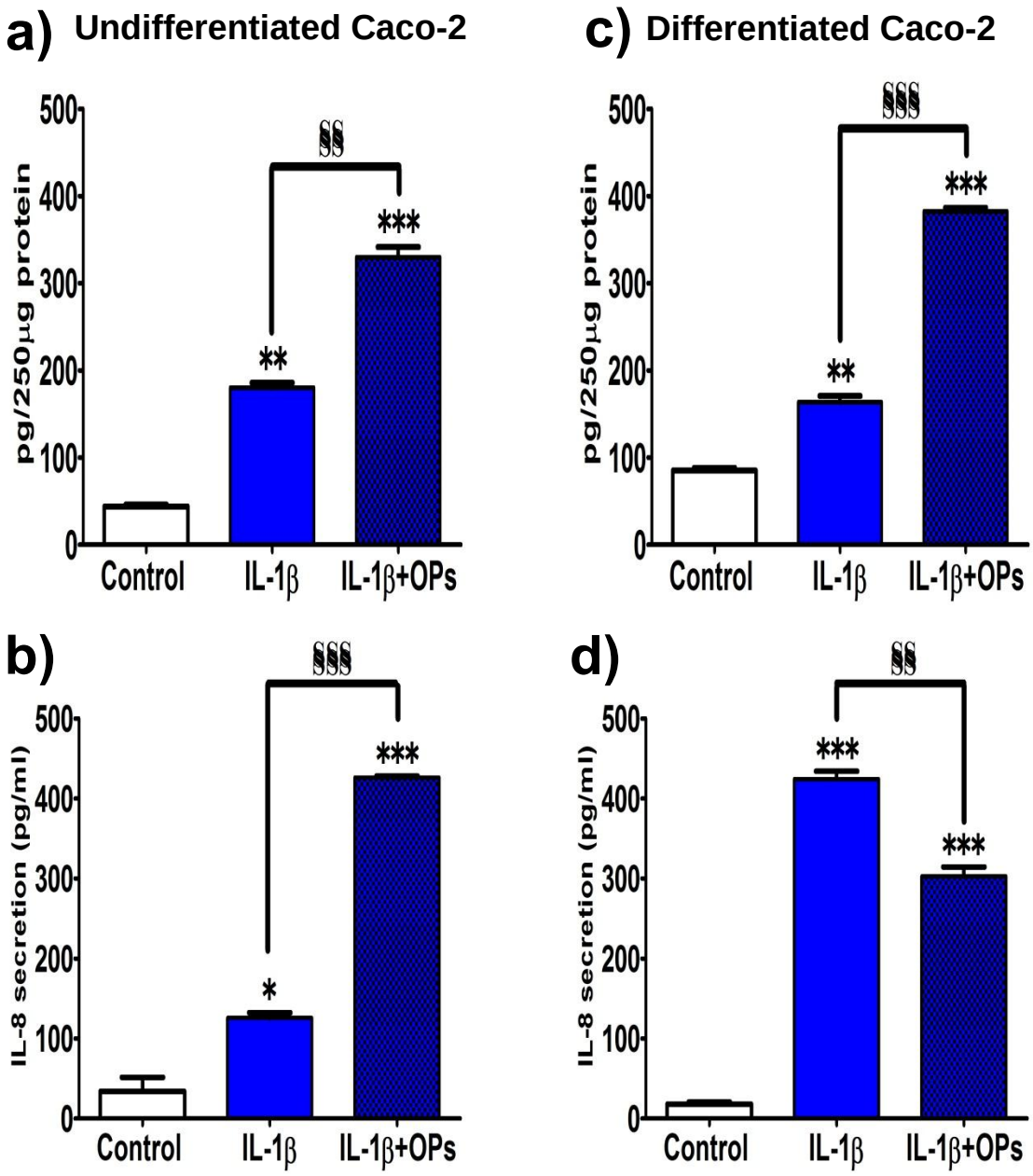


Fig11 Intracellular IL-8 protein accumulation and IL-8 secretion in both undifferentiated and differentiated Caco-2 cells stimulated with IL-1β The Caco-2 cells were seeded 6×10^5 cells/well on 24 wells plates, after 24 hours (undifferentiated state) or 18 days (differentiated state) cells were pre-treated for 1 hour with 10µg/ml of OPs. Then, we treated cells with 5ng/ml of IL-1β together with OPs for the next 6 hours. Then intracellular IL-8 protein contents were extracted with lysis buffer. Intracellular IL-8 protein contents and IL-8 secretion were measured by ELISA as described in material and methods. Data were expressed as mean ± SEM of and shown one representative result from 3 different experiments, n=4 per conditions, **p<0.05, ***p<0.0001 vs control, §§p<0.0001 vs stimulus. Data were analyzed by one-way analysis of variance (ANOVA), followed by Bonferroni as post-hoc test.

4.15. The OPs affect IL-8 mRNA stability

To gain further insight on the effect of OPs on IL-8 expression in the presence of IL-1β, we evaluated IL-8 mRNA stability. To this end, we measured the mRNA levels of IL-8, whose reported half-life is about 2 hours, in the presence of actinomycin D, a known inhibitor of gene transcription. In these conditions, the mRNA levels of a highly regulated gene should decay due to degradation while the mRNA level of a housekeeping gene such as GAPDH remain fairly constant. In the cells treated only with IL-1β and actinomcin D, IL-8 mRNA level was significantly decreased in both undifferentiated and differentiated Caco-2 cells. As shown in Fig 12 a and b (blue line) at 2 hours, IL-8 mRNA decreased by about 50% with respect to time 0, as a consequence of the inhibition of gene transcription. On the other hand, in the cells treated with both IL-1β and phenolic extracts, IL-8 mRNA levels at 2 hours were significantly higher than IL-1β alone. These results clearly indicate that the phenolic extract affects the stability of IL-8 mRNA. Importantly, these results could explain, at least in part, the discrepancy between the effects observed on IL-8 promoter activity and on mRNA levels in cells treated with both IL-1β and OPs (Fig. 12 a and b).

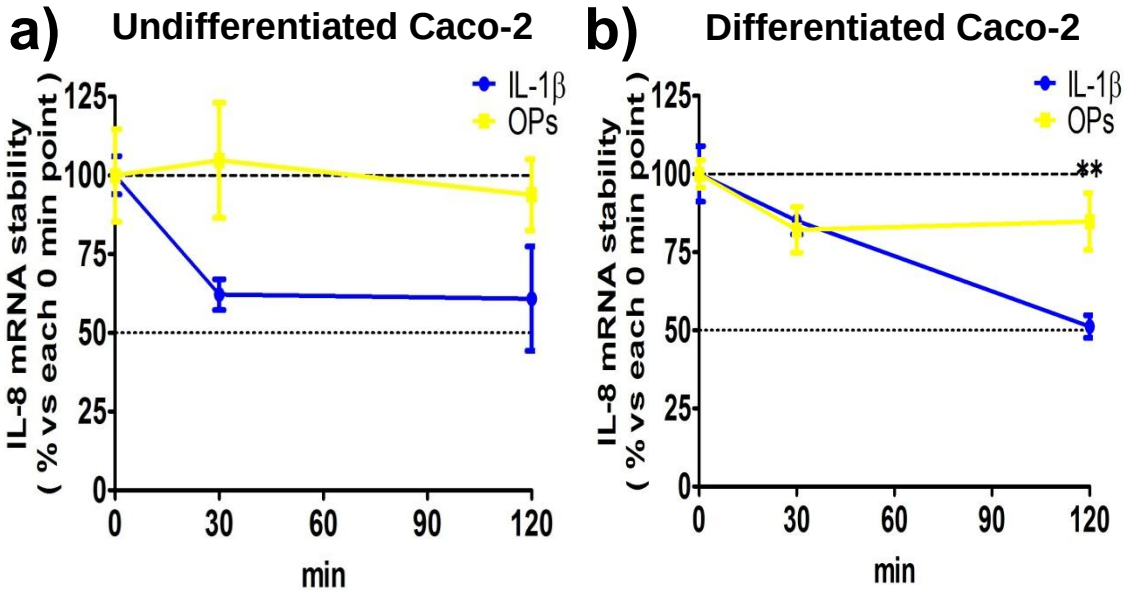


Fig 12 The IL-8 mRNA stabilization with OPs The Caco-2 cells were seeded 6×10^5 cells/well on 24 wells plates, after 24 hours (undifferentiated state) or 18 days (differentiated state) cells were pre-treated for 1 hour with 10μg/ml of OPs. Then, we treated cells with 5ng/ml of IL-1β together with OPs. After 6 hours the medium was replaced with 10μg/ml of actinomycin D till maximum 2 hours. The time 0 IL-8 mRNA levels were set at 100% by the following conditions. Data were expressed as mean ± SEM and shown representative results from one experiment repeated two-three times and yielding similar results, n=4 per conditions,**$p<0.01$ vs stimuli alone. Data were analyzed by two-way analysis of variance (ANOVA), followed by Bonferroni as post-hoc test.

4.16. Modulation of MAPK signalling by Phenolic extracts

As discussed in the introduction, different MAPKs cascades have been shown to be involved in the stabilization of IL-8 mRNA. More specifically, activation of these MAPKs is associated to increase stability of IL-8 transcripts [(79)]. To assess whether OPs increase IL-8 mRNA stability by means of these MAPKs, we used specific inhibitors of p38MAPK (SB203580), ERK (U-0126) and JNK (SP600125). We obtained that the inhibition of both p38MAPK and ERK signaling pathways prevents, at least in part, the induction of IL-8 mRNA expression by OPs, and this tendency was observed in both undifferentiated and differentiate Caco-2 cells (Fig 13 a and b). These results strongly suggest that activation of p38 and/or ERK contributes to the biological activity of OPs on IL-8 expression, probably at the level of mRNA stability.

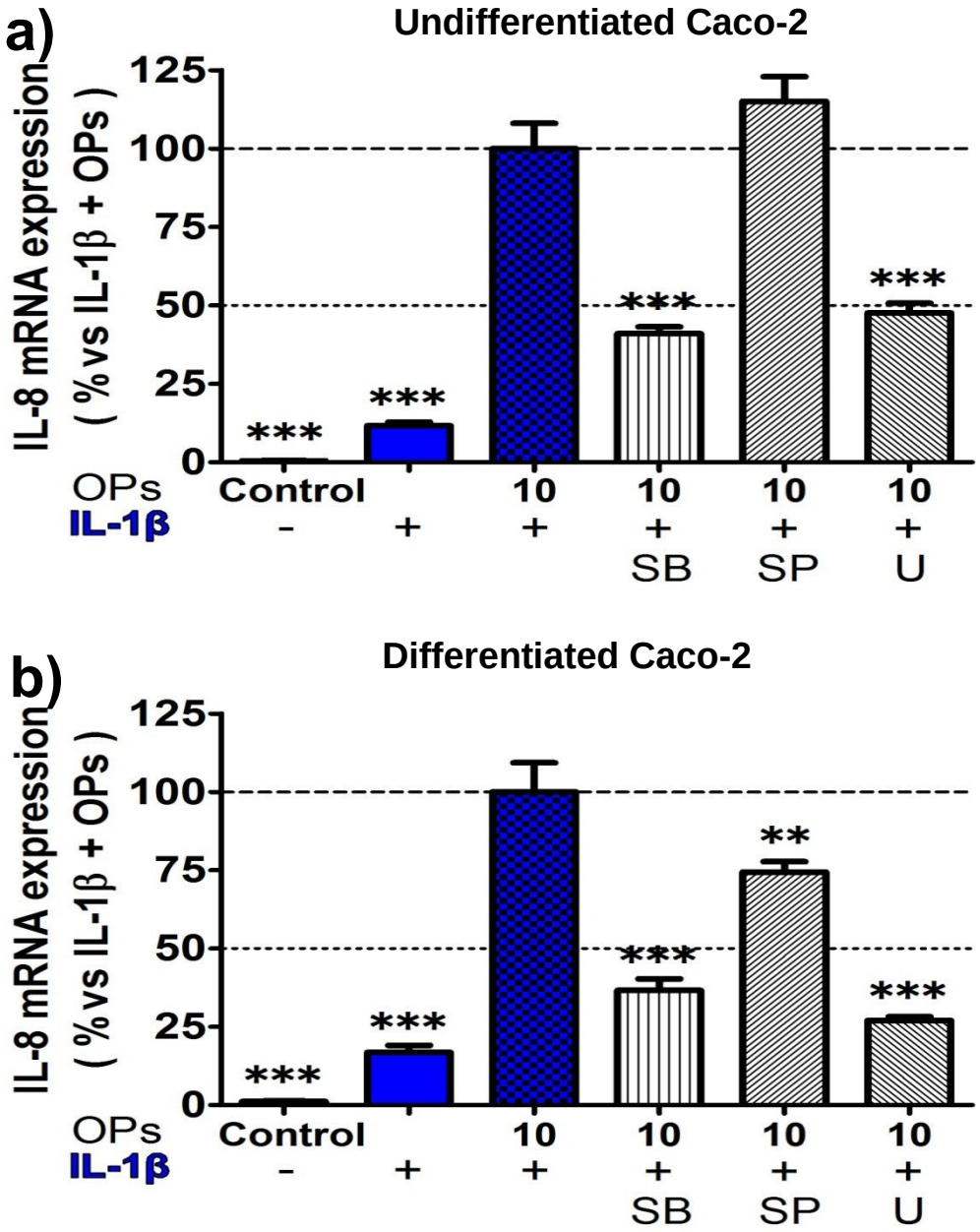


Fig13 Involvement of p38 MAPK and ERK pathway in the induction of IL-8 mRNA expression by OPs The undifferentiated (a) and differentiated (b) Caco-2 cells were pre-treated for 1 hour with 10 μg/ml of OPs together with individual MAPKs inhibitors; 30μM SB203580 (SB: p38 inhibitor), 10μM SP600125 (SP: JNK inhibitor), 10μM U0126 (U: ERK inhibitor) or as vehicle (DMSO). Then 1hour later inflammation states were induced by 5ng/ml of IL-1β together with OPs and individual inhibitors for the next 6 hours. The cells treated with IL-1β+ 10μg/ml of OPs were set at 100%. Data were expressed as mean ± SEM of and shown one representative result, n=3 per conditions, **p<0.01, ***p<0.0001 vs stimuli alone. Data were analyzed by one-way analysis of variance (ANOVA), followed by Bonferroni as post-hoc test.

CHAPTER 5

DISCUSSION

Olive oil has been recognized as an important component of a healthy diet, also because of its phenolic content[89]. In fact, previous studies have identified many properties of olive oil, including anti-inflammatory [43], anti-atherogenic [90], anti-cancer [91] and anti-microbial [92] activities. Heretofore, however, there are only few studies where the effects of olive oil phenols (OPs) have been examined *in vitro* on intestinal cells or *in vivo* in the gut [46; 93; 94].

The present study was aimed to elucidate how OPs interact with intestinal epithelial cells in the normal and inflamed conditions. Van De Wall *et al* previously demonstrated that the differentiation stage may significantly influence the inflammatory response in intestinal epithelial cells [95]. Therefore, we examined the effects of OPs on the inflammatory process in both undifferentiated and differentiated Caco-2 cells, a well established cellular model of intestinal epithelial cells. Interleukin-8 (IL-8) is rapidly induced in response to pro-inflammatory cytokines, bacterial and cellular stresses, and is involved in the acute phase of gastrointestinal inflammation. The synthesis of IL-8 is controlled by different signalling pathways including NF-κB and MAPKs. The present results strongly suggest that although OPs actually inhibited NF-κB-driven transcription and nuclear translocation, they increased IL-8 mRNA expression in both undifferentiated and differentiated cells stimulated with IL-1β. We next examined the molecular mechanisms whereby they affect expression of IL-8 mRNA and protein secretion in the medium by assessing the mRNA stability, intracellular protein translation and the effects of OPs on the MAPK signalling pathway.

5.1. Biological activity of OPs

So far, many studies demonstrated the anti-inflammatory effects of OPs as a mixture of pure compounds, often at concentrarions much higher than the dietary intake. Therefore, in the present study we investigated the effects of OPs isolated from olive oil as a mixture at concentrations close to those that could be found in the intestine of subjects consuming the Mediterranean diet.

We have already shown the effects of OPs on NF-κB driven transcription and its nuclear translocation in gastric adenocarcinoma cells (AGS) [(65)]. In the above study, we examined the biological activity of OPs obtained from different commercially available extra-virgin olive oils. In the present study, we used D.O.P. (Denominazione di Origine Protetta) olive oil from "PLANETA®", a small company which strictly controls the growing environment, origin of olive fruits and follows low scale producing procedures. As we mentioned in results 4.1. section, "PLANETA" olive oil exhibited high organoleptic quality, but total phenolic content and composition was similar to those found in large-scale produces olive oils. In the present study "PLANETA" olive oil suppressed NF-κB-driven transcription in AGS cells with an IC50 of 1.38μg/ml similar to the IC50s (0.86-1.28μg/ml) obtained previously with phenolic extracts derived from other olive oils produeced at a large scale [(65)].

As shown in Table 1, amoung phenols secoiridois (oleuropein-aglycon, ligstroside-aglycon) were the most abundant compounds present in the extract. In deed we previously demonstrated on the AGS cells, that secoiridoids significantly contributed to the biological activity of OPs and that the metabolite homovanillic

alcohol was also active, indicating that metabolic transformation does not impair the biological activity[65].

5.2. OPs suppressed the IL-8 expressions induced by bacterial LPS

Although intestine is usually highly tolerant to bacterial LPS, under certain conditions LPS derived from pathobiont microbiota could contribute to inflammatory diseases. As shown in Fig 2, LPS did not induce NF-κB-driven transcription but it induced IL-8 native promoter activity (Fig 3), mRNA expression and protein level (Fig 9). This indicated that LPS induced IL-8 expression not through NF-κB but through other pathways, most likely MAPK, AP-1, phosphatidylinositol 3-kinase (PI3K) and Akt [96]. Furthermore, OPs suppressed LPS-induced IL-8 native promoter activity (Fig 3), IL-8 mRNA and its protein expressions in both undifferentiated and differentiated Caco-2 (Fig 9). These data suggest that the phenolic extract modulates the IL-8 expression through an NF-κB-independent pathway. Therefore, when the inflammation state is caused by LPS, olive oil phenols may act as anti-inflammatory agents, thus suggesting that consumption of extra-virgin olive oil may attenuate the inflammatory response in the gut in a state of microbial imbalance

5. 3. OPs enhance the IL-8 mRNA expression induced by IL-1β

The gastrointestinal tract is composed of a single layer of intestinal epithelial cells, and this physical barrier separates subepithelial mucosal immune cells such as lymphocytes, macrophages, and dendritic cells from bacteria and food antigens [97]. However, in the cases of infection, stress, and food intoxication, this barrier becomes

weakened and bacterial LPS could pass through the barrier and activate macrophages. Then, the activated macrophages could amplify the signals generated by immune cells (neutrophils, dendritic cells, etc) by secreting many chemokines and cytokines such as, IL-6, TNF-α and IL-1β [98]. IL-1β is a prototypical proinflammatory cytokine that plays a central role in the inflammatory responses of the intestine [99]. Recently, Sanchez et al. reported that, supplementation of extra-virgin olive oil phenol extracts in an experimental model of colitis in mice, suppressed the inflammatory cascade through up-regulation of peroxisome proliferator-activated receptor gamma (PPAR$\boldsymbol{\gamma}$) and inhibition of NF-κB and MAPK signaling pathways[100]. Furthermore, Handa et al. demonstrated that the main polyphenol occurring in green tea, epigallocatechin-3-gallate (EGCG), inhibit IL-1β-induced IL-8 production and expression of cell surface adhesion molecule in a cell model of gastric inflammation[101]. On these premises we hypothesized that also OPs could suppress IL-8 expression under IL-1β-induced inflammatory state. Neverthethless even though the phenolic extract suppressed NF-κB-driven transcription (Fig 4), nuclear translocations (Fig 5) and IL-8 native promoter activity (Fig 6) in cells treated with IL-1β, it stimulated IL-8 mRNA levels (Fig 10 a and b) and intracellular IL-8 protein content (Fig 11 b) in both undifferentiated and differentiated Caco-2 cells. These data suggest that phenolic compounds might affect multiple signalling pathways contributing toIL-8 expression. Moreover, in differentiated Caco-2 cells, we found no correlation between IL-8 secretion (Fig 10 d) and mRNA expression (Fig 10 c), implaying a blockade in the secretion process in the presence of OPs. These observations strongly suggest that phenolic extract does not only affect transcription of IL-8 gene but it may also influence the secretion process.

5. 4. OPs increase IL-8 mRNA stability

As already discussed, we found a discrepancy between the effect of OPs on IL-8 promoter activity and mRNA levels in cells treated with IL-1β. To gain further insight on these contradictory results, we evaluated the effects of OPs on the stability of IL-8 mRNA and we did find that they delay the decay of IL-8 mRNA. So far, there is no information reporting the effect of OPs on mRNA stability.

Previous studies have suggested the involvement of MAPK and p38 pathways in IL-8 mRNA stabilization [102; 103]. Therefore, we examined the potential contribution of these pathways in OPs-induced IL-8 mRNA stability by using specific MAPK inhibitors in both undifferentiated and differentiated Caco-2 cells. As shown in Fig 13 a and b, inhibition of both p38 and ERK MAPKs significantly prevented the IL-8 mRNA expression induced by OPs. These results indicate that both p38 and ERK are involved in IL-8 mRNA stability and that the phenolic extract exhibits a profound effect on these pathways. A relevant role of p38 pathway in IL-8 mRNA expression and stability was put forward by Hoffmann et al.[79]. Moreover, other authors also demonstrated the involvement of ERK in IL-8 expression in addition to p38. Intriguingly, Corona *et al* recently demonstrated that olive oil phenol extract exerted their chemopreventive effects by the inhibition of phosphorylation of p38 kinase in human colon adenocarcinoma cells, which suggests that protein phosphorylation cascades of p38 could also be involved in the molecular actions of the phenolic extract [77]. At present, we do not know the effects on the phosphorylation states of p38 in the present experimental conditions and it may require further investigations.

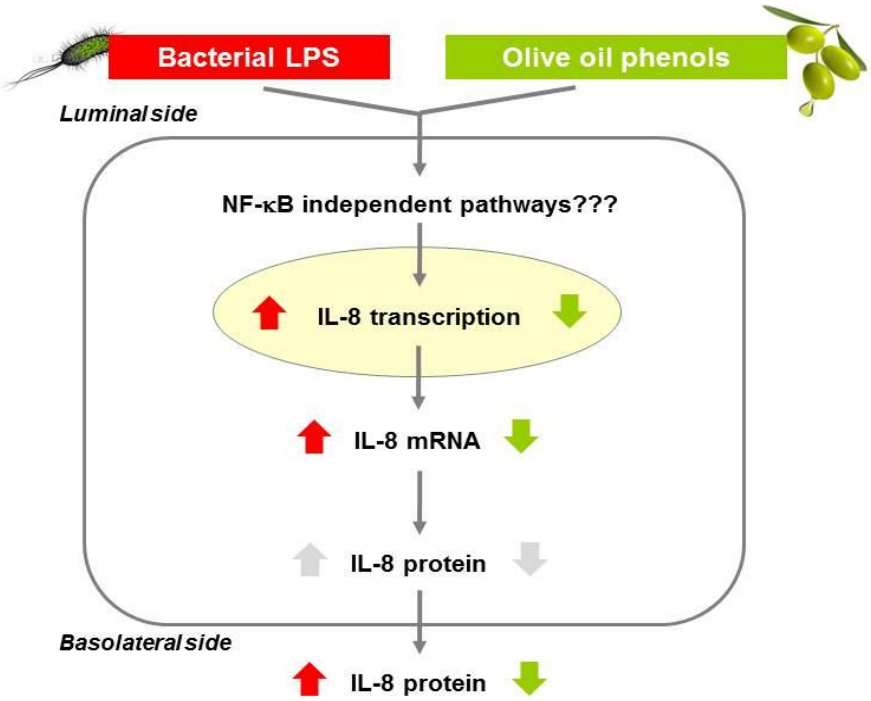
Our results also suggest that JNK does not seem to be responsible for the increased IL-8 mRNA stability induced by OPs (Fig 13 a and b); however we observed that JNK inhibitors significantly suppressed IL-8 secretion induced by OPs (Data not shown). These data imply that JNK pathway is not contributing to the stabilization of IL-8 mRNA , but it most likely influence the IL-8 secretion process.

5. 5. Limitations of NF-κB-based biological assays

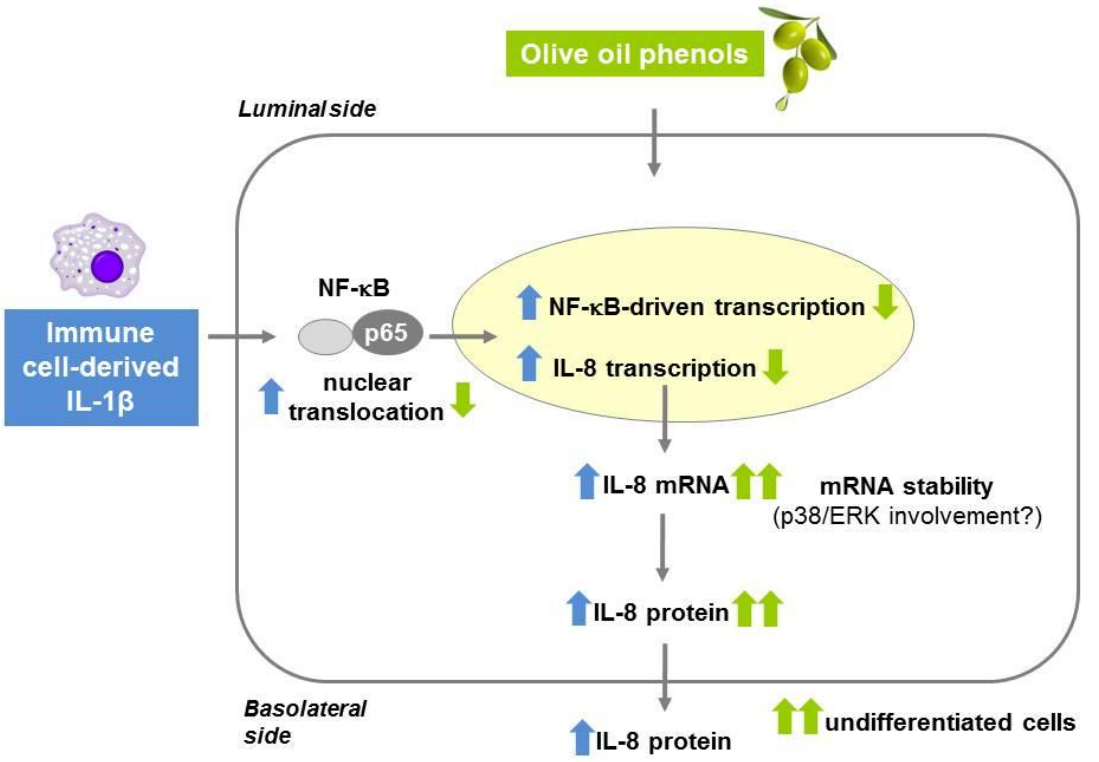
NF-κB has been recognized as a target of several antiinflammatory pharmacological interventions. Inhibition of the NF-κB activity suppresses the expression of various inflammatory mediators. Many previous studies have used biological assays based on NF-κB-driven transcription and nuclear translocation to assess the anti-inflammatory profile of drugs and natural products. The results obtained in the present study suggest that, additional pathways should be considered to test the anti-inflammatory properties of a given treatment, also in consideration of the inflammatory stimulus used in the assay.

5. 6. Conclusion

All data reported in the present thesis can be summarized in the schemes shown on the next page. OPs act through different molecular pathways on the inflammatory processes induced by different stimuli.



Red arrows, effects of the inflammatory stimulus; green arrows, effects of olive oil phenols; grey arrows, not evaluated



Blue arrows, effects of inflammatory stimulus; green arrows, effects of olive oil phenols

***Abbreviations*:**

AGS	human gastric adenocarcinoma cells
AP	apigenin
AP-1	activator protein 1
Caco-2	human epithelial colorectal adenocarcinoma
C/EBPβ	CCAAT-enhancer-binding protein-β
COX2	cyclooxygenase 2
CREB	cAMP response element binding protein
EGCG	epigallocatechin-3-gallate
ERK1/2	extracellular signal-regulated kinase 1/2
HY	hydroxytyrosol
IBD	inflammatory bowel disease
IC50	half maximal inhibitory concentration
iNOS	inducible nitric oxide synthase
IL-1β	interleukin-1beta
IL-6	interleukin-6
IL-8	Interleukin-8
JNK	c-Jun N-terminal kinase
LDL	low density lipoprotein
LigA	ligstroside aglicone
LU	luteolin
MAPKs	Mitogen-activated protein kinases
MMP-9	metalloproteinase-9
MUFA	monounsaturated fatty acids
NASH	nonalcoholic steatohepatitis
NF-κB	nuclear factor-kappa B
Nrf2	nuclear factor (erythroid-derived 2)-like 2
OleA	oleuropein aglicone
Ops	olive oil phenols
PGE2	prostaglandin E2
PI3K	phosphatidylinositol 3-kinase

PPAR$\boldsymbol{\gamma}$	peroxisome proliferator-activated receptor gamma
THP1	human monocytic cells
TLR4	toll-like receptor 4
TLR9	toll-like receptor 9
TNF-$\boldsymbol{\alpha}$	tumour-necrosis factor α
TY	tyrosol

CHAPTER 6

REFERENCES

1. Garrett WS, Gordon JI, Glimcher LH (2010) Homeostasis and inflammation in the intestine. *Cell* **140**, 859-870.
2. Cader MZ, Kaser A (2013) Recent advances in inflammatory bowel disease: mucosal immune cells in intestinal inflammation. *Gut* **62**, 1653-1664.
3. Breen DM, Rasmussen BA, Cote CD *et al.* (2013) Nutrient-sensing mechanisms in the gut as therapeutic targets for diabetes. *Diabetes* **62**, 3005-3013.
4. Henao-Mejia J, Elinav E, Jin C *et al.* (2012) Inflammasome-mediated dysbiosis regulates progression of NAFLD and obesity. *Nature* **482**, 179-185.
5. Koboziev I, Reinoso Webb C, Furr KL *et al.* (2013) Role of the enteric microbiota in intestinal homeostasis and inflammation. *Free radical biology & medicine* **68c**, 122-133.
6. Leaphart CL, Cavallo J, Gribar SC *et al.* (2007) A critical role for TLR4 in the pathogenesis of necrotizing enterocolitis by modulating intestinal injury and repair. *Journal of immunology* **179**, 4808-4820.
7. Sodhi CP, Shi XH, Richardson WM *et al.* (2010) Toll-like receptor-4 inhibits enterocyte proliferation via impaired beta-catenin signaling in necrotizing enterocolitis. *Gastroenterology* **138**, 185-196.
8. Martin GR, Wallace JL (2006) Gastrointestinal inflammation: a central component of mucosal defense and repair. *Experimental biology and medicine (Maywood, NJ)* **231**, 130-137.
9. Bruewer M, Utech M, Ivanov AI *et al.* (2005) Interferon-gamma induces internalization of epithelial tight junction proteins via a macropinocytosis-like process. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **19**, 923-933.
10. Beutler B, Rietschel ET (2003) Innate immune sensing and its roots: the story of endotoxin. *Nature reviews Immunology* **3**, 169-176.
11. Medzhitov R, Janeway C, Jr. (2000) Innate immune recognition: mechanisms and pathways. *Immunological reviews* **173**, 89-97.
12. Bocker U, Damiao A, Holt L *et al.* (1998) Differential expression of interleukin 1 receptor antagonist isoforms in human intestinal epithelial cells. *Gastroenterology* **115**, 1426-1438.

13. Reimund JM, Wittersheim C, Dumont S *et al.* (1996) Increased production of tumour necrosis factor-alpha interleukin-1 beta, and interleukin-6 by morphologically normal intestinal biopsies from patients with Crohn's disease. *Gut* **39**, 684-689.
14. Schuerer-Maly CC, Eckmann L, Kagnoff MF *et al.* (1994) Colonic epithelial cell lines as a source of interleukin-8: stimulation by inflammatory cytokines and bacterial lipopolysaccharide. *Immunology* **81**, 85-91.
15. Forsythe RM, Xu DZ, Lu Q *et al.* (2002) Lipopolysaccharide-induced enterocyte-derived nitric oxide induces intestinal monolayer permeability in an autocrine fashion. *Shock (Augusta, Ga)* **17**, 180-184.
16. Al-Sadi RM, Ma TY (2007) IL-1beta causes an increase in intestinal epithelial tight junction permeability. *Journal of immunology* **178**, 4641-4649.
17. Willett WC, Sacks F, Trichopoulou A *et al.* (1995) Mediterranean diet pyramid: a cultural model for healthy eating. *The American journal of clinical nutrition* **61**, 1402s-1406s.
18. Keys A, Menotti A, Karvonen MJ *et al.* (1986) The diet and 15-year death rate in the seven countries study. *Am J Epidemiol* **124**, 903-915.
19. Estruch R, Ros E, Salas-Salvadó J *et al.* (2013) Primary Prevention of Cardiovascular Disease with a Mediterranean Diet. *New England Journal of Medicine* **368**, 1279-1290.
20. Visioli F, Bellomo G, Montedoro G *et al.* (1995) Low density lipoprotein oxidation is inhibited in vitro by olive oil constituents. *Atherosclerosis* **117**, 25-32.
21. Pallauf K, Giller K, Huebbe P *et al.* (2013) Nutrition and healthy ageing: calorie restriction or polyphenol-rich "MediterrAsian" diet? *Oxidative medicine and cellular longevity* **2013**, 707421.
22. Corona G, Spencer JP, Dessi MA (2009) Extra virgin olive oil phenolics: absorption, metabolism, and biological activities in the GI tract. *Toxicology and industrial health* **25**, 285-293.
23. Bendini A, Cerretani L, Carrasco-Pancorbo A *et al.* (2007) Phenolic molecules in virgin olive oils: a survey of their sensory properties, health effects, antioxidant activity and analytical methods. An overview of the last decade. *Molecules (Basel, Switzerland)* **12**, 1679-1719.

24. Esti M, Cinquanta L, La Notte E (1998) Phenolic Compounds in Different Olive Varieties. *Journal of agricultural and food chemistry* **46**, 32-35.
25. Montedoro G, Servili M, Baldioli M *et al.* (1992) Simple and hydrolyzable phenolic compounds in virgin olive oil. 1. Their extraction, separation, and quantitative and semiquantitative evaluation by HPLC. *Journal of agricultural and food chemistry* **40**, 1571-1576.
26. Vissers MN, Zock PL, Roodenburg AJ *et al.* (2002) Olive oil phenols are absorbed in humans. *The Journal of nutrition* **132**, 409-417.
27. Vissers MN, Zock PL, Roodenburg AJC *et al.* (2002) Olive Oil Phenols Are Absorbed in Humans. *The Journal of nutrition* **132**, 409-417.
28. Tuck KL, Hayball PJ (2002) Major phenolic compounds in olive oil: metabolism and health effects. *The Journal of nutritional biochemistry* **13**, 636-644.
29. Buck K, Zaineddin AK, Vrieling A *et al.* (2010) Meta-analyses of lignans and enterolignans in relation to breast cancer risk. *The American journal of clinical nutrition* **92**, 141-153.
30. Hoffman R, Gerber M (2012) Virgin olive oil as a source of phytoestrogens. *European journal of clinical nutrition* **66**, 1180.
31. Granados-Principal S, Quiles JL, Ramirez-Tortosa CL *et al.* (2010) Hydroxytyrosol: from laboratory investigations to future clinical trials. *Nutrition reviews* **68**, 191-206.
32. Richard N, Arnold S, Hoeller U *et al.* (2011) Hydroxytyrosol is the major anti-inflammatory compound in aqueous olive extracts and impairs cytokine and chemokine production in macrophages. *Planta medica* **77**, 1890-1897.
33. Choi JS, Nurul Islam M, Yousof Ali M *et al.* (2013) Effects of C-glycosylation on anti-diabetic, anti-Alzheimer's disease and anti-inflammatory potential of apigenin. *Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association* **64c**, 27-33.
34. Wall C, Lim R, Poljak M *et al.* (2013) Dietary flavonoids as therapeutics for preterm birth: luteolin and kaempferol suppress inflammation in human gestational tissues in vitro. *Oxidative medicine and cellular longevity* **2013**, 485201.
35. Cicerale S, Conlan XA, Sinclair AJ *et al.* (2009) Chemistry and health of olive oil phenolics. *Critical reviews in food science and nutrition* **49**, 218-236.

36. Gill CI, Boyd A, McDermott E *et al.* (2005) Potential anti-cancer effects of virgin olive oil phenols on colorectal carcinogenesis models in vitro. *International journal of cancer Journal international du cancer* **117**, 1-7.
37. Goya L, Mateos R, Bravo L (2007) Effect of the olive oil phenol hydroxytyrosol on human hepatoma HepG2 cells. Protection against oxidative stress induced by tert-butylhydroperoxide. *European journal of nutrition* **46**, 70-78.
38. Di Benedetto R, Vari R, Scazzocchio B *et al.* (2007) Tyrosol, the major extra virgin olive oil compound, restored intracellular antioxidant defences in spite of its weak antioxidative effectiveness. *Nutrition, metabolism, and cardiovascular diseases : NMCD* **17**, 535-545.
39. Manna C, Galletti P, Cucciolla V *et al.* (1997) The protective effect of the olive oil polyphenol (3,4-dihydroxyphenyl)-ethanol counteracts reactive oxygen metabolite-induced cytotoxicity in Caco-2 cells. *The Journal of nutrition* **127**, 286-292.
40. Manna C, Galletti P, Cucciolla V *et al.* (1999) Olive oil hydroxytyrosol protects human erythrocytes against oxidative damages. *The Journal of nutritional biochemistry* **10**, 159-165.
41. Hashimoto T, Ibi M, Matsuno K *et al.* (2004) An endogenous metabolite of dopamine, 3,4-dihydroxyphenylethanol, acts as a unique cytoprotective agent against oxidative stress-induced injury. *Free radical biology & medicine* **36**, 555-564.
42. Scoditti E, Calabriso N, Massaro M *et al.* (2012) Mediterranean diet polyphenols reduce inflammatory angiogenesis through MMP-9 and COX-2 inhibition in human vascular endothelial cells: a potentially protective mechanism in atherosclerotic vascular disease and cancer. *Archives of biochemistry and biophysics* **527**, 81-89.
43. Visioli F, Bellosta S, Galli C (1998) Oleuropein, the bitter principle of olives, enhances nitric oxide production by mouse macrophages. *Life sciences* **62**, 541-546.
44. Rosignoli P, Fuccelli R, Fabiani R *et al.* (2013) Effect of olive oil phenols on the production of inflammatory mediators in freshly isolated human monocytes. *The Journal of nutritional biochemistry* **24**, 1513-1519.
45. Bartoli R, Fernandez-Banares F, Navarro E *et al.* (2000) Effect of olive oil on early and late events of colon carcinogenesis in rats: modulation of arachidonic acid metabolism and local prostaglandin E(2) synthesis. *Gut* **46**, 191-199.

46. Sanchez-Fidalgo S, Villegas I, Cardeno A *et al.* (2010) Extra-virgin olive oil-enriched diet modulates DSS-colitis-associated colon carcinogenesis in mice. *Clinical nutrition* **29**, 663-673.
47. Gong D, Geng C, Jiang L *et al.* (2009) Effects of hydroxytyrosol-20 on carrageenan-induced acute inflammation and hyperalgesia in rats. *Phytotherapy research : PTR* **23**, 646-650.
48. Acin S, Navarro MA, Arbones-Mainar JM *et al.* (2006) Hydroxytyrosol administration enhances atherosclerotic lesion development in apo E deficient mice. *Journal of biochemistry* **140**, 383-391.
49. Visioli F, Galli C, Bornet F *et al.* (2000) Olive oil phenolics are dose-dependently absorbed in humans. *FEBS letters* **468**, 159-160.
50. Caruso D, Visioli F, Patelli R *et al.* (2001) Urinary excretion of olive oil phenols and their metabolites in humans. *Metabolism: clinical and experimental* **50**, 1426-1428.
51. Sirtori CR, Gatti E, Tremoli E *et al.* (1992) Olive oil, corn oil, and n-3 fatty acids differently affect lipids, lipoproteins, platelets, and superoxide formation in type II hypercholesterolemia. *The American journal of clinical nutrition* **56**, 113-122.
52. Roche HM, Zampelas A, Knapper JM *et al.* (1998) Effect of long-term olive oil dietary intervention on postprandial triacylglycerol and factor VII metabolism. *The American journal of clinical nutrition* **68**, 552-560.
53. Kris-Etherton PM, Pearson TA, Wan Y *et al.* (1999) High-monounsaturated fatty acid diets lower both plasma cholesterol and triacylglycerol concentrations. *The American journal of clinical nutrition* **70**, 1009-1015.
54. Oliveras-Lopez MJ, Berna G, Carneiro EM *et al.* (2008) An extra-virgin olive oil rich in polyphenolic compounds has antioxidant effects in OF1 mice. *The Journal of nutrition* **138**, 1074-1078.
55. Eletto D, Leone A, Bifulco M *et al.* (2005) Effect of unsaturated fat intake from Mediterranean diet on rat liver mRNA expression profile: selective modulation of genes involved in lipid metabolism. *Nutrition, metabolism, and cardiovascular diseases : NMCD* **15**, 13-23.

56. Deng X, Elam MB, Wilcox HG *et al.* (2004) Dietary olive oil and menhaden oil mitigate induction of lipogenesis in hyperinsulinemic corpulent JCR:LA-cp rats: microarray analysis of lipid-related gene expression. *Endocrinology* **145**, 5847-5861.
57. Acin S, Navarro MA, Perona JS *et al.* (2007) Microarray analysis of hepatic genes differentially expressed in the presence of the unsaponifiable fraction of olive oil in apolipoprotein E-deficient mice. *The British journal of nutrition* **97**, 628-638.
58. Camargo A, Ruano J, Fernandez JM *et al.* (2010) Gene expression changes in mononuclear cells in patients with metabolic syndrome after acute intake of phenol-rich virgin olive oil. *BMC genomics* **11**, 253.
59. Bogani P, Galli C, Villa M *et al.* (2007) Postprandial anti-inflammatory and antioxidant effects of extra virgin olive oil. *Atherosclerosis* **190**, 181-186.
60. Perez-Martinez P, Lopez-Miranda J, Blanco-Colio L *et al.* (2007) The chronic intake of a Mediterranean diet enriched in virgin olive oil, decreases nuclear transcription factor kappaB activation in peripheral blood mononuclear cells from healthy men. *Atherosclerosis* **194**, e141-146.
61. Bellido C, Lopez-Miranda J, Blanco-Colio LM *et al.* (2004) Butter and walnuts, but not olive oil, elicit postprandial activation of nuclear transcription factor kappaB in peripheral blood mononuclear cells from healthy men. *The American journal of clinical nutrition* **80**, 1487-1491.
62. Lucas L, Russell A, Keast R (2011) Molecular mechanisms of inflammation. Anti-inflammatory benefits of virgin olive oil and the phenolic compound oleocanthal. *Current pharmaceutical design* **17**, 754-768.
63. Dell'Agli M, Fagnani R, Mitro N *et al.* (2006) Minor components of olive oil modulate proatherogenic adhesion molecules involved in endothelial activation. *Journal of agricultural and food chemistry* **54**, 3259-3264.
64. Dell'Agli M, Fagnani R, Galli GV *et al.* (2010) Olive oil phenols modulate the expression of metalloproteinase 9 in THP-1 cells by acting on nuclear factor-kappaB signaling. *Journal of agricultural and food chemistry* **58**, 2246-2252.
65. Sangiovanni E, Colombo E, Fumagalli M *et al.* (2012) Inhibition of NF- kappaB activity by minor polar components of extra-virgin olive oil at gastric level. *Phytotherapy research : PTR* **26**, 1569-1571.

66. Baltes S, Nau H, Lampen A (2004) All-trans retinoic acid enhances differentiation and influences permeability of intestinal Caco-2 cells under serum-free conditions. *Development, growth & differentiation* **46**, 503-514.

67. Ebert B, Seidel A, Lampen A (2007) Phytochemicals induce breast cancer resistance protein in Caco-2 cells and enhance the transport of benzo[a]pyrene-3-sulfate. *Toxicological sciences : an official journal of the Society of Toxicology* **96**, 227-236.

68. Sambuy Y, De Angelis I, Ranaldi G *et al.* (2005) The Caco-2 cell line as a model of the intestinal barrier: influence of cell and culture-related factors on Caco-2 cell functional characteristics. *Cell biology and toxicology* **21**, 1-26.

69. Buhrke T, Lengler I, Lampen A (2011) Analysis of proteomic changes induced upon cellular differentiation of the human intestinal cell line Caco-2. *Development, growth & differentiation* **53**, 411-426.

70. Pinto M, Robine-Leon, S., Appay, M. D., Kedinger, M., Triadou, N., Dussaulx, I'.. Lacroix, B., Simon-Assmann, P.. Haffen, K., Fogh, J., and, Zweibaum A (1983) Enterocyte-like differentiation and polarization of the human colon carcinoma cell line Caco-2 in culture. . *Biol Cell* **47**, 323-330.

71. Turck N, Richert S, Gendry P *et al.* (2004) Proteomic analysis of nuclear proteins from proliferative and differentiated human colonic intestinal epithelial cells. *Proteomics* **4**, 93-105.

72. Engle MJ, Goetz GS, Alpers DH (1998) Caco-2 cells express a combination of colonocyte and enterocyte phenotypes. *Journal of cellular physiology* **174**, 362-369.

73. Baldwin AS, Jr. (1996) The NF-kappa B and I kappa B proteins: new discoveries and insights. *Annual review of immunology* **14**, 649-683.

74. Hayward R, Lefer AM (1998) Time course of endothelial-neutrophil interaction in splanchnic artery ischemia-reperfusion. *American Journal of Physiology - Heart and Circulatory Physiology* **275**, H2080-H2086.

75. Hoesel B, Schmid JA (2013) The complexity of NF-kappaB signaling in inflammation and cancer. *Molecular cancer* **12**, 86.

76. Garrington TP, Johnson GL (1999) Organization and regulation of mitogen-activated protein kinase signaling pathways. *Current opinion in cell biology* **11**, 211-218.

77. Corona G, Deiana M, Incani A *et al.* (2007) Inhibition of p38/CREB phosphorylation and COX-2 expression by olive oil polyphenols underlies their anti-proliferative effects. *Biochemical and biophysical research communications* **362**, 606-611.
78. Holtmann H, Winzen R, Holland P *et al.* (1999) Induction of interleukin-8 synthesis integrates effects on transcription and mRNA degradation from at least three different cytokine- or stress-activated signal transduction pathways. *Molecular and cellular biology* **19**, 6742-6753.
79. Hoffmann E, Dittrich-Breiholz O, Holtmann H *et al.* (2002) Multiple control of interleukin-8 gene expression. *Journal of leukocyte biology* **72**, 847-855.
80. Obata T, Brown GE, Yaffe MB (2000) MAP kinase pathways activated by stress: the p38 MAPK pathway. *Critical care medicine* **28**, N67-77.
81. Zhang X, Chen X, Song H *et al.* (2005) Activation of the Nrf2/antioxidant response pathway increases IL-8 expression. *European journal of immunology* **35**, 3258-3267.
82. Aupperle KR, Bennett BL, Boyle DL *et al.* (1999) NF-kappa B regulation by I kappa B kinase in primary fibroblast-like synoviocytes. *Journal of immunology* **163**, 427-433.
83. Tamura Y, Ohta H, Torisu S *et al.* (2013) Markedly increased expression of interleukin-8 in the colorectal mucosa of inflammatory colorectal polyps in miniature dachshunds. *Veterinary immunology and immunopathology* **156**, 32-42.
84. Nahidi L, Leach ST, Mitchell HM *et al.* (2013) Inflammatory bowel disease therapies and gut function in a colitis mouse model. *BioMed research international* **2013**, 909613.
85. Nishitani Y, Zhang L, Yoshida M *et al.* (2013) Intestinal anti-inflammatory activity of lentinan: influence on IL-8 and TNFR1 expression in intestinal epithelial cells. *PloS one* **8**, e62441.
86. Montedoro G, Servili M, Baldioli M *et al.* (1992) Simple and hydrolyzable phenolic compounds in virgin olive oil. 2. Initial characterization of the hydrolyzable fraction. *Journal of agricultural and food chemistry* **40**, 1577-1580.
87. Folin O, Ciocalteu V (1927) ON TYROSINE AND TRYPTOPHANE DETERMINATIONS IN PROTEINS. *Journal of Biological Chemistry* **73**, 627-650.

88. Dell'Agli M, Maschi O, Galli GV *et al.* (2008) Inhibition of platelet aggregation by olive oil phenols via cAMP-phosphodiesterase. *The British journal of nutrition* **99**, 945-951.
89. Visioli F, Poli A, Gall C (2002) Antioxidant and other biological activities of phenols from olives and olive oil. *Medicinal research reviews* **22**, 65-75.
90. Carluccio MA, Siculella L, Ancora MA *et al.* (2003) Olive oil and red wine antioxidant polyphenols inhibit endothelial activation: antiatherogenic properties of Mediterranean diet phytochemicals. *Arteriosclerosis, thrombosis, and vascular biology* **23**, 622-629.
91. Owen RW, Giacosa A, Hull WE *et al.* (2000) Olive-oil consumption and health: the possible role of antioxidants. *The lancet oncology* **1**, 107-112.
92. Tripoli E, Giammanco M, Tabacchi G *et al.* (2005) The phenolic compounds of olive oil: structure, biological activity and beneficial effects on human health. *Nutrition research reviews* **18**, 98-112.
93. Campolo M, Di Paola R, Impellizzeri D *et al.* (2013) Effects of a polyphenol present in olive oil, oleuropein aglycone, in a murine model of intestinal ischemia/reperfusion injury. *Journal of leukocyte biology* **93**, 277-287.
94. Sanchez-Fidalgo S, Sanchez de Ibarguen L, Cardeno A *et al.* (2012) Influence of extra virgin olive oil diet enriched with hydroxytyrosol in a chronic DSS colitis model. *European journal of nutrition* **51**, 497-506.
95. Van De Walle J, Hendrickx A, Romier B *et al.* (2010) Inflammatory parameters in Caco-2 cells: effect of stimuli nature, concentration, combination and cell differentiation. *Toxicology in vitro : an international journal published in association with BIBRA* **24**, 1441-1449.
96. Liu HT, Huang P, Ma P *et al.* (2011) Chitosan oligosaccharides suppress LPS-induced IL-8 expression in human umbilical vein endothelial cells through blockade of p38 and Akt protein kinases. *Acta pharmacologica Sinica* **32**, 478-486.
97. Haller D, Jobin C (2004) Interaction between resident luminal bacteria and the host: can a healthy relationship turn sour? *Journal of pediatric gastroenterology and nutrition* **38**, 123-136.

98. Meng F, Lowell CA (1997) Lipopolysaccharide (LPS)-induced macrophage activation and signal transduction in the absence of Src-family kinases Hck, Fgr, and Lyn. *The Journal of experimental medicine* **185**, 1661-1670.

99. Dinarello CA (1994) Interleukin-1 in disease. *The Keio journal of medicine* **43**, 131-136.

100. Sanchez-Fidalgo S, Cardeno A, Sanchez-Hidalgo M *et al.* (2013) Dietary extra virgin olive oil polyphenols supplementation modulates DSS-induced chronic colitis in mice. *The Journal of nutritional biochemistry* **24**, 1401-1413.

101. Gutierrez-Orozco F, Stephens BR, Neilson AP *et al.* (2010) Green and black tea inhibit cytokine-induced IL-8 production and secretion in AGS gastric cancer cells via inhibition of NF-kappaB activity. *Planta medica* **76**, 1659-1665.

102. Bhattacharyya S, Gutti U, Mercado J *et al.* (2011) MAPK signaling pathways regulate IL-8 mRNA stability and IL-8 protein expression in cystic fibrosis lung epithelial cell lines. *American journal of physiology Lung cellular and molecular physiology* **300**, L81-87.

103. Dauletbaev N, Eklove D, Mawji N *et al.* (2011) Down-regulation of cytokine-induced interleukin-8 requires inhibition of p38 mitogen-activated protein kinase (MAPK) via MAPK phosphatase 1-dependent and -independent mechanisms. *The Journal of biological chemistry* **286**, 15998-16007.