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Cycle XXX

Modulation of nitric oxide production by olive oil phenols and their metabolites

SCIENTIFIC SECTOR OF AFFERENCE

MED/04

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Abstract

Nitric oxide (NO) is an important signaling molecule involved in many physiological and pathological processes. NO contributes to vessels vasodilatation and blood flow increasing, but also to the vessel homeostasis by inhibiting vascular smooth muscle contraction and growth, platelet aggregation, and leukocyte adhesion to the endothelium. In the intestinal epithelium, NO may play, at low levels, a protective role against the inflammatory process, while after acute or chronic exposure a toxic mechanism of action leads to injurious effects. Dietary polyphenols and their metabolites have been found to be directly involved in the modulation of the intracellular signals that lead to the production of NO. The purpose of this study was to investigate the mechanism of the modulatory action of extra-virgin olive oil (EVOO) phenylethanoids, tyrosol (Tyr) and hydroxytyrosol (HT), and hydroxycinnamates, ferulic acid (FA), hydroferulic acid (DHFA) and isoferulic acid (IFA), in comparison to their glucuronide and sulfate metabolites, on the production of NO both at vascular and intestinal level. The first part of this investigation was assessed in endothelial cells (HUVEC and HAEC) to evaluate the superoxide production, the release of NO and cGMP and the activation of eNOS via Akt phosphorylation in endothelial cells. It was observed that the tested phenolic compounds enhanced NO concentration inhibiting its depletion caused by superoxide overproduction. Moreover, some of them enhanced its production through the modulation of Akt activation and of eNOS phosphorylation. DHFA and IFA showed better effectiveness than FA, as well as HT, which worked better than Tyr either as superoxide inhibitor or as eNOS enhancer. Interestingly, some of the tested metabolites worked in the same way of their parent compounds: it is the case of HT, whose metabolites were significantly effective in Akt and eNOS activation, as well as in superoxide counteraction.

Overall, obtained data showed that these compounds promote NO production and availability, so their intake by EVOO dietary consumption can help in the prevention of cardiovascular diseases such as hypertension, thrombosis, hypercholesterolaemia and atherosclerosis.

The second part of this study was focused on the modulation of NO and cGMP production in intestinal cells (Caco-2), with the aim of investigating the ability of EVOO phenolics to inhibit iNOS activation and the underlying mechanism of action. Treatments with pathological concentrations of lypopolysaccharide (LPS) were carried out to stimulate iNOS activation pathway, which involves NF-κB activation through IκBα and Akt phosphorylation, and evaluate the transepithelial resistance changes on Caco-2 monolayers. It was overally observed that all the tested compounds inhibited NO release induced by LPS, acting as inhibitors of iNOS expression. Considering hydroxycinnamates, they were able to down-regulate Akt phoshorylation. IFA sulf was surprisingly the most effective in the Akt inhibition, while DHFA and its glucuronide better inhibited IκBα degradation and iNOS. No one of the phenylethanoids instead were able to inhibit Akt activation. However, they were effective in the inhibition of IκBα degradation, suggesting a modulatory action on a parallel mechanism upstream NF-κB translocation to the nucleus. All the tested phenolic compounds counteracted the deleterious effect of LPS on Caco-2 cell monolayer permeability, suggesting the ability of preserving intestinal barrier integrity. Thus, it can be assumed that reaching relevant concentrations of EVOO phenols in the gut lumen may exert beneficial effects against intestinal inflammation, which is one of the major features of many intestinal diseases.

1. Introduction

1.1. Nitric oxide (NO)

Nitric oxide (**NO**) is a free radical compound which plays an important cellular signaling role within the mammalian body **(Habib et al., 2011; Hou et al., 1999)**. NO is composed of an atom each of nitrogen and oxygen such that seven electrons from nitrogen and eight electrons from oxygen are involved to form an uncharged molecule **(Fig. 1) (Habib et al., 2011)**.

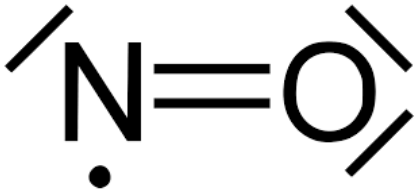


Figure 1. Molecular structure of nitric oxide

The reactivity of NO towards biological molecules is not linked with its unpaired electron reactivity, but rather depends on its physical properties, such as its small size, high diffusion rate, and lipophilicity **(Hughes, 2008)**. Its functions in the human body have been shown by several researchers only in the last three decades, during which in 1998 has been recognized as a signaling molecule with pivotal roles in many physiological and pathological processes **(Habib et al., 2011)**. NO is not a classical intercellular messenger, which is secreted by a transporter and binds to a membrane-bound receptor. Instead, it is freely diffusible and its biological effects are determined by its chemical reactivity. NO has a short biological half-life (estimated to be a few seconds) due to its rapid reaction with a variety of molecules **(Csonka et al., 2015)**. Indeed, as a free radical, it reacts rapidly with species containing unpaired electrons, such as molecular oxygen, superoxide anion and metals **(Mayer et al., 1997)**.

The major pathway for the metabolism of NO is its oxidation to nitrite and nitrate, eventually followed by their urinary excretion. NO in the presence of molecular oxygen is oxidized to nitrogen-dioxide (NO_2), which, by reacting with another NO molecule, forms N_2O_3, an intermediate that participates in nitrosation reactions. N_2O_3 may be decomposed to nitrite and a one-electron reduction of NO_2 may also lead to nitrite formation. Nitrite has a half-life of a few minutes in the circulation as it can be further oxidized to the more stable nitrate by oxyhaemoproteins such as oxygenated haemoglobin or myoglobin **(Csonka et al., 2015)**. Nitrite, nitrate and nitroso species, all coming from the NO metabolization, can themselves mediate NO-like bioactivities along the cardiovascular system, including vascular tone **(Rassaf et al., 2006)**.

1.1.1. NO synthesis in human tissues

The biosynthesis of NO takes place in a NADPH-dependent manner by oxidation of the guanidino nitrogen of L-arginine in the presence of molecular oxygen and several cofactors (FMN, FAD, tetrahydrobiopterin, heme moiety), producing L-citrulline. This oxidation is catalyzed in humans by nitric oxide synthase (NOS) **(Fig. 2) (Wolf, 1997)**. This enzyme, which is almost ubiquitous, has three isoforms: NOS1 or neuronal nitric oxide synthase (nNOS), NOS2 or inducible nitric oxide synthase (iNOS), and NOS3 endothelial nitric oxide synthase (eNOS) **(Habib et al., 2011)**. nNOS and eNOS are also recognized as constitutive isoforms, unlike the iNOS which is not continuously present in tissues. Constitutive NOS are calcium dependent and continuously present whereas iNOS is Ca^{2+} independent and is expressed only after cytokine exposure **(Nathan et al., 1994)**. On the contrary, nNOS and eNOS are constitutively expressed and need elevated levels of Ca^{2+} along with activation of calmodulin in order to produce NO for brief periods of time **(Moncada et al., 1991)**.

The three NOS isoforms are characterised by regions of high homology (51-57%) **(Kleinert et al., 2003)**, namely the oxygenase reductase domains, but at the same time each isoform exhibits distinctive features which reflect their specific *in vivo* functions. **(Andrew et al., 1999)**. Indeed, each NOS isoform has the same layout of catalytic domains: a C-terminal reductase with one binding site each for FAD, FMN and NADPH, and an N-terminal oxygenase section. Conversely, each isoenzyme has a different N-terminal extension, not essential for catalysis that probably functions in the intracellular localisation of the enzyme **(Mayer et al., 1997)**.

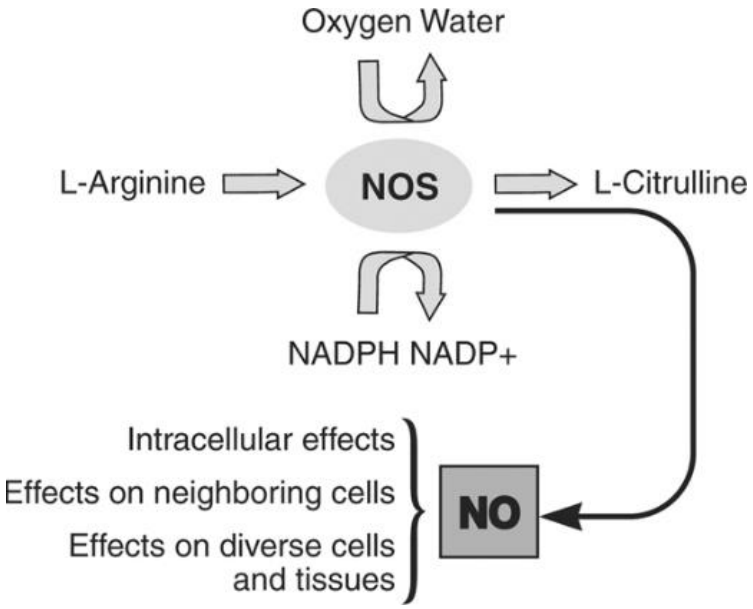


Figure 2. NO production by nitric oxide synthase (from Mackenzie et al., 2008).

Between these two regions, just at the N-terminal side of the reductase, lies the calmodulin (CaM) binding domain, which plays a key role in both the structure and function of the enzyme **(Andrew et al., 1999)**. The oxygenase domain contains bound heme and the binding site for the cofactor tetrahydrobiopterin (H_4biopterin). H_4biopterin is essential for the coupling of NADPH dependent O_2 activation to NO synthesis. **(Mayer et al., 1997)**.

1.1.1.1. Endothelial nitric oxide synthase (eNOS): localization and regulation

The eNOS is constitutively expressed in endothelial cells and synthesizes the NO needed for regulation of blood pressure, which is the main objective of its production **(Tuteja et al., 2004)**. The eNOS is expressed mainly in the endothelium of large arteries with the expression decreasing in smaller arteries and not expressed in capillary endothelial cells (ECs). Furthermore, eNOS is also expressed in cardiomyocites and in essentially all blood cells, including erythrocytes, leucocytes, platelets, and CAC (circulating angiogenic cells) **(Cortese-Krott et al., 2012; Heiss et al., 2010)**. This enzyme is composed of two identical monomers, and each monomer contains the amino-terminal oxidase domain and carboxy-terminal reductase domain. In order to produce NO from substrates O_2 and L-arginine, electron flux has to occur from the reductase domain of one monomer to the oxygenase domain of the other monomer. Ca^{2+}/CaM binding to eNOS facilitates the electron transfer from NADPH to the reductase domain flavins or from the flavins to the oxygenase domain heme iron **(Boo et al., 2003)**. The binding of the cofactor BH4 is essential for eNOS to efficiently generate NO **(Dudzinski et al., 2006)**. In the absence of this cofactor, eNOS shifts from a dimeric to a monomeric form, thus becoming uncoupled. In this conformation, instead of synthesizing NO, eNOS produces superoxide anion, a highly reactive free radical with deleterious consequences to the cardiovascular system **(Luo et al., 2014)**.

eNOS is a membrane protein mainly found in plasma membrane caveolae or on the Golgi apparatus. Caveolae are relatively rich in cholesterol and sphingolipids which lend these membrane compartments a distinct fluidity and create a unique environment for an efficient control of protein-protein interactions and signal transduction. In resting endothelial cells, eNOS strongly and directly interacts with caveolin-1 in the caveolae, a protein-protein interaction which tonically inhibits eNOS activity by masking the calmodulin binding site **(Heiss et al., 2014)**.

Several signaling molecules which play an important role in the regulation of eNOS enzyme activity, such as G-protein coupled receptors, modulators of calcium flux or Akt kinase, are also compartmentalized in caveolae or recruited to caveolae or nearby membrane regions upon activation. Thus, caveolar localization of eNOS appears easy for regulation of the enzyme by hormonal prompts, such as oestrogen or insulin **(Heiss et al., 2014)**.

The particular properties of eNOS which enable it to perform its specialised functions include Ca^{2+} sensitivity, and the post-translational modifications which mediate subcellular localisation, such as phosphorylation and S-nitrosylation. These enable the enzyme to respond not only to a variety of neurohormonal agents (either endogenous or exogenous), but also to haemodynamic forces. In these respects, eNOS differs significantly from the other isoforms. The signaling events which lead to the eNOS activation are secondary to increased shear stress inside the vases and involve deformation of the ECs and their glycocalyx due to viscous drag of the flowing blood **(Fig. 3)**. This leads to activation of cell adhesion proteins, including integrins **(Loufrani et al., 2008a)**, platelet endothelial cell adhesion molecule **(Otte et al., 2009)**, and cytoskeletal proteins **(Loufrani et al., 2008b)**. Then, subsequently to the activation of these proteins, PI3 kinase is activated and gives way to the phosphorylation of protein kinase B (Akt), which phosphorylates eNOS, rendering it effective in producing NO **(Heiss et al., 2010)**.

As stated before, the activation of eNOS is also induced by an increase in intracellular Ca^{2+}, resulting from either activation of diverse G-protein-coupled receptors (GPCR) by various ligands or from mobilization of intracellular Ca^{2+} stores. Thus, the activation of eNOS, via receptor-mediated activation of G proteins, can even be carried out by hormones as catecholamines and vasopressin, autacoids such as bradykinin and histamine, and platelet-derived mediators such as serotonin and ADP **(Boulanger et al., 1997)**. Endothelin-1 also

activates eNOS via heterotrimeric G-protein beta-gamma subunit signaling to protein kinase B/Akt.

An increase in the association of heat shock protein 90 (HSP90) with eNOS is also well recognized for increasing NO production. **(Liu et al., 2003b)**.

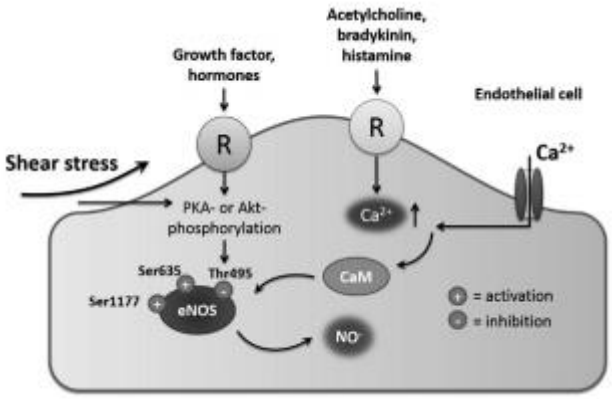


Figure 3. eNOS activation scheme in the endothelial cell (from Zhao et al., 2015).

The actual intracellular Ca^{2+} that is required to activate this enzyme may be significantly different from that released from subcellular compartments as well as the average Ca^{2+} concentration **(Liu et al., 2003a)**.

Regulation of intracellular calcium levels provides the most rapid induction of eNOS activity, and a multitude of diverse pathways mobilize calcium to activate eNOS. In general, calcium is released from cellular storage pools as the downstream effect of phospholipase C (PLC)-mediated cascades. Once activated, PLC cleaves phosphatidylinositol 4,5-triphosphate into diacylglycerol and inositol 1,4,5-triphosphate (PI3), the former leads to the activation of protein kinase C and the latter to the activation of PI3-receptors. Activation of PI3-receptors leads to the increase of intracellular calcium concentration in a complex way, involving the activation of numerous ion channels **(Fujimoto et al., 1992)**.

In addition to post-translational regulatory pathways involving acylation and calcium/calmodulin, the dynamic regulation of eNOS mainly involves numerous pathways of phosphorylation and dephosphorylation, influenced by the caveolar localization of the enzyme. Indeed, eNOS is known to be phosphorylated at multiple sites, including Ser 1177 and Ser 633, which are stimulatory, and Thr 495 and Ser 114, which are inhibitory **(Heiss et al., 2014)**. In particular, phosphorylation at Ser 114 is often assumed to inhibit eNOS enzyme activity. Dephosphorylation at this site has been observed after stimulation with agonists, such as vascular endothelial growth factor (VEGF) **(Heiss et al., 2014)**. The protein kinase Akt is a key determinant of eNOS phosphorylation at Ser 1177 and consequent eNOS activation at basal levels and in response to agonists. Moreover, phosphorylation of Ser 1177 is catalyzed by a number of distinct kinases including PKA, AMPK **(Chen et al., 1999a)**, calcium-calmodulin kinase kinase (CaMKK) and II beta and checkpoint kinase (CHK) **(Park et al., 2011**). Akt kinase is under the direct control of phosphoinositide-3-kinase (PI3K) **(Vanhaesebroeck et al., 1997)**. PI3K is itself controlled by a number of eNOS agonists, and these PI3 kinase/Akt pathways are significant in the coordinate regulation of eNOS phosphorylation state and enzymatic activity.

Ser 633 may represent a second stimulatory phosphorylation site responsive to basal stimuli, such as shear stress, and agonists downstream of protein kinase A **(Heiss et al., 2014)**. Instead, Ser 615 may be another Akt phosphorylation site and may serve to sensitize eNOS to calmodulin binding and modulate phosphorylation at other eNOS sites **(Michell et al., 2002)**.

The S-nitrosylation of the eNOS protein is one of the other levels of dynamic receptor-mediated post-translational control of eNOS activity **(Erwin et al., 2005)**. The source of NO for nitrosylation is eNOS itself, in a process that occurs when eNOS is membrane localized.

Quiescent eNOS in endothelial cells is inhibited as a result of tonic S-nitrosylation at two of the cysteine residues, Cys 94 and Cys 99 (Cys 96 and Cys 101 in bovine eNOS), that form the zinc tetrathiolate cluster. In response to eNOS agonists, eNOS is rapidly but transiently denitrosated on a time course that parallels the increase in eNOS catalytic activity **(Erwin et al., 2005; Ravi et al., 2004)**.

1.1.1.2. Neuronal nitric oxide synthase (nNOS): localization and regulation

The nNOS is found in a variety of immature and mature neurons in both the central and peripheral nervous systems and is a constitutionally expressed enzyme, though it can also be induced in neurons by certain treatments **(Jiang et al., 2004; Tuteja et al., 2004)**. In addition, nNOS is also present in skeletal muscle, cardiac muscle and smooth muscle **(Schwarz et al., 1999; Xu et al., 1999)**, where NO controls blood flow and muscle contractility **(Lau et al., 2000; Stamler et al., 2001a)**. nNOS is active only in its dimer form while monomer is inactive, and the dimerization requires tetrahydrobiopterin (BH4), heme and L-arginine binding **(Reif et al., 1999)**. nNOS monomer exhibits a bi-domain structure containing an oxygenase domain (N-terminal) and a reductase domain (C-terminal) which can be separated by a calmodulin binding place. The oxygenase domain which binds the substrate L-arginine contains a tetrahydrobiopterin (BH4) binding site and a cytochrome P-450-type heme active site; there is also a binding site for Zn which facilitates nNOS dimerization. The reductase domain which binds the substrate NADPH contains a binding site for FMN and FAD **(Hemmens et al., 2000; Noguchi et al., 2001; Sagami et al., 2001)**. Within the FMN binding domain, there is an autoinhibitory loop which controls nNOS activity. The above-mentioned dimerization increases nNOS activity by creating high-affinity binding sites for L-arginine and BH4, thus facilitating electron flow. Interestingly,

the electron appears to flow from one monomer to another **(Siddhanta et al., 1996)**, which may be a major reason why the nNOS monomer is inactive. More importantly, dimer stabilization protects nNOS from proteolysis. Destabilization of dimeric nNOS makes it more susceptible to be phosphorylated by protein kinase C and hydrolyzed by trypsin. Recently, it has been shown that phosphorylation of nNOS is important for nNOS activity as well as what happens for eNOS. This protein phosphorylation is regulated by some kinases and phosphatases, for example, PKA, calmodulin-dependent kinases, PKC, phosphatase 1, which are in turn regulated by extracellular and intracellular prompts **(El-Milli et al., 2008)**.

However, phosphorylation at different sites of nNOS affects its activity differently. Of particular note, the protein kinase CaM-KII phosphorylates nNOS at Ser847, which reduces nNOS activity by inhibiting Ca^{2+}-CaM binding. As we know, Ser847-PO_4 is located within the autoinhibitory loop which functions to prevent the displacement of the loop even in the presence of high concentrations of Ca^{2+}-CaM, thus reducing nNOS activity. By contrast, the protein phosphatase 1 decreases the level of phosphorylation of nNOS at Ser847, leading to an increased nNOS activity **(Rameau et al., 2004)**. Another phosphorylation site of nNOS is at Ser1412 which is analogous to the established phosphorylation site of Akt present in endothelial nitric oxide synthase **(Adak et al., 2001)**. Phosphorylation at this site increases nNOS activity, whereas, dephosphorylation by phosphatases decreases nNOS activity **(Rameau et al., 2007)**. Apart from the above mentioned two phosphorylation sites, phosphorylation at Ser741 by CaM-KI found in transfected cells also deregulates nNOS activity **(Song et al., 2004)**.

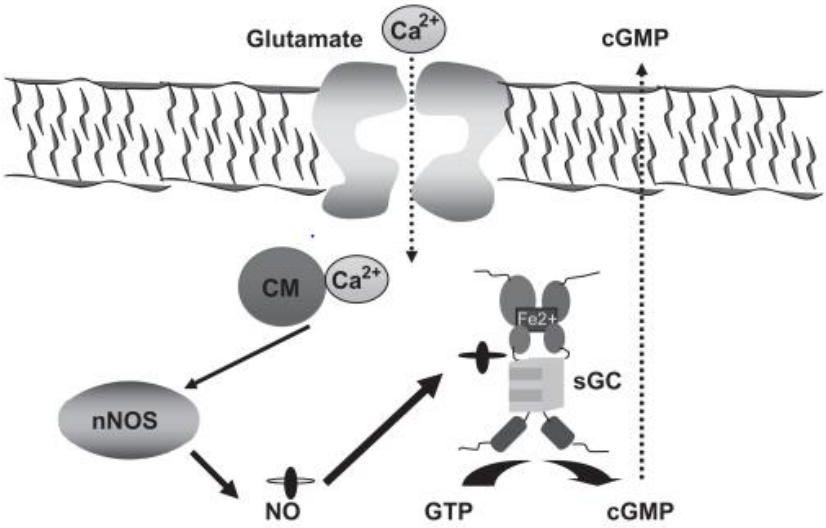


Figure 4. nNOS activation scheme in the neuronal cell (from Rodrigo et al., 2006).

Many studies suggest that also calmodulin is effective as an activator of nNOS functioning as an allosteric modulator. When CaM and Ca^{2+} are absent, electron flow from FAD to FMN slows down. Binding of CaM to nNOS facilitates electron flow transferring from NADPH to the reductase domain flavins and from the reductase domain to the heme centre. Usually, nNOS is inactive at basal intracellular Ca^{2+} concentrations **(Zhou et al., 2009)**. While stimulating factors render intracellular Ca^{2+} levels increased, calmodulin binds to nNOS, activating nNOS. In contrast, when intracellular Ca^{2+} concentrations decrease to basal levels, calmodulin dissociates from nNOS, and it becomes inactive again **(Zhou et al., 2009)**. Thus, nNOS activity is mainly regulated by intracellular Ca^{2+} concentrations, which activate or inhibit nNOS activity through calmodulin binding **(Fig. 4)**.

1.1.1.3. Inducible nitric oxide synthase (iNOS): localization and regulation

As previously stated, iNOS is mostly transcriptionally regulated and is not normally produced in most of the cells **(Soskic et al., 2011)**. iNOS is usually induced by cytokines in almost every cell and generates locally high level of NO for prolonged periods of time **(Messmer et al., 1995)**. It is mainly expressed in macrophages, neutrophils and epithelial cells. It has also been reported that in mice oestrogen and progesterone-induced iNOS expression is different in different cell population, i.e. estrogen induces it in myometrial mast cell whereas progesterone induces it in epithelial cells **(Habib et al., 2011)**.

The iNOS activity depends on arginine availability, so its functions depend on the regulation of arginine transport or consumption of arginine by other biochemical pathways (e.g. arginase) **(Sudar et al., 2010)**. The active iNOS enzyme is a homodimer, as happens for the constitutive forms of the enzyme **(Soskic et al., 2011)**.

The iNOS is primarily regulated at the expression level by transcriptional and post-transcriptional mechanisms. There is a marked heterogeneity of signal transduction pathways or transcription factors involved in the induction of iNOS expression, with a cell and species specificity **(Kleinert et al., 2000)**. For example, iNOS expression in murine and rat cells is induced by incubation with bacterial lipopolysaccharide (LPS), stimulatory cytokines such as interferon-γ (IFN-γ), interleukin- 1β (IL-1β), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α) or other compounds. In contrast, the majority of human cells requires a complex cytokine combination including IFN-γ, LPS, IL-1β and TNF-α for iNOS induction **(Kleinert et al., 2003)**. Many different compounds (more or less specific) and also molecular activators or inhibitors (permanent active or dominant negative isoforms of kinases, etc.) have been shown to induce, enhance or inhibit iNOS expression by activating or blocking a wide variety of signal transduction pathways **(Kleinert et al., 2000)**.

NO, itself with different mechanisms, but also protein kinase A (PKA), protein kinase C (PKC), the p42/p44 MAPK/ERK pathway and other specific kinases and phosphatases, have been showed to be involved in both induction and inactivation of iNOS depending on the cell type which have been exposed to the iNOS inducers **(Kleinert et al., 2003)**.

The iNOS promoters from all species contain a TATA box about 30 bp from the transcription start site. Near the TATA box, all mammalian promoters contain binding sites for the transcription factors, NF-IL6, octamer factors and for transcription factors induced by TNF-α. NF-κB is a central target for activators or inhibitors of iNOS expression. Indeed, LPS, IL-1β, TNF-α and oxidative stress for instance have been shown to induce iNOS expression in different cell types by activating NF-κB. Also, inhibition of iNOS expression by numerous agents, such as glucocorticoids, TGF-β1, antioxidants (e.g. pyrrolidine dithiocarbamate, PDTC) and inhibitors of PC-PLC has been shown to result from inhibition of NF-κB activation.

This inhibition can result from direct capture of NF-κB by protein-protein interactions **(Kleinert et al., 1996)**, inhibition of nuclear translocation of NF-κB **(Jeon et al., 1998)**, inhibition of NF-κB transactivation activity **(Yu et al., 2002)** or from enhancement of the expression of IκB, the specific inhibitor of NF-κB **(Saura et al., 1998)**. This inhibitor is present in three different isoforms, IκBα, IκBβ, and IκBε **(Kanarek et al., 2010)**. The proximal region of the promoter interacts with the NF-κB trans-acting factor. This DNA-binding protein complex resides in the cytosol of quiescent cells bound to the IκB inhibitory complex. In fact, in most cells NF-κB exists in the cytoplasm in an inactive complex bound to IκB. Most agents that activate NF-κB do so through a common pathway based on phosphorylation-induced, proteasome-mediated degradation of IκB **(Fig. 5)**. The key regulatory step in this pathway involves activation of a high molecular weight IκB kinase

(IKK) complex, whose catalysis is generally carried out by a heterodimeric kinase consisting of IKKα and IKKβ subunits.

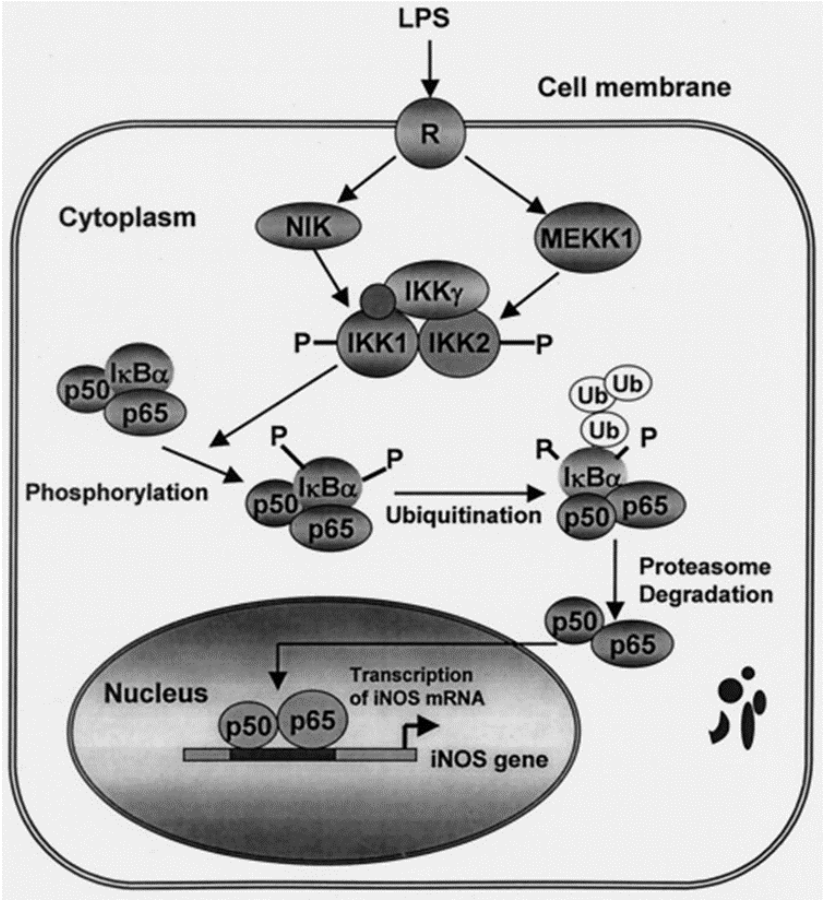


Figure 5. iNOS expression after LPS stimulus in intestinal cells (from Pan et al., 2000).

So, following the activation of cells by several agents (e.g., LPS and TNF-α), IκB dissociates and degrades after phosphorylation and the DNA-binding complex translocates to the nucleus, where it binds to cis-regulatory regions of the iNOS promoter **(Wang et al., 1995)**. Potent NF-κB activators, such as TNF-α and IL-1, cause almost complete degradation of IκBs (especially IκBα) within minutes. This process, which is mediated by the 26S

proteasome, depends on phosphorylation of two conserved serines (S32 and S36 in human IκBα) in the N-terminal regulatory domain of IκBs **(Karin, 1999)**.

The enzymes that catalyze the ubiquitination of phospho-IκB are constitutively active. Thus, the only regulated step that dictates the fate of IκB and NF-κB is in most cases the phosphorylation of the two N- terminal serines in the IκB molecule.

The phosphorylation step is the one that provides specificity to this signaling pathway **(Karin, 1999)**. There are only two exceptions to this pathway for NF-κB activation. The first is activation of NF-κB in response to UV radiation, which although dependent on IκB degradation does not involve N-terminal IκB phosphorylation while the second exception is anoxia, which stimulates phosphorylation of IκBa at tyrosine 42 **(Bender et al., 1998; Imbert et al., 1996)**.

The extent to which IKK is activated by a given stimulus seems to dictate the kinetics of IκB degradation. IKKα was identified as a protein that interacts with the MAP kinase kinase kinase (MAP3K), and NIK (NF-κB inducing kinase) **(Règnier et al., 1997)**. In tissue culture cells, NIK acts as a potent activator of both IKK and NF-κB, but it requires an activation stimulus by cytokines such as TNFα or IL-1 **(Karin, 1999)**. The above mentioned MAP3K could be activated through the PI3/Akt pathway. Akt can also regulate signaling pathways that lead to induction of the NF-κB family of transcription factors, at least in part, at the level of degradation of the NF-κB inhibitor IκB, and is specific for NF-κB, as other inducible transcription factors are not affected by Akt overexpression.

However, Akt does not act alone to induce cytokine promoters and NF-κB reporters, because signals from other pathways are required to observe the effect **(Kane et al., 1999).**

1.1.2. Mechanisms of action of NO

NO is a reactive molecule which has a variety of effects depending on the relative concentrations of NO and the surrounding environment in which NO is produced. There are both direct effects of NO, that are mediated by the NO molecule itself, and indirect effects of NO that are mediated by reactive nitrogen species produced by the interaction of NO with superoxide anion or with oxygen.

For example, cGMP, that is produced by the interaction of NO with soluble guanylate cyclase, mediates many of the physiological effects of NO, and it is also an important example of the direct effects of NO. Furthermore, NO can react with several metalloproteins. For example, in cells that can synthesize the inducible NO synthase isoenzyme, NO binding converts the iron-sulphur enzyme *cis*-aconitase to an mRNA-binding form that regulates cellular iron metabolism **(Hentze et al., 1996).** The molecular mechanisms that mediate the biological activities of NO are various and can be allocated into different categories. In fact, NO reacts readily with transition metals, such as iron, copper and zinc. These metals are abundantly present in prosthetic groups of enzymes and other proteins, and by that mechanism, NO regulates the activity of various enzymes. Moreover, NO is able to induce the formation of S-nitrosothiols from cysteine residues in a reaction called S-nitrosylation. Nitrosylation has been shown to modify the activity of several proteins involved in cellular regulatory mechanisms **(Stamler et al., 2001b)**. Lastly, NO reacts very quickly with superoxide anion ($O_2^{-\cdot}$), resulting in the formation of peroxynitrite ($ONOO^-$). Peroxynitrite is a nitrating agent and a powerful oxidant that is able to modify proteins, lipids and nucleic acids. The first mechanism represents direct effects of NO and the two latter mechanisms are referred as indirect effects of NO.

At low concentrations of NO (< 1 μM) the direct effects predominate, whereas at higher concentrations (> 1 μM) the indirect effects become more important **(Davis et al., 2001)**.

1.1.2.1. NO signaling through transition metals: the cGMP production

Due to its chemical structure, NO can act as an electron donor in chemical reactions, and thereby is prone to react with transition metals resulting in the formation of metal-nitrosyl complexes. In biological systems, important transition metals that react with NO are iron, copper and zinc. As mentioned before, one of the main targets in NO signaling is soluble guanylate cyclase (sGC). Indeed, once released, NO binds, at diffusion-controlled rates, to the soluble guanylate cyclase (sGC), which is the major target for the physiological effects of NO. sGC is an enzyme containing a heme structure with ferrous iron, and it converts GTP to the intracellular signaling molecule cGMP. The basal activity of sGC is low, but it is rapidly activated by even low concentrations of NO (10-100 nM). NO binds directly to the heme in sGS to form a ferrous-nitrosyl-heme complex resulting in changes in the porphyrin ring structure. This leads to the activation of sGC and 400-500- fold increase in the rate of cGMP synthesis **(Fig. 6) (Davis et al., 2001)**. The principal mediator of cGMP signals is cGMP dependent protein kinase (PKG). PKG is a serine/threonine kinase that is activated upon binding of cGMP.

Two types of PKG have been characterized. PKG I is a cytosolic enzyme that is ubiquitously expressed with particularly high expression levels in cerebellum, platelets and smooth muscle cells. PKG I has various targets which are related to smooth muscle relaxation and to platelet and neutrophil activation **(Lucas et al., 2000)**.

Conversely, PKG-II has been detected in renal cells, zona glomerulosa cells of the adrenal cortex, intestinal mucosa, pancreatic ducts, parotid and submandibular glands, chondrocytes, and several brain nuclei, but not in cardiac and vascular myocytes **(Lucas et al., 2000)**.

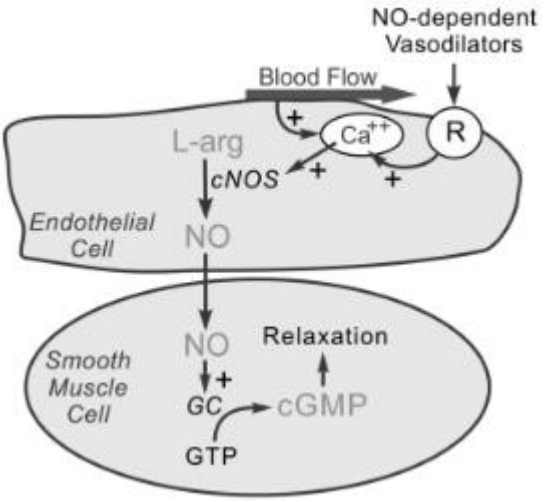


Figure 6. cGMP production after NO release (from Klabunde, 2016)

Other factors that convey cGMP mediated signaling are cyclic nucleotide gated channels (CNG), cAMP dependent protein kinase (PKA) and phosphodiesterases. CNGs are voltage gated cation channels that are involved in the processing of visual information in retina **(Korhonen et al., 2005)**. Because the cyclic nucleotide binding domains of PKG and PKA have significant homology, cGMP is able to activate PKA, although with a 50- fold lower selectivity than cAMP **(Lucas et al., 2000)**.

Another target of cGMP are phosphodiesterases (PDEs), which catalyze the conversion / inactivation of cAMP or cGMP to 5'AMP and 5'GMP, respectively. Different families of PDEs are either regulated (stimulated or inhibited) by cGMP or they target cGMP. PDE 5 is specific for cGMP, and selective inhibitors of this enzyme are widely used in the treatment of erectile dysfunction. PDE 5 inhibitors, such as sildenafil, enhance cGMP levels and by that mechanism, augment NO-induced vasodilatation in penile vessels **(Lucas et al., 2000)**.

1.1.2.2. NO signaling through S-Nitrosylation

S-nitrosylation of proteins has been recognized as an important mechanism that regulates the functions of the target proteins, and it has been even compared to protein phosphorylation **(Stamler et al., 2011b)**. As already discussed in **1.1. section**, in aqueous solutions NO reacts readily with molecular oxygen (O_2) forming dinitrogen trioxide (N_2O_3) in a process called as NO autoxidation. N_2O_3 is decomposed rapidly to nitrosonium ion (NO^+) and nitrite. Nitrosonium ion is responsible for the nitrosylation of thiols, secondary amines and phenolics. Rate of autoxidation is mainly dependent on the concentrations of NO and oxygen, and it is dramatically accelerated within lipid membranes. Therefore, the rate of formation of N_2O_3 is high at the site of NO synthesis. This highlights the importance of the distance between the site of NO synthesis and target molecules and subcellular environment and conditions in NO signaling **(Korhonen et al., 2005)**. Although nitrosylation of proteins is a chemical reaction, there seems to be specificity, which is a requirement for a proper signaling mechanism. For example, p21ras, a kinase involved in the MAP kinase cascade activation, is a target for NO-based signaling. p21ras contains four cysteine residues, but only cysteine 118 is nitrosylated resulting in the activation **(Lander et al., 1997)**.

Generally, the group of cellular target proteins that are regulated by S-nitrosylation is various, and includes transcription factors, kinases involved in signaling cascades, caspases, ion channels and metabolic proteins. The S-nitrosylation has not a univocal role when it occurs in the before mentioned substrates, but rather it can activate or inactivate different signal proteins, or blocking the DNA binding as what happens for the NF-κB transcription factor **(Korhonen et al., 2005)**.

1.1.2.3. NO signaling through peroxynitrite formation

Reaction between NO and superoxide anion ($O_2^{-\cdot}$) occurs forming peroxynitrite ($ONOO^-$), which is a reactive molecule able to nitrate and oxidize proteins, lipids, and nucleotides. The reaction between NO and superoxide anion is very rapid and the rate constant of the reaction is about three times greater than the rate of superoxide decomposition by superoxide dismutase (SOD). The rate of $ONOO^-$ production is strongly dependent on the presence of NO and superoxide, and $ONOO^-$ formation is favoured in an environment containing equivalent amounts of NO and superoxide **(Fig. 7)**.

Sources of superoxide production are mainly considered to be mitochondria and immune cells (in particular macrophages and granulocytes), and the synthesis of both NO and superoxide results in increased inflammation **(Davis et al., 2001)**. Excessive peroxynitrite formation leads to nitrated proteins, inhibition of mitochondrial respiration, depletion of cellular energetics, DNA damage, apoptosis and necrotic cell death, resulting in cellular/tissue injury **(Virag et al., 2003)**.

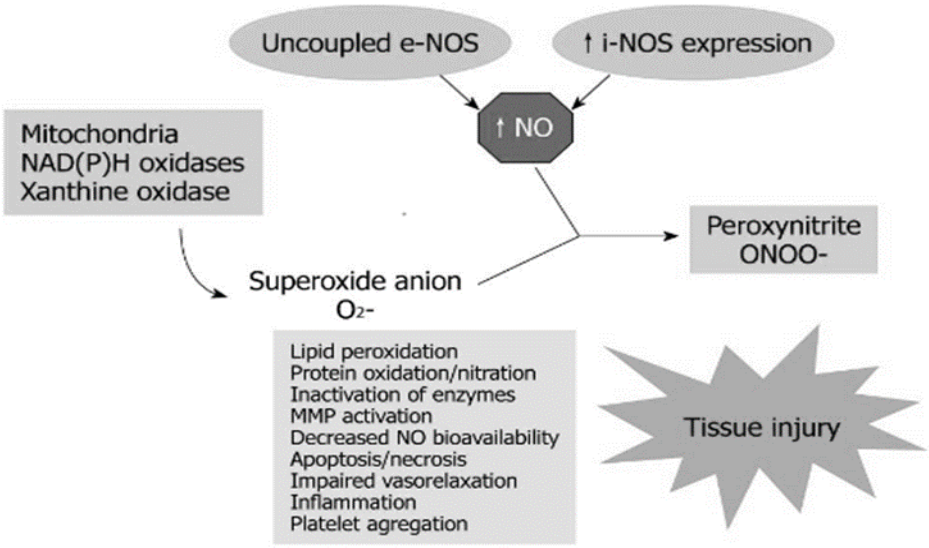


Figure 7. Peroxynitrite formation after NO release (from Abdennebi et al., 2011)

Nitrotyrosine has been used as a marker of peroxynitrite formation and tissue injury. Nitration of proteins and enzymes modulates catalytic activity, cell signaling and cytoskeletal organization **(Virag et al., 2003)**. It is of interest that also the activity of iNOS is regulated by $ONOO^-$ – mediated nitration. Nitration of iNOS decreases catalytic activity, and this may be a regulatory mechanism in patients with sepsis **(Lanone et al., 2002)**. NO can also nitrate tyrosine residues within proteins without formation of $ONOO^-$. For example, NO has been shown to nitrate tyrosine residues and thereby inhibit activity of enzymes cyclooxygenase-1 **(Goodwin et al., 1998)** and cyclooxygenase-2 **(Gunther et al., 1997)**.

1.1.3. Physiopathology of NO in the cardiovascular system

The cells of the vascular endothelium transmit circulatory stimuli to the arterial wall leading to the regulation of vessel tone, haemostasis, blood pressure and vascular remodelling. It is the ability of the endothelium to synthesise and release NO that accounts for

the regulation of these physiological processes. Atherosclerosis, and diseases which predispose to atherosclerosis such as hypercholesterolaemia, diabetes, and hypertension, are characterised by endothelial dysfunction. Here endothelial cell function is compromised, which in turn facilitates altered activity of platelets, neutrophils and the underlying vascular smooth muscle cells (VSMC) **(Naseem, 2005)**. Therefore, the NO regulation in the cardiovascular system plays a fundamental role in the maintenance of the normal vascular functions, in particular, the regulation of the vascular tone, the main action of NO in the vessels, carried on by different mechanisms.

Low levels of NO (nanomolar concentrations) produced by eNOS in endothelial cells cause relaxation of vascular smooth muscle cells and consequent vasodilation. This basal release of NO is important in order to maintain the basal vascular tone.

Thus, the inhibition of NO synthesis in the vascular system leads to elevate blood pressure **(Walford et al., 2003)**. On the contrary, high levels of NO (micromolar concentrations) produced by inducible NOS lead to an exaggerated vasodilatory response and hypotension **(Kilbourn et al., 1990)**.

The modulation of vascular smooth muscle relaxation occurs via activation of the eNOS, which releases short amounts for a short time of NO which activates the soluble guanylyl cyclase to increase the cytosolic cGMP concentration and decrease Ca^{2+} flux. This causes a decrease in the formation of the calcium-calmodulin myosin light chain kinase complex in the vascular smooth muscle cells and promotes vasorelaxation by altering phosphorylation of the regulatory myosin light chains **(Horowitz et al., 1996)**. cGMP decreases intracellular calcium flux by directly inhibiting voltage-gated Ca^{2+} channels **(Bolotina et al., 1994)** and by activating protein kinases, of which protein kinase G I (PKG I) is of primary importance **(Massberg et al., 1999)**. PKG I phosphorylates proteins in the sarcoplasmic reticulum,

including Ca^{2+}-dependent potassium channels **(Bolotina et al., 1994)**, 1,4,5 inositoltriphosphate (IP3) receptor-associated cGMP kinase substrate (IRAG) **(Schlossmann et al., 2000)** and phospholamban **(Cornwell et al., 1991)**. Phospholamban, in turn, activates sarcoplasmic reticulum ATPase (SERCA) **(Simmerman et al., 1998)**. The phosphorylation of these sarcoplasmic proteins leads to the sequestration of Ca^{2+} in the sarcoplasmic reticulum, thereby decreasing intra-cellular Ca^{2+} flux **(Schlossmann et al., 2000)**. Interestingly, NO can also directly inhibit Ca^{2+} dependent potassium channels in VSMC **(Bolotina et al., 1994)**. This cGMP-independent mechanism leads to a fall in Ca^{2+} flux and promotes vasodilation through altered phosphorylation of myosin light chains **(Walford et al., 2003)**.

Another important function of NO in the vascular system is the inhibition of adhesion, aggregation and recruitment of the platelets to the growing thrombus associated to a platelet hyperactivity **(Loscalzo, 2001)**.

NO exerts its anti-platelet actions through cGMP. Indeed, the cGMP-mediated decrease of Ca^{2+} flux inhibits the formation of the active conformation of glycoprotein IIb/IIIa **(Michelson et al., 1996)**, and decrease platelet association with fibrinogen **(Mendelsohn et al., 1990)**.

Furthermore, cGMP itself showed two different mechanisms by which it is effective as antithrombotic agent. Firstly, cGMP inhibits thrombin-mediated activation of phosphoinositide 3-kinase (PI-3 kinase) **(Pigazzi et al., 1999)**. Activated PI-3 kinase promotes the active conformation of glycoprotein IIb/IIIa **(Zhang et al., 1996),** where its inhibition decreases the effectively irreversible association of fibrinogen with glycoprotein IIb/IIIa, leading to platelet disaggregation. Secondly, cGMP can also increase intracellular cAMP indirectly by inhibiting the degradation of cAMP by phosphodiesterase III (PDE III). Moreover, an increased cAMP, like cGMP, has been associated with phospholamban phosphorylation and decreased intracellular Ca^{2+} flux **(Walford et al., 2003)**. It has also been

demonstrated that cGMP- and cAMP-elevating agents, such as prostacyclin, can act synergistically to inhibit platelet aggregation **(Stamler et al., 1989)**.

NO is also capable of inhibiting the VSMC proliferation, which is associated to the narrowing of vessels observed in the atherosclerotic process **(Ross, 1999)**. This inhibition is carried out by both dependent and independent cGMP mechanisms. The cGMP dependent mechanism is related, as happens for the inhibition of the platelet aggregation, to the decrease of Ca^{2+} flux, which conversely promotes proliferation when it is increased in an eNOS-impairment state **(Baran, 1996)**. Other proposed but less well studied cGMP-dependent mechanisms include the downregulation of c-myc **(Bennett et al., 1994)** and upregulation of p21 **(Ishida et al., 1997)**, both of which inhibit cell cycling.

The independent-cGMP action against vascular proliferation involves NO in the inhibition of the formation of polyamines, compounds which are needed for DNA synthesis. In addition, NO can promote VSMC apoptosis through, in part, the upregulation of Fas expression on the VMSC surfaces **(Boyle et al., 2002)**. In any case, apoptosis is promoted only at high concentration of NO, whilst low concentrations protect endothelial cells from the apoptotic stimuli **(Shen et al., 1998)**.

NO has been studied for its capacity in promoting angiogenesis. In fact, NO cannot only inhibit endothelial cell apoptosis **(Dimmeler et al., 1999)** but also enhance endothelial cell migration **(Ziche et al., 1994)**. This latter effect is mediated by upregulation of urokinase-type plasminogen activator **(Ziche et al., 1997)** which facilitates matrix metallo-proteinase activation via plasmin to dissolve the extracellular matrix, and by the stimulation of endothelial cell pseudopod formation **(Noiri et al., 1998)**. Moreover, NO may suppress the formation of angiostatin **(Matsunaga et al., 2002)**, a known inhibitor of angiogenesis **(O'Reilly et al., 1994)**.

The NO release by eNOS alters the ability of leukocytes to adhere to the vascular endothelium, which is a critical step in the inflammatory response, where an increased adhesion is associated with atherogenesis **(Ross, 1999)**. NO can limit leukocyte adhesion to the vascular endothelium through the stabilization of the expression of IκBα, so inhibiting the NF-κB, which normally increases the expression of the chemokine, monocyte chemotactic protein 1 (MCP-1), and of the glycoprotein adhesion molecule, VCAM-1 **(Chen et al., 1999b)**.

These factors increase the recruitment of leukocytes and their adhesion to the endothelium. NO, additionally, decreases polyunsaturated fatty acids oxidation, which is another stimulus for NF-κB activation **(Khan et al., 1996)**. Monocytes, when activated, release ROS (reactive oxygen species), which contribute to oxidative stress, as well as growth factors and chemokines, which stimulate cell proliferation. Thus, through the inhibition of leukocyte adhesion, NO also exerts antioxidant and antiproliferative effects. Indeed, as a free radical, it has also the ability to modify the redox environment of vascular cells. As in the modulation of endothelial cell apoptosis, NO has opposing, dose-dependent, effects on the intracellular redox environment. In particular, low levels of NO released by eNOS reduce the presence of intracellular reactive oxygen species **(Garg et al., 1990)**, but high levels of NO, through the formation of peroxynitrite, may promote oxidative damage **(Radi et al., 1991)** and progression of cardiovascular diseases **(Kojda et al., 1999)**. A primary mechanism by which NO supports antioxidant effects in the intracellular environment is through the reduction of $O_2^{\cdot -}$. NO induces the expression of extracellular superoxide dismutase (ecSOD), which catalyzes the dismutation of $O_2^{\cdot -}$ to hydrogen peroxide **(Fukai et al., 2000)**. Interestingly, hydrogen peroxide induces the expression and activation of eNOS **(Drummond et al., 2000)**, an action that increases the ability of a cell to reduce oxidative stress. NO exerts additional antioxidant effects by increasing the expression of heme-oxygenase I **(Durante et**

al., 1997) and ferritin **(Recalcati et al., 1998)**. Heme-oxygenase I catalyzes the formation of bilirubin **(Maines, 1997)**, which scavenges $O_2^{\cdot -}$, while ferritin binds ferric ions and reduces $O_2^{\cdot -}$ formation **(Balla et al., 1992)**. At high concentrations, NO combines rapidly with $O_2^{\cdot -}$ to form $ONOO^-$, and it is through this powerful oxidant that NO may contribute to oxidative stress. The formation of peroxynitrite is normally limited because the relatively high concentrations of SOD outcompete NO for $O_2^{\cdot -}$ **(Koppenol, 1998)**; however, when intracellular levels of NO are elevated, as during the expression of iNOS, the formation of $ONOO^-$ predominates. Peroxynitrite and its radical derivatives oxidize free thiols, which normally serve as a source of intracellular antioxidants, and promote lipid peroxidation and protein damage **(Radi et al., 1991)**. Interestingly, $ONOO^-$ can also react with thiols to form S-nitrosothiols, and thus promote effects such as vasodilation and inhibition of platelet aggregation **(Stamler et al., 1992)**. This reaction may serve as a direct way by which NO diminishes oxidative stress and, thus, exerts cyto-protective effects.

1.1.4. NO in the intestinal tract

NO has several important functions in the intestinal environment, including immune responses, antimicrobial activity, neurotransmission, regulation of vascular functions and regulation of the barrier function and epithelial integrity **(Witthoft et al., 1998)**. However, excessive and prolonged NO produced by iNOS can lead to inflammatory conditions that increase mucosal permeability, triggering the inflammation which chronically leads to the inflammatory bowel diseases (IBDs) **(Alican et al., 1996; Marion et al., 2003)**.

Therefore, the role of NO in intestinal inflammation is still controversial: it may play a protective role at low concentrations, while after acute or chronic exposure a toxic mechanism of action is postulated, leading to hypersecretory or injurious effects **(Alican et al., 1996)**. In

the intestine, increases in NO levels have been found in ulcerative colitis **(Alican et al., 1996)**, in celiac disease and following entero-invasive bacterial infection **(Witthoft et al., 1998).**

NO regulates major epithelial functions involved in host defence, such as mucus production and epithelial fluid secretion. It induces gastric mucus secretion via the activation of soluble guanylate cyclase **(Brown et al., 1993)** and its production appears to be caused by the activation of cholinergic receptors **(Wilson et al., 1996).** Furthermore, a constitutive production of NO by eNOS inhibits neutrophil b2 integrin function, decreases endothelial P-selectin expression and reduces chemotactic responses to various chemokines, such as interleukin (IL)-8 and monocyte chemotactic protein-1 (MCP-1). Therefore, it reduces leucocyte chemotaxis, adhesion and recruitment in tissues **(Banick et al., 1997; Davenpeck et al., 1994; Sato et al., 1999; Sato et al., 2000)**.

Additionally, the constitutive production of NO exerts its anti-inflammatory effects via modulation of platelet homotypic aggregation and platelet adhesion to vessel walls **(Radomski et al., 1987).** NO has also been found to alter the cytokine profile released by macrophages, so that following a T helper 1 (Th1) response, Th1-associated cytokines are down-regulated and T helper 2 (Th2) cytokines are favoured **(Huang et al., 1998)**.

Under pathological conditions, the production of NO is upregulated, due to an enhancement of the inducible NOS expression. This condition leads to a loss of barrier function with epithelial disruption and bacterial translocation, which triggers the inflammation **(Witthoft et al., 1998; Lu et al., 2014)**. Indeed, under abnormal conditions, loss of GI barrier integrity can permit the penetration of normally excluded luminal substances (e.g., endotoxin and microbes) into or across the mucosa, which can lead to the initiation and/or perpetuation of inflammatory processes and mucosal injury. This injury and

the ensuing loss of mucosal barrier integrity have been implicated in the pathogenesis of a wide range of inflammatory disorders including IBDs **(Banan et al., 2001)**.

Crohn's disease and ulcerative colitis are the principal types of inflammatory bowel disease. They are characterized by the destruction of the gastrointestinal tract integrity, the intestinal mucosa infiltration of monocytes and neutrophils, the excessive production of cytokines and the subsequent chronic intestinal inflammation **(Lu et al., 2014)**. NO has been recognized as involved in the etiology of this kind of mucosal inflammation **(Chen et al. 2011)**. Indeed, the upregulated production of NO causes deleterious effects indirectly through the generation of reactive nitric oxygen species (RNOS), such as peroxynitrite anion ($ONOO^-$), the nitroxyl anion (NO-) and dioxide nitrogen (NO_2), responsible for oxidative stress **(Dijkstra et al., 1998)**. Hence, the elevated levels of peroxynitrite formed after the reaction of large quantities of NO with the superoxide anion, may be an essential factor in tissue injury during these diseases **(Banan et al., 2001)**.

Macrophages and polymorphonuclear cells were initially thought to be the main source of NO during inflammatory processes. It is now clear that intestinal and colonic epithelial cells are a major site of iNOS expression, in animal models of gut inflammation and in human IBD **(Tepperman et al., 1994; Singer et al., 1996; Morin et al., 1998)**, as well as in human intestinal epithelial cell lines **(Kolios et al., 1995; Salzman et al., 1996)**. Since the first demonstration of increased calcium-independent iNOS activity in colonic tissue from patients with ulcerative colitis **(Boughton-Smith et al., 1993)**, other studies have confirmed the increase in iNOS activity or NO release **(Rachmilewitz et al., 1995)**, in iNOS protein **(Singer et al., 1996)**, and in iNOS mRNA level **(McLaughlan et al., 1997)**, in patients with inflammatory bowel disease (IBD). Studies using animal models of IBD have also shown iNOS involvement **(Cavicchi et al., 1999)**.

Human colon epithelial cell lines are known to upregulate iNOS expression and the production of NO and its redox products (NOx) in response to stimulation with a combination of interferon-γ (IFN-γ) and interleukin-1 (IL-1), lipopolysaccharide (LPS) or tumor necrosis factor-α (TNF-α) **(Jenkins et al., 1994; Kolios et al., 1995; Salzman et al., 1996)**. Increased iNOS expression was also noted in the surface colon epithelium of patients during acute *Shigella* colitis **(Islam et al., 1997)**, and epithelial cells from rats challenged *in vivo* with LPS from *Escherichia coli* manifested increased iNOS activity **(Tepperman et al., 1994)**. However, *Salmonella dublin*, an enteroinvasive bacteria that is known to be a potent agonist of the intestinal epithelial cell proinflammatory gene program **(Jung et al., 1995; Huang et al., 1996; Eckmann et al., 1997)**, did not upregulate iNOS expression or NO production in human colon epithelial cell lines in the absence of costimulation of those cells with IFN-γ **(Scharton-Kersten et al., 1997)**. Several findings suggest that bacterial infection alone is not a sufficient stimulus to upregulate epithelial iNOS expression and NO production in the absence of concurrent mucosal inflammation and the associated increased production of cytokines such as IFN-γ and IL-1 or TNF-α **(Witthoft et al., 1998)**.

Increased NO levels have also been found in human tumors **(Thomsen et al., 1994; Klotz et al., 1998; Klotz et al., 1999)**. Most of the cellular components of the tumor mass (the tumor cells themselves, the immune cell infiltrate, and the stromal cells) have been shown to generate NO *in vitro*. Although a body of evidence indicates a role for NO in tumor growth, the end point of NO up-regulation is matter of dispute because increased NO levels have been equally documented to favor and to impair tumor progression **(Donnini et al., 2002)**.

Nitrogen species produced by inflammatory cells are known to regulate the expression of genes that encode tumour suppressors, such as p53, DNA mismatch-repair proteins and

DNA base excision-repair proteins, transcription factors such as NF-κB or signalling proteins such as cyclooxygenases. Moreover, by reacting with oxygen or superoxide radical, NO generates highly reactive species, such as nitrous anhydride (N_2O_3) and peroxynitrite that cause direct and indirect DNA damage, such as nitrosative deamination of nucleic acid bases, transition and/or transversion of nucleic acids, alkylation and DNA strand breakage **(Burney et al., 1999)**. This circuitry of inflammation and cancer modifies apoptosis and autophagy and promotes cellular cycle progression, invasion and angiogenesis **(Ullman et al., 2011)**. The role of iNOS in different stages of colon carcinogenesis has been highlighted in numerous studies in humans and mice **(Ohshima et al., 1994; Mannick et al., 1996)** and several data have also demonstrated increased iNOS activity in a high percentage of colon adenomas **(Nosho et al., 2005).**

Interestingly, it has been suggested that encounters between bacteria and intestinal stem cells and their niche at the bottom of the colonic crypts may induce activation of NF-κB in these cells, resulting in the release of high concentrations of reactive oxygen species (ROS) and NO **(Puglisi et al., 2015)**. This event, following further mutagenesis, triggers expansion and neoplastic transformation of the stem cell compartment, leading subsequently to the development of adenomatous polyps **(Puglisi et al., 2015)**.

1.2. The Mediterranean diet

The Mediterranean diet (MD) has long been celebrated as the gold standard of healthy diets for its highly palatable nature and favourable impact on the prevention of chronic diseases, promotion of greater longevity, and quality of life. A large quantity of scientific evidence has been accumulated over the past several decades showing that Mediterranean-type diets are highly protective against the development of cardiovascular disease, metabolic

disorders, and certain cancers **(Brill, 2008; D'Alessandro et al., 2014; Dontas et al., 2007)**. Defining the MD as a single entity is difficult because an assortment of Mediterranean-style eating patterns has emerged in the literature suggesting that there is no single ideal **MD (Noah et al., 2001)** but rather a Mediterranean style of eating that shares several key dietary components. In addition, to use the term Mediterranean diet to describe the eating pattern of the people living around the Mediterranean Sea would be a mistake, as there are at least 20 countries with coastlines bordering the Mediterranean Sea, each with their own eating habits, religions, and ethnic and cultural customs **(Brill, 2008)**.

Several researchers have suggested that the health effects of the MD are due to a dietary pattern of eating, comprising a variety of foods with the interaction of multiple synergistic protective factors as opposed to choose individual nutrients or foods. Hence, the combined effect of many constituents of the Mediterranean pattern of eating can possibly explain the favourable effect on health and longevity that this lifestyle confers **(Trichopoulou et al., 2003)**.

1.2.1. Food composition of the Mediterranean diet

Likely the most unifying theme of all the Mediterranean dietary patterns described in literature is that they consist mainly of foods from plant origin, with only little amounts of animal food sources. A characteristic of the traditional MD is the high intake of fruits, vegetables, nuts, legumes, wholegrain cereals and, mainly, olive oil **(Fig. 8) (Trichopoulou et al., 2003)**, all of which are derived from the plant kingdom. The Mediterranean dietary pattern also includes moderate consumption of fish and shellfish, white meat, eggs, alcohol through wine intake, and dairy products. Moreover, in the MD the consumption of traditional and local food products is recommended, as well as the seasonal choice of fresh and locally grown

produce as far as possible, according to the seasonal availability and the biodiversity of food **(Del Chierico et al., 2014; García-Fernández et al., 2014)**. On the contrary, consumption of red meat, processed meats, and foods rich in sugars and in fats should be small in both quantity and frequency **(Castro-Quezada et al., 2014)**.

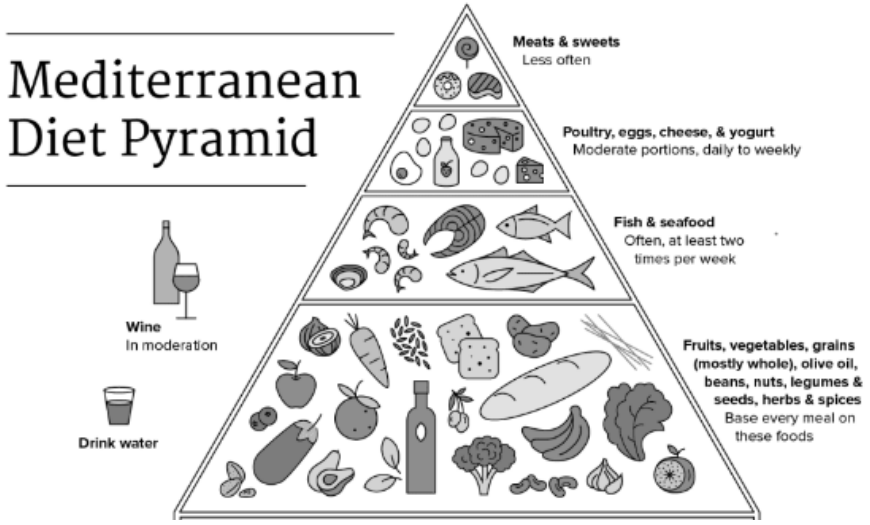


Figure 8. Scheme of the typical Mediterranean diet food composition (from America's test kitchen, 2017).

1.2.2. Healthy benefits of the Mediterranean Diet

Plant foods are rich in dietary fiber, vitamins, minerals, and polyphenols, dietary compounds that confer numerous health benefits, among which most notably is the protection from cancer, including upper digestive tract, stomach, colorectal, pancreas, liver, and selected hormone-related cancers such as endometrial cancer **(Dernini et al., 2017)**, and from coronary health diseases (CHD) **(Kushi et al., 1995a; Kapiszewska et al., 2005)**. Moreover, a low consumption of animal products in particular has been cited as a strong contributing factor for the excellent health of the Mediterranean people **(Kushi et al., 1995b)**. The MD is renowned for its abundance of plant-based foods, filled with an

extraordinary array of antioxidants. Antioxidants counteract excessive production of reactive oxygen species (ROS), which include both free radicals and non-radicals. Pathologies arise in the body when the production of ROS exceeds the body's antioxidant capacity **(Bogani et al., 2007)**. Thus, a large intake of antioxidants should provide the underlying protective mechanism related to their ability to fight DNA damage and oxidative stress and therefore provides an explanation for the significant reduction in the incidence of chronic disease associated with the MD **(Brill, 2008)**. The main source of fat in the Mediterranean diet is olive oil, which contains oleic acid and phenolic compound that reduce oxidative stress and consequently ageing, which is promoted by free radicals **(Covas, 2008)**. Moreover, MD includes low-glycemic index and low-glycemic load foods, derived from the whole grain and other fiber-rich product intake, which have been associated with a lower risk of diabetes, mainly type 2, CHD and cancer, while refined grain has been linked to the risk of diabetes, obesity, CHD and other chronic diseases **(Barclay et al., 2008)**.

It has been found that the Mediterranean population has a greater longevity than many other developed countries **(Panagiotakos et al., 2004)**. The hypothesis that Mediterranean diet would have a positive association with longevity has been demonstrated by **Trichopoulou (2004)**. The main explanation is the fact that this type of diet protects against chronic diseases such as cancer and coronary diseases **(Karampola et al., 2011)**, hence reducing death's risks.

1.3. Olive oil

Olive oil is the most important element in the Mediterranean diet, not only for its appreciable taste and usefulness in flavouring a large variety of foods, but also because it has several beneficial properties due to its chemical composition **(Cicerale et al., 2012)**. It is the main product obtained by olives, fruits which come from the *Olea europaea* evergreen trees

(Boskou et al., 1996). This plant is being cultivated worldwide, but the Mediterranean area is the most favourable place where to grow it, because of its dry summers and mild winters **(Huang et al., 2008)**.

In fact, the oil's chemical composition and sensory properties depends on the climate, but also on the growing procedures, the cultivar of the olive and the production techniques **(Cicerale et al., 2009)**. The oil extraction from olives is usually conducted through pressure, centrifugation, and percolation **(Huang et al., 2008)**. The olive oil's quality is determined taking into account its free acidity as a parameter. The highest quality olive oil (Extra-Virgin olive oil, EVOO) must feature a free acidity lower than 0.8%. Virgin olive oil is characterized by acidity between 0.8% and 2%, while lampante olive oil (a low-quality oil which is not edible) features a free acidity higher than 2% **(Regulation (EEC) No 2568/91)**. The increase of this free acidity in olive oil is due to free fatty acids that are released from triglycerides. Virgin olive oil is obtained under mechanical conditions that do not alter its composition and it is not mixed with other oils, as well as EVOO, which accounts for only 10% of oil produced **(Huang et al., 2008)**.

1.3.1. Olive oil chemical composition

EVOO chemical composition is characterized by two major components, the saponifiables and the unsaponifiables compounds. The first group comprises triacylglycerols (TAG), partial glycerides, esters of fatty acids or free fatty acids and phosphatides, and represent nearly 98% of the oil chemical composition, while the second is mainly formed by minor components such as hydrocarbons (squalene), phytosterols (β-sitosterol, stigmasterol, and campersterol), tocopherols (α-tocopherol), carotenoids (β-carotene), pigments (chlorophylls), aliphalic and triterpenic alcohols, volatile compounds and polyphenols. All of these compounds contribute, though differently, to the oil flavour and to its health benefits

(Boskou et al., 1996). Countless studies have demonstrated that the protective effects of olive oil in the context of the Mediterranean diet is due to its high monounsaturated fatty acids (MUFA) content and to the beneficial effects of the minor components, mainly to the phenolic fraction **(Covas et al., 2006a)**.

1.3.2. Olive oil phenolic composition

As stated in the previous chapter, the phenolic fraction is only a small proportion compared to the other compounds, which characterize the olive oil composition. Nevertheless, EVOO polyphenols play a key role in the beneficial effects on human health attributed to EVOO. Indeed, they have been shown to possess antimicrobial, antioxidant and anti-inflammatory properties, *in vivo* and *in vitro* **(Cicerale et al., 2012)**. The phenolic compounds contribute to the sensory properties of EVOO **(Uylaser et al., 2014)**. They confer a bitter, pungent taste and a strong, fruity flavour to the oil, indicating a high sensory quality **(Visioli et al., 2002)**. EVOOs with high phenol levels exhibit a high stability because of their antioxidant capacity, so significantly contributing to determine the shelf-life of the oil, preventing its autoxidation **(Fito et al., 2007)**.

At least, 36 phenolic compounds have been identified in EVOO, but not all of these are present in every oil **(Cicerale et al., 2009)**. The large variety of polyphenols found in olive oil is different in chemical structures and concentrations (0.02–600 mg/kg), depending on numerous factors including: variety, region in which the olive is grown, agricultural techniques used to cultivate the olive, maturity of the olive fruit at harvest, and processing **(Cicerale et al., 2009)**. Indeed, different varieties, cultivated in the same environment and processed at a fixed ripening stage, produce olive oil with different total polyphenols content **(Vitaglione et al., 2013)**.

The main classes of olive oil polyphenols are the following:

- secoiridoids, where the most abundant are the dialdehydic form of decarboxymethyl elenolic acid linked to hydroxytyrosol (3,4-DHPEA) or tyrosol (p-HPEA), (3,4-DHPEA-EDA or p-HPEA-EDA), oleuropein, an isomer of the oleuropein aglycon (hydroxytyrosol linked to elenolic acid) (3,4-DHPEA-EA), and ligstroside aglycon (tyrosol linked to elenolic acid) (p-HPEA-EA). p-HPEA-derivates and dialdehydic forms of oleuropein and ligstroside aglycon have also been detected as minor hydrophilic phenols of olive oil **(Bendini et al., 2007)**;
- phenylethanoids, which possess a hydroxyl group attached to an aromatic hydrocarbon group, such as hydroxytyrosol (3,4-dihydroxyphenyl-ethanol or 3,4-DHPEA) and tyrosol (p-hydroxyphenyl-ethanol or p-HPEA) **(Rodriguez-Morato et al., 2016) (Fig. 9)**. Their concentration is usually low in fresh oils but increases during oil storage **(Obied et al., 2008)** due to the hydrolysis of secoiridoids;

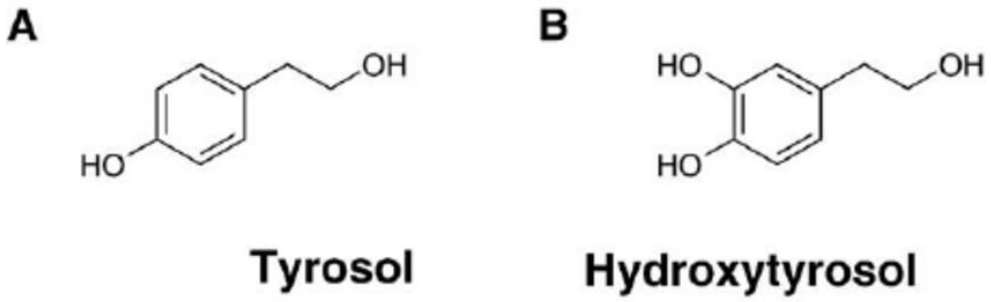


Figure 9. Chemical structure of phenylethanoids tyrosol (A) and hydroxytyrosol (B).

- phenolic acids, which can be divided into two subgroups: hydroxybenzoic acid derivatives and hydroxycinnamic acid derivatives, such as gallic acid, protocatechuic

acid, p-hydroxybenzoic acid, vanillic acid, caffeic acid, syringic acid, p- and o-coumaric acid, ferulic acid, and cinnamic acid **(Bendini et al., 2007) (Fig. 10)**;

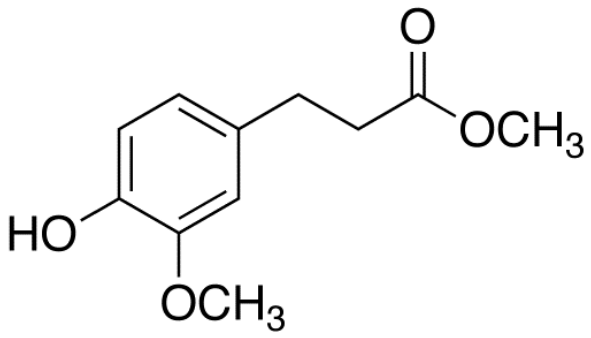


Figure 10. Chemical structure of the hydroxycinnamic derivative ferulic acid.

- flavonoids, which contain two benzene rings joined by a linear three carbon chain. Flavonoids are largely planar molecules and their structural variation comes in part from the pattern of modification by hydroxylation, methoxylation, prenylation, or glycosylation. Flavonoid aglycones are subdivided into flavones, flavonols, flavanones, and flavanols depending upon the presence of a carbonyl carbon at C-4, an OH group at C-3, a saturated single bond between C-2 and C-3, and a combination of carbonyl at C-4 with an OH group at C-3, respectively. Luteolin and apigenin are the most concentrated **(Bendini et al., 2007)**;
- hydroxy-isocromans, a class which consists in only two compounds: 1-phenyl-6,7-dihydroxy-isochroman and 1-(39-methoxy-49-hydroxy) phenyl-6,7-dihydroxy-isochroman **(Bianco et al., 2001)**;
- lignans, whose structure is not well understood, but is based on the condensation of aromatic aldehydes. (+)-1-Acetoxypinoresinol and (+)-1-pinoresinol were characterized as the most concentrated lignans in extra virgin olive oil **(Owen et al., 2000)**.

The phenolic acids were the first group of phenolic compounds found in extra virgin olive oil; these compounds together with phenyl-alcohols, hydroxy-isochromans and flavonoids **(Bianco et al., 2001)**, are present in small amounts in extra virgin olive oil, while secoiridoids and lignans are the most prevalent ones **(Bendini et al., 2007)**. Phenolic acids such as ferulic acid (FA) can also be found in large concentration in vegetables, fruits, cereals, and coffee **(Zhao et al., 2008).**

1.3.3. Absorption and metabolism of olive oil phenolic compounds

The degree to which phenolic compounds are bioavailable (absorbed, metabolised, distributed and eliminated) is fundamental in understanding and evaluating the health benefits associated with EVOO's consumption; to achieve an effect in specific tissues or organs, in fact, they must be bioavailable **(Soler et al., 2001)**. The majority of researches regarding the bioavailability of these compounds has focused on the absorption and excretion of two major phenolics: hydroxytyrosol (HT) and tyrosol (Tyr), and significant absorption (~40–95%) of these compounds has been demonstrated in humans **(Cicerale et al., 2010)**. Furthermore, other minor compounds' absorption and metabolism have been studied, such as hydroxycinnamic and hydroxybenzoic acid derivatives, because of their largely proved health benefits **(Feliciano et al., 2016; Zhao et al., 2008)**.

The dietary intake of EVOO polyphenols has been estimated to be around 9 mg, within 25–50 mL of olive oil per day, where at least 1 mg of them is derived from free hydroxytyrosol and tyrosol, and 8 mg are related to their elenolic esters and to the oleuropein- and ligstroside-aglycons **(De la Torre, 2008)**. On the contrary, hydroxycinnamic acids such as FA and caffeic acid are poorly found in olive oil (0.01- 0.03 mg/100 g olive oil).

1.3.3.1. Absorption and metabolism of hydroxytyrosol and tyrosol

Concerning HT and Tyr, several clinical and animal studies have provided evidence that they are absorbed, and exert their biological effects, in a dose-dependent manner. Even from moderate doses (25 mL/d), which are lower than the traditional daily dietary intake in Mediterranean countries **(Weinbrenner et al., 2004)**, around 98 % of these phenolics were present in plasma and urine in conjugated forms, mainly glucuronides and sulphate conjugates **(Miró-Casas et al., 2003)**. After ingestion, EVOO polyphenols can be partially modified in the acidic environment of the stomach. Aglycone secoiridoids are subjected to a time-dependent hydrolysis in the acidic gastric environment, leading to a significant increase in the amount of free hydroxytyrosol and tyrosol after 30 min. This decomposition of secoiridoid aglycones increased with increased gastric residency, though under normal pH conditions (pH 2.0) and normal physiological time frames (up to 4 h) some remain intact and enter the small intestine un-hydrolyzed **(Corona et al., 2006)**. On the contrary, if the ingested secoiridoid is glycosilated, it is not subjected to gastric hydrolysis **(Vissers et al., 2002)**. Hence phenolics such as the glucosides of oleuropein enter the small intestine unmodified, along with high amounts of free HT and Tyr. Following ingestion of olive oil, the levels of HT and Tyr increase rapidly achieving a peak concentration at approximately 1 h in plasma **(Miro-Casas et al., 2003)** and around 2 h in urine **(Miro-Casas et al., 2003)**, which supports the evidence that the small intestine is the major site of absorption for these compounds.

A minor metabolic pathway of hydroxytyrosol gives rise to the formation of 3-hydroxy-4-methoxyphenylethanol (homovanillic alcohol) in a reaction regulated by the catecholmethyltransferase (COMT). Therefore, in the process of crossing epithelial cells of the GI tract, phenolic compounds from olive oil are subjected to an important first pass metabolism through a classic phase I/II biotransformation. This process is very relevant, to the extent that polyphenols in their free form are deemed undetectable in biological matrices.

It is not surprising that some authors caution that the attained concentrations after their ingestion are too low to explain the observed biological activities in *in vitro* and *in vivo* models at higher doses/concentrations **(Vissers et al., 2004)**.

Nevertheless, in the case of poorly absorbed phenolic compounds, it has been suggested that these components may exert local antioxidant activities in the gastrointestinal tract and this proposal is supported by research demonstrating the free radical scavenging capacity of olive oil phenolics in both the faecal matrix and intestinal epithelial cells **(De la Torre, 2008)**.

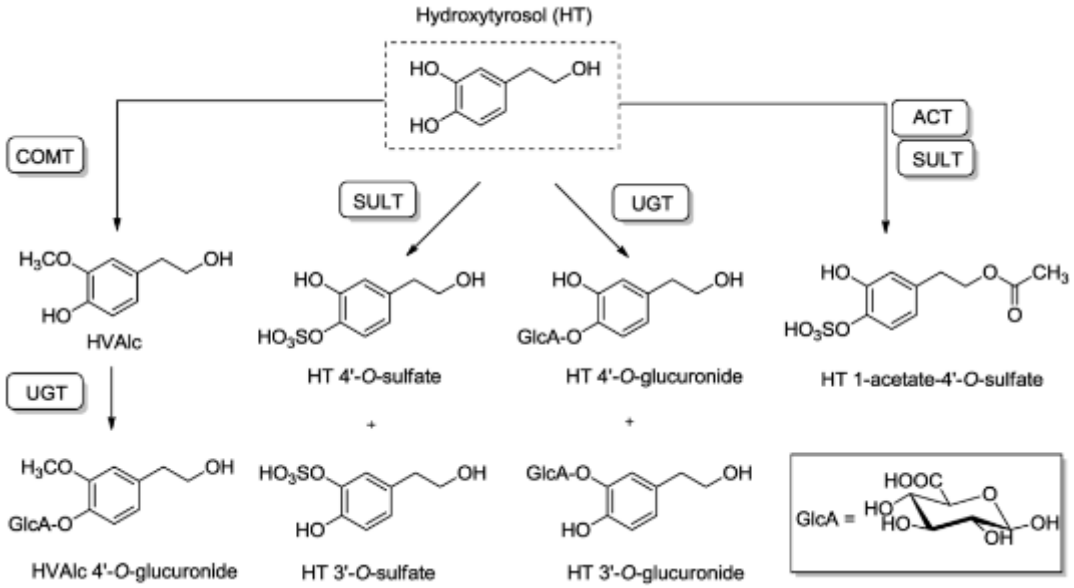


Figure 11. Biotransformation pathways of hydroxytyrosol (HT) (from Rodriguez-Morato et al., 2015). Abbreviations: COMT: Catechol-O-methyltransferase; UGT: UDP-glucuronosyltransferase; SULT: Sulfotransferase; ACT: O-Acetyltransferase; GlcA: Glucuronic acid; HVAlc: Homovanillyl alcohol.

The analysis of human plasma and urine **(Miro-Casas et al., 2003)** has demonstrated that both HT and Tyr are dose-dependently absorbed and are metabolized primarily to *O*-glucuronidated conjugates **(Caruso et al., 2001; Visioli et al., 2000; Visioli et al., 2001)**. In

fact, sulfated and glucuronidated HT and Tyr were the predominant metabolites found in human plasma and urine, and they have also been shown to concentrate in the intestinal epithelium, since glucuronidation and sulfation are the major pathways of phase II xenobiotic metabolism in the human intestine **(Fig. 11) (Miro-Casas et al., 2003)**.

The mechanism by which absorption occurs after olive oil phenolic compounds intake remains unclear. However, the different polarities of the various phenolics has been postulated to play a role in the absorption of these compounds **(Vissers et al., 2002)**.

A pioneering work by **Manna et al. (2000)** using differentiated Caco-2 cell monolayers as a model system examined the mechanisms of HT absorption. They concluded that HT transport occurs via a bidirectional passive diffusion mechanism, and estimated that the molecule was quantitatively absorbed at the intestinal level.

As stated before, it is understandable that the bioavailability of HT and Tyr is poor due to an extensive metabolism; more than 10 metabolites have been found in previous studies. These metabolites include *O*-methylated forms **(Caruso et al., 2001)**, aldehydes and acids formed via oxidation of the aliphatic alcohol **(D'Angelo et al., 2001)**, sulfates **(Tuck et al., 2002)**, glucuronides **(Khymenets et al., 2011)**, and acetylated and sulfated derivatives **(Rubio et al., 2014)** as well as an N-acetylcysteine derivative **(Kotronoulas et al., 2013)**. Once absorbed, HT and Tyr are widely distributed throughout the organism. The pharmacokinetic analysis indicated an extensive and fast uptake of this antioxidant by different organs including skeletal muscle, kidneys, liver, lungs, heart and brain **(Rodriguez-Morato et al., 2016)**.

1.3.3.2. Absorption and metabolism of ferulic acid and its derivatives

In situ or *ex vivo* absorption models suggested that, after ingestion, FA can be absorbed from the stomach **(Zhao et al., 2004)**, jejunum **(Spencer et al., 1999)**, and ileum **(Spencer et al., 1999)**. After a 25-min incubation of FA in the rat stomach, about 70% of the FA disappeared from the stomach and it was recovered in the gastric mucosa, blood, bile and urine, suggesting a gastric absorption of FA **(Zhao et al., 2004)**. In the same way, FA quickly disappeared from the jejunum and to a significantly lesser extent from the ileum, when it was perfused in an isolated rat intestine model **(Spencer et al., 1999)**.

Only 0.5–0.8% of ingested FA was found in feces of rats, indicating a very efficient absorption rate for FA **(Adam et al., 2002; Zhao et al., 2003a)**. The exact mechanism for this high absorption rate is not known. The fact that FA could move into the gastric mucosa even at 0°C suggests that FA might diffuse across the stomach mucosa **(Zhao et al., 2004)**.

Several metabolic studies **(Zhang et al., 2005; Zhao et al., 2003b; Zhao et al., 2004)** have shown that FA can be metabolized *in vivo* into a number of metabolites including FA-glucuronide, FA-sulfate, FA- diglucuronide, FA-sulfoglucuronide (FA-diconjugate with sulfate and glucuronide), m-hydroxyphenylpropionic acid, feruloylglycine, dihydroferulic acid, isoferulic acid, vanillic acid and vanilloylglycine. Conjugated FA including FA-glucuronide, FA-sulfate and FA-sulfoglucuronide are the major metabolites in the plasma and urine of rats **(Fig. 12) (Rondini et al., 2002; Zhao et al., 2003b)**.

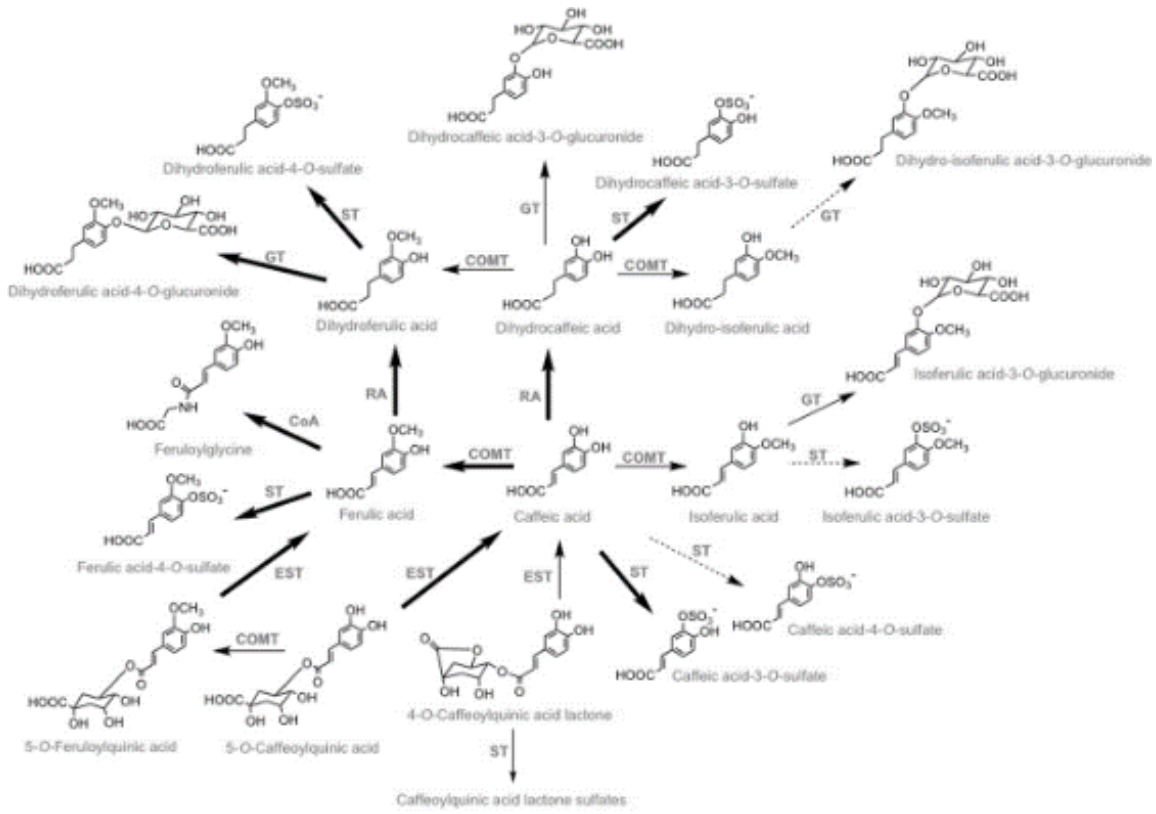


Figure 12. Proposed metabolism of ferulic acid and caffeic acid (from Stalmach et al., 2009).

These results suggest that the conjugation reaction with glucuronic acid and/or sulfate is the main pathway of *in vivo* FA metabolism. The conjugation of FA took place mainly in the liver through the activities of sulfotransferases and UDP glucuronosyl transferases **(Zhao et al., 2004)**. Intestinal mucosa **(Kern et al., 2003; Spencer et al., 1999)** and kidney **(Zhang et al., 2005; Zhao et al., 2003b)** may also, at least in part, contribute to this conjugation process. In fact, FA found in food esterified with carboxylic acids such as quinic acid **(Herrmann, 1989)** could also be released and absorbed in the colon, providing these esters reached this segment intact. Moreover, FA has been found to be metabolized into hydroferulic acid, vanillic acid, 3-hydroxyphenylacetic acid and protocatechuic acid by colonic microflora **(Ekbatan et al., 2016)**. It cannot be excluded that they could be unstable during their passage

through the upper part of the gastrointestinal tract or could be absorbed intact or as free forms in the stomach and the small intestine after lysis of the ester bond by local esterases **(Poquet et al., 2007)**.

The conjugation of FA may be dose dependent as very high doses of FA may saturate the conjugation enzymes, leading to accumulation of free FA in plasma. This was evidenced by recovery of free FA in the plasma of rats after high doses of FA (up to 70 µmol/ kg), but not after low doses (up to 7 µmol/kg) **(Adam et al., 2002; Rondini et al., 2002; Zhao et al., 2003b)**. Similarly, the urinary excretion of free FA was found to be dose dependent after oral administration of a relative lower dose (70 µmol/kg) **(Zhao et al., 2003b)** and a higher dose (462 µmol/kg) **(Zhang et al., 2005)** in rats; 2.9% of the lower dose and 14.3% of the higher dose was detected as free FA in the urine samples. More free FA was found in the urine after intraperitoneal injection **(Teuchy et al., 1971)** than oral administration **(Zhao et al., 2003b)**. Furthermore, an intravenous administration of FA was associated with very high levels of free FA in the urine **(Choudhury et al., 1999)**, suggesting a rapid filtration of free FA by the kidneys. FA is also metabolized into *m*-hydroxyphenylpropionic acid by the intestinal microflora through reduction, demethylation, and dihydroxylation at C4 **(Chesson et al., 1999)**.

Generally, the bioavailability of free FA is very low due to its rapid conjugation process in the liver **(Zhang et al., 2005; Zhao et al., 2004)**. In addition, the cumulative urinary excretion of total FA may be used to estimate its absorbability, because absorbed dietary FA is excreted mainly through urine **(Rondini et al., 2002; Zhao et al., 2003b)**. The absorbability can partly reflect the bioavailability of FA. The urinary excretion in rats suggests the following order for absorbability of dietary FA: free FA > feruloyl mono-, di-saccharides > feruloyl polysaccharides **(Zhao et al., 2003a, 2003b)**. This is because simple sugar FA esters are easily hydrolyzed by esterases and/or microflora in the intestine.

Feruloyl polysaccharides with complex structure may reduce the interactions between the hydrolyzing enzymes (xylanases, ferulate esterases) and the polymers of FA, resulting in reducing the release of FA **(Zhao et al., 2005)**.

Concerning the distribution of FA and its derivatives, serum albumin seems to be the major carrier of FA **(Zhang et al., 2005; Kang et al., 2004)**. **Zhao et al. (2004)** estimated that approximately 4%, 10% and 53% of the orally administered FA can be found in the gastric mucosa, blood and other tissues including liver and kidney, respectively. **Adam et al. (2002)** also determined that about the 49% of perfused FA in the rat intestine might be distributed in the liver and peripheral tissues. Another study assessed the quick distribution of FA in almost all the rat tissues. Indeed, **Zhang et al. (2005)** reported that free FA was recovered in the kidney, lung, liver, spleen, heart, uterus and brain, at approximately 30 min after an oral administration of 521 μmol/kg BW of FA in rats.

1.3.4. Biological activity of olive oil polyphenols

The consumption of EVOO has been associated with many health benefits including lower incidence of coronary heart disease and cancer. Studies conducted so far (including human trials, animal, *in vivo* and *in vitro* experiments) have demonstrated that olive oil phenolic compounds have positive effects on various physiological biomarkers, implicating phenolic compounds as partially responsible for the health benefits associated with the Mediterranean diet **(Cicerale et al., 2010)**. Thus, the researchers have mainly been focused on the biological activities of these compounds, assessing the healthy benefits of HT, Tyr and other minor components like the hydroxycinnamic derivative FA.

1.3.4.1. Biological activities of hydroxytyrosol and tyrosol

As phenolic compounds HT and Tyr are expected to have strong antioxidant activities **(Carrasco-Pancorbo et al., 2005)** due to the potent redox properties of the phenolic hydroxyl groups and the structural relationship in the chemical configuration of the molecules **(Cheng et al., 2002)**. Among the free radicals, the 'reactive oxygen species' (ROS), which are continuously formed as a result of normal metabolic processes, can oxidize and damage cellular macromolecules, possibly leading to the development of degenerative diseases (for example, atherosclerosis, cancer, diabetes, rheumatoid arthritis and inflammatory diseases). Exogenous antioxidants such as HT and Tyr are important because they have a twofold function, preventing food oxidation – and in particular lipid oxidation – and at the same time increasing the amount of antioxidant agents present in the organism, protecting against degenerative diseases. Olive oil phenolic compounds can act as antioxidants in various ways. In oxidative systems where transition metals, such as Cu and Fe, are present they can chelate metallic ions, thus preventing their involvement in Fenton reactions that can generate high concentrations of hydroxyl radicals **(Halliwell et al., 1995)**. However, the most important antioxidant activity is related to the free radical-scavenging ability, by breaking the chain of reactions triggered by free radicals. The antioxidant properties of the *o*-diphenols such as HT are associated with their ability to form intramolecular hydrogen bonds between the hydroxyl group and the phenoxylic radicals **(Visioli et al., 1998)**. By estimating the antioxidant activity of these polyphenolic compounds on the basis of their ability to inhibit the formation of peroxides, it has been shown that HT and hydroxycinnamates have the highest efficacy **(Papadopoulos et al., 1991)**. The antioxidant activity of HT has also been demonstrated in cellular models and animals **(Manna et al., 1997; Speroni et al., 1998)**. Tyr, although has been reported to have a low antioxidant capacity as compared to HT, is able to scavenge peroxynitrite **(De la Puerta et al., 2001)** and $O_2^{-\cdot}$ **(Bertelli et al., 2002)**.

HT and Tyr biological activities *in vivo*, however, are mediated by mechanisms other than scavenging free radicals **(Forman et al., 2014)**. In experimental studies (*in vivo* and *in vitro*), they have been shown to beneficially alter blood lipid composition **(Vissers et al., 2001, Visioli et al., 2005)**, inducing a significant increase in circulating HDL **(Marrugat et al. 2004, Weinbrenner et al., 2004, Covas et al., 2006b)**, and significantly decreasing LDL, total cholesterol and triglycerides **(Gimeno et al., 2002, Gorinstein et al., 2002)**. Numerous studies have shown that these phenols are potent inhibitors of LDL oxidation in vitro **(Visioli et al., 1994; Visioli et al., 1995)**. The *in vivo* oxidation of LDL is linked to the formation of atherosclerotic plaques, which are postulated to contribute to the development of coronary heart disease. In particular, HT has been reported to reduce the risk of coronary heart disease and atherosclerosis **(Grignaffini et al., 1994; Salami et al., 1995)**. It has also been postulated that HT inhibits arachidonic acid lipoxygenase **(Petroni et al., 1997)** and inhibits platelet aggregation **(Petroni et al., 1994; Petroni et al., 1995)**. It is presumed that HT penetrates in cell membranes and consequently can inhibit the production of leukotriene B4 (LTB4) from endogenous arachidonic acid **(Kohyama et al., 1997)**. Furthermore, the prevention of cardiovascular disease by HT is also linked to a regulation of several enzymatic activities. For example, its inhibition on the inducible form of NO synthase and proinflammatory enzymes such as 5-lipoxygenase have been demonstrated in vitro **(Hu et al., 2014)**.

HT showed to exert anti-proliferative effects, via its ability to strongly inhibit ERK1/2 phosphorylation and downstream cyclin D1 expression **(Corona et al., 2009)**. These findings are of particular relevance due to the high colonic bioavailability of HT when compared to the other olive oil polyphenols and may help explain the inverse link between colon cancer and olive oil consumption. Furthermore, HT inhibits colon cancer cell proliferation **(Fabiani et al., 2002)** and induces cancer cell apoptosis **(Guichard et al., 2006)** through a mechanism of action linked to a prolonged stress of the endoplasmic reticulum (activation of unfolded

proteins) and over-expression of proapoptotic factors, such as Ser/Thr phosphatase 2A, a key protein involved in the induction of apoptosis in colon cancer cells **(Guichard et al., 2006)**. In addition, **Zhao et al. (2014)** showed that HT inhibited the proliferation and induced apoptosis in hepato cellular carcinoma (HCC) *in vitro* and in an orthotopic tumor model of HCC. The underlying mechanisms may be, at least in part, due to HT suppression of Akt-mediated NF-κB activation, down-regulating NF-κB-regulated gene products. This inhibition of the NF-κB activation was also observed in a rodent model of pulmonary edema induced by hypobaric hypoxia after Tyr pretreatments **(Wang et al., 2017)**.

As previously stated, the concentrations of HT and Tyr themselves in biological fluids are extremely low compared to their metabolites **(Gonzalez-Santiago et al., 2010; Kotronoulas et al., 2013; Pastor et al., 2016)**.

So, there is a good reason to take into account the participation of metabolites, along with their parent compounds, with respect to the beneficial effects reported for dietary consumed HT and Tyr.

Khymenets et al. (2010, 2011) highlighted a loss of antioxidant activity of HT 3'-*O*- and 4'-*O*-glucuronides and Tyr 4'-*O*-glucuronide. Such results were in line with a previous theoretical study in which phase II metabolites glucuronides (and also sulfates) were predicted to lose the antiradical activity characteristic of their parent compound **(Nenadis et al., 2005)**.

In contrast to the previously mentioned reports, another study **(Deiana et al., 2011)** reported that HT glucuronide metabolites, specifically the mix of 3'-*O*-ß-D and 4'-*O*-ß-D-glucuronidated isoforms, could protect renal cells (LLC-PK1 cells as a culture model) against H_2O_2-induced lipid peroxidation-related membrane oxidative damage by reducing malondialdehyde production and modifying the profile of the major oxidizable membrane lipids, unsaturated fatty acids and cholesterol. Nonetheless, glucuronide metabolites acted to

lesser extent than parent HT. In a different study, HT glucuronides were also shown to protect red blood cells (RBC) from *in vitro* H_2O_2-induced oxidative injury at low concentrations **(Paiva-Martins et al., 2013)**. At higher concentrations, however, the effect of protection for HT glucuronides remained practically the same as at low doses. The authors postulated that some sort of saturation of glucuronide-specific RBC transporters could have occurred, limiting the availability of glucuronides inside RBC, or/and the glucuronides may have undergone a restricted hydrolysis to liberate active HT, which could then be absorbed by the cells. Recent HT bioavailability data demonstrated that sulphated metabolites appear to be the most abundant among the HT phase II metabolites **(Gonzalez-Santiago et al., 2010; Kotronoulas et al., 2013; Perez-Maňa et al., 2015)**, thus it is also important to consider this sulfoconjugated forms as bioactive compounds.

Interestingly, a recent report evaluating the antioxidant effect of HT and Tyr sulfate metabolites in intestinal cells (Caco-2) found that HT and Tyr sulfates displayed an efficiency comparable to that of the parent compounds **(Atzeri et al., 2016)**. Moreover, Tyr glucuronide and sulfate showed anti-inflammatory and antioxidant activities preventing the rise of reactive oxygen species, the depletion of glutathione, and the down-regulation of glutathione peroxidase 1, glutamate-cysteine ligase catalytic subunit, and heme oxygenase-1 genes **(Muriana et al., 2017)**. Tyr sulfate and glucuronide were also able to prevent the phosphorylation of NF-κB signaling proteins, as well as the over-expression of adhesion molecules at gene, protein, and secretory levels, and the adhesion of human monocytes to the endothelial cells **(Muriana et al., 2017)**.

1.3.4.2. Biological activities of ferulic acid and its derivatives

One of the best documented biological activity of FA is its antioxidant property. Due to its phenolic nucleus and an extended side chain, FA readily forms a resonance stabilized phenoxy radical which accounts for its free radical-scavenging effect **(Graf, 1992)**. This enables FA to protect DNA and lipids against oxidation by reactive oxygen species (ROS) **(Andreasen et al., 2001; Anselmi et al., 2004; Dinis et al., 2002)**. Thus, FA may be beneficial in the prevention and/or treatment of disorders linked to oxidative stress, including Alzheimer's disease **(Jin et al., 2005; Kim et al., 2004; Ono et al., 2005)**, diabetes **(Balasubashini et al., 2003; Balasubashini et al., 2004)**, cancers **(Asanoma et al., 1994; Chang et al., 2006; Kampa et al., 2004; Kawabata et al., 2000)**, hypertension **(Suzuki et al., 2002; Suzuki et al., 2007)**, and atherosclerosis **(Dinis et al., 2002; Wang et al., 2005; Wang et al., 2004)**. The strong link between inflammation and oxidative stress suggests that FA may also be effective against inflammatory diseases **(Fernandez et al., 1998; Murakami et al., 2002)**. Furthermore, the special structure of FA endows its strong UV absorptive ability, making it an important skin protecting agent **(Chan et al., 2004; Lin et al., 2005)**. The preventive/therapeutic efficacy of FA is dependent on its physiological concentrations, which is predominated by its pharmacokinetic properties (absorption, metabolism, distribution and elimination).

Several studies investigated the biological properties of the FA metabolites, often to a greater extent with respect to the parent compound. FA-sulfate, better than FA, elicited a concentration-dependent vasorelaxation of saphenous and femoral arteries and aortae. In anesthetized mice, intravenous injection of FA-sulfate decreased mean arterial pressure, whereas FA had no effect, confirming the results obtained *ex vivo*. FA-sulfate is probably one of the major metabolites accounting for the blood pressure-lowering effects associated with FA consumption **(Van Rymenant et al., 2017)**. IFA, one of the FA isomeric forms,

proved to be a multifunctional compound, having anti-inflammatory **(Sakai et al., 2001)**, antiviral **(Wang et al., 2011)**, antioxidative **(Wang et al., 2011)** and antidiabetic properties **(Liu et al., 2000)**. Quite recently, IFA also showed antiglycation properties against fructose and glucose-mediated glycation and oxidation of bovine serum albumin **(Meeprom et al., 2013)**. The isomer hydroferulic acid (DHFA) have proved to be an inhibitor of *in vitro* platelet activation more effective than its phenolic precursor FA **(Baeza et al., 2017)**. A mixture of several FA metabolites totally abrogated superoxide production in isolated aortic rings **(Perez-Ternero et al., 2017)**.

2. Aim of the study

The Mediterranean diet has long been celebrated as the healthiest diet for its highly palatable nature and favourable impact on the prevention of chronic diseases, promotion of greater longevity, and quality of life **(Brill, 2008)**. In particular, its benefits have been linked to the consumption of its main food components, among which olive oil plays a pivotal role. The regular consumption of olive oil helps in increasing the intake of bioactive substances such as phenolic compounds. In particular, HT and Tyr, as well as FA and its derivatives, have been largely studied for their importance either as radical scavengers or in the modulation of abnormal signaling pathways which are peculiar of several diseases **(Han et al., 2007)**. Furthermore, the poor bioavailability of these compounds usually does not affect their biological significance, because many studies highlighted that their metabolization leads to the production of derivatives which are effective as the parent compounds **(Rodriguez-Morato et al., 2016)**. HT, TYR and FA have already demonstrated to be effective in the prevention of many pathologies, such as cardiovascular diseases and inflammatory bowel diseases. **(Corona et al., 2009; Lv et al., 2012; Bulotta et al., 2014; Fuentes et al., 2014; Incani et al., 2016; Ghosh et al., 2017).**

Cardiovascular diseases (CVDs) such as atherosclerosis and hypertensive heart disease, and inflammatory bowel diseases (IBDs) as well as colon cancer, share a common point: the dysregulation of NO production, though in different ways. In CVDs the down-regulation of NO production, through an affection of eNOS activation or a depletion of NO by the increase of superoxide release, plays a pivotal role in the impairment of the endothelial function **(Förstermann et al., 2011)**. Indeed, a dysfunctional vascular endothelium related to NO breakdown, promotes atherosclerosis through vasoconstriction, platelet aggregation and adhesion to the vascular wall, leukocyte adhesion, thrombogenesis, inflammation, smooth-muscle cell proliferation and collagen breakdown **(Le Mellédo et al., 2004)**.

Conversely, in IBDs has been found an abnormal up-regulation of the iNOS transcription, which is related to a significant hyper production of NO **(Banan et al., 2011)**. Once released in large amounts, it reacts with superoxide forming peroxynitrite, a high reactive and dangerous molecule which can damage a wide array of molecules in cells, including DNA and proteins **(Szabò et al., 2007)**. Furthermore, NO at high concentration can alter membrane permeability, hence giving way to the bacterial translocation, triggering the inflammation status **(Witthoft et al., 1998; Lu et al., 2014)**.

NO release modulation has widely been under investigation in the last 25 years **(Bohlen, 2015)** and many bioactive compounds have been evaluated as effective in enhancing or counteracting NO presence in the cells environment. In particular, a variety of phenolic compounds have been the object of this kind of surveys, proving their capability in modulating NO production in pathophysiological conditions.

Within this context, the goal of this study was to evaluate how dietary polyphenols, in particular the main ones coming from olive oil intake, are involved in the regulation of NO production, both in the endothelial vessels and in the gastrointestinal tract. In particular, this study focused on the mechanisms by which hydroxycinnamates, HT and Tyr, and phenylethanoids, FA, and derivatives IFA and DHFA, in comparison to their metabolites tyrosol glucuronide (Tyr glu), tyrosol sulfate (Tyr sulf), 3'-hydroxytyrosol 3'-glucuronide (HT glu), hydroxytyrosol 3'-sulfate (HT sulf), ferulic acid 4-*O*-β-D-glucuronide (FA glu), isoferulic acid 3-*O*-sulfate (IFA sulf), and dihydroferulic acid 4-*O*-β-D-glucuronide (DHFA glu), are responsible for the enhancement or reduction of NO presence in the systemic and in the intestinal environment, respectively. These metabolites are the main products of the phase I metabolism in the liver and of colonic microbiota metabolism in the small intestine of HT, Tyr and FA **(Lewandowska et al., 2016; Rodriguez-Morato et al., 2016; Perez-Ternero et al., 2017)**.

Almost all the polyphenols circulating in blood are glucuronidated and/or sulfated, and free aglycones are found in plasma to a lesser extent **(Lewandowska et al., 2016)**. Thus, the participation of metabolites, along with their parent compounds, should be taken into consideration regarding the beneficial effects reported for dietary consumed olive oil phenolic compounds.

To assess the endothelial NO release and the modulation of the eNOS by the tested compounds, Human Umbilical Vein Endothelial Cell (HUVEC) monolayers and Human Aortic Endothelial Cells (HAEC) were used. The former are isolated from the vein of the umbilical cord and were used in a first set of experiments to test hydroxycinnamate compounds, while HAEC are isolated from aortic endothelium and were used in a second set of trials to test phenylethanoid compounds. These cell types are commonly used as a laboratory model for endothelial physiological and pharmacological investigations, such as macromolecule transport, blood coagulation, angiogenesis, and fibrinolysis. Moreover, they have widely been used to evaluate the eNOS expression and activity **(Chung et al., 2017; Hung et al., 2016; Krause et al., 2012; Steffen et al., 2007; Steffen et al., 2008; Tsui et al., 2015)**. In this study, both HUVEC and HAEC, showing the same pattern of NO production, were used to evaluate either the possible activation of eNOS via Akt1 phosphorylation or the suppression of NADPH oxidase's superoxide production, both leading to an increase of NO concentration and to an increase of the vasorelaxation second messenger cGMP, after treatments with the olive oil phenolics and their metabolites.

As regards the gut environment, the Caco-2 cells monolayer model was used to simulate the intestinal tract. Caco-2 cells are an adenocarcinoma colon cell line that spontaneously undergo differentiation into enterocyte-like cells at about 21 days after plating. Differentiated Caco-2 cells express microvilli, basolateral membranes separated by tight

junctions, intestine-specific antioxidant enzymes and possess absorptive properties similar to those of normal intestine.

The overexpression of iNOS was induced in Caco-2 by exposure to lipopolysaccharide (LPS). This toxin, also known as lipoglycan and endotoxin, is a large molecule consisting of a lipid and a polysaccharide composed of O-antigen, outer core and inner core joined by a covalent bond; it is usually found in the outer membrane of Gram-negative bacteria, and elicits strong immune responses and inflammation in animals **(Kulp et al., 2010)**. After stimulation of Caco-2 cells by 1 µg/mL of LPS, IκB is usually phosphorylated and degraded; so, free NF-κB translocates into the nucleus to regulate the expression of multiple NF-κB-dependent genes, such as iNOS **(Panaro et al., 2012)**. Therefore, NO modulation by olive oil phenols and their metabolites have been studied as ability to inhibit iNOS expression through NF-κB modulation. The related signaling pathway together with the modulation of cGMP production and the consequent intestinal membrane permeability, measured as transepithelial resistance (TEER), were also evaluated.

3. Materials and methods

3.1. Chemicals and reagents

Bradford reagent, lipopolysaccharide from *Escherichia coli*, angiotensin II, L-arginine, apocynin, sodium nitrite, cytochrome c from bovine heart, hydroxytyrosol, tyrosol, ferulic acid, isoferulic acid, dihydroferulic acid, superoxide dismutase from bovine erythrocytes, Hanks' Balanced Salt solution, CellLytic-M, Nω-Nitro-L-arginine (L-NNA) and Griess' reagent were purchased from Sigma Aldrich (Milan, Italy). Hanks' Balanced Salt solution with sodium bicarbonate (HBSS) without phenol red, Dulbecco's Phosphate Buffered Saline with $MgCl_2$ and $CaCl_2$, Dulbecco's Phosphate Buffered Saline without $CaCl_2$ and $MgCl_2$ were purchased from Euroclone (Milan, Italy). Tyrosol glucuronide, tyrosol sulfate sodium salt, 3'-hydroxytyrosol 3'-glucuronide, hydroxytyrosol 3-sulfate sodium salt, ferulic acid 4-*O*-β-D-glucuronide, isoferulic acid 3-*O*-sulfate disodium salt, and dihydroferulic acid 4-*O*-β-D-glucuronide were obtained from LGC standards (Sesto San Giovanni, Italy). Cyclic GMP EIA Kit 96 well, Nitrate/Nitrite Fluorometric Assay Kit, EZBlock™ Phosphatase Inhibitor Cocktail II, and EZBlock™ Protease Inhibitor Cocktail were purchased from Cambridge Biosciences (Cambridge, UK).

3.2. Human umbilical vein endothelial cells (HUVEC) and Human Aortic Endothelial Cells (HAEC) culture

3.2.1. Material for cell culture

The HUVEC cell line (Human Umbilical Vein Endothelial Cells), the HAEC cell line (Human Aortic Endothelial Cells), the medium EBM-2 with and without phenol red, the BulletKit™ – basal medium and SingleQuots™ and ReagentPack Subculture Reagents with

Trypsin/EDTA, TNS (Trypsin Neutralizer solution) and HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) solutions were obtained from Lonza (Basel, Switzerland).

3.2.2. Maintenance of cell culture

HUVEC and HAEC cells were maintained in T75 flasks until they reached confluence in EBM-2 supplemented with 2% FBS, 0.2% heparin, 0.2% hydrocortisone, 0.2% hFGFb (Human Fibroblast Growth Factor basic), 0.2% hVEGF (Human Vascular Endothelial Growth Factor), 0.2% long R3-IGF-1 (analog of Human Insulin-Like Growth Factor-1), 0.2% ascorbic acid, 0.2% hEGF (Human Epidermal Growth Factor) and 0.2% of GA 1000 (gentamycin sulfate) at 37°C in a 5% CO_2 humidified atmosphere. The subcultures were prepared by removing the cells with trypsin solution at 1% after washing with HEPES, and then seeded into 6-well plates for different experiments. Cells were cultured for 7 days, replacing the medium three times a week.

3.2.3. MTT viability test

The MTT assay **(Schiller et al., 1992)** was assessed on HUVEC and HAEC cells in order to evaluate any toxic activity of the tested compounds. Cells were seeded in 96-well plates ($5x10^4$ cells/mL in 100 µL) and, when fully differentiated, were exposed to various concentrations of the compounds (1–10 µM, in serum free medium), or an equivalent volume of MeOH for the controls, and incubated for 24 h. After that, the medium was removed and 100 µL of MTT solution (5 mg/mL of HBSS) was added and left for 6 h at 37°C. The medium was then aspirated, 100 µL of DMSO were added in each well and the absorbance

was read at 570 nm by using a micro plate reader (Infinite 200, Tecan, Salzburg, Austria). The absorbance was proportional to the cells viability.

3.2.4. $O_2^{\cdot-}$ release from intact cells

To measure $O_2^{\cdot-}$ release in intact cells, confluent HUVEC and HAEC cells (approximately $7.5x10^5$ cells/well) were treated with phenolics (1 µM) in phenol free EBM-2 medium. After 24 h of incubation, cells were shortly washed with 50 mM phosphate buffer, pH 7.4, and subsequently exposed to 40 µmol/L ferricytochrome c for 6 h at 37 °C in HEPES-buffered isotonic salt medium (133 mM NaCl, 6.5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5.5 mM glucose, 50 µM L-arginine, 20 mM HEPES, pH 7.4). Reduction of ferricytochrome c was measured in the supernatant at 550 nm ($e = 21.1\ mM^{-1}cm^{-1}$). Specificity of the assay for $O_2^{\cdot-}$ was ascertained by co-incubation with superoxide dismutase (SOD; 200 U/mL). $O_2^{\cdot-}$ release was calculated from the difference in the setups without and with SOD and related to the cell number in each dish (mmol $O_2^{\cdot-}$/million of cells).

3.2.5. Production of cGMP and evaluation of NO release

In order to evaluate the cGMP production in HUVEC and HAEC cells, treatments with phenolics (1 µM) in phenol free EBM-2 medium were assessed. After 24 h of incubation, some wells were treated for 30 minutes with apocynin (100 µM) and L-NNA (100 µM) and all the mediums were collected from the wells for the NO analysis carried out by using the Nitrate/Nitrite Fluorometric Assay Kit (Cayman Chemicals, Ann Arbor, Michigan, USA). Then, cells were treated with 300 µL of HCl 0.1 µM and after 15 minutes were scraped

and centrifuged in small Eppendorf tubes in order to collect supernatants for the analysis of cGMP by using the Cyclic GMP EIA Kit 96 well (Vinci-Biochem, Vinci, Italy).

3.2.6. Detection of eNOS and Akt1 proteins by Western Blotting

Endothelial cells were lysed in lysis buffer supplemented with phosphatase and protease inhibitor. Protein concentration was determined by the Bradford assay **(Bradford, 1976)**. Aliquots (10 µg) of reduced and denatured proteins were separated on 9% polyacrylamide gels and thereupon transferred into nitrocellulose membranes. These membranes were treated with 25 mL of TTBS (Tris/HCl, pH 7.5, 100 mM NaCl, 0.1% Tween 20) 4% dry milk for 30 min at room temperature. β-actin, anti-p-eNOS, anti-Akt1 and anti-p-Akt1 polyclonal antibody (Santa Cruz Biotechnology, Dallas, Texas, USA) in TTBS 1% dry milk (dilution 1:1000 v/v) were added to the membranes and kept overnight at 4°C. Membranes were washed two times with TTBS before incubation with IRDye 680rd donkey anti-goat and IRDye 800cw donkey anti-rabbit (LI-COR, Lincoln, Nebraska, USA) for 45 min at room temperature and then washed three times with TTBS. The bands were visualized by using the Odyssey® Fc imaging system (LI-COR, Lincoln, Nebraska, USA).

3.3. Human colon carcinoma cell line (Caco-2) culture

3.3.1. Material for cell culture

Fetal Bovine serum and Dulbecco's Modified Eagle's Medium Low Glucose w/L-Glutamine were purchased from Euroclone (Milano, Italy). Transwell inserts were obtained from Corning Costar Corp. (New York, N.Y., USA).

3.3.2. Maintenance of intestinal cell culture

Caco-2 cells (ECACC, Salisbury, UK) were cultured in monolayers at 37°C in a humidified atmosphere at 5% of CO_2 **(Incani et al., 2016)**. At passage 45-60, cells were plated at a density of about 5×10^4/mL and used when fully differentiated (14-21d postseeding), replacing the medium twice a week.

3.3.3. MTT viability test

The MTT assay **(Schiller et al., 1992)** was assessed on Caco-2 cells in order to evaluate any toxic activity of the compounds tested. Cells were seeded in 96-well plates ($5x10^4$ cells/mL in 100 µL), then cells were exposed to various concentrations of the compounds (1-10 µM, in serum free medium), or an equivalent volume of MeOH for the controls, and incubated for 24 h. After that, the same procedure for the HUVEC viability assessment was followed.

3.3.4. Production of cGMP and evaluation of NO release

In order to evaluate the cGMP production in Caco-2 cells, treatments with 1 µM of phenolic compound and with LPS (1 µg/mL) in PBS were assessed. After different incubation times (2-48h), PBS was collected from the wells for the NO analysis. Then, cells were treated with 300 µL of HCl 0.1 µM and after 15 minutes were scraped and centrifuged in small Eppendorf tubes to collect supernatants for the cGMP analysis by using the Cyclic GMP EIA Kit 96 well (Vinci-Biochem, Vinci, Italy). The nitrite concentration was determined by mixing 100 µL of the collected PBS with an equal volume of Griess' reagent and incubating

for 20 minutes at room temperature. Then, plate was read at 540 nm, and the nitrite levels were determined with a sodium nitrite standard curve ranging from 0.1 to 10 μM.

3.3.5. Determination of transepithelial electrical resistance (TEER)

Differentiated Caco-2 cells seeded in transwell inserts (polycarbonate membrane, 0.4 μm pore size, Sigma-Aldrich) were used to determine the transepithelial electrical resistance (TEER) as described by **Serreli et al. (2017)**. Briefly, cells in inserts with TEER values >300 Ω/cm^2 were pretreated with the phenolic extract dissolved in MeOH (1 μM) for 30 minutes. Then, LPS (1 μg/mL) was added and cells were incubated for 48 h. TEER was measured every 15 min and reported as percentage of the corresponding TEER value at time zero (T = 0).

3.3.6. Western blot detection of IκBα, Akt and iNOS

Caco-2 cells were lysed in lysis buffer supplemented with phosphatase and protease inhibitor. Protein concentration was determined by the Bradford assay **(Bradford, 1976)**. Aliquots (20 μg) of reduced and denatured proteins were separated on 10% polyacrylamide gels and thereupon transferred into nitrocellulose membranes. These membranes were blocked with 25 mL of TTBS (Tris/HCl, pH 7.5, 100 mM NaCl, 0.1% Tween 20) 4% dry milk for 30 min at room temperature. Primary polyclonal anti-IκBα, anti-phospho IκBα, anti-Akt, anti-phospho Akt, anti-β actin and anti-iNOS antibodies (Cell Signaling Technology, Inc., Danvers, Massachusetts, USA) in TTBS 1% dry milk (dilution 1:1000 v/v) were added to the membranes and kept overnight at 4°C.

Membranes were washed two times with TTBS before incubation with secondary antibody anti-mouse in TTBS 1% milk (dilution 1:2000 v/v) for 45 min at room temperature and then washed twice with TTBS and once with TBS. The bands were visualized by using the ChemiDoc™ XRS+ System (Bio-Rad Laboratories, Inc., Hercules, California, USA).

3.4. Statistics

One-way analysis of variance (ANOVA) followed by Tukey's test was performed in order to highlight possible significant differences between groups using the GraphPad Prism 5 software (GraphPad software, San Diego, CA, USA).

4. Results

4.1. Modulation of NO production in endothelial cells

4.1.1. Cell viability

Cell viability was assessed in HUVEC and HAEC in order to evaluate the possible cytotoxic effects of the EVOO phenolic compounds tested. The reduction of the tetrazolium dye MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to the insoluble formazan reflected the number of viable cells present. The purple color intensity of formazan was analyzed spectrophotometrically and the cell viability were expressed as percentage of viability compared to the control (100% viability).

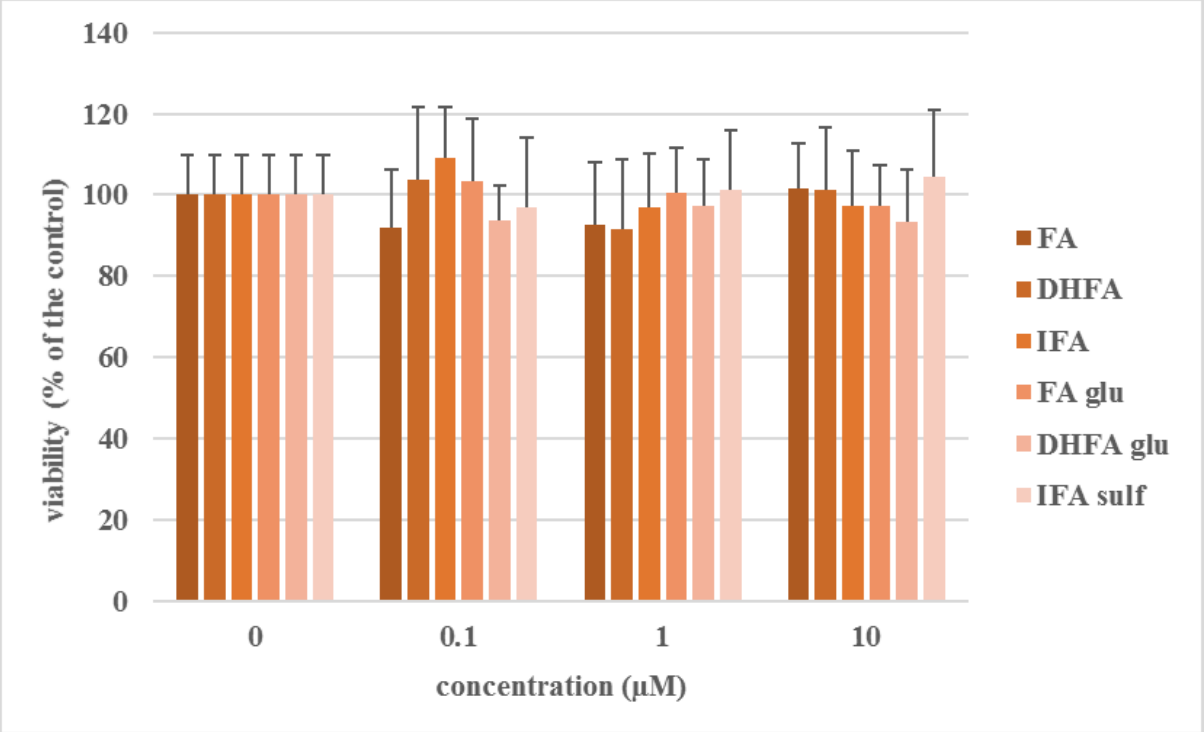


Figure 13. Percentage of cell viability compared to control (100%) of HUVEC incubated for 24 h with different concentrations of ferulic acid (FA), hydroferulic acid (DHFA), isoferulic acid (IFA) and their glucuronide and sulfate metabolites (0.1-10 µM) (n=9).

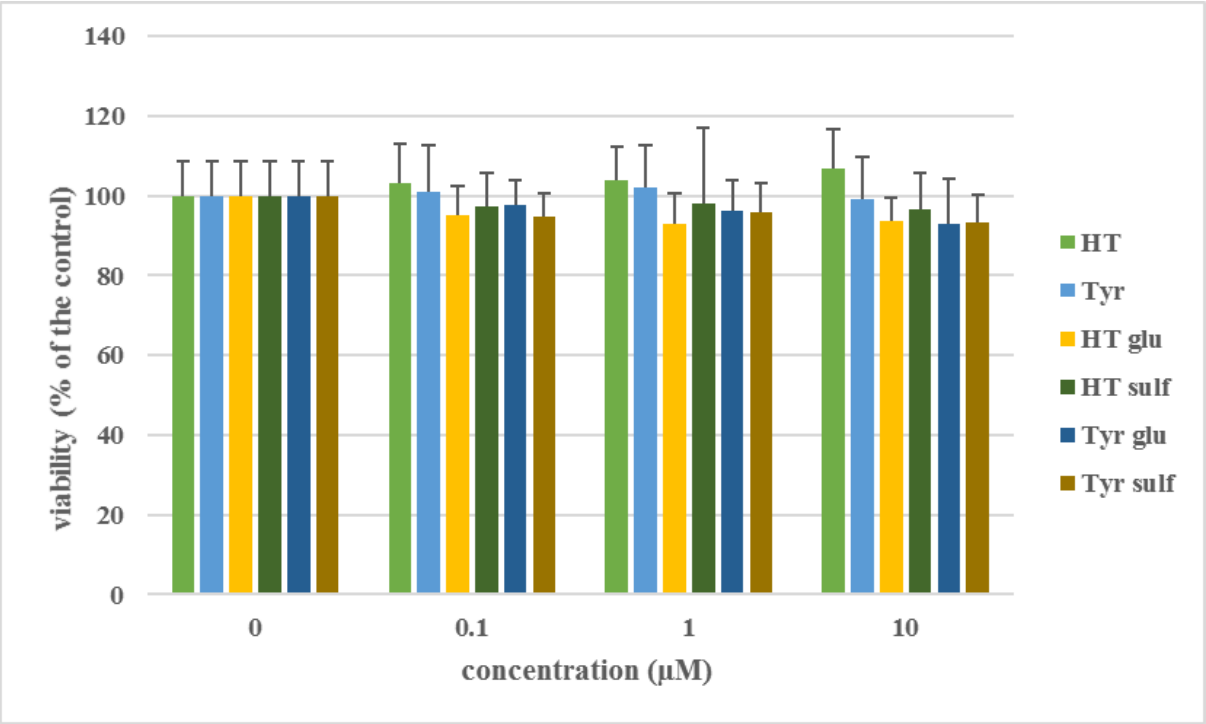


Figure 14. Percentage of cell viability compared to control (100%) of HAEC incubated for 24 h with different concentrations of hydroxytyrosol (HT), tyrosol (Tyr) and their glucuronide and sulfate metabolites (0.1-10 µM) (n=9)

Figures 13 and **14** show the percentage of HUVEC and HAEC viability incubated for 24 h with increasing concentrations of the olive oil polyphenols (0.1-10 µM). No one of the compounds affected cell viability at all tested concentrations.

4.1.2. Hydroxycinnamates

4.1.2.1. $O_2^{\cdot -}$ release from intact cells

Figure 15 shows the effects of various compounds tested on release of $O_2^{\cdot -}$ from intact HUVEC cells. This process was evaluated through ferricytochrome c reduction analysis: it can be seen that $O_2^{\cdot -}$ release was enhanced by the eNOS inhibitor L-NNA and inhibited by apocynin. All the tested compounds, significantly inhibited $O_2^{\cdot -}$ release. In particular, IFA and DHFA were significantly the most effective.

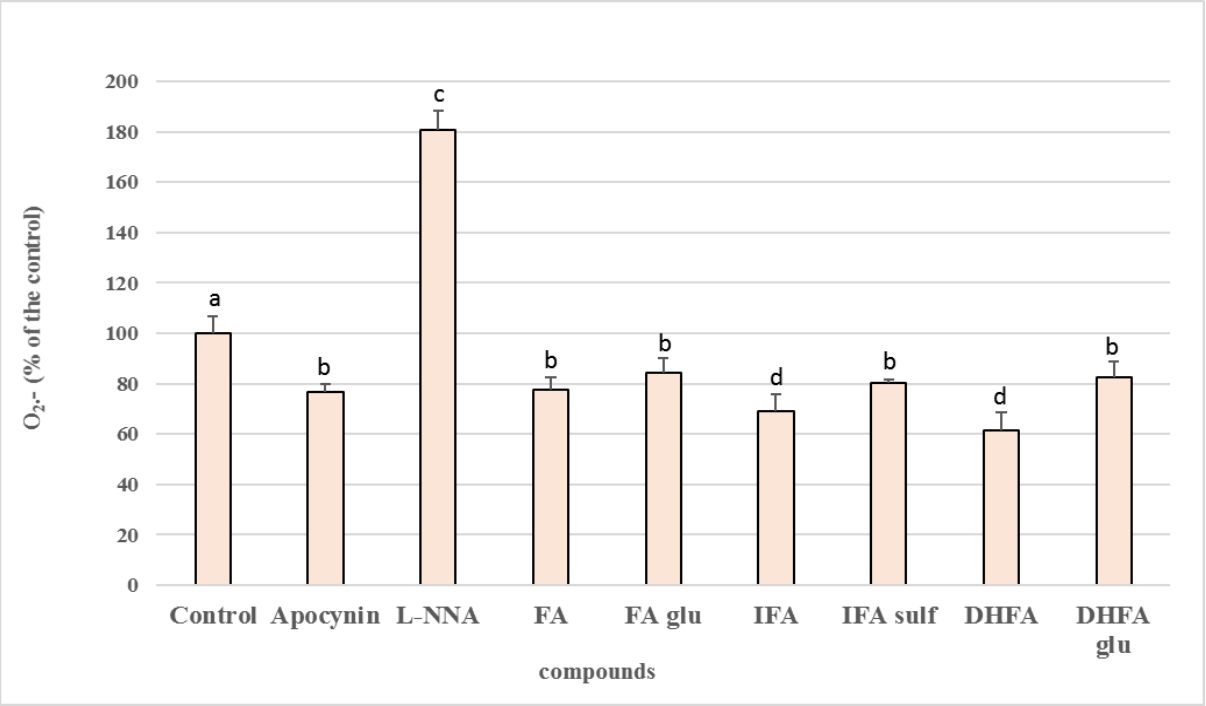


Figure 15. Percentage of $O_2^{\cdot -}$ release compared to control (100%) of HUVEC incubated for 24 h with L-arginine (100 µM) and apocynin, L-NNA, ferulic acid (FA), hydroferulic acid (DHFA), isoferulic acid (IFA) and their glucuronide and sulfate metabolites (1 µM) (n=9). Values not sharing a superscript letter are significantly different ($p < 0.05$).

4.1.2.2. Evaluation of NO release

NO release was measured as nitrite concentration in HUVEC supernatants after 24 h phenolic treatments. **Figure 16** shows that NO production was considerably declined in cells treated with L-NNA, whereas apocyin improved its concentration. Interestingly, IFA and DHFA, increased significantly nitrite concentration respect to the control and to those cells treated with apocynin, which elicited NO release, at the same rate of IFA and DHFA metabolites. FA and FA glu did not significantly enhanced NO production with respect to the control.

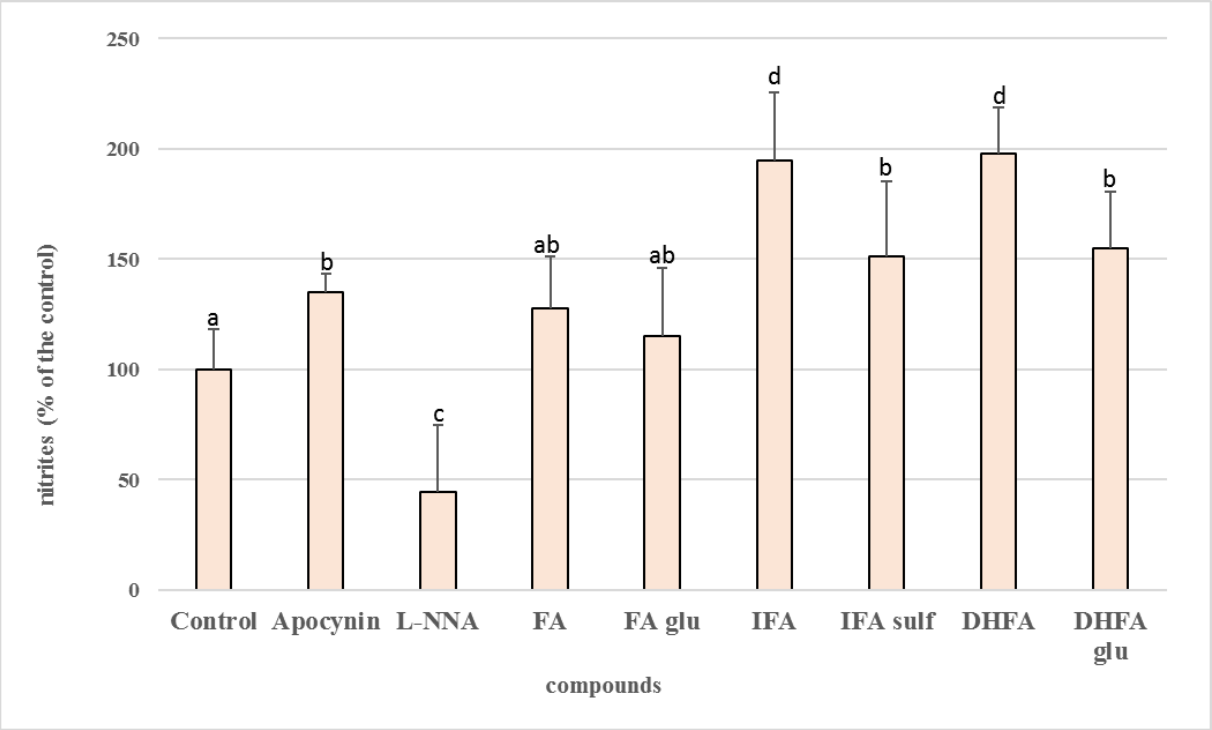


Figure 16. NO release measured as nitrites percentage compared to control (100%) from HUVEC incubated for 24 h with L-arginine (100 µM) and apocynin, L-NNA, ferulic acid (FA), hydroferulic acid (DHFA), isoferulic acid (IFA) and their glucuronide and sulfate metabolites (1 µM) (n=9). Values not sharing a superscript letter are significantly different ($p < 0.05$).

4.1.2.3. Production of cGMP

cGMP production is usually linked to the NO concentration, and, as expected, its analysis showed it was almost specular to that of NO, as shown in **Figure 17**. Cells treated with L-NNA showed the lowest level of cGMP, while cells treated with apocynin and with all the compounds significantly increased cGMP. The most effective compound was DHFA, while FA, IFA and their metabolites were as effective as apocynin.

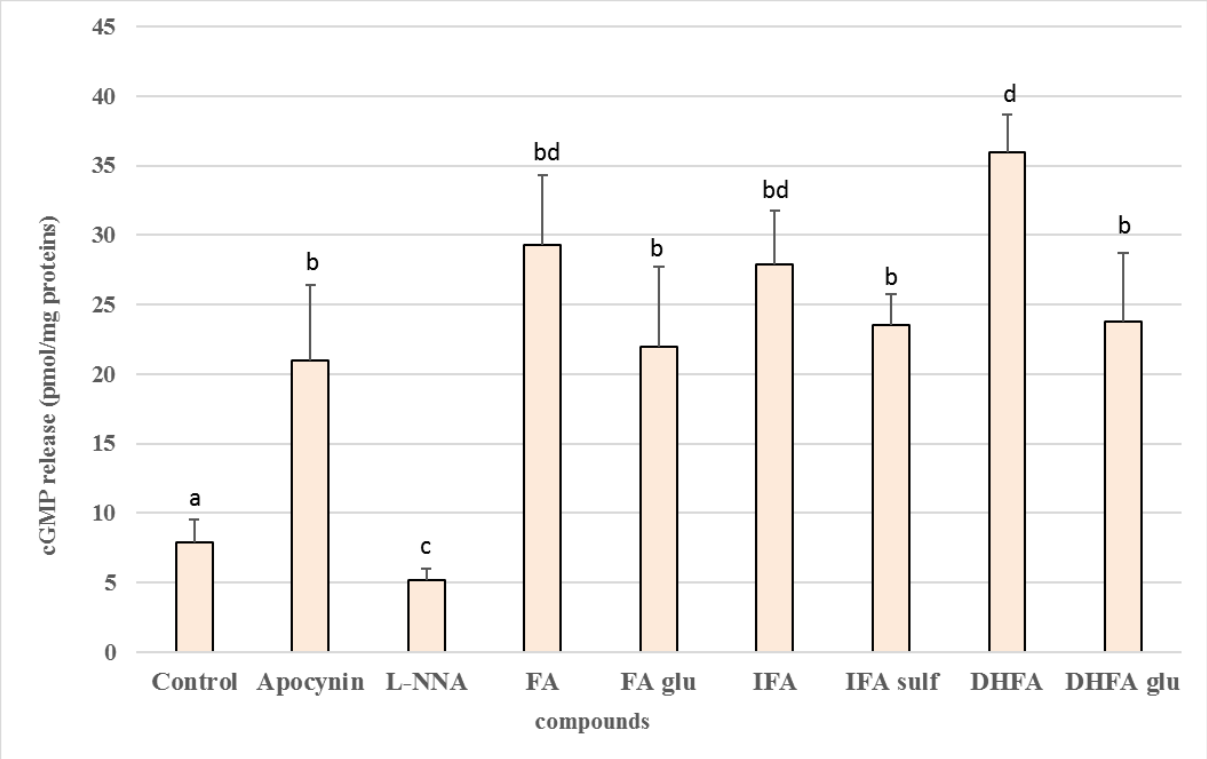


Figure 17. cGMP release (pmol/mg proteins) from HUVEC incubated for 24 h with L-arginine (100 µM) and apocynin, L-NNA, ferulic acid (FA), hydroferulic acid (DHFA), isoferulic acid (IFA) and their glucuronide and sulfate metabolites (1 µM) (n=9). Values not sharing a superscript letter are significantly different ($p < 0.05$).

4.1.2.4. Modulation of eNOS activation pathway

The endothelial NOS activation pathway was evaluated after olive oil phenolics treatments, determining the enzyme phosphorylation state and the modulation of Akt kinase, a key signal in its activation pathway.

4.1.2.4.1. Evaluation of eNOS activation

eNOS activation was assessed through western blot analysis of its phosphorylated form, compared with β-actin content. **Figure 18** shows that among phenolic acids tested IFA, DHFA and their metabolites were the most effective in improving eNOS phosphorylation, together with FA glu, whose parent compound was not as effective as it.

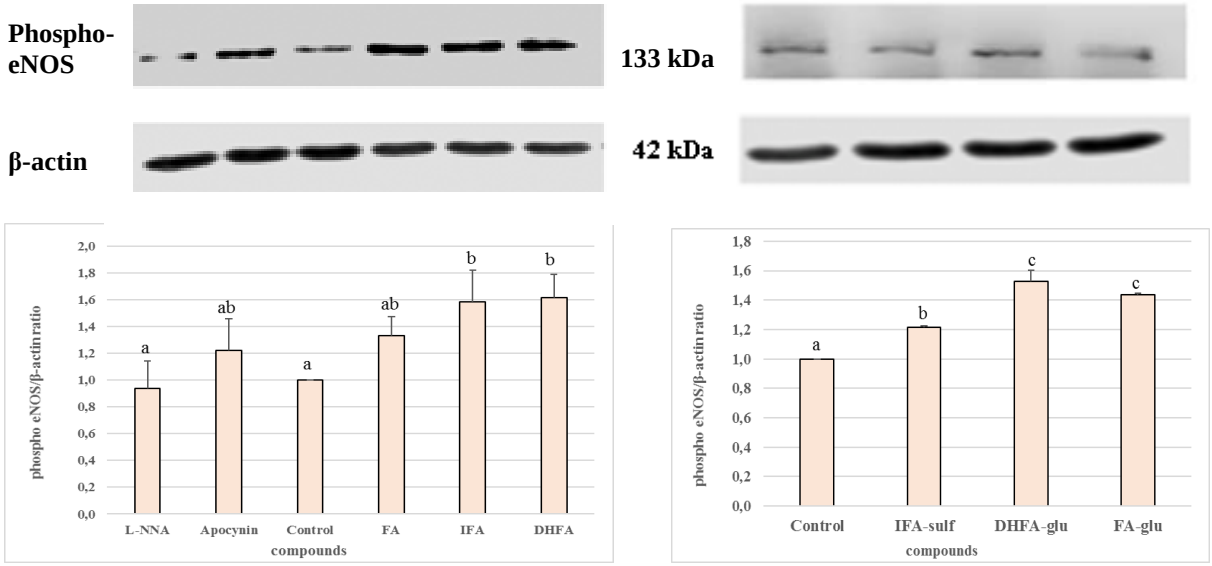


Figure 18. Activation of eNOS showed as phospho eNOS/β actin ratio in HUVEC treated with apocynin, L-NNA, ferulic acid (FA), hydroferulic acid (DHFA), isoferulic acid (IFA) and their glucuronide and sulfate metabolites (1 μM) (n=6). Values not sharing a superscript letter are significantly different ($p < 0.05$).

4.1.2.4.2. Assessment of Akt phosphorylation

eNOS is usually activated by viscous drag/shear stress in blood vessels to produce NO continuously, a process mediated by the phosphatidylinositol 3-kinase (PI3kinase)/Akt pathway. Akt activation through phosphorylation after cell treatments was assessed by western blot analysis.

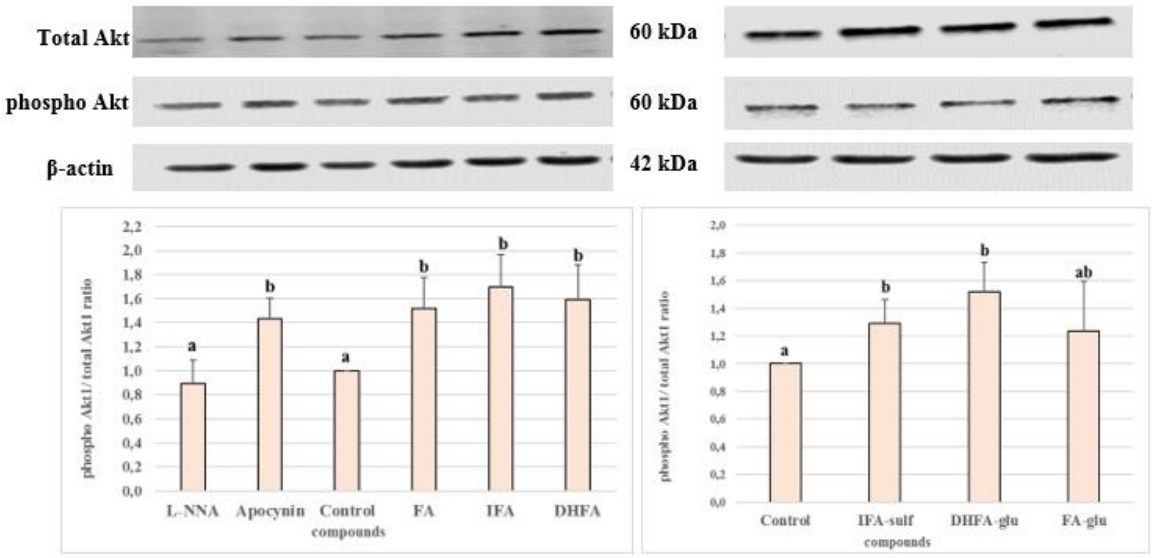


Figure 19. Modulation of Akt showed as phospho Akt/total Akt ratio in HUVEC treated with apocynin, L-NNA, ferulic acid (FA), hydroferulic acid (DHFA), isoferulic acid (IFA) and their glucuronide and sulfate metabolites (1 µM) (n=6). Values not sharing a superscript letter are significantly different ($p < 0.05$).

As shown in the **Figure 19**, cells treated with apocynin showed a significant increase of the Akt phosphorylation, which was also ascertain in those cells treated with almost all the compounds, with the exception of FA glu.

4.1.3. Phenylethanoids

4.1.3.1. $O_2^{\cdot -}$ release from intact cells

Figure 20 shows the effects of phenylethanoids tested on release of $O_2^{\cdot -}$ from intact HAEC. This process was evaluated through ferricytochrome c reduction analysis: it can be seen that $O_2^{\cdot -}$ release was enhanced by the eNOS inhibitor L-NNA and inhibited by apocynin. All the tested compounds significantly inhibited $O_2^{\cdot -}$ release. HT was significantly the most effective, while other compounds were as effective as apocynin.

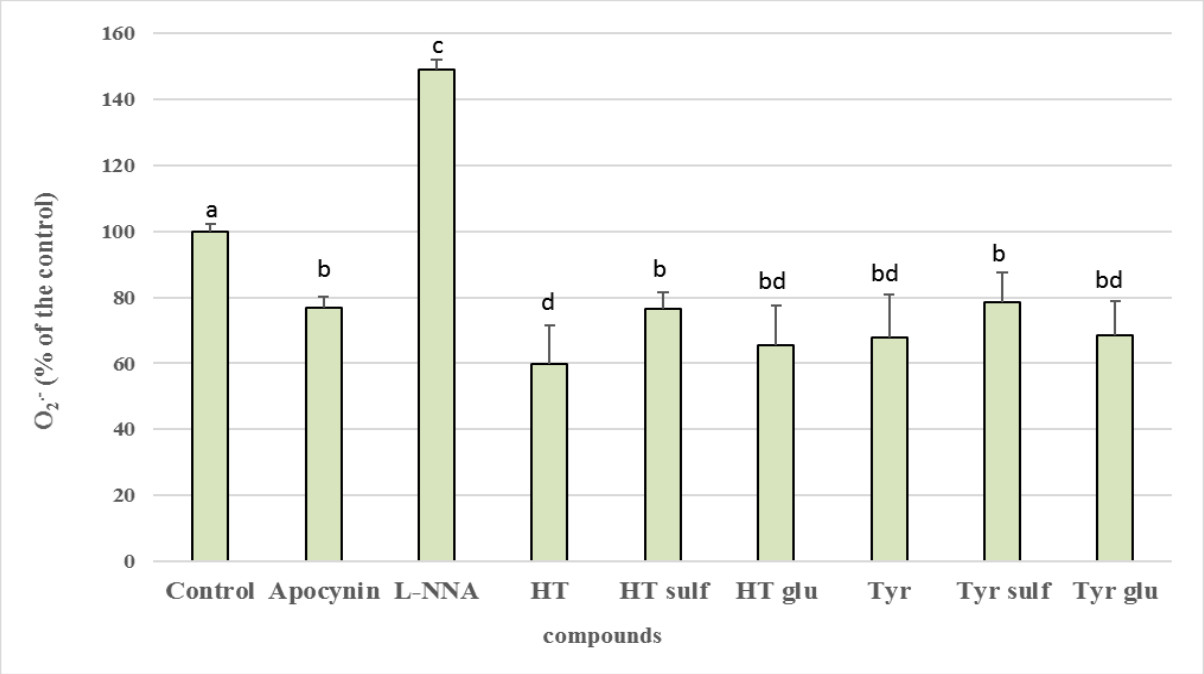


Figure 20. Percentage of $O_2^{\cdot -}$ release compared to control (100%) of HAEC incubated for 24 h with L-arginine (100 µM) and apocynin, L-NNA, hydroxytyrosol (HT), tyrosol (Tyr), and their glucuronide and sulfate metabolites (1 µM) (n=9). Values not sharing a superscript letter are significantly different ($p < 0.05$).

4.1.3.2. Evaluation of NO release

NO release was measured as nitrite concentration in HAEC supernatants after 24h phenolic treatments. **Figure 21** shows that NO production decreased in cells treated with L-NNA, while apocyin improved its concentration. Tyr and HT, together with their metabolites (except Tyr glu), elicited NO release, at the same rate of apocynin.

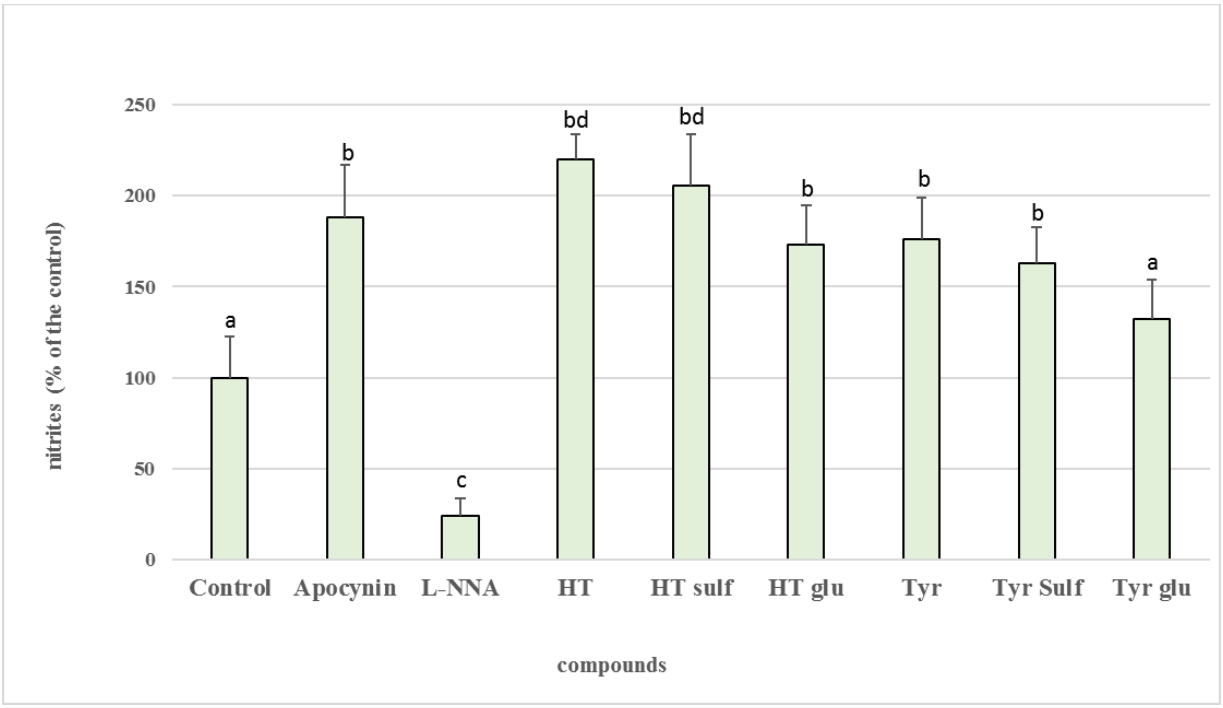


Figure 21. NO release measured as nitrites percentage compared to control (100%) from HAEC incubated for 24 h with L-arginine (100 µM) and apocynin, L-NNA, hydroxytyrosol (HT), tyrosol (Tyr), and their glucuronide and sulfate metabolites (1 µM) (n=9). Values not sharing a superscript letter are significantly different ($p < 0.05$).

4.1.3.3. Production of cGMP

Figure 22 shows that cells treated with L-NNA showed the lowest level of cGMP, while cells treated with apocynin and with all the compounds significantly increased cGMP as expected. The most effective compounds in increasing cGMP concentration was the glucuronide of HT, while the other phenylethanoids (except Tyr glu) were as effective as apocynin.

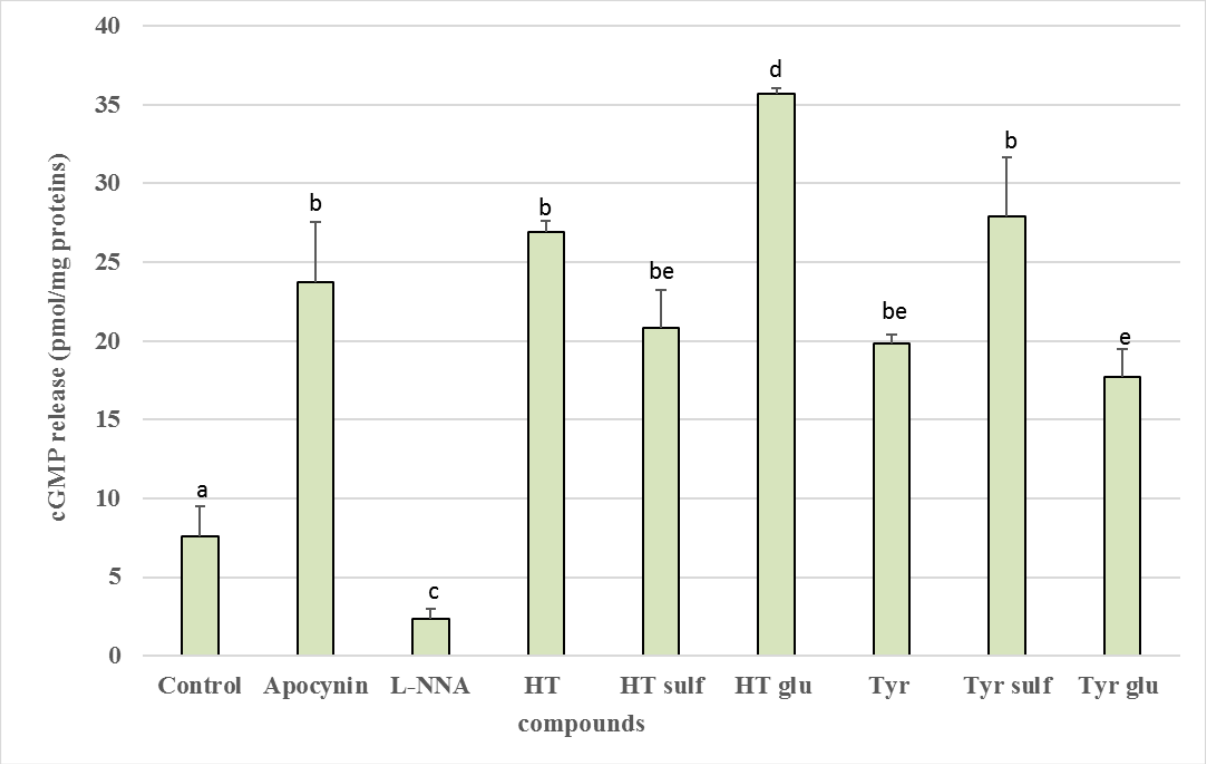
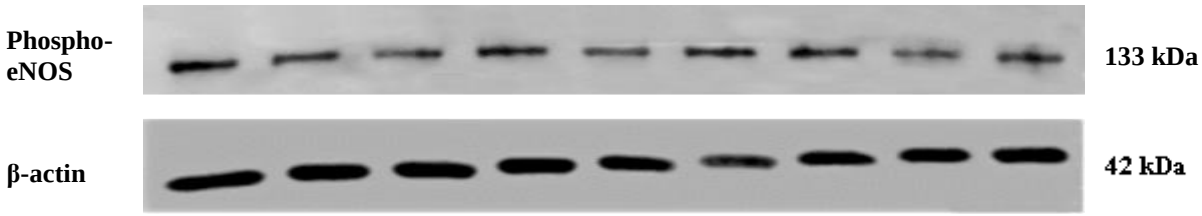


Figure 22. cGMP release (pmol/mg proteins) from HAEC incubated for 24 h with L-arginine (100 µM) and apocynin, L-NNA, hydroxytyrosol (HT), tyrosol (Tyr), and their glucuronide and sulfate metabolites (1 µM) (n=9). Values not sharing a superscript letter are significantly different ($p < 0.05$).

4.1.3.4. Modulation of eNOS activation pathway

4.1.3.4.1. Evaluation of eNOS activation

eNOS activation was assessed through western blot analysis of its phosphorylated form, compared with β-actin content. **Figure 23** shows that among phenylethanoids, HT and HT glu significantly elicited eNOS activation, while it is interesting to highlight that eNOS phosphorylation was not enhanced in those cells treated with HT sulf and Tyr together with its metabolites, where eNOS phosphorylation state was similar to that of the control.



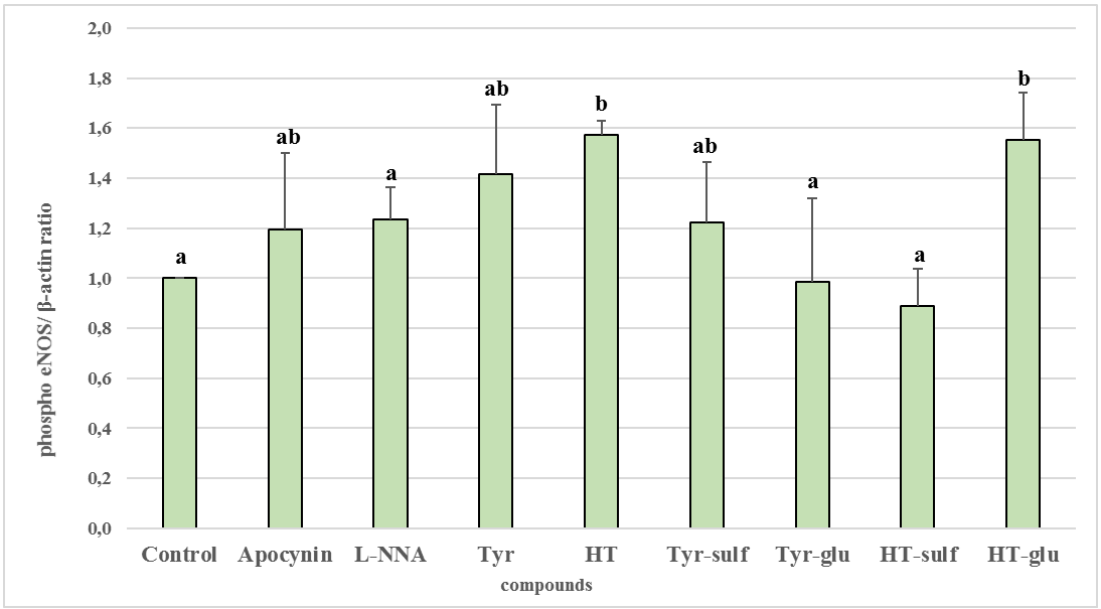


Figure 23. Modulation of eNOS showed as phospho eNOS/β actin ratio in HAEC cells treated with apocynin, L-NNA, hydroxytyrosol (HT), tyrosol (Tyr), and their glucuronide and sulfate metabolites (1 µM) (n=6). Values not sharing a superscript letter are significantly different ($p < 0.05$).

4.1.3.4.2. Assessment of Akt phosphorylation

As shown in the **Figure 24**, cells treated with apocynin showed a significant increase of the Akt phosphorylation, which was also ascertain in those cells treated with almost all the compounds. It is interesting to note that HT glucuronide and sulfate were as effective as HT, better than Tyr and its metabolites which did not exert any significant activity in enhancing Akt activation

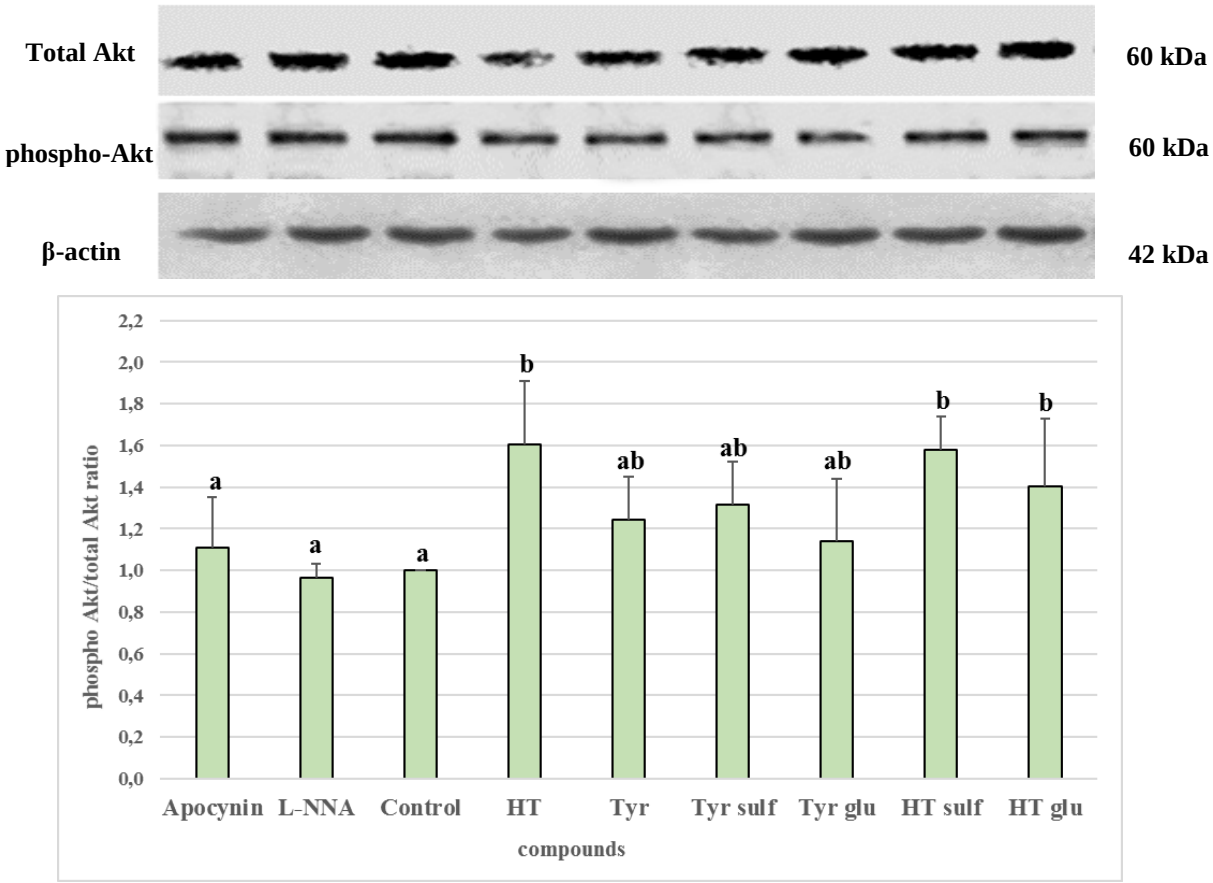


Figure 24. Modulation of Akt showed as phospho Akt/total Akt ratio in HAEC treated with apocynin, L-NNA, hydroxytyrosol (HT), tyrosol (Tyr), and their glucuronide and sulfate metabolites (1 μM) (n=6). Values not sharing a superscript letter are significantly different ($p < 0.05$).

4.2. Modulation of NO production in Caco-2 cells

4.2.1. Cell viability

Figures 25 and **26** show the percentage of Caco-2 cells viability incubated for 24 hours with increasing concentrations of the olive oil polyphenols (0.1-10 µM) as already done in the endothelial cells. Even in this case, no one of the compounds affected cells viability at all the tested concentrations.

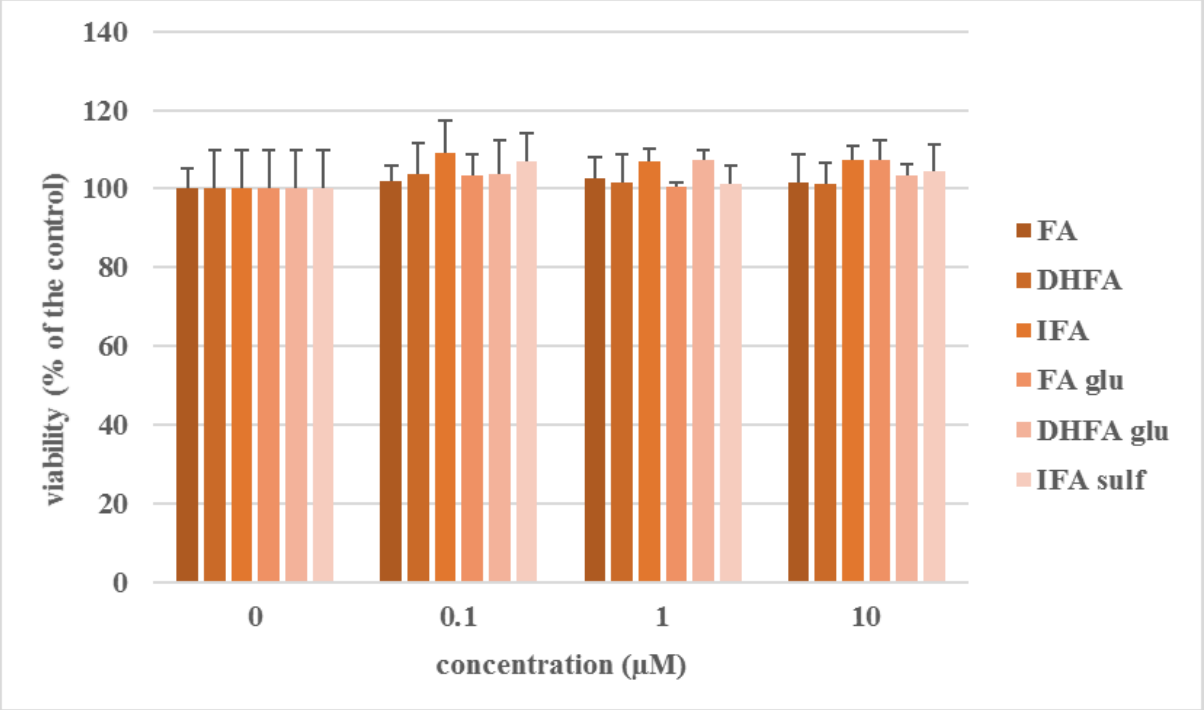


Figure 25. Percentage of cell viability compared to control (100%) of Caco-2 cells incubated for 24 h with different concentrations of ferulic acid (FA), hydroferulic acid (DHFA), isoferulic acid (IFA) and their glucuronide and sulfate metabolites (0.1-10 µM) (n=9).

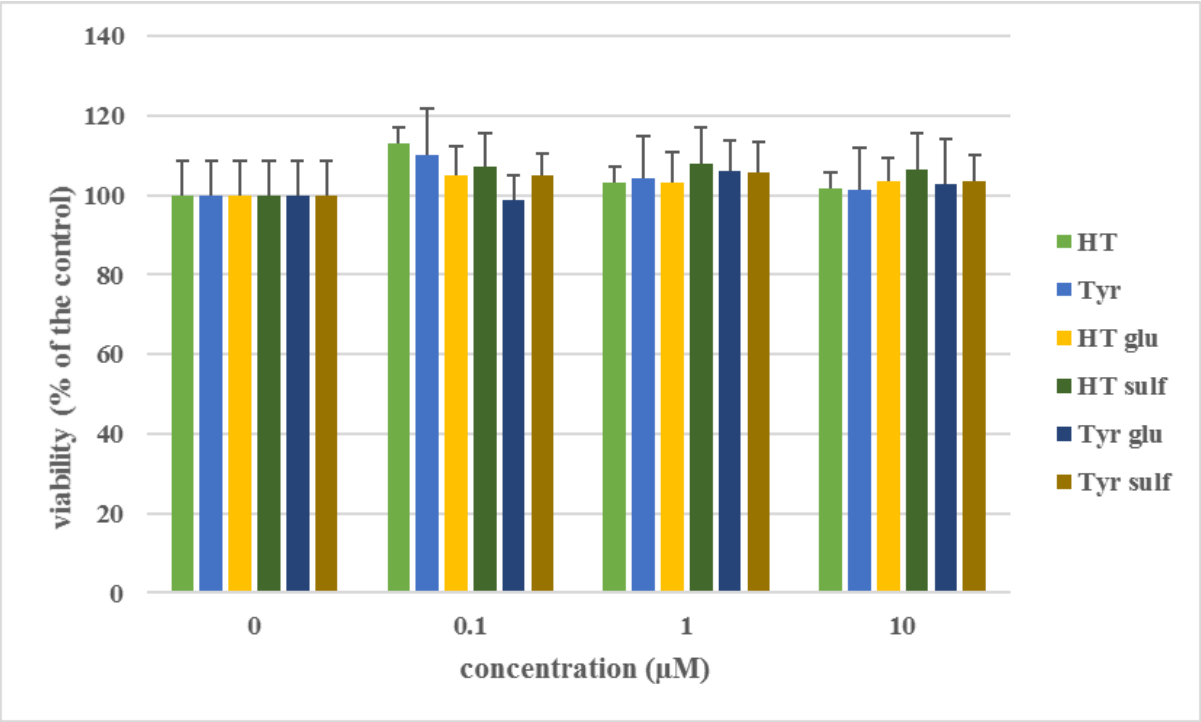


Figure 26. Percentage of cell viability compared to control (100%) of Caco-2 cells incubated for 24 h with different concentrations of hydroxytyrosol (HT), tyrosol (Tyr) and their glucuronide and sulfate metabolites (0.1-10 µM) (n=9).

4.2.2. Evaluation of NO release in Caco-2 cells

NO production in Caco-2 cells after 48 h LPS exposure with and without phenolic pretreatment was analyzed as nitrites content in cells supernatant. **Figures 27** and **28** show that cell treated with LPS released NO twofold more than control cells. All the compounds tested significantly counteracted NO production. Among them, DHFA, HT and HT sulf were the most effective in limiting NO release, which level was similar to that of control cells.

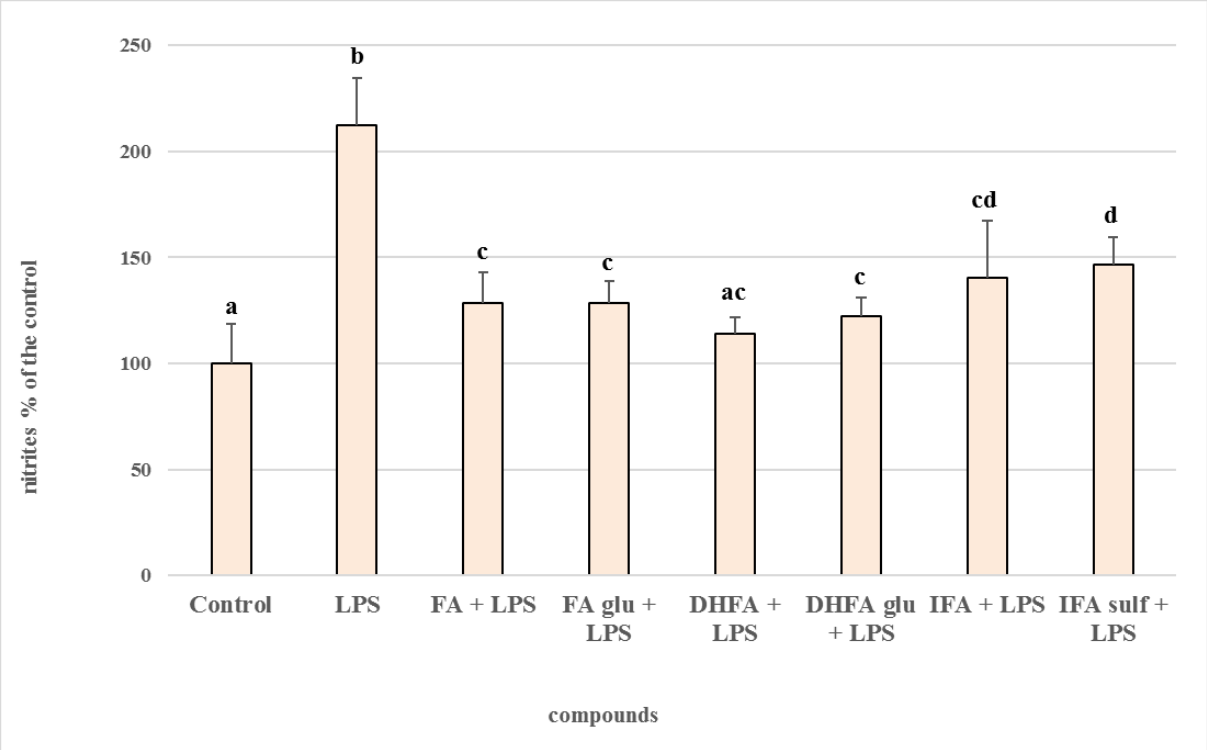


Figure 27. NO release measured as nitrites percentage compared to control (100%) of Caco-2 cells incubated for 48 h with L-arginine (100 µM), LPS (1 µg/mL) after pretreatment with ferulic acid (FA), hydroferulic acid (DHFA), isoferulic acid (IFA) and their glucuronide and sulfate metabolites (1 µM) (n=9). Values not sharing a superscript letter are significantly different ($p < 0.05$).

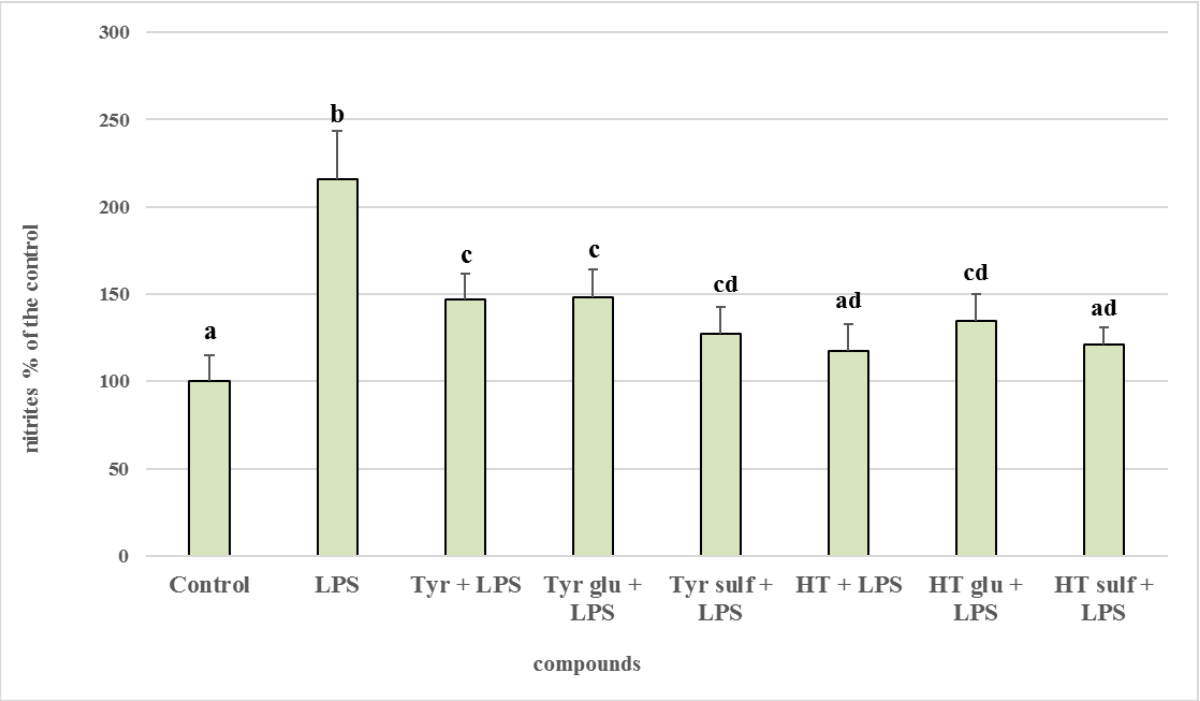


Figure 28. NO release measured as nitrites percentage compared to control (100%) of Caco-2 cells incubated for 24 h with L-arginine (100 µM), LPS (1 µg/mL) after pretreatment with hydroxytyrosol (HT), tyrosol (Tyr), and their glucuronide and sulfate metabolites (1 µM) (n=9). Values not sharing a superscript letter are significantly different ($p < 0.05$).

4.2.3. Production of cGMP by Caco-2 cells

The LPS treatment in Caco-2 cells elicited NO production and, consequently, cGMP release by the guanylate cyclase, as shown in **Figures 29 and 30**. Overall, phenolic pretreatments significantly reduced NO and cGMP release, which were led to lesser amounts particularly in cells treated with DHFA and HT sulf.

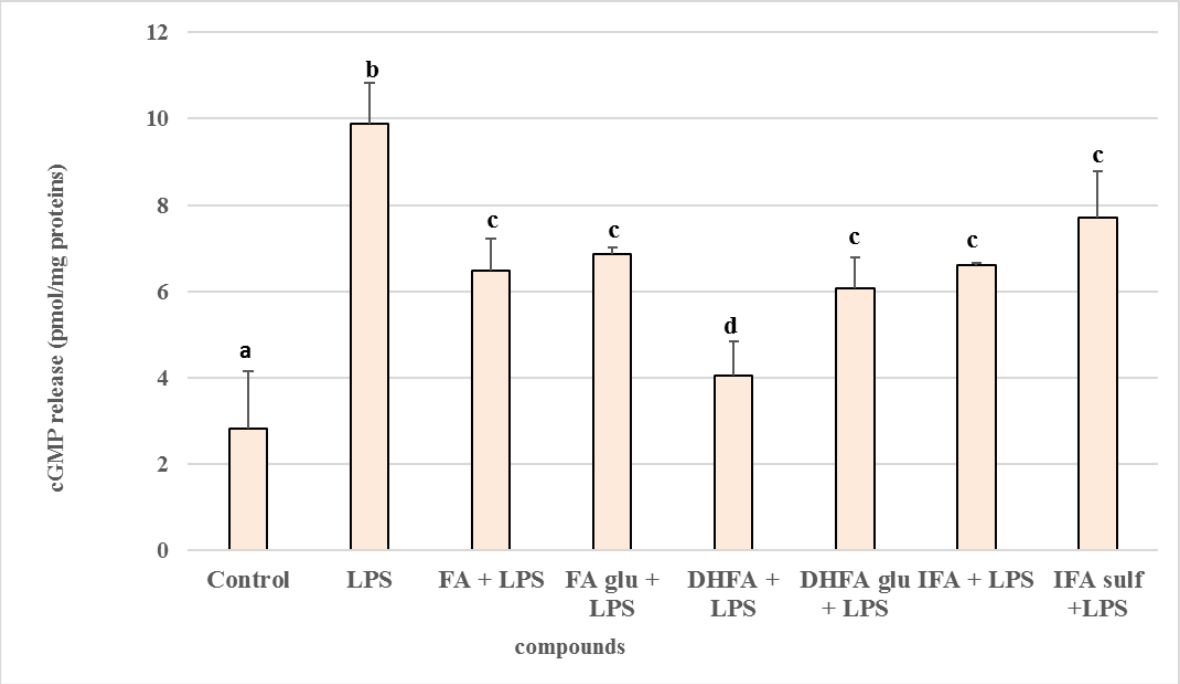


Figure 29. cGMP release (pmol/mg proteins) of Caco-2 cells incubated for 48 h with L-arginine (50 µM), LPS (1 µg/mL) after pretreatment with ferulic acid (FA), hydroferulic acid (DHFA), isoferulic acid (IFA) and their glucuronide and sulfate metabolites (1 µM) (n=9). Values not sharing a superscript letter are significantly different ($p < 0.05$).

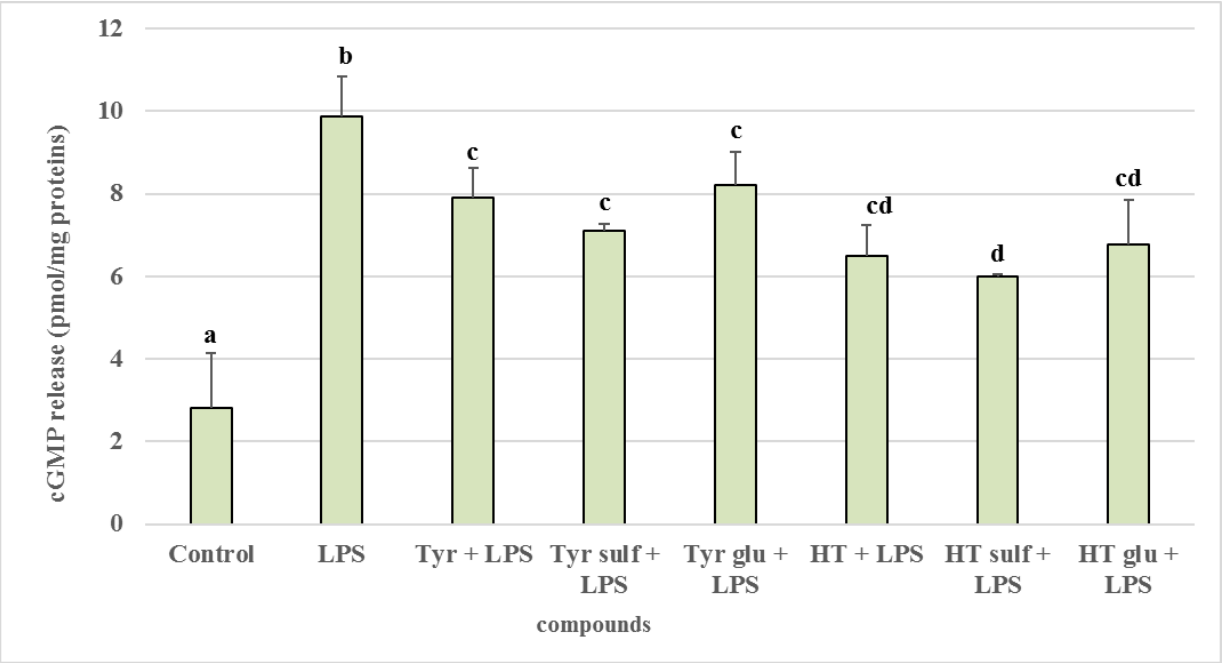


Figure 30. cGMP release (pmol/mg proteins) of Caco-2 cells incubated for 48 h with L-arginine (100 µM), LPS (1 µg/mL) after pretreatment with hydroxytyrosol (HT), tyrosol (Tyr), and their glucuronide and sulfate metabolites (1 µM) (n=9). Values not sharing a superscript letter are significantly different ($p < 0.05$).

4.2.4. Determination of transepithelial electrical resistance (TEER)

The transepithelial electrical resistance (TEER) was evaluated in Caco-2 cells monolayers in order to highlight the alteration of permeability due to LPS pro-inflammatory effect. Measures were taken every 15 min until 48 h of treatment (data not shown); at 6 h the TEER determined in cells treated with LPS showed the lowest value. Phenolic pretreatment limited the increase of permeability, which was 20% less than that of non-pretreated cells **(Fig. 31-32)**. This remarkable protection against TEER decrease was observed almost equally for all the tested phenolic compounds.

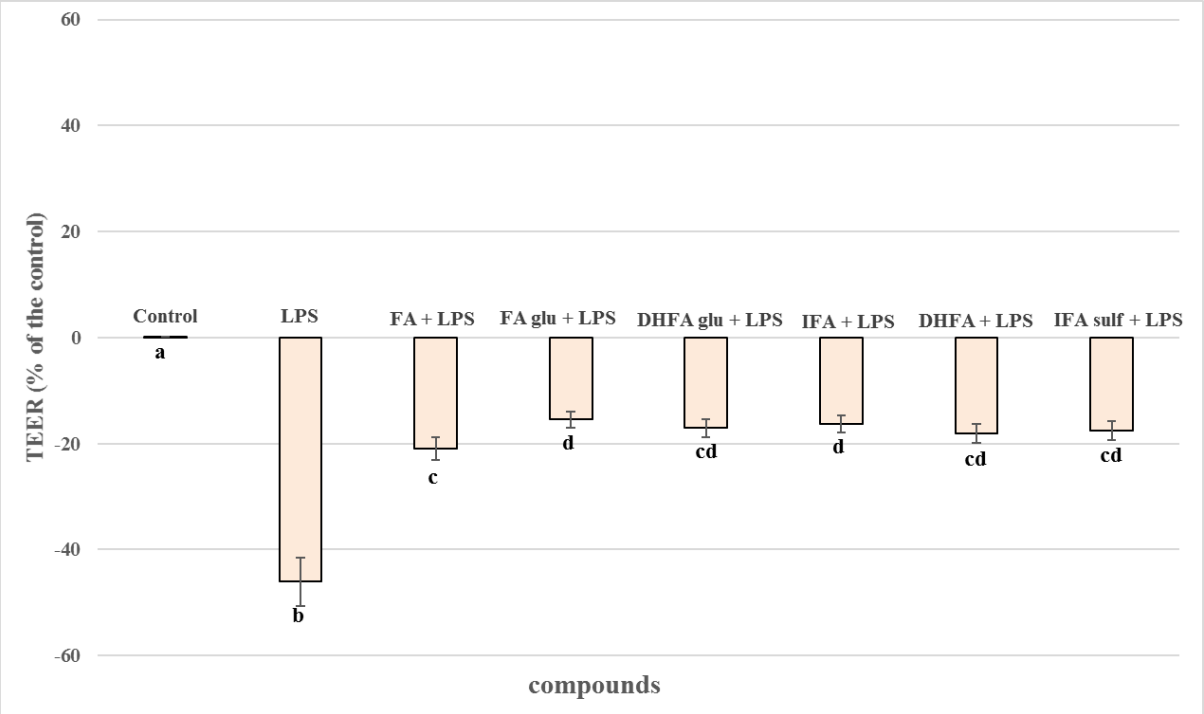


Figure 31. TEER value in Caco-2 cell monolayers incubated for 6 h with L-arginine (100 µM), LPS (1 µg/mL) and ferulic acid (FA), hydroferulic acid (DHFA), isoferulic acid (IFA) and their glucuronide and sulfate metabolites (1 µM) (n=6). Values are shown as percentage of TEER decrease respect to the control. Values not sharing a superscript letter are significantly different ($p < 0.05$).

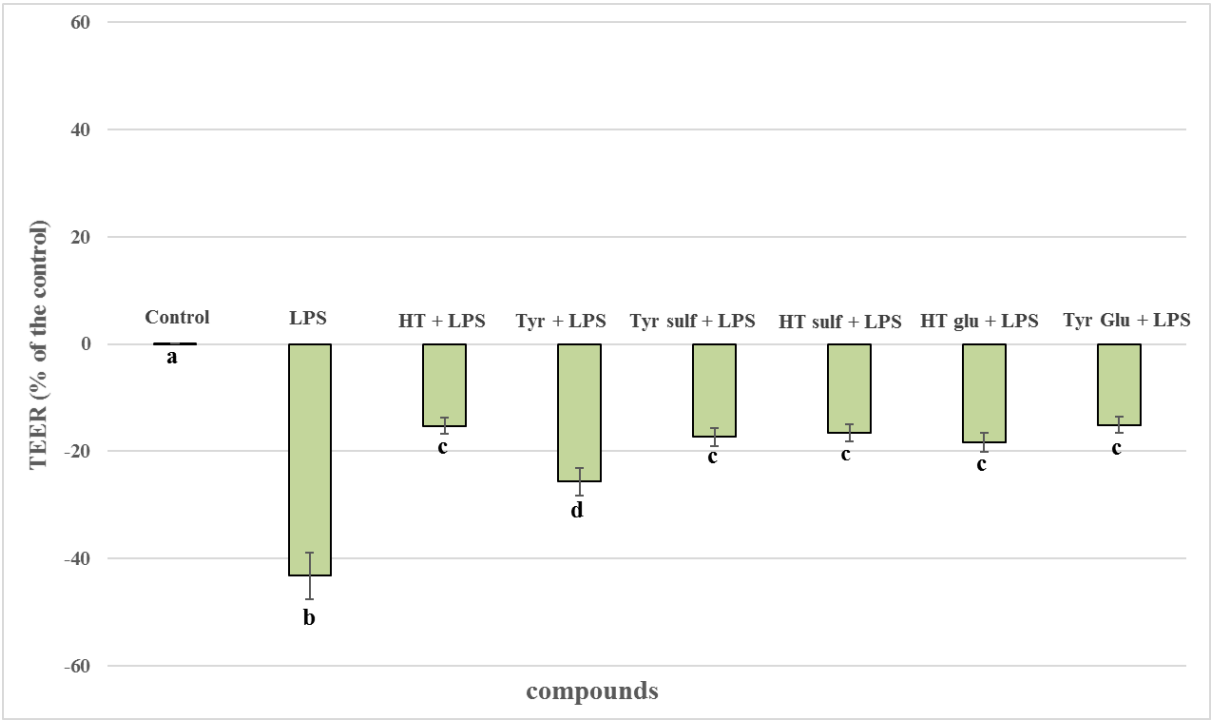


Figure 32. TEER value in Caco-2 cell monolayers incubated for 6 h with L-arginine (100 µM), LPS (1 µg/mL) and hydroxytyrosol (HT), tyrosol (Tyr), and their glucuronide and sulfate metabolites (1 µM) (n=6). Values are shown as percentage of TEER decrease respect to the control. Values not sharing a superscript letter are significantly different ($p < 0.05$).

4.2.5. Modulation of the iNOS activation pathway

iNOS expression pathway after LPS and olive oil phenolics treatments was evaluated. The modulation of iNOS expression was assessed by western blotting analyses, together with Akt phosphorylation and IκB degradation, linked to the NF-κB translocation into the nucleus and therefore to iNOS expression.

4.2.5.1. Analysis of iNOS expression

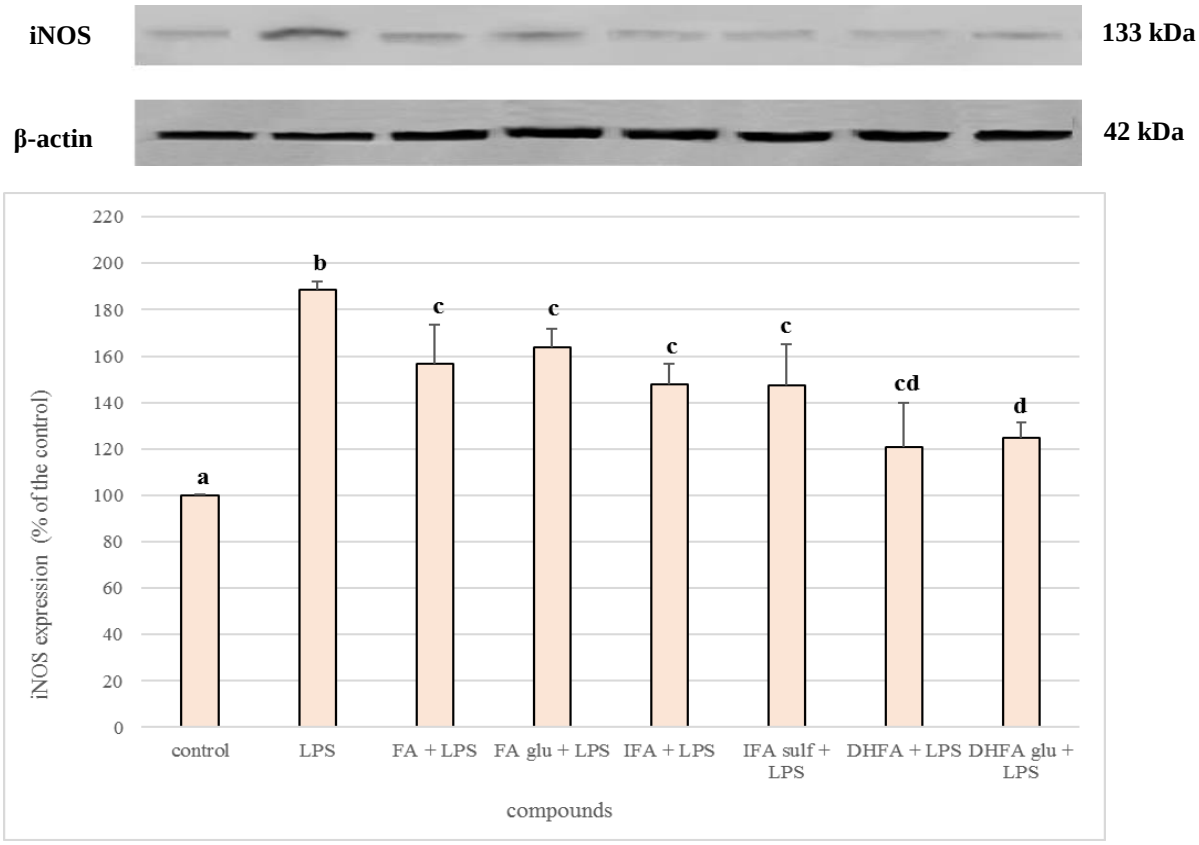


Figure 33. Activation of iNOS measured as percentage of the control cells expression in Caco-2 cells treated with LPS (1 µg/mL) and phenolic compounds ferulic acid (FA), isoferulic acid (IFA), hydroferulic acid (DHFA) and their glucuronide and sulfate metabolites (1 µM) for 48 h. Values not sharing a superscript letter are significantly different (n=3) ($p < 0.05$).

The induction of iNOS expression gives rise to the massive NO production in intestinal cells, and it is elicited by different stimuli such as LPS. In **Figure 33** and **34** are shown the results of the western blot analysis assessed to study iNOS expression after 48 h of LPS incubation in Caco-2 cells. LPS alone increased iNOS expression about twofold more than that of control cells, while almost all the tested phenolic compounds could inhibit this process. HT, IFA and DHFA were the most effective in inhibiting iNOS activation.

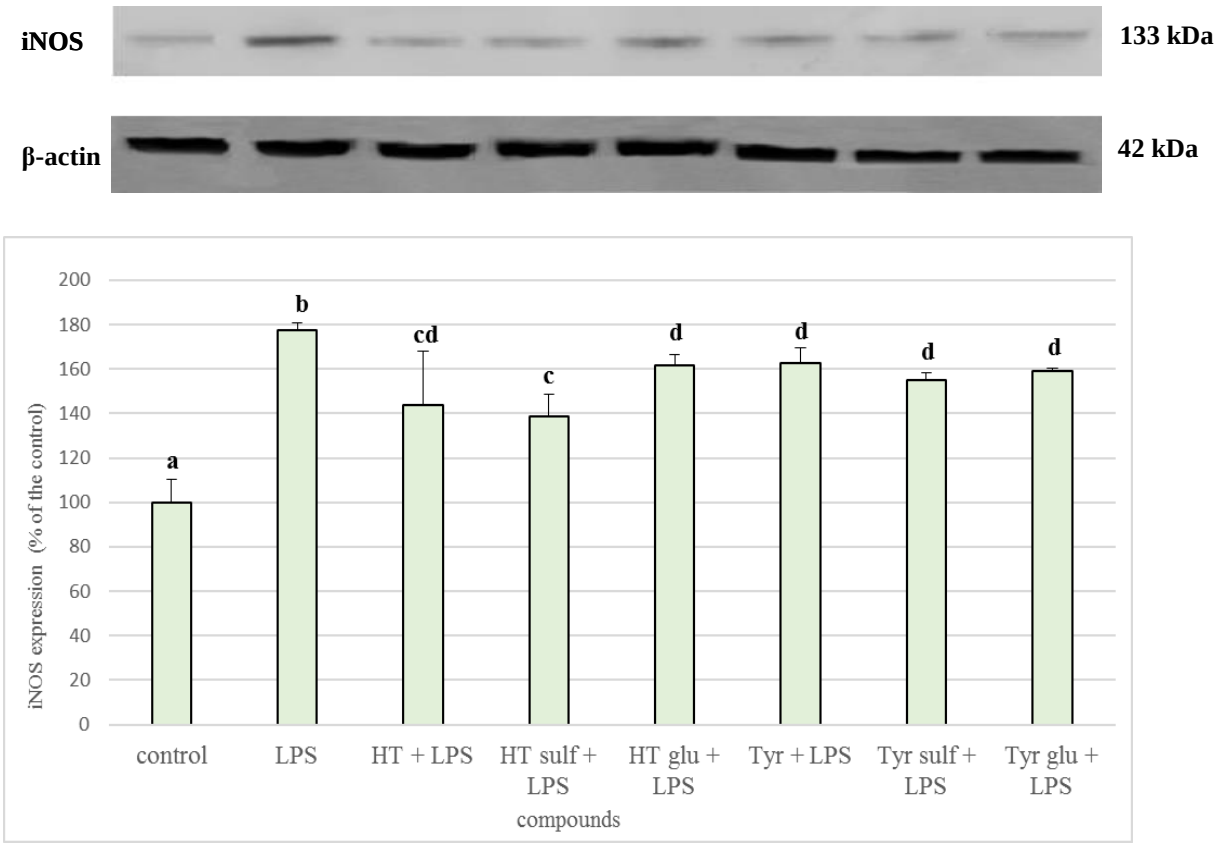
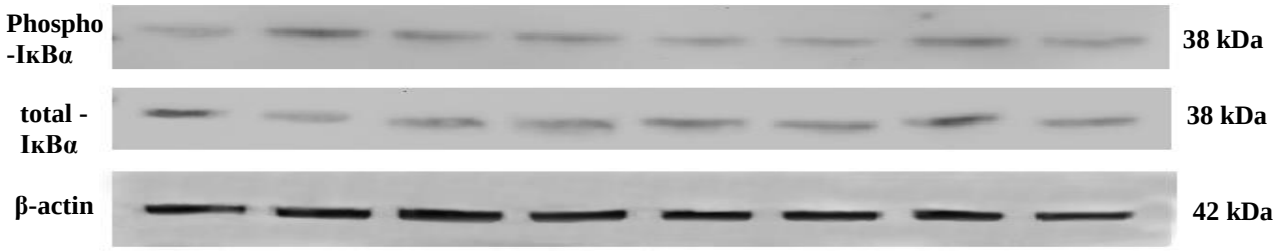


Figure 34. Activation of iNOS measured as percentage of the control cells expression in Caco-2 cells treated with LPS (1 µg/mL) and phenolic compounds hydroxytyrosol (HT), tyrosol (Tyr) and their glucuronide and sulfate (1 µM) for 48 h. Values not sharing a superscript letter are significantly different (n=3) ($p < 0.05$).

4.2.5.2. Assessment of IκBα phosphorilation



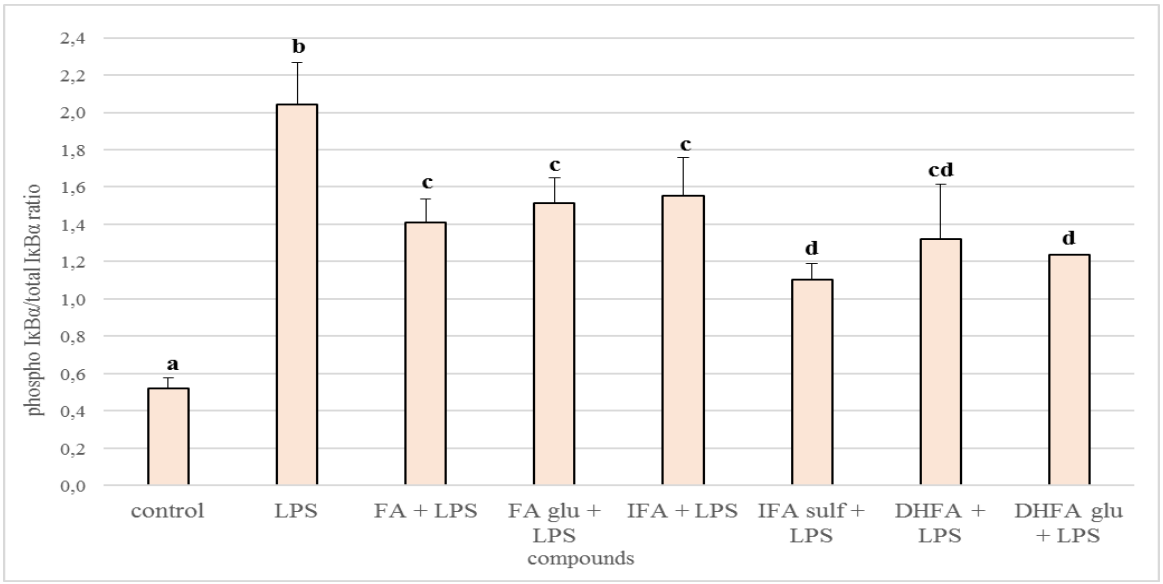


Figure 35. Degradation of IκBα measured as phospho IκBα/total IκBα ratio in Caco-2 cells treated with LPS (1 µg/mL) and phenolic compounds ferulic acid (FA), isoferulic acid (IFA), hydroferulic acid (DHFA) and their glucuronide and sulfate metabolites (1 µM) for 48 h. Values not sharing a superscript letter are significantly different (n=3) ($p < 0.05$).

IκBα phosphorylation by IKK is a main step which allows NF-κB to modulate iNOS expression. **Figure 35** and **36** report the results of IκBα modulation occurred after LPS treatments. A western blot analysis was assessed to evaluate the ratio between IκBα and its phosphorylated form. The latter was predominant in cells treated only with LPS, while pretreatment with phenolics significantly inhibited IκBα phsphorylation, thus blocking its degradation. It can be seen that metabolites worked similarly to their parent compounds, and the best inhibitory activity was observed in those cells treated with IFA, HFA and DHFA.

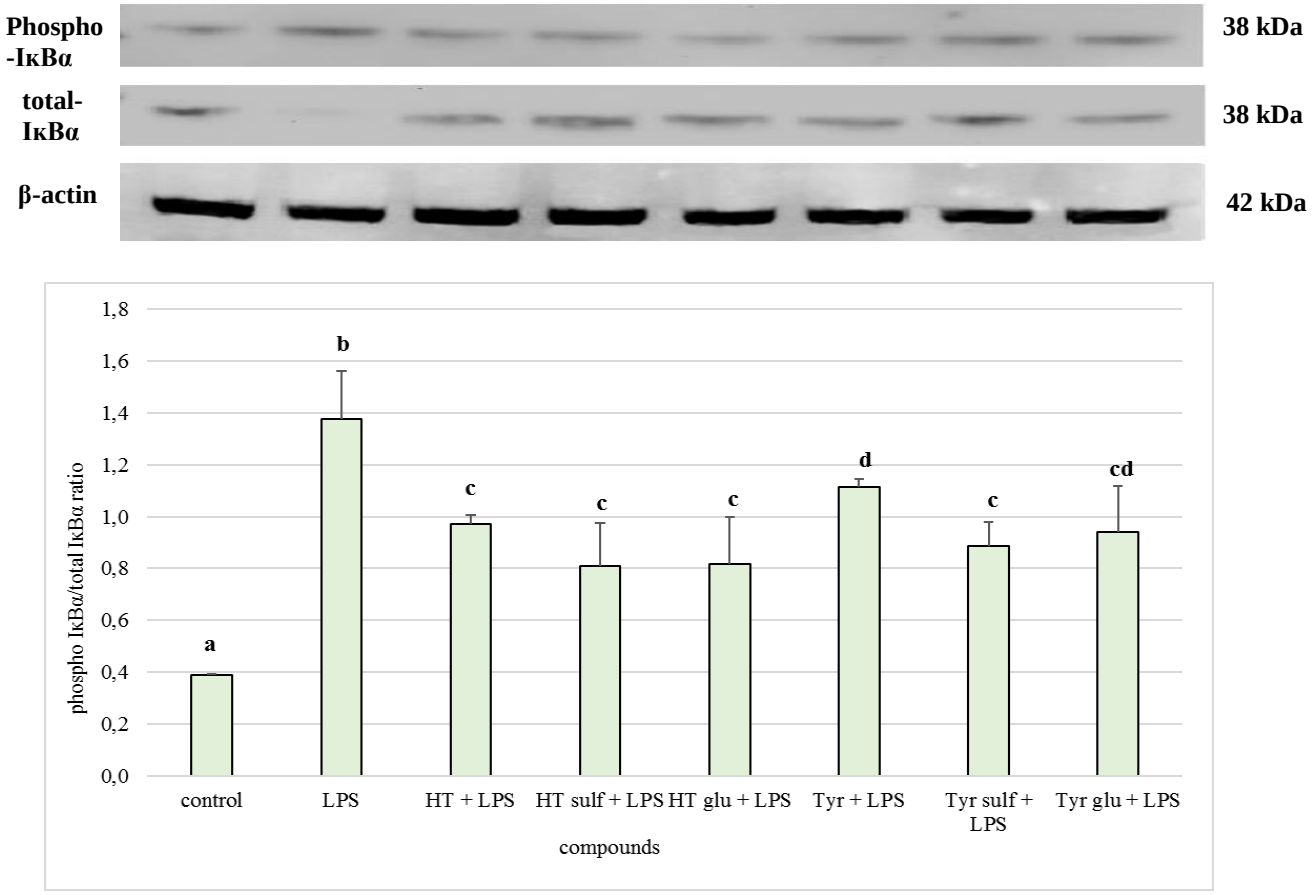


Figure 36. Degradation of IκBα measured as phospho IκBα/total IκBα ratio in Caco-2 cells treated with LPS (1 µg/mL) and phenolic compounds hydroxytyrosol (HT), tyrosol (Tyr) and their glucuronide and sulfate metabolites (1 µM) for 48 h. Values not sharing a superscript letter are significantly different (n=3) ($p < 0.05$).

4.2.5.3. Evaluation of Akt phosphorylation

Western blot analyses allowed the evaluation of the Akt phosphorylation status after LPS treatments. As expected, LPS increased Akt activation through phosphorylation, which was twofold higher than that of control cells (**Fig. 37** and **38**). Overall, pretreatment with hydroxycinnamates (particularly with IFA sulf) counteracted this activation, while phenylethanoids seemed not to inhibit this process **(Fig. 38).**

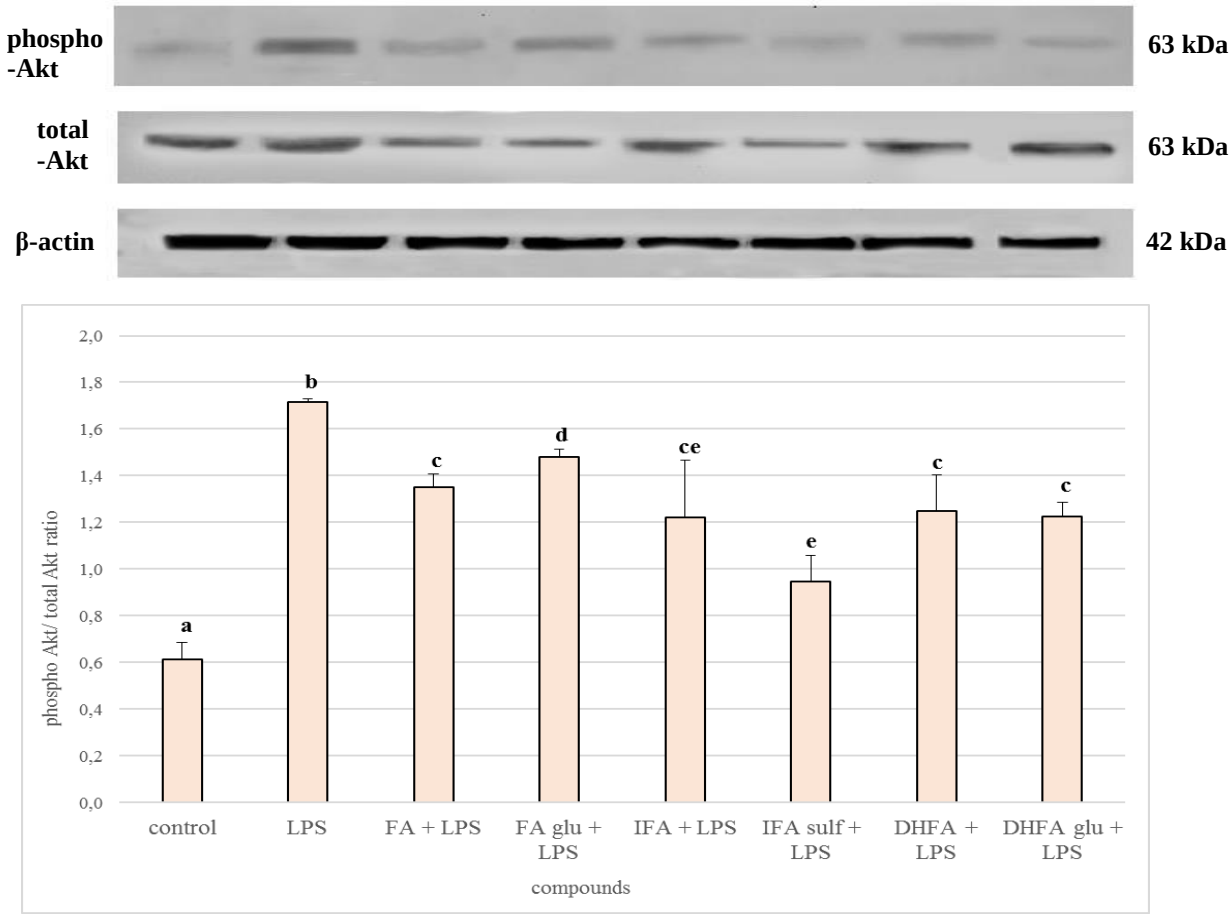


Figure 37. Activation of Akt measured as phospho Akt/total Akt ratio in Caco-2 cells treated with LPS (1 µg/mL) and phenolic compounds ferulic acid (FA), isoferulic acid (IFA), hydroferulic acid (DHFA) and their glucuronide and sulfate metabolites (1 µM) for 48 h. Values not sharing a superscript letter are significantly different (n=3) ($p < 0.05$).

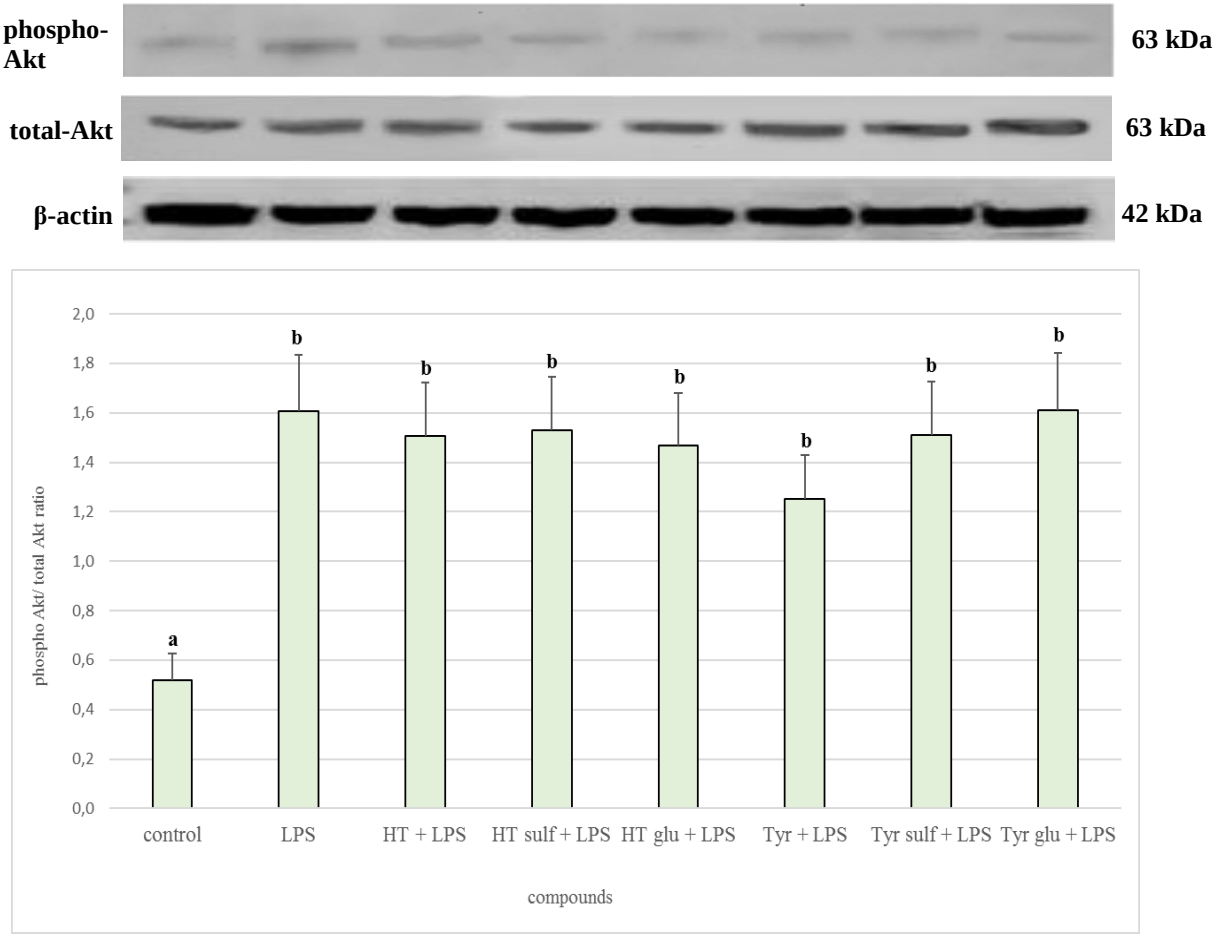


Figure 38. Activation of Akt measured as phospho Akt/total Akt ratio in Caco-2 cells treated with LPS (1 µg/mL) and phenolic compounds hydroxytyrosol (HT), tyrosol (Tyr) and their glucuronide and sulfate metabolites (1 µM) for 48 h. Values not sharing a superscript letter are significantly different (n=3) ($p < 0.05$).

5. Discussion

NO has been largely studied over the last three decades for its implication as a messenger in a wide variety of physiological and pathological processes in the human body **(Bian et al., 2008; Butler et al., 1993; Moncada et al., 1991)**.

At the cardiovascular level, NO is strictly involved in the local regulation of basal arterial tone acting as a vasodilator in different ways **(Furchgott et al., 1980; Hentze et al., 1996)**. In addition to its vasodilatory properties, NO mediates several protective functions of the endothelium by inhibiting neutrophil activation and adhesion, platelet adhesion and aggregation, vascular smooth muscle cell proliferation, and the expression of proinflammatory cytokines **(Bian et al., 2008)**. Thus, injuries or dysfunctional endothelial cells with a loss of endothelium-derived NO are major critical factors in the pathogenesis of vascular diseases, including hypertension, atherosclerosis, and vasospasm with compromised blood flow **(Bian et al., 2008)**. In these pathological conditions, NO bioavailability is reduced by oxidation due to excessive production of superoxide anions in the vascular wall, suggesting that antioxidant compounds may have a role in the modulation of endothelial-dependent vasodilatation **(Migliori et al., 2015)**.

NO production in the intestinal epithelium is related to a protective role at low concentrations **(Alican et al., 1996)**. In fact, infection of the gut results in a iNOS activation which contributes to the cellular defense against pathogenic microorganisms through NO release. Conversely, an excessive and prolonged NO produced by iNOS can lead to inflammatory conditions that increase mucosal permeability, thus triggering inflammatory bowel diseases **(Chen et al., 2015)**.

Experimental studies widely highlighted a significant interaction between NO modulation and the phenolic compounds ingested after consumption of certain foods such as vegetables, wine and olive oil, which are constituents of the MD **(Khurana et al., 2013; Panaro et al., 2012)**. These compounds are different in chemical structure and, therefore, in their biological activity **(Rice-Evans et al., 1996)**.

Among the notable bioactivities of phenolic compounds, the antioxidant activity, in terms of scavenging of free radicals, inhibiting lipid oxidation, reducting hydroperoxide formation, and so on, has largely been studied **(Li et al., 2014)**. Moreover, their structure allows them to be involved in the modulation of intracellular signaling, also including NO production and inhibition **(Hussain et al., 2016; Panaro et al., 2012; Santangelo et al., 2007)**. However, the relevance of *in vitro* data for the interpretation of *in vivo* effects of phenols is controversial, since the oral bioavailability of these compounds is rather limited due to their metabolization mainly to sulfates and glucuronides **(Kroon et al., 2004)**. In any case, some phenols' glucuronides and sulfates have demonstrated to be effective as well as their parent compound in the regulation of many cell signals **(Heleno et al., 2015)**, such as the enhancement of NO production at the endothelial level **(Ladurner et al., 2014; Lodi et al., 2009)**.

Olive oil polyphenols are some of the most studied phenols for their health properties **(Bendini et al., 2007; De la Torre, 2008)**. Among them, hydroxytyrosol (HT) and tyrosol (Tyr) are highly concentrated and have been recognized as having the best antioxidant properties and protective effects against many diseases, acting not only as radical scavengers but also influencing gene expression and intracellular signals **(Rodriguez-Morato et al., 2016)**.

In addition, less concentrated compounds in olive oil like the hydroxycinnamic derivative FA showed beneficial effects such as anti-inflammatory, anti-apoptotic, anticarcinogenic, anti-diabetic, hepatoprotective, cardioprotective and neuroprotective actions **(Ghosh et al., 2017)**.

The present study aimed to explore the ability of olive oil phenolic compounds HT, Tyr and FA in comparison to their glucuronides and sulfates metabolites, to influence NO production at both endothelial and gut levels. These compounds were tested in different cell models which simulated the vessel endothelium and the intestinal epithelium, at 1 μM concentration, which could be found in circulation after ingestion of foods containing these phenolic compounds **(Manach et al., 2004)**, as well as in the gut lumen, when they can reach higher concentrations (up to 500 μM) **(Jenner et al., 2005).**

The first part of the project was focused on the investigation of the possible mechanisms by which olive oil phenols are effective in the enhancement of NO release in HUVEC and HAEC coltures, models which simulate vessel endothelium **(Chung et al., 2017; Krause et al., 2012; Steffen et al., 2007; Steffen et al., 2008)**. The first set of trials, where the hydroxycinnamate compounds were tested, was performed on HUVEC, the most used cell line to study the endothelium pathophysiology, easy to handle, coming from a venous endothelium. HAEC come from an arterial endothelium and are thus considered an experimental model more suitable for studies on endothelial damage leading to atherosclerosis **(Rabini et al., 2002)**. Both cell lines, treated with L-NNA (Nγ-nitro-L-Arginine), which is a competitive inhibitor of nitric oxide synthase (NOS) with selectivity for the neuronal and endothelial isoforms, and with apocynin, an inhibitor of NADPH oxidase activity, showed the same reduction of NO release. Thus, to have a more complete pool of data, the second set of trials, where the phenylethanoid compounds were tested, was performed on HAEC.

Endothelial cells were treated for 24 h with 1 μM of each phenolic compound (a non-toxic concentration as indicated by the MTT test) to measure endothelial NO production and cGMP, as well as superoxide production. The cGMP and NO production are strictly correlated: one of the main targets in NO signaling is in fact soluble guanylate cyclase (sGC), which converts GTP to the intracellular signaling molecule cGMP, a molecule which is responsible for many of the physiological effects of NO **(Hentze et al., 1996)**. Superoxide production is also a key event in the modulation of NO level. Once released by NADPH oxidase, superoxide removes NO via diffusion-controlled formation of peroxynitrite followed by spontaneous isomerization to nitrate or oxidation and nitration of biomolecules. By eliminating a major source of $O_2^{\cdot -}$, the loss of NO via this route is prevented, thus leading to an increase in the cellular steady-state level of NO, even with no change in the generation of NO via NO synthases or by other routes **(Steffen et al., 2007).**

NO production in endothelial cells was studied assessing its byproduct nitrite concentration in the cell medium after 24 h incubation with phenols. To better elucidate the mechanism underliyng NO modulation by the phenolic compounds, a set of cells were treated with L-NNA, and another set was treated with apocynin, effective in preventing the production of the superoxide, being an inhibitor of NADPH oxidase. As expected, cells treated with L-NNA produced less than a half of the NO produced by control cells, while the apocynin inhibition of NADPH significantly counteracted the superoxide-mediated NO depletion. Nitrite concentration in the cell supernatant after 24 h treatments with olive oil phenolic compounds was averagely higher than that of controls; in particular, HT, DHFA and IFA were the most effective in increasing nitrite concentration. Moreover, it is interesting to note that HT sulf was as effective as its parent compound.

$O_2^{\cdot-}$ release in endothelial cells after phenolic pretreatments was quantified by one-electron oxidation with ferricytochrome c with intact cells, in presence or absence of superoxide dismutase, which helped to discriminate $O_2^{\cdot-}$ oxidation from the other oxygen reactive species. In this study, the production of superoxide was highly elicited by L-NNA because of the inhibition of NO production which did not react with $O_2^{\cdot-}$ to form peroxynitrite. On the contrary, treatments with apocynin considerably reduced the superoxide release by inhibiting NADPH oxidase activity. All the compounds significantly proved to be effective as apocynin in counteracting $O_2^{\cdot-}$ production. Some of the compounds, namely HT, IFA and DHFA, were even more effective than apocynin. The tested polyphenols could have been involved in the superoxide suppression in different ways. As polyphenols, they possess scavenging activity **(Andreasen et al., 2001; Anselmi et al., 2004; Dinis et al., 2002; Rodriguez-Morato et al., 2016)** so they could have decreased $O_2^{\cdot-}$ concentration by acting as primary antioxidants. Moreover, depending on their molecular structure, they could also act as NADPH oxidase inhibitors: indeed, **Steffen et al. (2008)** showed that (-)-epicatechin, a flavan-3-ol, was effective in inhibiting $O_2^{\cdot-}$ production working as antioxidant but not as NAPDH oxidase inhibitor, whereas the converse pattern was observed for the metabolites 3'- and 4'-*O*-methyl epicatechin, with an apocynin-like mode of inhibition. Therefore, the mechanisms by which the tested phenols and their metabolites influence $O_2^{\cdot-}$ production needs to be better investigated and will be the focus of further experiments.

NO production by endothelial cells is also related to cGMP release. Thus, we investigated the variation of cGMP concentration in the cells pretreated with the phenolic compounds compared to controls. As expected, the observed trend paralleled that of NO production. In fact, hydroxycinnamates FA, and IFA enhanced cGMP activity as well as their

metabolites, while DHFA was significantly the most effective above all the hydroxycinnamates.

However, HT glu was unexpectedly the most effective among the phenylethanoids, despite it did not show the same efficacy in enhancing NO level. Beyond the possible collateral interactions between this compound and the enzymes involved in cGMP degradation, this result could possibly be due to the inability of the experimental system to highligh small differences of nitrite concentration among samples.

In the context of NO modulation, eNOS activation through phosphorylation is the main event, which is usually carried out after different stimuli and intracellular signals activation **(Heiss et al., 2014)**. One of the key signals involved in eNOS activation is the phosphorylation of the kinases Akt which, once activated by intracellular signaling modulation or by different agonists, phosphorylates eNOS giving way to NO release **(Heiss et al., 2010; Shiojima et al., 2002)**. Different polyphenols coming from different foods (fruits and vegetables) have already proved to induce Akt phosphorylation in endothelial cells **(Chang et al., 2011; Mansuri et al., 2014)**.

Olive oil phenols tested in this study were able to induce Akt phosphorylation, though to a different extent. Whereas almost all the hydroxycinnamates (except FA), parent compounds and metabolites to the same extent, significantly activated Akt, the best activity among phenylethanoids was detected in HT and their metabolites in the same way, while Tyr did not exert any significant Akt activation. These results are in contrast with *in vivo* experiments in rat models, where Akt activation by Tyr as well as by HT was ascertained **(Pei et al., 2016; Samuel et al., 2008).** However, it should be considered the possible HT endogenous formation from Tyr, which could have influenced the evaluation of intracellular Tyr activity **(Rodriguez-Morato et al., 2016)**. In HUVECs, Tyr and its metabolites did not

improve eNOS activation, whilst HT and its glucuronide and sulfate stimulated eNOS phosphorylation, reflecting what observed in the case of Akt modulation.

However, HT modulatory action on eNOS activity is still not clear: **Schmitt et al. (2007)** underlined the absence of activity of HT in eliciting eNOS activity in different endothelial models, while a more recent study highlighted the enhancement of NO production by HT conjugated with elenolic acid **(Segade et al., 2016)**.

As regards hydroxycinnamates, DHFA and IFA together with their metabolites and with FA glu showed the best activity, while FA did not significantly activate eNOS. The activity of FA different analogues in activating eNOS was also demonstered by **Zhao et al. (2016)** who showed that these compounds were able to restore eNOS activitation which was previously impaired by TNF-α treatment.

Unlike eNOS which is constitutive and needs to be activated mainly through phosphorylation to produce physiological small amounts of NO, iNOS is not usually expressed in cells but its expression can be induced by bacterial LPS, cytokines, and other agents **(Förstermann et al., 2012)**. Once expressed, iNOS is constantly active and not regulated by intracellular Ca^{2+} concentrations, and produces large amounts of NO **(Förstermann et al., 2012)**.

This NO overproduction in the intestine, if prolonged, can damage cells through peroxynitrite formation and lead to several inflammatory disorders **(Banan et al., 2001)**.

The second part of this study was focused on the modulation of NO production at intestinal levels, with the aim of investigating the ability of olive oil phenolics, which showed modulatory activities towards eNOS, to inhibit iNOS activation and the underlying mechanism of action. As a model of human intestinal epithelium Caco-2 cell line was used. These cells derive from a human colon adenocarcinoma; once in culture they undergo a

process of spontaneous differentiation into normal mature enterocytes, expressing microvilli, basolateral membranes separated by tight junctions, intestine-specific antioxidant enzymes such as superoxide dismutase, glutathione peroxidase, glutathione reductase and catalase **(Baker et al., 1992; Baker et al., 1993)**. Caco-2 cell line represents a well-established model for the study of intestinal transport of nutrients and xenobiotics, and it is widely used in pharmacology and toxicology **(Artursson, 1990; Artursson et al. 2001).**

In order to activate the pathway which leads to iNOS activation, Caco-2 cells were treated with a pro-inflammatory agent, LPS, given at the pathological concentration of 1 µg/mL **(Guo et al., 2015)**, which has already been used for this purpose in many researches **(Panaro et al., 2012; Van De Walle et al., 2010; Romier et al., 2008).** LPS, the major cell wall component of Gram-negative bacteria, is abundant in the gut lumen and it is correlated to the pathogenesis of IBD when it reaches elevated circulating levels (over 10 ng/mL) **(Guo et al., 2013)**.

This investigation was firstly focused on NO release in the cell supernatant after 48 h of LPS exposure, and on the consequent cGMP release. NO production, measured as nitrite concentration, was twofold higher in those cells treated only with LPS, with respect to the controls, while the cGMP release was even threefold higher. NO production was considerably reduced in cells pretreated with phenolics (1 µM, non-toxic concentration), and the same results were observed in the case of cGMP release. Cells incubated with DHFA, HT and its sulfate metabolites show the lowest nitrite concentration and, at the same time, the lowest cGMP production among all the tested compounds. The abnormal cGMP production after iNOS overexpression at the intestinal level caused by LPS is known to contribute to diarrhea in ulcerative colitis **(Arora et al., 2015),** thus keeping lower levels, as happened after phenolic treatments in this study, could be of great importance to keep gut homeostasis **(Wang et al., 2014)**.

NO and cGMP release in Caco-2 cells are consequences of LPS triggered iNOS activation: the best iNOS expression, assessed by western blotting, was found in LPS-treated cells, while it was led back to a lesser concentration by phenolic compounds though with a different extent. Among the hydrxycinnamates tested, DHFA and its glucuronide were the most effective in inhibiting iNOS activation, better than FA, IFA and their metabolites. Regarding phenylethanois compounds, the highest iNOS down-regulation was observed in cells treated with HT and HT sulf. The iNOS down-regulation by HT, HT acetate and some other HT derivatives was already demonstered by **Maiuri et al. (2005)** and **Aparicio-Soto et al. (2015),** though in two different macrophage cell lines. Moreover, hydroxycinnamic derivatives were found to be able to inhibit iNOS activation in a colitis rat model **(Sadar et al., 2016)** as well as in macrophages **(Kim et al., 2012; Lampiasi et al., 2016; Nagasaka et al., 2007)** and in neuronal cells **(Kikugawa, et al., 2017; Kim et al., 2004; Wu et al., 2015)**. Other studies highlighted the capacity of other polyphenols, with different chemical structures, to inhibit iNOS expression: some of them, such as resveratrol, acted counteracting IκBα **(Panaro et al., 2012),** while another studied highlighted the possible activity of resveratrol as direct iNOS inhibitor **(Cosan et al., 2010)**.

iNOS activation and NO release are key events in the oneset and progression of intestinal inflammation **(Alican et al., 1996)** and are strictly linked to the central event that is the alteration of intestinal epithelium permeability **(Unno et al., 1997)**.

LPS itself has been demonstrated to disrupt the tight junctions (TJ) structure and cause mucosal hyperpermeability directly or indirectly, through the secretion of cytokine and inflammatory mediators like NO **(Guo et al., 2013; Han et al., 2004).**

Han et al. (2004) showed that an increased production of NO associated with lipopolysaccharide (LPS)-induced systemic inflammation leads to functionally significant

alterations in the expression and/or targeting of key TJ proteins in ileal and colonic epithelium, with an important decrease of the membranes permeability. In this study, LPS significantly reduced Caco-2 cell monolayer permeability, leading TEER values at the lowest level after 6-12 h of treatment (below 60% of the control values), as was previously observed by **Yang et al. (2016).** Pretreatments with the tested phenolic compounds counteracted the LPS-induced permeability decrease, keeping TEER values close to those of the control monolayers. This effect was observed for all the compounds tested almost in the same way, with no significant differences between parent compounds and their glucuronides and sulfates. This finding could be explained by the fact that several pathways are involved in the intestinal permeability decrease, so the compounds tested could have influenced the release of cytokines and protected the TJ injuries with a NO-independent mechanism, but still linked with the NF-κB signaling pathway activation by LPS **(Tu et al., 2016).** The NF-κB translocation into the nucleus is allowed when its inhibitor IκB gets phosphorylated by its kinase IKK **(Pan et al., 2000)** which, in turn, could be activated by phosphorylated Akt/PKB or other kinases **(Kane et al., 1999).** It was previously shown that polyphenols can modulate this pathway, influencing NF-κB-induced iNOS gene expression **(Bognar et al. 2013; Panaro et al., 2012).**

IκBα degradation through previous phosphorylation was assessed by western blot analysis, which showed an increase of the phospho IκBα/total IκBα ratio in those cells incubated with LPS alone. It is interesting to note that compounds which better inhibited NO production (DHFA, HT and HT sulf), together with IFA sulf, were the most effective in blocking IκBα phosphorylation and then NF-κB activation. The outstanding activity of these compounds in counteracting IκBα degradation could be linked to the inhibition of the LPS-induced Akt phosphorylation. Indeed, intestinal cells treated with LPS had, as expected, a significant increase in Akt phosphorylation, which was partially counteracted by pretreatment

with hydroxycinnamic derivatives, but especially by IFA sulf. As regards phenylethanoids pretreatments, western blot analyses revealed an absence of Akt inhibition with respect to the LPS alone treated samples. Further studies are needed to explain how HT, Tyr and their metabolites counteracted IκBα phosphorylation and in which pathway they may be effective as antagonists/inhibitors. In fact, beyond the Akt-induced IKK activation, LPS is known to induce IκB phosphorylation also activating key protein kinases such as mitogen-activated protein kinases (MAPKs) p38, JNK1/2, and ERK1/2, all of which are involved in activating key transcription factors, including NF-κB **(Murakami, 2009).** In support of the MAPK down-regulation in the context of IκBα phosphorylation, **Cardeno et al. (2014)** proved that EVOO phenolic extracts reduced LPS-induced oxidative stress and inflammatory responses through decreasing NO with a significant down-regulation of iNOS which was linked to a reduced MAPK phosphorylation and nuclear NF-κB translocation in murine macrophages. In fact, simple phenols such as HT and its metabolite homovanillic alcohol were studied for their capacity to interact with MAPK, and they proved to be effective in the prevention of ERK1/2 and JNK1/2/3 phosphorylation in renal cells **(Incani et al., 2010)**. Considering this, the MAPK involvement in intestinal IκBα modulation by EVOO phenols needs to be the focus of further investigations.

This study pointed out for the first time the ability of some of the most important polyphenols found in olive oil to enhance NO production in physiological conditions at the endothelial level, and to prevent an abnormal and pathological NO overproduction at the intestinal level, investigating the underlying intracellular mechanism involved. This dual activity of the phenolic compounds was already observed by **Duarte et al. (2014)** in the case of flavonoids, which showed to be modulators of NO, inhibiting or enhancing its production, depending on the tissue location and on the NOS isoform. One of the main mechanisms by which dietary polyphenols are thought to influence cardiovascular diseases is via protection of

the bioactivity of the endothelium-derived NO. Additionally, polyphenols may also interfere with the signalling cascades of inflammation and prevent overproduction of NO by iNOS and its deleterious consequences which can lead to chronic pathologies such as IBD (**Duarte et al., 2014).**

Another key feature of this study was the demonstration that the major metabolites of the olive oil phenolic compounds, glucuronides and sulfates, which are usually found at higher concentrations than the parent compounds at systemic and intestinal level after olive oil ingestion, essentially retain parental biological activity.

Concerning NO modulation in endothelial cells, it was observed that the tested phenolic compounds enhanced NO concentration inhibiting its depletion caused by superoxide overproduction. Moreover, some of them enhanced its production through the modulation of Akt activation and of eNOS phosphorylation. It is interesting to note that compounds with similar structure, such as FA and IFA, showed different activities. Indeed, DHFA and IFA showed better effectiveness than FA in activating eNOS, as well as HT, which worked better than Tyr either as superoxide inhibitor or as eNOS enhancer. It could be explained considering that these slight differences influence the antioxidant capacity of the compounds which can act as superoxide scavengers, but also their interaction with proteins and enzymes.

For instance, **Zhao et al. (2016)** suggested that FA and coumaric acid showed different activities in enhancing NO availbility because of the methoxy group and the 2-propenoic moieties which are present in FA and in apocynin chemical structure, which is known to be a powerful NADPH inhibitor **(Steffen et al., 2007)**.

Moreover, it is noticeable that some of the tested metabolites worked in the same way of their parent compounds: it is the case of HT, whose glucuronide was significantly effective

in Akt and eNOS activation, and showed the same efficacy in superoxide counteraction. This latter finding was not expected, because of the loss of antioxidant activity of the metabolites tested, which was previously verified by **Nenadis et al. (2005)** and **Khymenets et al., (2010)**. The poor activity of Tyr in comparison with that of HT could instead be explained considering its different chemical structure (it has only one hydroxyl group) which, as stated before, could influence its interaction with intracellular signals and its antiradical activity, that is required to better scavenge an abnormal superoxide release. Overall, this study showed that these compounds promote NO production and availability as well as other polyphenols previously studied **(Biegańska-Hensoldt et al., 2017; Steffen et al., 2007, Steffen et al., 2008)**, so their intake by EVOO dietary consumption can help in the prevention of cardiovascular diseases such as hypertension, thrombosis, hypercholesterolaemia and atherosclerosis, which are linked to a loss of physiological NO concentration due to an aberrant eNOS expression and activity and to superoxide overproduction **(Albrecht et al., 2003).**

Regarding the intestinal NO overproduction induced by pathological concentration of LPS, it was overally observed that all the tested compounds inhibited NO release, acting as inhibitors of iNOS expression. Considering hydroxycinnamates, they showed the same mechanism of action highlited in HUVEC, being able to modulate Akt phoshorilation. IFA sulf was surprisingly the most effective in the Akt inhibition, while DHFA and its glucuronide better inhibited IκBα degradation and iNOS.

No one of the phenylethanoids instead were able to inhibit Akt activation, however, they were effective in the inhibition of IκBα degradation, suggesting a modulatory action on a parallel mechanism upstream NF-κB translocation to the nucleus. Lastly, all the tested phenolic compounds counteracted the deleterious effect of LPS on Caco-2 cell monolayer permeability, suggesting the ability of preserving intestinal barrier integrity.

The polyphenols' activity in restoring epithelial impermeability, previously impaired by different inflammatory stimuli, was also verified by **Kim et al. (2017)** and **Carrasco-Pozo (2013)**. Moreover, **Serreli et al. (2017)** already showed that table olive phenolic extracts rich in phenols like those tested in this investigation were able to counteract the permeability decrease in intestinal cell monolayers. Thus, it can be assumed that reaching relevant concentrations of EVOO phenols in the gut lumen may exert beneficial effects against intestinal inflammation which is one of the major features of many intestinal diseases.

Although further researches are needed to better understand the mechanisms underlying the action of the tested phenolic compounds, the obtained data are of great biological relevance, adding evidences to the hypothesised importance of EVOO phenolics in the prevention of both cardiovascular and inflammatory bowel diseases. This study highlighted new properties of those EVOO compounds which are already known to possess manifold activities in the prevention of chronic diseases, thus further exploiting and boosting EVOO consumption in the context of a balanced diet as the Mediterranean one.

References

Abdennebi, H. B., Zaouali, M. A., Alfany-Fernandez, I., Tabka, D., & Rosellò-Catafau, J. (2011). How to protect liver graft with nitric oxide. *World Journal of Gastroenterology, 17(24)*, 2879-2889.

Adak, S., Santolini, J., Tikunova, S., Wang, Q., Johnson, J. D., & Stuehr, D. J. (2001). Neuronal nitric-oxide synthase mutant (Ser-1412 → Asp) demonstrates surprising connections between heme reduction, NO complex formation, and catalysis. *Journal of Biological Chemistry, 276*, 1244-1252.

Adam, A., Crespy, V., Levrat-Verny, M. A., Leenhardt, F., Leuillet, M., Demigne, C., et al. (2002). The bioavailability of ferulic acid is governed primarily by the food matrix rather than its metabolism in intestine and liver in rats. *The Journal of Nutrition, 132*, 1962-1968.

Albrecht, E. W. J. A., Stegeman, C. A., Heeringa, P., Henning, R. H. & van Goor, H. (2003). Protective role of endothelial nitric oxide synthase. *Journal of Pathology, 199(1)*, 8-17.

Alican, I., & Kubes, P. (1996) A critical role for nitric oxide in intestinal barrier function and dysfunction. American Journal of Physiology. *Gastrointestinal and Liver Physiology, 33*, 225-237.

Andreasen, M. F., Landbo, A. K., Christensen, L. P., Hansen, A., & Meyer, A. S. (2001). Antioxidant effects of phenolic rye (Secale cereal L.) extracts, monomeric hydroxycinnamates, and ferulic acid dehydrodimers on human low-density lipoproteins. *Journal of Agricultural and Food Chemistry, 49*, 4090-4096.

Andrew, P. J., & Mayer, B. (1999). Enzymatic function of nitric oxide synthases. *Cardiovascular Research, 43*, 521-531.

Anselmi, C., Centini, M., Granata, P., Sega, A., Buonocore, A., Bernini, A., et al. (2004). Antioxidant activity of ferulic acid alkyl esters in a heterophasic system: a mechanistic insight. *Journal of Agricultural and Food Chemistry, 52*, 6425-6432.

Aparicio-Soto, M., Sanchez-Fidalgo, S., Gonzalez-Benjumea, A., Maya, I., Fernandez-Bolaňos, J. G., & Alarcon-de-la-Lastra, C. (2015). *Journal of Agricultural and Food Chemistry, 63*, 836-846.

Arora, K., Sinha, C., Zhang, W., Moon, C. S., Ren, A., Yarlagadda, S., et al. (2015). Altered cGMP dynamics at the plasma membrane contribute to diarrhea in ulcerative colitis. *The American Journal of Pathology, 185(10)*, 2790-2804.

Artursson, P. (1990). Epithelial transport of drugs in cell culture. I: A model for studying the passive diffusion of drugs over intestinal absorptive (Caco-2) cells. *Journal of Pharmaceutical Sciences, 79(6)*, 476-482.

Artursson, P., Palm, K., & Luthman, K. (2001). Caco-2 monolayers in experimental and theoretical predictions of drug transport. *Advanced Drug Delivery Reviews, 46(1-3)*, 27-43.

Asanoma, M., Takahashi, K., Miyabe, M., Yamamoto, K., Yoshimi, N., Mori, H., et al. (1994). Inhibitory effect of topical application of polymerized ferulic acid, a synthetic lignin, on tumor promotion in mouse skin two-stage tumorigenesis. *Carcinogenesis, 15*, 2069-2071.

Atzeri, A., Lucas, R., Incani, A., Peñalver, P., Zafra-Gómez, A., Melis, M. P., et al. (2016). Hydroxytyrosol and tyrosol sulfate metabolites protect against the oxidized cholesterol pro-oxidant effect in Caco-2 human enterocyte-like cells. *Food and Function, 7*, 337-346.

Baeza, G., Bachmair, E. M., Wood, S., Mateos, R., Bravo, L., & de Roos, B. (2017). The colonic metabolites dihydrocaffeic acid and dihydroferulic acid are more effective inhibitors of in vitro platelet activation than their phenolic precursors. *Food and Function, 22, 8(3)*, 1333-1342.

Baker, S. S. & Baker, R. D. (1992). Antioxidant enzymes in the differentiated Caco-2 cell line. *In Vitro Cellular & Developmental Biology, 28A(9-10),* 643-647.

Baker, S. S., & Baker, R. D. (1993). Caco-2 cell metabolism of oxygen-derived radicals. *Digestive Diseases and Sciences, 38(12),* 2273-2280.

Balasubashini, M. S., Rukkumani, R., & Menon, V. P. (2003). Protective effects of ferulic acid on hyperlipidemic diabetic rats. *Acta Diabetologica, 40*, 118-122.

Balasubashini, M. S., Rukkumani, R., Viswanathan, P., & Menon, V. P. (2004). Ferulic acid alleviates lipid peroxidation in diabetic rats. *Phytotherapy Research, 18*, 310-314.

Balla, G., Jacob, H. S., Balla, J., Rosenberg, M., Nath, K., Apple, F., et al. (1992). Ferritin: a cytoprotective antioxidant strategem ofendothelium. *Journal of Biological Chemistry, 267*, 18148-18153.

Banan, A., Fields, J. Z., Zhang Y., & Keshavarzian A. (2001). iNOS upregulation mediates oxidant-induced disruption of F-actin and barrier of intestinal monolayers. *American Journal of Physiology. Gastrointestinal and Liver Physiology, 280*, 1234-1246.

Banick, P. D., Chen, Q., Xu, Y. A., & Thom, S. R. (1997). Nitric oxide inhibits neutrophil beta 2 integrin function by inhibiting membrane associated cyclic GMP synthesis. *Journal of Cell Physiology, 172*, 12-24.

Baran, I. (1996). Calcium and cell cycle progression: possible effects of external perturbations on cell proliferation. *Biophysical Journal, 70*, 1198-1213.

Barclay, A. W., Petocz, P., McMillan-Price, J., Flood, V. M., Prvan, T., Mitchell, P., et al. (2008). Glycemic index, glycemic load, and chronic disease risk--a meta-analysis of observational studies. *American Journal of Clinical Nutrition 87(3)*, 627-637.

Bender, K., Gottlicher, M., Whiteside, S., Rahmsdorf, H. J. & Herrlich, P. (1998). Sequential DNA damage-independent and -dependent activation of NF-kappaB by UV. *EMBO Journal, 17*, 5170-5181.

Bendini, A., Cerretani, L., Carrasco-Pancorbo, A., Gómez-Caravaca, A. M., Segura-Carretero, A., Fernández-Gutiérrez, 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*, *12*, 1679-1719.

Bennett, M. R., Anglin, S., McEwan, J. R., Jagoe, R., Newby, A. C., & Evan, G. I. (1994). Inhibition of vascular smooth muscle cell proliferation in vitro and in vivo by c-myc antisense oligodeoxynucleotides. *Journal of Clinical Investigation*, *93*, 820-828.

Bertelli, A. A., Migliori, M., Panichi, V., Longoni, B., Origlia, N., Ferretti, A., et al. (2002). Oxidative stress and inflammatory reaction modulation by white wine. *Annals of the New York Academy of Sciences, 957*, 295-301.

Bian, K., Doursout, M-F., & Murad, F. (2008). Vascular system: role of nitric oxide in cardiovascular diseases. *The Journal of Clinical Hypertension*, *10(4)*, 304-310.

Bianco, A., Coccioli, F., Guiso, M., & Marra, C. (2001). The occurrence in olive oil of a new class of phenolic compounds: Hydroxy-isochromans. *Food and Chemical Toxicology, 77*, 405-411.

Biegańska-Hensoldt, S., & Rosołowska-Huszcz, D. (2017). Polyphenols in preventing endothelial dysfunction. *Postepy higieny i medycyny doswiadczalnej, 71*, 227-235.

Bogani, P., & Visioli, F. (2007). Antioxidants in the Mediterranean diets: an update. *World Review of Nutrition and Dietetics, 97*, 162-179.

Bognar, E., Sarszegi, Z., Szabo, A., Debreceni, B., Kalman, N., Tucsek, S., et al. (2013). Antioxidant and anti-inflammatory effects in Raw264.7 macrophages of malvidin, a major red wine polyphenol. *Plos One, 8(6)*, e65355.

Bohlen, H. G. (2015). Nitric Oxide and the Cardiovascular System. *Comprehensive Physiology, 5*, 803-828.

Bolotina, V. M., Najibi, S., Palacino, J. J., Pagano, P. J., & Cohen, R. A. (1994). Nitric oxide directly activates calcium-dependent potassium channels in vascular smooth muscle. *Nature, 368*, 850-853.

Boo, Y. C., & Hanjoong, J. (2003). Flow-dependent regulation of endothelial nitric oxide synthase: role of protein kinases. *American Journal of Physiology - Cell Physiology, 285*, 499-508.

Boskou, D., Blekas, G., & Tsimidou, M. (1996). History and characteristics of the olive tree. *In Olive Oil Chemistry and Technology*. D. Boskou, Ed. American Oil Chemists' Society. Press: Champaign, IL, USA.

Boughton-Smith, N. K., Evans, S. M., Hawkey, C. J., Cole, A. T., Balsitis, M., Whittle, B. J. R. et al. (1993). Nitric oxide synthase activity in ulcerative colitis and Crohn's disease. *Lancet, 342*, 338-340.

Boulanger, C. M., & Vanhoutte, P. M. (1997). G proteins and endothelium-dependent relaxations. *Journal of Vascular Research, 34*, 175-185.

Boyle J. J., Weissberg P. L., & Bennett M. R. (2002). Human macrophage-induced vascular smooth muscle cell apoptosis requires NO enhancement of Fas/Fas-L interactions. *Arteriosclerosis, Thrombosis, and Vascular Biology, 22*, 1624-1630.

Bradford, M. M. A. (1976). Rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry, 72*, 248-254.

Brill, J. B. (2008). The Mediterranean Diet and your health. *American Journal of Lifestyle Medicine, 3(1)*, 44-56.

Brown, J. F., Keates, A. C., Hanson, P. J., & Whittle, B. J. (1993). Nitric oxide generators and cGMP stimulate mucus secretion by rat gastric mucosal cells. *American Journal of Physiology, 265*, 418-422.

Bulotta, S., Celano, M., Lepore, S. M., Montalcini, T., Pujia, A., & Russo, D. (2014). Beneficial effects of the olive oil phenolic components oleuropein and hydroxytyrosol: focus on protection against cardiovascular and metabolic diseases. *Journal of Translational Medicine, 12*, 219-218.

Burney, S., Niles, J. C., Dedon, P. C., & Tannenbaum, S. R. (1999). DNA damage in deoxynucleosides and oligonucleotides treated with peroxynitrite. *Chemical Research in Toxicology, 12*, 513-520.

Butler, A. R., & Williams, L. H. (1993). The physiological role of nitric oxide. *Chemical Society Reviews, 22*, 233-241.

Cardeno, A., Sánchez-Hidalgo, M., Aparicio-Soto, M., Sánchez-Fidalgo, S., & Alarcón-de-la-Lastra, C. (2014). Extra virgin olive oil polyphenolic extracts downregulate inflammatory responses in LPS-activated murine peritoneal macrophages suppressing NFκB and MAPK signalling pathways. *Food and Function, 5(6)*, 1270-1277.

Carrasco-Pancorbo, A., Cerretani, L., Bendini, A., Segura-Carretero, A., Del Carlo, M., Gallina-Toschi, T., et al. (2005). Evaluation of the antioxidant capacity of individual phenolic compounds in virgin olive oil. *Journal of Agricultural and Food Chemistry, 53*, 8918-8925.

Carrasco-Pozo, C., Morales, P., & Gotteland, M. (2013). Polyphenols protect the epithelial barrier function of Caco-2 cells exposed to indomethacin through the modulation of occludin and zonula occludens-1 expression. *Journal of Agricultural and Food Chemistry, 61*, 5291-5297.

Caruso, D., Visioli, F., Patelli, R., Galli, C., & Galli, G. (2001). Urinary excretion of olive oil phenols and their metabolites in humans. *Metabolism, 50(12)*, 1426-1428.

Castro-Quezada, I., Román-Viñas, B., & Serra-Majem, L. (2014). The Mediterranean Diet and Nutritional Adequacy: A Review. *Nutrients, 6*, 231-248.

Cavicchi, M., & Whittle, B. J. R. (1999). Regulation of induction of nitric oxide synthase and the inhibitory actions of dexamethasone in the human intestinal epithelial cell line, Caco-2: influence of cell differentiation. *British Journal of Pharmacology, 128*, 705-715.

Chan, M., Rocha, S., Lehman, M., White, S., Santana, I., Nip, J., et al. (2004). Clinical and in vitro investigation of the effects of ferulic acid on human skin pigmentation. *Journal of Investigative Dermatology, 122*, A157-A157.

Chang, C. J., Chiu, J. H., Tseng, L. M., Chang, C. H., Chien, T. M., Wu, C. W., et al. (2006). Modulation of HER2 expression by ferulic acid on human breast cancer MCF7 cells. *European Journal of Clinical Investigation, 36*, 588–596.

Chang, C. W-J., Edirisinghe, I., Jablonski J. E., Tadapaneni, R. K., Tulio, A. Z., Burton-Freeman, B., et al. (2011). Polyphenol-rich fruit modulate endothelial cell function via PI3 Kinase/Akt Pathway in Vitro in Human Umbilical Vein Endothelial Cell (HUVEC). *The FASEB Journal, 25(1)*.

Chen, F., Castranova, V., Shi, X., & Demers, L. M. (1999b). New insights into the role ofnuclear factor-kappaB, a ubiquitous transcription factor in the initia-tion of diseases. *Clinical Chemistry, 45*, 7-17.

Chen, X. M., & Kitts, D. D. (2011). Antioxidant and anti-inflammatory activities of Maillard reaction products isolated from sugar-amino acid model systems. *Journal of Agricultural and Food Chemistry, 59*, 11294-11303.

Chen, X. M., & Kitts, D. D. (2015). Evidence for inhibition of nitric oxide and inducible nitric oxide synthase in Caco-2 and RAW 264.7 cells by a Maillard reaction product [5-(5,6-dihydro-4H-pyridin-3-ylidenemethyl)furan-2-yl]-methanol. *Molecular and Cellular Biochemistry, 406*, 205-215.

Chen, Z. P., Mitchelhill, K. I., Michell, B., Stapleton, D., J., Rodriguez-Crespo, I., Witters, L. A., et al. (1999a). AMP-activated protein kinase phosphorylation of endothelial NO synthase. *FEBS Letters, 443*, 285-289.

Cheng, Z., Ren, J., Li, Y., & Chen, Z. (2002). Study on the multiple mechanisms underlying the reaction between hydroxyl radical and phenolic compounds by qualitative structure and activity relationship. *Bioorganic & Medicinal Chemistry, 10*, 4067-4073.

Chesson, A., Provan, G. J., Russell, W. R., Scobbie, L., Richardson, A. J., & Stewart, C. (1999). Hydroxycinnamic acids in the digestive tract of livestock and humans. *Journal of the Science of Food and Agriculture, 79*, 373-378.

Choudhury, R., Srai, S. K., Debnam, E., & Rice-Evans, C. A. (1999). Urinary excretion of hydroxycinnamates and flavonoids after oral and intravenous administration. *Free Radical Biology and Medicine, 27*, 278-286.

Chung, C.-P., Chang, Y.-C., Ding, Y., Lim, K., Liu, Q., Zhu, L., et al. (2017). α-Klotho expression determines nitric oxide synthesis in response to FGF-23 in human aortic endothelial cells. *PLoS ONE, 12(5)*, Article number e0176817.

Cicerale, S., Lucas, L., & Keast, R. (2010). Biological activities of phenolic compounds present in virgin olive oil. *International Journal of Molecular Sciences, 11*, 458-479.

Cicerale, S., Conlan, X. A., Sinclair, A. J., & Keast, R. S. (2009). Chemistry and health of olive oil phenolics. *Critical Reviews in Food Science and Nutrition, 49*, 218-236.

Cicerale, S., Lucas, L. J., & Keast, R. S. J. (2012). Antimicrobial, antioxidant and anti-inflammatory phenolic activities in extra virgin olive oil. *Current Opinion in Biotechnology, 23*, 129-135.

Cornwell, T. L., Pryzwansky, K. B., Wyatt T. A., & Lincoln T. M. (1991). Regulation of sarcoplasmic reticulum protein phosphorylation by localized cyclicGMP-dependent protein kinase in vascular smooth muscle cells. *Molecular Pharmacology, 40*, 923-931.

Corona, G., Tzounis, X., Dessi, M. A., Deiana, M., Debnam, E. S., Visioli, F. et al. (2006). The fate of olive oil polyphenols in the gastrointestinal tract: implications of gastric and colonic microflora-dependent biotransformation. *Free Radical Research, 40(6),* 647-658.

Corona, G., Spencer, J. P. E., & Dessì, M. A. (2009). Extra virgin olive oil phenolics: absorption, metabolism, and biological activities in the GI tract. *Toxicology and Industrial Health, 25*, 285-293.

Cortese-Krott, M. M., Rodriguez-Mateos, A., Sansone, R., Kuhnle, G. G., Thasian-Sivarajah, S., Krenz, T., et al., (2012). Human red blood cells at work: identification and visualization of erythrocytic eNOS activity in health and disease. *Blood, 120*, 4229-4237.

Cosan, D. T., Bayram, B., Soyocak, A., Basaran, A., Gunes, H. V., Degirmenci, I., et al. (2010). Role of phenolic compounds in nitric oxide synthase activity in colon and breast adenocarcinoma. *Cancer Biotherapy and Radiopharmaceuticals, 25(5)*, 577-580.

Covas, M. I., Nyyssonen, K., Poulsen, H. E., Kaikkonen, J., Zunft, H. J., Kiesewetter, H., et al. (2006b). The effect of polyphenols in olive oil on heart disease risk factors: a randomized trial. *Annals of Internal Medicine, 145(5)*, 333-341.

Covas, M. I., Ruiz-Gutierrez, V., de la Torre, R., Kafatos, A., Lamuela-Raventos, R. M. Osada, J., et al. (2006a). Minor components of olive oil: evidence to date of health benefits in humans. *Nutrition Reviews, 64(10),* 20-30.

Covas, M. I. (2008). Bioactive effects of olive oil phenolic compounds in humans: reduction of heart disease factors and oxidative damage. *Inflammopharmacology, 16(5),* 216-218.

Csonka, C., Pali, T., Bencsik, P., Görbe, A., Ferdinandy, P., & Csont, T. (2015). Measurement of NO in biological samples. *British Journal of Pharmacology, 172*, 1620-1632.

D'Alessandro, A., & De Pergola, G. (2014). Mediterranean Diet Pyramid: A Proposal for Italian People. *Nutrients, 6*, 4302-4316.

D'Angelo, S., Manna, C., Migliardi, V., Mazzoni, O., Morrica, P., Capasso, G. et al. (2001). Pharmacokinetics and metabolism of hydroxytyrosol, a natural antioxidant from olive oil. *Drug Metabolism Disposition, 29(11),* 1492-1498.

Davenpeck, K. L., Gauthier, T. W., & Lefer, A. M. (1994). Inhibition of endothelial-derived nitric oxide promotes P-selectin expression and actions in the rat microcirculation. *Gastroenterology, 107*, 1050-1058.

Davis, K. L., Martin, E., Turko, I. V., & Murad, F. (2001). Novel effects of nitric oxide. *Annual Review of Pharmacology and Toxicology, 41*, 203-236.

Deiana, M., Incani, A., Rosa, A., Atzeri, A., Loru, D., Cabboi, B., et al. (2011). Hydroxytyrosol glucuronides protect renal tubular epithelial cells against H_2O_2 induced oxidative damage. *Chemico-Biological Interactions, 193*, 232-239.

De la Puerta, R., Martinez Dominguez, M. E., Ruiz-Gutierrez, V., Flavill, J. A., &. Hoult, J. R. (2001). Effects of virgin olive oil phenolics on scavenging of reactive nitrogen species and upon nitrergic neurotransmission. *Life Sciences, 69(10),* 1213-1222.

De la Torre, R. (2008). Bioavailability of olive oil phenolic compounds in humans. *Inflammopharmacology, 16*, 245-247.

Del Chierico, F., Vernocchi, P., Dallapiccola, B. & Putignani, L. (2014). Mediterranean diet and health: food effects on gut microbiota and disease control. *International Journal of Molecular Sciences, 15(7)*, 11678-11699.

Dernini, S., Berry, E. M., Serra-Majem, L., La Vecchia, C., Capone, R., Medina, F. X., et al. (2017). Med Diet 4.0: the Mediterranean diet with four sustainable benefits. *Public Health Nutrition, 20(7)*, 1322-1330.

Dijkstra, G., Moshage, H., van Dullemen, H. M., de Jager-Krikken, A., Tiebosch, A. T., Kleibeuker, J. H., et al. (1998). Expression of nitric oxide synthases and formation of nitrotyrosine and reactive oxygen species in inflammatory bowel disease. *Journal of Pathology, 186*, 416-421.

Dimmeler, S., Hermann, C., Galle, J., & Zeiher, A. M. (1999). Upregulation of super-oxide dismutase and nitric oxide synthase mediates the apoptosis-suppressive effects of shear stress on endothelial cells. *Arteriosclerosis, Thrombosis, and Vascular Biology, 19*, 656-664.

Dinis, T. C., Santosa, C. L., & Almeida, L. M. (2002). The apoprotein is the preferential target for peroxynitrite-induced LDL damage protection by dietary phenolic acids. *Free Radical Research, 36*, 531-543.

Donnini, S., & Ziche, M. (2002). Constitutive and inducible nitric oxide synthase: role in angiogenesis. *Antioxidants & Redox Signaling, 4(5)*, 817-823.

Dontas, A. S., Zerefos, N. S., Panagiotakos, D. B., & Valis, D. A. (2007). Mediterranean diet and prevention of coronary heart disease in the elderly. *Clinical Interventions in Aging, 2(1)*, 109-115.

Drummond, G. R., Cai, H., Davis, M. E., Ramasamy, S., & Harrison, D. G. (2000). Transcriptional and posttranscriptional regulation of endothelial nitricoxide synthase expression by hydrogen peroxide. *Circulation Research, 86*, 347-354.

Duarte, J., Francisco, V., & Perez-Vizcaino, B. (2014). Modulation of nitric oxide by flavonoids. *Food & Function, 5*, 1653-1668.

Dudzinski, D. M., Igarashi, J., Greif, D., & Michel, T. (2006). The regulation and pharmacology of endothelial nitric oxide synthase. *Annual Review of Pharmacology and Toxicology, 46*, 235-276.

Durante, W., Kroll, M. H., Christodoulides, N., Peyton, K. J., & Schafer, A. I. (1997). Nitric oxide induces heme oxygenase-1 gene expression and carbon monoxide production in vascular smooth muscle cells. *Circulation Research, 80*, 557-564.

Eckmann, L. W. Stenson, F., Savidge, T. C., Lowe, D. C., Barrett, K. E., Fierer, J., et al. (1997). Role of intestinal epithelial cells in the host secretory response to infection by invasive bacteria. Bacterial entry induces epithelial prostaglandin H synthase-2 expression and prostaglandin E2 and F2a production. *Journal of Clinical Investigation, 100*, 296-309.

Ekbatan, S. S., Sleno, L., Sabally, K., Khairallah, J., Azadi, B., Rodes, L. et al. (2016). Biotransformation of polyphenols in a dynamic multistage gastrointestinal model. *Food Chemistry, 204*, 453-462.

El-Milli, N., Rodrigo, R., Naghizadeh, B., Cauli, O., & Felipo, V. (2008). Chronic hyperammonemia reduces the activity of neuronal nitric oxide synthase in cerebellum by altering its localization and increasing its phosphorylation by calcium-calmodulin kinase II. *Journal of Neurochemistry, 106*, 1440-1449.

Erwin, P. A., Lin, A. J., Golan, D. E., & Michel, T. (2005). Receptor-regulated dynamic S-nitrosylation of endothelial nitric-oxide synthase in vascular endothelial cells. *Journal of Biological Chemistry, 280*, 19888-19894.

Fabiani, R., De Bartolomeo, A., Rosignoli, P., Servili, M., Montedoro, G. F., Morozzi, G., et al. (2002). Cancer chemoprevention by hydroxytyrosol isolated from virgin olive oil through G1 cell cycle arrest and apoptosis. *European Journal of Cancer Prevention, 11*, 351-358.

Feliciano, R. P., Boeres, A., Massacessi, L., Istas, G., Ventura, M. R., Nunes dos Santos, G., et al. (2016). Identification and quantification of novel cranberry-derived plasma and urinary (poly)phenols. *Archives of Biochemistry and Biophysics, 599*, 31-41.

Fernandez, M. A., Saenz, M. T., & Garcia, M. D. (1998). Anti-inflammatory activity in rats and mice of phenolic acids isolated from Scrophularia frutescens. *The Journal of Pharmacy and Pharmacology, 50*, 1183-1186.

Fito, M., de la Torre, R., & Covas, M. I. (2007). *Olive oil and oxidative stress. Molecular Nutrition and Food Research, 51(10),* 1215-1224.

Forman, H. J., Davies, K. J. A., & Ursini, F. (2014). How do nutritional antioxidants really work: Nucleophilic tone and para-hormesis versus free radical scavenging in vivo. *Free Radical Biology & Medicine, 66*, 24-35.

Förstermann, U. & Li, H. (2011). Therapeutic effect of enhancing endothelial nitric oxide synthase (eNOS) expression and preventing eNOS uncoupling. *British Journal of Pharmacology, 164*, 213-223.

Förstermann, U., & Sessa, W. C. (2012). Nitric oxide synthases: regulation and function. *European Heart Journal, 33*, 829-837.

Fuentes, E., & Palomo, I. (2014). Mechanisms of endothelial cell protection by hydroxycinnamic acids. *Vascular Pharmacology, 63*, 155-161.

Fujimoto, T., Nakade, S., Miyawaki, A., Mikoshiba, K., & Ogawa, K. (1992). Localization of inositol 1,4,5-trisphosphate receptor-like protein in plasmalemmal caveolae. *Journal of Cell Biology, 119(6)*, 1507-1513.

Fukai, T., Siegfried, M. R., Ushio-Fukai, M., Cheng, Y., Kojda, G., & Harrison, D. G. (2000). Regulation of the vascular extracellular superoxide dismutase bynitric oxide and exercise training. *Journal of Clinical Investigation, 105*, 1631-1639.

Furchgott, R. F., & Zawadzki, J. V. (1980). The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature, 288*, 373-376.

García-Fernández, E., Rico-Cabanas, L., Rosgaard, N., Estruch, R., & Bach-Faig, A. (2014). Mediterranean Diet and Cardiodiabesity: A Review. *Nutrients, 6*, 3474-3500.

Garg, U. C., & Hassid, A. (1990). Nitric oxide-generating vasodilators inhibit mitogenesis and proliferation of BALB/C 3T3 fibroblasts by a cyclicGMP-independent mechanism. *Biochemical and Biophysical Research Communications, 171*, 474-479.

Ghosh, S., Basak, P., Dutta, S., Chowdhury, S., & Sil, P. C. (2017). New insights into the ameliorative effects of ferulic acid in pathophysiological conditions. *Food and Chemical Toxicology, 103*, 41-55.

Gimeno, E., Fito, M., Lamuela-Raventos, R. M., Castellote, A. I., Covas, M., Farre, M., et al. (2002). Effect of ingestion of virgin olive oil on human low-density lipoprotein composition. *European Journal of Clinical Nutrition, 56(2),* 114-120.

Gonzalez-Santiago, M., Fonolla, J., & Lopez-Huertas, E. (2010). Human absorption of a supplement containing purified hydroxytyrosol, a natural antioxidant from olive oil, and evidence for its transient association with low-density lipoproteins. *Pharmacology Research, 61*, 364-370.

Goodwin, D. C., Gunther, M. R., Hsi, L. C., Crews, B. C., Eling, T. E., Mason, R. P., et al. (1998). Nitric oxide trapping of tyrosyl radicals generated during prostaglandin endoperoxide synthase turnover. Detection of the radical derivative of tyrosine 385. *Journal of Biological Chemistry, 273*, 8903-8909.

Gorinstein, S., Leontowicz, H., Lojek, A., Leontowicz, M., Ciz, M., Krzeminski, R., et al. (2002). Olive oils improve lipid metabolism and increase antioxidant potential in rats fed diets containing cholesterol. *Journal of Agricultural and Food Chemistry, 50(21)*, 6102-6108.

Graf, E. (1992). Antioxidant potential of ferulic acid. *Free Radical Biology and Medicine, 13*, 435-448.

Grignaffini, P., Roma, P., Galli, C., & Catapano, A. L. (1994). Protection of low-density lipoprotein from oxidation by 3,4-dihydroxyphenylethanol. *Lancet, 343*, 1296-1297.

Guichard, C., Pedruzzi, E., Fay, M., Marie, J. C., Braut-Boucher, F., Daniel, F., et al. (2006) Dihydroxyphenylethanol induces apoptosis by activating serine/threonine protein phosphatase PP2A and promotes the endoplasmic reticulum stress response in human colon carcinoma cells. *Carcinogenesis, 27*, 1812-1827.

Gunther, M. R., Hsi, L. C., Curtis, J. F., Gierse, J. K., Marnett, L. J., Eling, T. E., et al. (1997). Nitric oxide trapping of the tyrosyl radical of prostaglandin H synthase-2 leads to tyrosine iminoxyl radical and nitrotyrosine formation. *Journal of Biological Chemistry*, 272, 17086-17090.

Guo, S., Al-Sadi, R., Said, H. M., & Ma, T. Y. (2013). Lipopolysaccharide causes an increase in intestinal tight junction permeability in vitro and in vivo by inducing enterocyte membrane expression and localization of TLR-4 and CD14. *American Journal of Pathology, 182*, 375-387.

Guo, S., Nighot, M., Al-Sadi, R., Alhmoud, T., Nighot, P., & Ma, T. Y. (2015). Lipopolysaccharide regulation of intestinal tight junction permeability is mediated by TLR4 signal transduction pathway activation of FAK and MyD88. *Journal of Immunology, 195(10)*, 4999-5010.

Habib, S., & Ali, A. (2011). Biochemistry of Nitric Oxide. *Indian Journal of Clinical Biochemistry, 26(1)*, 3-17.

Halliwell, B., Aeschbach, R., Loliger, J. & Aruoma, O. I. (1995). The characterization of antioxidants. *Food and Chemical Toxicology, 33*, 601-617.

Han, X., Fink, M. P., Yang, R., & Delude, R. L. (2004). Increased inos activity is essential for intestinal epithelial tight junction dysfunction in endotoxemic mice. *Shock, 21(3)*, 261-270.

Han, X., Shen, T., & Lou, H. (2007). Dietary Polyphenols and Their Biological Significance. *International Journal of Molecular Sciences, 8*, 950-988.

Hentze, M. W., Kuhn, L. C., (1996). Molecular control of vertebrate iron metabolism: mRNA-based regulatory circuits operated by iron, nitric oxide, and oxidative stress. *National Academy of Sciences of the United States of America*, *93*, 8175-8182.

Heiss, C., Schanz, A., Amabile, N., Jahn, S., Chen, Q., Wong, M. L., et al. (2010). Nitric oxide synthase expression and functional response to nitric oxide are both important modulators of circulating angiogenic cell response to angiogenic stimuli. *Arteriosclerosis, Thrombosis, and Vascular Biology, 30*, 2212-2218.

Heiss, E. H., & Dirsch, V. M. (2014). Regulation of eNOS enzyme activity by posttranslational modification. *Current Pharmaceutical Design, 20(22)*, 3503-3513.

Heleno, S. A., Martins, A., Queiroz, M. J. R. P., & Ferreira, I. C. F. R. (2015). Bioactivity of phenolic acids: Metabolites versus parent compounds: A review. *Food Chemistry, 173(15)*, 501-513.

Hemmens, B., Goessler, W., Schmidt, K., & Mayer B. (2000). Role of bound zinc in dimer stabilization but not enzyme activity of neuronal nitric-oxide synthase. *Journal of Biological Chemistry, 275,* 35786-35791.

Herrmann, K. (1989) Occurrence and content of hydroxycinnamic and hydroxybenzoic acid compounds in foods. *Critical Reviews in Food Science and Nutrition, 28,* 315-347.

Horowitz, A., Menice, C. B., Laporte, R., & Morgan, K. G. (1996). Mechanisms of smooth muscle contraction. *Physiological Reviews, 76,* 967-1003.

Hou, Y. C., Janczuk, A., & Wang, P. G. (1999). Current trends in the development of nitric oxide donors. *Current pharmaceutical design,* 5*(6),* 417-441.

Hu, T., He, X-W., Jiang, J-G., & Xu, X-L. (2014). Hydroxytyrosol and its potential therapeutic effects. *Journal of Agricultural and Food Chemistry, 62,* 1449-1455.

Huang, G. T., Eckmann, L., Savidge, T. C., & Kagnoff, M. F. (1996) Infection of human intestinal epithelial cells with invasive bacteria upregulates apical intercellular adhesion molecule-1 (ICAM-1) expression and neutrophil adhesion. *Journal of Clinical Investigation, 98,* 572-583.

Huang, C. L., & Sumpio, E. B. (2008). Olive Oil, the Mediterranean Diet, and Cardiovascular Health. *Journal of the American College of Surgeons, 207(3),* 407-416.

Huang, F. P., Niedbala, W., Wei, X. Q., Xu, D., Feng, G. J., & Robinson, J. H. (1998). Nitric oxide regulates Th1 cell development through the inhibition of IL-12 synthesis by macrophages. *European Journal of Immunology, 28,* 4062-4070.

Hughes, M. N. (2008). Chemistry of nitric oxide and related species. *Method Enzymology, 436,* 3-19.

Hung, M.-J., Kao, Y.-C., Mao, C.-T., Chen, T.-H., & Chen, W.-S. (2016). Aliskiren attenuates the effects of interleukin-6 on endothelial nitric oxide synthase and caveolin-1 in human aortic endothelial cells. *Nitric Oxide - Biology and Chemistry, 61,* 45-54.

Hussain, T., Tan, B., Yin, Y., Blachier, F., Tossou, M. C. B. & Rahu, N. (2016). Oxidative Stress and Inflammation: What Polyphenols Can Do for Us? *Oxidative Medicine and Cellular Longevity, Article ID 7432797,* 1-9.

Imbert, V., Rupec, R. A., Livolsi, A., Pahl, H. L., Traenckner, E. B., Mueller-Dieckmann, C., et al. (1996). Tyrosine phosphorylation of I kappa B-alpha activates NF-kappa B without proteolytic degradation of I kappa B-alpha. *Cell, 86,* 787-798.

Incani, A., Deiana, M., Corona, G., Vafeiadou, K., Vauzour, D., Dessì, M. A., et al. (2010). Involvement of ERK, Akt and JNK signalling in H2O2-induced cell injury and protection by hydroxytyrosol and its metabolite homovanillic alcohol. *Molecular Nutrition and Food Research, 54(6),* 788-796.

Incani, A., Serra, G., Atzeri, A., Melis, M. P., Serreli, G., Bandino, G., et al. (2016). Extra virgin olive oil phenolic extracts counteract the pro-oxidant effect of dietary oxidized lipids in human intestinal cells. *Food and Chemical Toxicology, 90,* 171-180.

Ishida, A., Sasaguri, T., Kosaka, C., Nojima, H., & Ogata, J. (1997). Induction of the cyclin-dependent kinase inhibitor p21 (Sdi1/Cip1/Waf1) by nitric oxide-generating vasodilator in vascular smooth muscle cells. *Journal of Biological Chemistry, 272,* 10050-10057.

Islam, D., Veress, B., Bardhan, P. K., Lindberg, A. A., & Christensson, B. (1997). In situ characterization of inflammatory responses in the rectal mucosae of patients with shigellosis. *Infection and Immunity, 65,* 739-749.

Jenkins, D. C., Charles, I. G., Baylis, S. A, Lelchuk, R., Radomski, M. W., & Moncada, S. (1994). Human colon cancer cell lines show a diverse pattern of nitric oxide synthase gene expression and nitric oxide generation. *British Journal of Cancer, 70*, 847-849.

Jenner, A. M., Rafter, J., & Halliwell, B. (2005). Human fecal water content of phenolics: The extent of colonic exposure to aromatic compounds. *Free Radical Biology & Medicine, 38,* 763-772.

Jeon, Y. J., Han, S. H., Lee, Y. W., Yea, S. S., & Yang, K. H. (1998). Inhibition of NF-κB/Rel nuclear translocation by dexamethasone: mechanism for the inhibition of iNOS gene expression. *Biochemistry and Molecular Biology International, 45*, 435-441.

Jiang, X., Mu, D., Manabat, C., Koshy, A. A., Christen, S., Täuber, M. G. et al. (2004). Differential vulnerability of immature murine neurons to oxygen-glucose deprivation. *Experimental Neurology, 190*, 224-232.

Jin, Y., Yan, E. Z., Fan, Y., Zong, Z. H., Qi, Z. M., & Li, Z. (2005). Sodium ferulate prevents amyloid-beta-induced neurotoxicity through suppression of p38 MAPK and upregulation of ERK-1/2 and Akt/protein kinase B in rat hippocampus. *Acta Pharmacologica Sinica, 26*, 943-951.

Jung, H. C., Eckmann, L., Yang, S. K., Panja, A., Fierer, J., Morzycka-Wroblewska, E., et al. (1995). A distinct array of proinflammatory cytokines is expressed in human colon epithelial cells in response to bacterial invasion. *Journal of Clinical Investigation, 95*, 55-65.

Kampa, M., Alexaki, V. I., Notas, G., Nifli, A. P., Nistikaki, A., Hatzoglou, A., et al. (2004). Antiproliferative and apoptotic effects of selective phenolic acids on T47D human breast cancer cells: Potential mechanisms of action. *Breast Cancer Research, 6*, 63-74.

Kanarek, N., London, N., Schueler-Furman, O., & Ben-Neriah, Y. (2010). Ubiquitination and degradation of the inhibitors of NF-kappaB. *Cold Spring Harbor Perspectives in Biology, 2(2).*

Kane, L. P., Shapiro, V. S., Stokoe, D., & Weiss, A. (1999). Induction of NF-kappaB by the Akt/PKB kinase. *Current Biology, 9(11),* 601-604.

Kang, J., Liu, Y., Xie, M. X., Li, S., Jiang, M., & Wang, Y. D. (2004). Interactions of human serum albumin with chlorogenic acid and ferulic acid. *Biochimica Et Biophysica Acta – General Subjects, 1674*, 205-214.

Kapiszewska, M., Soltys, E., Visioli, F., Cierniak, A., & Zaja, G. (2005). The protective ability of the Mediterranean plant extracts against the oxidative DNA damage: the role of the radical oxygen species and the polyphenol content. *Journal of Physiology and Pharmacology, 56,* 183-197.

Karampola, M., Papandreou, D., & Makedou, K. (2011). The role of Mediterranean diet in health and disease: an updated mini review. *Nutrition & Food Science, 41(1),* 63-72.

Karin, M. (1999). How NF-kappaB is activated: the role of the IkappaB kinase (IKK) complex. *Oncogene, 18(49),* 6867-6874.

Kawabata, K., Yamamoto, T., Hara, A., Shimizu, M., Yamada, Y., Matsunaga, K., et al. (2000). Modifying effects of ferulic acid on azoxymethane-induced colon carcinogenesis in F344 rats. *Cancer Letters, 157*, 15-21.

Kern, S. M., Bennett, R. N., Needs, P. W., Mellon, F. A., Kroon, P. A., & Garcia-Conesa, M. T. (2003). Characterization of metabolites of hydroxycinnamates in the in vitro model of human small intestinal epithelium caco-2 cells. *Journal of Agricultural and Food Chemistry, 51,* 7884-7891.

Khan, B. V., Harrison, D. G., Olbrych, M. T., Alexander, R. W., & Medford, R. M. (1996). Nitric oxide regulates vascular cell adhesion molecule 1 gene expression and redox-sensitive transcriptional events in human vascularendothelial cells. *Proceedings of the National Academy of Sciences of the United States of America*, 93, 9114-9119.

Khurana, S., Venkataraman, K., Hollingsworth, A., Piche, M., & Tai, T. C. (2013). Polyphenols: Benefits to the Cardiovascular System in Health and in Aging. *Nutrients, 5(10)*, 3779-3827.

Khymenets, O., Farre, M., Pujadas, M., Ortiz, E., Joglar, J., & Covas, M. I. (2011). Direct analysis of glucuronidated metabolites of main olive oil phenols in human urine after dietary consumption of virgin olive oil. *Food Chemistry, 126*, 306-314.

Khymenets, O., Fito, M., Tourino, S., Muñoz-Aguayo, D., Pujadas, M., Torrese, J. L., et al. (2010). Antioxidant activities of hydroxytyrosol main metabolites do not contribute to beneficial health effects after olive oil ingestion. *Drug Metabolism & Disposition, 38*, 1417-1421.

Kikugawa, M., Ida, T., Ihara, H., Sakamoto, T. (2017). Ferulic acid and its water-soluble derivatives inhibit nitric oxide production and inducible nitric oxide synthase expression in rat primary astrocytes. *Bioscience, Biotechnology and Biochemistry, 81(8)*, 1607-1611.

Kilbourn, R. G., Jubran, A., Gross, S. S., Griffith, O. W., Levi, R., Adams, J., et al. (1990). Reversal of endotoxin-mediated shock by NG-methyl-L-arginine, an inhibitor of nitric oxide synthesis. *Biochemical and Biophysical Research Communications, 172*, 1132-1138.

Kim, E. O., Min, K. J., Kwon, T. K., Um, B. H., Moreau, R. A., & Choi, S. W. (2012). Anti-inflammatory activity of hydroxycinnamic acid derivatives isolated from corn bran in lipopolysaccharide-stimulated Raw 264.7 macrophages. *Food and Chemical Toxicology, 50(5)*, 1309-1316.

Kim, H. S., Cho, J. Y., Kim, D. H., Yan, J. J., Lee, H. K., Suh, H. W., et al. (2004). Inhibitory effects of long-term administration of ferulic acid on microglial activation induced by intracerebroventricular injection of beta-amyloid peptide (1–42) in mice. *Biological & Pharmaceutical Bulletin, 27*, 120-121.

Kim, Y., Kim, D-M., & Kim, J. Y. (2017). Ginger extract suppresses inflammatory response and maintains barrier function in human colonic epithelial Caco-2 cells exposed to inflammatory mediators. *Journal of Food Science, 82(5)*, 1264-1270.

Klabunde, R. E. (2016). Nitric oxide. Cardiovascular Physiology Concepts, URL http://www.cvphysiology.com/Blood%20Flow/BF011. Achieved 05/09/2017.

Kleinert, H., Euchenhofer, C., Ihrig Biedert, I., & Förstermann, U. (1996). Glucocorticoids inhibit the induction of nitric oxide synthase II by down-regulating cytokine-induced activity of transcription factor nuclear factor-κB. *Molecular Pharmacology, 49*, 15-21.

Kleinert, H., Boissel, J. P., Schwarz, P. M., & Förstermann, U. (2000). Regulation of the expression of nitric oxide synthase isoforms. *In: Nitric Oxide: Biology and Pathobiology, L.J. Ignarro, ed. (New York, USA: Academic Press)*, 105-128.

Kleinert, H., Schwarz, P. M., & Forstermann, U. (2003). Regulation of the expression of inducible Nitric Oxide Synthase. *Biological Chemistry, 384*, 1343-1364.

Klotz, T., Bloch, W., Jacobs, G., Niggermann, S., Englemann, U., & Addicks, K. (1999). Immunolocalization of inducible and constitutive nitric oxide synthases in human bladder cancer. *Urology, 54*, 416-419.

Klotz, T., Bloch, W., Volberg, C., Engelmann, U., & Addicks, K. (1998). Selective expression of inducible nitric oxide synthase in human prostatic carcinoma. *Cancer, 82*, 1897-1903.

Kojda, G., & Harrison, D. (1999). Interactions between NO and reactive oxygenspecies: pathophysiological importance in atherosclerosis, hypertension, diabetes and heart failure. *Cardiovascular Research, 43*, 562-571.

Kolios, G., Brown, Z., Robson, R. L., Robertson, D. A. F. & Westwick, J. (1995). Inducible nitric oxide synthase activity and expression in a human colonic epithelial cell line, HT-29. *British Journal of Pharmacology, 116*, 2866-2872.

Kohyama, N., Nagata, T., Fujimoto, S., & Sekiya, K. (1997). Inhibition of arachidonate lipoxygenase activities by 2-(3,4-dihydroxyphenyl)ethanol, a phenolic compound from olives. *Bioscience, Biotechnology, and Biochemistry, 61*, 347-350.

Koppenol, W. H. (1998). The basic chemistry of nitrogen monoxide and peroxynitrite. *Free Radical Biology & Medicine, 25*, 385-391.

Korhonen, R., Lahti, A., Kankaanranta, H., & Moilanen, E. (2005). Nitric Oxide Production and Signaling in Inflammation. *Current Drug Targets - Inflammation & Allergy, 4*, 471-479.

Kotronoulas, A., Pizarro, N., Serra, A., Robledo, P., Joglar, J., Rubió, L., et al. (2013). Dose-dependent metabolic disposition of hydroxytyrosol and formation of mercapturates in rats. *Pharmacology Research, 77*, 47-56.

Krause, B. J., Prieto, C. P., Muñoz-Urrutia, E., San Martín, S., Sobrevia, L., & Casanello, P. (2012). Role of arginase-2 and eNOS in the differential vascular reactivity and hypoxia-induced endothelial response in umbilical arteries and veins. *Placenta, 33*, 360-366.

Kroon, P. A., Clifford, M. N., Crozier, A., Day, A. J., Donovan, J. L., Manach, C., et al. (2004). How should we assess the effects of exposure to dietary polyphenols in vitro? *American Journal of Clinical Nutrition, 80(1)*, 15-21.

Kulp, A., & Kuehn, M. J. (2010). Biological functions and biogenesis of secreted bacterial outer membrane vesicles. *Annual Reviews of Microbiology, 64*, 163-184.

Kushi, L. H., Lenart, E. B., & Willett, W. C. (1995a). Health implications of Mediterranean diets in light of contemporary knowledge: 1. Plant foods and dairy products. *American Journal of Clinical Nutrition, 61*, 1407-1415.

Kushi, L. H., Lenart, E. B., & Willett, W. C. (1995b). Health implications of Mediterranean diets in light of contemporary knowledge: 2. Meat, wine, fats and oils. *American Journal of Clinical Nutrition, 61*, 1416-1427.

Ladurner, A., Schachner, D., Schueller, K., Pignitter, M., Heiss, E. H., Somoza, V., et al. (2014). Impact of trans-resveratrol-sulfates and -glucuronides on endothelial nitric oxide synthase activity, nitric oxide release and intracellular reactive oxygen species. *Molecules, 19(10),* 16724-16736.

Lampiasi, N., & Montana, G. (2016). The molecular events behind ferulic acid mediated modulation of IL-6 expression in LPS-activated Raw 264.7 cells. *Immunobiology, 221(3)*, 486-493.

Lander, H. M., Hajjar, D. P. Hempstead, B. L. Mirza, U. A., Chait, B. T., Campbell, S., et al. (1997). A molecular redox switch on p21ras structural basis for the nitric oxide-p21ras interaction. *Journal of Biological Chemistry, 272*, 4323-4236.

Lanone, S., Manivet, P., Callebert, J., Launay, J. M., Payen, D., Aubier, M., et al., (2002). Inducible nitric oxide synthase (NOS2) expressed in septic patients is nitrated on selected tyrosine residues: implications for enzymic activity. *The Biochemical Journal, 366*, 399-404.

Lau, K. S., Grange, R. W., Isotani, E., Sarelius, I. H., Kamm, K. E., Huang, P. L., et al., (2000). nNOS and eNOS modulate cGMP formation and vascular response in contracting fast-twitch skeletal muscle. *Physiological Genomics, 2*, 21-27.

Le Mellédo, J-M., Mahil, N., & Baker, G. B. (2004). Nitric oxide: A key player in the relation between cardiovascular disease and major depressive disorder? *Journal of Psychiatry & Neuroscience, 29(6),* 414-416.

Lewandowska, K., Kalinowska, M., Lewandowski, W., Stępkowski, T. M., & Brzóska, M. (2016). The role of natural polyphenols in cell signaling and cytoprotection against cancer development. *Journal of Nutritional Biochemistry, 32,* 1-19.

Lin, F. H., Lin J. Y., Gupta, R. D., Tournas, J. A., Burch, J. A., Selim, M. A., et al. (2005). Ferulic acid stabilizes a solution of vitamins C and E and doubles its photoprotection of skin. *Journal of Investigative Dermatology, 125(4),* 826-832.

Liu, D., & Liman, E. R. (2003a). Intracellular Ca^{2+} and the phospholipid PIP2 regulate the taste transduction ion channel TRPM5. *Proceedings of the National Academy of Sciences of the United States of America, 100(25),* 15160-15165.

Liu, I. M., Hsu, F. L., Chen, C. F., & Cheng, J. T. (2000). Antihyperglycemic action of isoferulic acid in streptozotocin-induced diabetic rats. *British Journal of Pharmacology, 129,* 631-636.

Liu, S., Premont, R. T., Kontos, C. D., Huang, J., Rockey, D. C. (2003b). Endothelin-1 activates endothelial cell nitric-oxide synthase via heterotrimeric G-protein betagamma subunit signaling to protein kinase B/Akt. *The Journal of Biological Chemistry, 278(50),* 49929-49935.

Lodi, F., Jimenez, R., Moreno, L., Kroon P. A., Needs, P. W., Hughes, D. A., et al. (2009). Glucuronidated and sulfated metabolites of the flavonoid quercetin prevent endothelial dysfunction but lack direct vasorelaxant effects in rat aorta. *Atherosclerosis, 204(1),* 34-39.

Loscalzo, J. (2001). Nitric oxide insufficiency, platelet activation, and arterial thrombosis. *Circulatory Research, 88,* 56-62.

Loufrani, L., Retailleau, K., Bocquet, A., Dumont, O., Danker, K., Louis, H., et al. (2008a). Key role of alpha(1)beta(1)-integrin in the activation of PI3-kinase-Akt by flow (shear stress) in resistance arteries. *American Journal of Physiology. Heart and Circulatory Physiology, 294*, 1906-1913.

Loufrani, L., & Henrion, D. (2008b). Role of the cytoskeleton in flow (shear stress)-induced dilation and remodeling in resistance arteries. *Medical & Biological Engineering & Computing, 46(5),* 451-460.

Lu, Y., Zhu, M., Chen, W., Yin, L., Zhu, J., Chen N., et al. (2014). Tetramethylpyrazine improves oxazolone-induced colitis by inhibiting the NF-kB pathway. *Clinical & Investigative Medicine, 37(1*), 1-9.

Lucas, K. A., Pitari, G. M., Kazerounian, S., Ruiz-Stewart, I., Park, J., Schulz, S., et al. (2000). Guanylyl Cyclases and Signaling by Cyclic GMP. *Pharmacology Reviews, 52,* 375-413.

Luo, S., Lei, H., Qin, H., & Xia, Y. (2014). Molecular Mechanisms of Endothelial NO Synthase Uncoupling. *Current Pharmaceutical Design, 20*, 3548-3553.

Lv, J., Yu, L., Lu, Y., Niu, Y., Liu, L., Costa, J., et al. (2012). Phytochemical compositions, and antioxidant properties, and antiproliferative activities of wheat flour. *Food Chemistry, 135*, 325-331.

Mackenzie, I. S., Rutherford, D., & Macdonald, T. (2008). Nitric oxide and cardiovascular effects: New insights in the role of nitric oxide for the management of osteoarthritis. *Arthritis Research & Therapy, 10,* Suppl 2, S3.

Maines, M. D. (1997). The heme oxygenase system: a regulator of second messenger gases. *Annual Review of Pharmacology and Toxicology, 37*, 517-554.

Maiuri, M. C., De Stefano, D., Di Meglio, P., Irace, C., Savarese, M., Sacchi, R., et al. (2005). Hydroxytyrosol, a phenolic compound from virgin olive oil, prevents macrophage activation. *Naunyn-Schmiedeberg's Archives of Pharmacology, 371(6)*, 457-465.

Manach, C., Scalbert, A., Morand, C., Remesy, C., & Jimenez, L. (2004). Polyphenols: food sources and bioavailability. *American Journal of Clinical Nutrition, 79(5)*, 727-747.

Manna, C., Galletti, P., Cucciolla, V., Moltedo, O., Leone, A. & Zappia, V. (1997). The protective effect of the olive oil polyphenol (3,4-dihydroxyphenyl)ethanol counteracts reactive oxygen metabolite-induced cytotoxicity in Caco-2 cells. *Journal of Nutrition, 127*, 286-292.

Manna, C., Galletti, P., Maisto, G., Cucciolla, V., D'Angelo, S., & Zappia, V. (2000). Transport mechanism and metabolism of olive oil hydroxytyrosol in Caco-2 cells. *FEBS Letters, 470(3)*, 341-344.

Mannick, E. E., Bravo, L. E., Zarama G, Realpe, J. L., Zhang, X. J., Ruiz, B., et al. (1996). Inducible nitric oxide synthase, nitrotyrosine. and apoptosis in *Helicobacter pylori* gastritis: effect of antibiotics and antioxidants. *Cancer Research, 56*, 3238-3243.

Mansuri, M. L., Parihar, P., Solaski, I., & Parihar, M. S. (2014). Flavonoids in modulation of cell survival signalling pathways. *Genes & Nutrition, 9*, 400-409.

Marion, R., Coeffier, M., Leplingard, A., Favennec, L., Ducrotte, P., & Dechelotte, P. (2003). Cytokine-stimulated nitric oxide production and inducible NO-synthase mRNA level in human intestinal cells: lack of modulation by glutamine. *Clinical Nutrition, 22*, 523-528.

Marrugat, J., Covas, M. I., Fito, M., Schroder, H., Miro-Casas, E., Gimeno, E., et al. (2004). Effects of differing phenolic content in dietary olive oils on lipids and LDL oxidation-a randomized controlled trial. *European Journal of Nutrition, 43(3)*, 140-147.

Massberg, S., Sausbier, M., Klatt, P., Bauer, M., Pfeifer, A., Siess, W., et al., (1999). Increased adhesion and aggregation of platelets lacking cyclic guanosine 3',5'-monophosphate kinase I. *The Journal of Experimental Medicine, 189*, 1255-1264.

Matsunaga, T., Weihrauch, D. W., Moniz, M. C., Tessmer, J., Warltier, D. C., & Chilian, W. M. (2002). Angiostatin inhibits coronary angiogenesis during im-paired production of nitric oxide. *Circulation, 105*, 2185-2191.

Mayer, B., & Hemmnes, B. (1997). Biosynthesis and action of nitric oxide in mammalian cells. *Trends in Biochemical Sciences, 22(12)*, 477-481.

McLaughlan, J. M., Seth, R., Vautier, G., Robins, R. A., Scott, B. B., Hawkey, C. J. et al. (1997). Interleukin-8 and inducible nitric oxide synthase mRNA levels in inflammatory bowel disease at first presentation. *Journal of Pathology, 181*, 87-92.

Messmer, U. K., Lapetina, E. G., & Brune, B. (1995). Nitric oxide induced apoptosis in RAW 264.7 macrophages is antagonized by protein kinase C and protein kinase A activating compounds. *Molecular Pharmacology, 47*, 757-765.

Meeprom, A., Sompong, W., Chan, C. B., & Adisakwattana, S. (2013). Isoferulic acid, a new antiglycation agent, inhibits fructose- and glucose-mediated protein glycation in vitro, *Molecules, 18*, 6439-6454.

Mendelsohn, M. E., O'Neill, S., George, D., & Loscalzo, J. (1990). Inhibition of fibrinogen binding to human platelets by S-nitroso-N-acetylcysteine. *Journal of Biological Chemistry, 265*, 19028-19034.

Michell, B. J., Harris, M. B., Chen, Z. P., Ju, H., Venema, V. J., Blackstone, M. A., et al. (2002) Identification of regulatory sites of phosphorylation of the bovine endothelial nitric-oxide synthase at serine 617 and serine 635. *Journal of Biological Chemistry, 277*, 42344-42351.

Michelson A. D, Benoit, S. E., Furman, M. I., Breckwoldt, W. L., Rohrer, M. J., Barnard M. R., et al. (1996). Effects of nitric oxide/EDRF on platelet surface glycoproteins. *American Journal of Physiology, 270*, 1640-1648.

Migliori, M., Cantaluppi, V., Mannari, C., Bertelli, A. A. E., Medica, D., Quercia, A. D., et al. (2015). Caffeic acid, a phenol found in white wine, modulates endothelial nitric oxide production and protects from oxidative stress-associated endothelial cell injury. *PLoS ONE, 10(4)*, e0117530.

Miro-Casas, E., Covas, M. I., Farre, M., Fito, M., Ortuno, J., Weinbrenner, T., et al. (2003). Hydroxytyrosol disposition in humans. *Clinical Chemistry, 49(6)*, 945-952.

Moncada, S., Palmer R. M. J., & Higgs E. A. (1991). Nitric oxide physiology pathophysiology and pharmacology. *Pharmacological Reviews, 43*, 109-142.

Morin, M. J., Unno, N., Hodin, R. A. & Fink, M. P. (1998). Differential expression of inducible nitric oxide synthase messenger RNA along the longitudinal and crypt-villus axes of the intestine in endotoxemic rats. *Critical Care Medicine, 26*, 1258-1264.

Murakami, A. (2009). Chemoprevention with phytochemicals targeting inducible nitric oxide synthase. *Forum of Nutrition, 61*, 193-203.

Murakami, A., Nakamura, Y., Koshimizu, K., Takahashi, D., Matsumoto, K., Hagihara, K., et al. (2002). FA15, a hydrophobic derivative of ferulic acid, suppresses inflammatory responses and skin tumor promotion: Comparison with ferulic acid. *Cancer Letters, 180*, 121-129.

Muriana, F. J. G., Montserrat-de la Paz, S., Lucas, R., Bermudez, B., Jaramillo, S., Morales, J. C., et al. (2017). Tyrosol and its metabolites as antioxidative and antiinflammatory molecules in human endothelial cells. *Food and Function, 8(8)*, 2905-2914.

Nagasaka, R., Chotimarkorn, C., Shafiqul, I. M., Hori, M., Ozaki, H., & Ushio, H. (2007). Anti-inflammatory effects of hydroxycinnamic acid derivatives. *Biochemical and Biophysical Research Communications, 358(2)*, 615-619.

Naseem, K. M. (2005). The role of nitric oxide in cardiovascular diseases. *Molecular Aspects of Medicine, 26*, 33-65.

Nathan, C., & Xie, Q. (1994). Regulation of biosynthesis of nitric oxide. *Journal of Biological Chemistry, 269*, 13725-13728.

Nenadis, N., Wang, L. F., Tsimidou, M. Z., & Zhang, H. Y. (2005). Radical scavenging potential of phenolic compounds encountered in O. europaea products as indicated by calculation of bond dissociation enthalpy and ionization potential values. *Journal of Agricultural and Food Chemistry, 53*, 295-299.

Noah, A., & Trustwell, A. S. (2001). There are many Mediterranean diets. *Asia Pacific Journal of Clinical Nutrition, 10*, 2-9.

Noguchi, T., Sagami, I., Daff, S., & Shimizu, T. (2001). Important role of tetrahydrobiopterin in NO complex formation and interdomain electron transfer in neuronal nitric-oxide synthase. *Biochemical and Biophysical Research Communications, 282*, 1092-1097.

Noiri, E., Lee, E., Testa, J., Quigley, J., Colflesh, D., Keese, C. R., et al. (1998). Podokinesis in endothelial cell migration: role of nitric oxide. *American Journal of Physiology, 274*, 236-244.

Nosho, K., Yoshida, M., Yamamoto, H., Taniguchi, H., Adachi, Y., Mikami, M., et al. (2005). Association of Ets-related transcriptional factor E1AF expression with overexpression of matrix metalloproteinases, COX-2 and iNOS in the early stage of colorectal carcinogenesis. *Carcinogenesis, 26*, 892-899.

Obied, H. K., Prenzler, P. D., Ryan, D., Servili, M., Taticchi, A., Esposto, S., et al. (2008). Biosynthesis and biotransformations of phenol-conjugated oleosidic secoiridoids from *Olea europaea L*. *Natural Product Reports, 25*, 1167-1179.

Ono, K., Hirohata, M., & Yamada, M. (2005). Ferulic acid destabilizes preformed beta-amyloid fibrils in vitro. *Biochemical and Biophysical Research Communications, 336*, 444-449.

O'Reilly, M. S., Holmgren, L., Shing, Y., Chen, C., Rosenthal, R. A., Moses, M., et al. (1994). Angiostatin: a novel angiogen-esis inhibitor that mediates the suppression of metastases by a Lewislung carcinoma. *Cell, 79*, 315-328.

Ohshima, H., & Bartsch, H. (1994). Chronic infections and inflammatory processes as cancer risk factors: possible role of nitric oxide in carcinogenesis. *Mutation Research, 305(2)*, 253-264.

Otte, L. A., Bell, K. S., Loufrani, L., Yeh, J. C., Melchior, B., Dao, D. N., et al. (2009). Rapid changes in shear stress induce dissociation of a G alpha(q/11)-platelet endothelial cell adhesion molecule-1 complex. *Journal of Physiology, 587*, 2365-2373.

Owen, R. W., Mier, W., Giacosa, A., Hull, W. E., Spiegelhalder B. & Bartsch, H. (2000). Phenolic compounds and squalene in olive oils: the concentration and antioxidant potential of total phenols, simple phenols, secoiridoids, lignansand squalene. *Food and Chemical Toxicology, 38(8)*, 647-659.

Paiva-Martins, F., Silva, A., Almeida, V., Carvalheira, M., Serra, C., Rodrígues-Borges, J. E., et al. (2013). Protective activity of hydroxytyrosol metabolites on erythrocyte oxidative-induced hemolysis. *Journal of Agricultural and Food Chemistry, 61*, 6636-6642.

Pan, M-H., Lin-Shiau, S-Y., & Lin, J-K. (2000). Comparative studies on the suppression of nitric oxide synthase by curcumin and its hydrogenated metabolites through down-regulation of IκB kinase and NFκB activation in macrophages. *Biochemical Pharmacology, 60(11)*, 1665-1676.

Panagiotakos, D. B., Pitsavos, C., Polychronopoulos, E., Chrysohoou, C., Zampelas, A., & Thrichopoulou, A. (2004), Can a Mediterranean diet moderate the development and clinical progression of coronary heart disease? A systematic review. *Medical Science Monitor, 10(8)*, 193-198.

Panaro, M. A., Carofiglio, V., Acquafredda, A., Cavallo, P., & Cianciulli, A. (2012). Anti-inflammatory effects of resveratrol occur via inhibition of lipopolysaccharide-induced NF-kB activation in Caco-2 and SW480 human colon cancer cells. *British Journal of Nutrition, 108*, 1623-1632.

Papadopoulos, G., & Boskou, D. (1991). Antioxidant effect of natural phenols on olive oil. *Journal of the American Oil Chemists Society, 68*, 669-671.

Park, J. H., Kim, W. S., Kim, J. Y., et al. (2011). Chkl and Hsp90 cooperatively regulate phosphorylation of endothelial nitric oxide synthase at serine 1179. *Free Radical Biology and Medicine, 51*, 2217-2226.

Pastor, A., Rodrıguez-Morato, J., Olesti, E., Pujadas, M., Pérez-Mañá, C., Khymenets, O., et al. (2016). Analysis of free hydroxytyrosol in human plasma following the administration of olive oil. *Journal of Chromatography A, 1437*, 183-190.

Pei, Y-H., Chen, J., Xie, L., Cai, X-M., Yang, R-H., Wang, X. (2016). Hydroxytyrosol protects against myocardial ischemia/reperfusion injury through a PI3K/Akt-Dependent mechanism. *Mediators of Inflammation, 2016*, Article ID 1232103, 9 pages.

Perez-Maňa, C., Farre, M., Rodrıguez-Morato, J., Papaseit, E., Pujadas, M., Fitó, M. et al. (2015). Moderate consumption of wine, through both its phenolic compounds and alcohol content, promotes hydroxytyrosol endogenous generation in humans. A randomized controlled trial. *Molecular Nutrition & Food Research, 59*, 1213-1216.

Perez-Ternero, C., Macià, A., Alvarez de Sotomayor, M., Parrado, J., Motilva, M.-J., & Herrera, M.-D. (2017). Bioavailability of the ferulic acid-derived phenolic compounds of a rice bran enzymatic extract and their activity against superoxide production. *Food and Function, 21*, 8(6), 2165-2174.

Petroni, A., Blasevich, M., Papini, N., Salami, M., Sala, A., & Galli, C. (1997). Inhibition of leukocyte leukotriene B-4 production by an olive oil derived phenol identified by mass-spectrometry. *Thrombosis Research, 87*, 315-322.

Petroni, A., Blasevich, M., Salami, M., Papini, N., Montedoro, G. F., & Galli, C. (1995). Inhibition of platelet aggregation and eicosanoid production by phenolic components of olive oil. *Thrombosis Research, 78*, 151-160.

Petroni, A., Blasevich, M., Salami, M., Servili, M., Montedoro, G. F., & Galli, C. (1994). A phenolic antioxidant extracted from olive oil inhibits platelet aggregation and arachidonic acid metabolism in vitro. *World Review of Nutrition and Dietetics, 75*, 169-172.

Pigazzi, A., Heydrick, S., Folli, F., Benoit, S., Michelson, A., Loscalzo, J. (1999). Nitric oxide inhibits thrombin receptor-activating peptide-induced phosphoinositide 3-kinase activity in human platelets. *Journal of Biological Chemistry, 274*, 14368-14375.

Poquet, L., Clifford, M. N., & Williamson, G. (2007). Transport and metabolism of ferulic acid through the colonic epithelium. *Drug metabolism and disposition, 36*, 190-197.

Puglisi, M. A., Cenciarelli, C., Tesori, V., Cappellari, M., Martini, M., Di Francesco, A. M., et al. (2015). High nitric oxide production, secondary to inducible nitric oxide synthase expression, is essential for regulation of the tumour-initiating properties of colon cancer stem cells. *Journal of Pathology, 236*, 479-490.

Rabini, R. A., Vignini, A., Salvolini, E., Staffolani, R., Martarelli, D., Moretti, N., et al. (2002). Activation of human aortic endothelial cells by LDL from Type 1 diabetic patients: an in vitro study. *Atherosclerosis, 165(1)*, 69-77.

Rachmilewitz, D., Stamler, J. S., Bachwich, D., Karmeli, F., Ackerman, Z. & Podolsky, D. K. (1995). Enhanced colonic nitric oxide generation and nitric oxide synthase activity in ulcerative colitis and Crohn's disease. *Gut, 36*, 718-723.

Radi, R., Beckman, J. S., Bush, K. M., & Freeman, B. A. (1991). Peroxynitrite oxidation of sulfhydryls. The cytotoxic potential of superoxide and nitric oxide. *Journal of Biological Chemistry, 266*, 4244-4250.

Radomski, M. W., Palmer, R. M., & Moncada, S. (1987). Endogenous nitric oxide inhibits human platelet adhesion to vascular endothelium. *Lancet, 2*, 1057-1058.

Rameau, G. A., Chiu, L. Y., Ziff, E. B. (2004). *Journal of Biological Chemistry, 279*, 4307-4314.

Rameau, G. A., Tukey, D. S., Garcin-Hosfield, E. D., Titcombe, R. F., Misra, C., & Khatri, L., et al. (2007). Biphasic coupling of neuronal nitric oxide synthase phosphorylation to the NMDA receptor regulates AMPA receptor trafficking and neuronal cell death. *Journal of Neurosciences, 27*, 3445-3455.

Rassaf, T., Heiss, C., Hendgen-Cotta, U., Balzer, J., Matern, S., Kleinbongard, P., et al (2006). Plasma nitrite reserve and endothelial function in the human forearm circulation. *Free Radical Biology & Medicine, 41*, 295-301.

Ravi, K., Brennan, L. A., Levic, S., Ross, P. A., & Black, S. M. (2004). S-nitrosylation of endothelial nitric oxide synthase is associated with monomerization and decreased enzyme activity. *Proceedings of the National Academy of Sciences of the United States of America, 101*, 2619-2624.

Recalcati, S., Taramelli, D., Conte, D., & Cairo, G. (1998). Nitric oxide-mediated induction of ferritin synthesis in J774 macrophages by inflammatory cytokines: role of selective iron regulatory protein-2 downregulation. *Blood, 91*, 1059-1066.

Régnier, C. H., Song, H. Y., Gao, X., Goeddel, D. V., Cao, Z., & Rothe, M. (1997). Identification and characterization of an IkappaB kinase. *Cell, 90(2)*, 373-383.

Regulation (EEC) No 2568/91 on the characteristics of olive oil and olive-residue oil and on the relevant methods of analysis. http://eur-lex.europa.eu/legal-content/IT/TXT/PDF/?uri=CELEX:01991R2568-20151016&from=EN Accessed 12/06/2017.

Reif, A., Fröhlich, L. G., Kotsonis, P., Frey, A., Bömmel, H. M., Wink, D. A., et al. (1999). Tetrahydrobiopterin inhibits monomerization and is consumed during catalysis in neuronal NO synthase. *Journal of Biological Chemistry, 274*, 24921-24929.

Rice-Evans, C. A., Miller, N. J., & Paganga, G. (1996). Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Radical Biology and Medicine, 20(7)*, 933-956.

Rodrigo, R., Monfort, P., Cauli, O., Erceg, S., & Felipo, V. (2006). Pharmacological manipulation of cyclic GMP levels in brain restores learning ability in animal models of hepatic encephalopathy: Therapeutic implications. *Neuropsychiatric Disease and Treatment, 2(1)*, 53-63.

Rodriguez-Morato, J., Boronat, A., Kotronoulas, A., Pujadas, M., Pastor, A., Olesti, E., et al. (2016). Metabolic disposition and biological significance of simple phenols of dietary origin: hydroxytyrosol and tyrosol. *Drug Metabolism Reviews, 48(2)*, 218-236.

Rodriguez-Morato, J., Xicota, L., Fitò, M., Farrè, M., Dierssen, M., & De la Torre, R. (2015). Potential role of olive oil phenolic compounds in the prevention of neurodegenerative diseases. *Molecules, 20(3)*, 4655-4680.

Romier, B., Van De Walle, J., During, A., Larondelle, Y., & Schneider, Y-J. (2008). Modulation of signalling nuclear factor-kB activation pathway by polyphenols in human intestinal Caco-2 cells. *British Journal of Nutrition, 100*, 542-551.

Rondini, L., Peyrat-Maillard, M. N., Marsset-Baglieri, A., & Berset, C. (2002). Sulfated ferulic acid is the main in vivo metabolite found after short-term ingestion of free ferulic acid in rats. *Journal of Agricultural and Food Chemistry, 50*, 3037-3041.

Ross, R. (1999). Atherosclerosis - an inflammatory disease. *The New England Journal of Medicine, 340*, 115-126.

Rubio, L., Serra, A., Chen, C. Y., Macia, A., Romero, M. P., Covas, M. I., et al. (2014). Effect of the co-occurring components from olive oil and thyme extracts on the antioxidant status and its bioavailability in an acute ingestion in rats. *Food and Function, 5(4)*, 740-747.

Sadar, S. S., Vyawahare, N. S., & Bodhankar, S. L. (2016). Ferulic acid ameliorates tnbs-induced ulcerative colitis through modulation of cytokines, oxidative stress, iNOS, COX-2, and apoptosis in laboratory rats. *EXCLI Journal, 15*, 482-499.

Sagami, I., Daff, S., & Shimizu, T. (2001). Intra-subunit and inter-subunit electron transfer in neuronal nitric-oxide synthase: effect of calmodulin on heterodimer catalysis. *Journal of Biological Chemistry, 276*, 30036-30042.

Sakai, S., Ochiai, H., Mantani, N., Kogure, T., Shibahara, N., & Terasawa, K. (2001). Administration of isoferulic acid improved the survival rate of lethal influenza virus pneumonia in mice. *Mediators of Inflammation, 10*, 93-96.

Salami, M., Galli, C., De Angelis, L., & Visioli, F. (1995). Formation of F2-isoprostanes in oxidized low-density lipoprotein: inhibitory effect of hydroxytyrosol. *Pharmacology Research, 31*, 275-279.

Salzman, A. L., Denenberg, A. G., Ueta, I., O'Connor, M., Linn, S. C. & Szaboâ, C. (1996). Induction and activity of nitric oxide synthase in cultured human intestinal epithelial monolayers. *American Journal of Physiology, 270*, 565-573.

Samuel, S. M., Thirunavukkarasu, M., Penumathsa, S. V., Paul, D., & Maulikm M. (2008). Akt/FOXO3a/SIRT1 mediated cardioprotection by n-tyrosol against ischemic stress in rat in vivo model of myocardial infarction: switching gears towards survival and longevity. *Journal of Agricultural and Food Chemistry, 56(20)*, 9692-9698.

Santangelo, C., Varì, R., Scazzocchio, B., Di Benedetto, R., Filesi, C., & Masella, R. (2007). Polyphenols, intracellular signalling and inflammation. *Annali dell'Istituto Superiore di Sanita, 43(4)*, 394-405.

Sato, E, Simpson, K. L., Grisham, M. B., Koyama, S., & Robbins, R. A. (1999). Effects of reactive oxygen and nitrogen metabolites on MCP-1-induced monocyte chemotactic activity in vitro. *American Journal of Physiology, 277*, 543-549.

Sato, E., Simpson, K. L., Grisham, M. B., Koyama, S., & Robbins, R. A. (2000). Reactive nitrogen and oxygen species attenuate interleukin-8-induced neutrophil chemotactic activity in vitro. *Journal of Biological Chemistry, 275*, 10826-10830.

Saura, M., Zaragoza, C., Diaz-Cazorla, M., Hernandez-Perera, O., Eng, E., Lowenstein, C.J., et al. (1998). Involvement of transcriptional mechanisms in the inhibition of NOS2 expression by dexamethasone in rat mesangial cells. *Kidney International, 53*, 38-49.

Scharton-Kersten, T. M., Yap, G., Magram, J., & Sher, A. (1997). Inducible nitric oxide is essential for host control of persistent but not acute infection with the intracellular pathogen *Toxoplasma gondii*. *Journal of Experimental Medicine, 185*, 1261-1273.

Schiller, C. J., Klainz, A., Mynett, K., & Gescher, A. (1992). Assessment of viability of hepatocytes in suspension using the MTT assay. *Toxicology In Vitro, 6*, 575-578.

Schmitt, C. A., Handler, N., Heiss, E. H., Erker, T., & Dirsch, V. M. (2007). No evidence for modulation of endothelial nitric oxide synthase by the olive oil polyphenol hydroxytyrosol in human endothelial cells. *Atherosclerosis, 195*, 58-64.

Schwarz, P M., Kleinert, H., & Forstermann, U. (1999). Potential functional significance of brain-type and muscle-type nitric oxide synthase I expressed in adventitia and media of rat aorta. *Arteriosclerosis, Thrombosis, and Vascular Biology, 19*, 2584-2590.

Schlossmann, J., Ammendola, A., Ashman, K., Zong, X., Huber, A., Neubauer, G., et al., (2000). Regulation of intracellular calcium by a signalling complex of IRAG, IP3 receptor and cGMP kinase Ibeta. *Nature, 404*, 197-201.

Segade, M., Bermejo, R., Silva, A., Paiva-Martins, F., Gil-Longo, J., & Campos-Toimil, M. (2016). Involvement of endothelium in the vasorelaxant effects of 3,4-DHPEA-EA and 3,4-DHPEA-EDA, two major functional bioactives in olive oil. *Journal of Functional Foods, 23*, 637-646.

Serreli, G., Incani, A., Atzeri, A., Angioni, A., Campus, M., Cauli, E., et al. (2017). Antioxidant effect of natural table olives phenolic extract against oxidative stress and membrane damage in enterocyte-like cells. *Journal of Food Science, 82*(2), 380-385.

Shen, Y. H., Wang, X. L., & Wilcken, D. E. (1998). Nitric oxide induces and inhibits apoptosis through different pathways. *FEBS Letters, 433*, 125-131.

Shiojima, I., & Walsh, K. (2002). Role of Akt signaling in vascular homeostasis and angiogenesis. *Circulatory Research, 90*, 1243-1250.

Siddhanta, U., Wu, C., Abu-Soud, H. M., Zhang, J., Ghosh, D. K., & Stuehr, D. J. (1996). Heme iron reduction and catalysis by a nitric oxide synthase heterodimer containing one reductase and two oxygenase domains. *Journal of Biological Chemistry, 271*, 7309-7312.

Simmerman, H. K, & Jones, L. R. (1998). Phospholamban: protein structure, mechanism of action, and role in cardiac function. *Physiological Reviews, 78*, 921-947.

Singer, I. I., Kawka, D. W., Scott, S., Weidner, J. R., Mumford, R. A., Riehl, T. E. et al. (1996). Expression of inducible nitric oxide synthase and nitrotyrosine in colonic epithelium in inflammatory bowel disease. *Gastroenterology, 111*, 871-885.

Soler, A., Romero, M. P., Macia, A., Saha, S., Furniss, C. S. M., Kroon, P. A., et al. (2001). Digestion stability and evaulation of the metabolism and transport of olive oil phenols in the human small-intestinal epithelial Caco-2/TC7 cell line. *Food Chemistry, 119*, 703-714.

Song, T., Hatano, N., Horii, M., Tokumitsu, H., Yamaguchi, F., Tokuda, M., et al. (2004). Calcium/calmodulin-dependent protein kinase I inhibits neuronal nitric-oxide synthase activity through serine 741 phosphorylation. *FEBS Letters, 570*, 133-137.

Soskic, S. S., Dobutovic, B. D., Sudar, E. M., Obradovic, M. M., Nikolic, D. M., Djordjevic, J. D., et al. (2011). Regulation of inducible nitric oxide synthase (iNOS) and its potential role in insulin resistance, diabetes and heart failure. *The Open Cardiovascular Medicine Journal, 5*, 153-163.

Speroni, E., Guerra, M. C., Minghetti, A., Crespi-Perellino, N., Pasini, P., Piazza, F., et al. (1998) Oleuropein evaluated in vitro and in vivo as an antioxidant. *Phytotherapy Research, 12*, 98–100.

Spencer, J. P., Chowrimootoo, G., Choudhury, R., Debnam, E. S., Srai, S. K., & Rice-Evans, C. (1999). The small intestine can both absorb and glucuronidate luminal flavonoids. *FEBS Letters, 458*, 224-230.

Stalmach, A., Mullen, W., Barron, D., Uchida, K., Yokota, T., Cavin, C., et al. (2009). Metabolite profiling of hydroxycinnamate derivatives in plasma and urine after the ingestion of coffee by humans: identification of biomarkers of coffee consumption. *Drug Metabolism and Disposition, 37(8)*, 1749-1758.

Stamler, J. S., Lamas, S., & Fang, F. C. (2001b). Nitrosylation: The Prototypic Redox-Based signaling mechanism. *Cell, 106*, 675-683.

Stamler, J. S., & Meissner G. (2001a). Physiology of nitric oxide in skeletal muscle. *Physiological Reviews, 81*, 209-237.

Stamler, J. S., Simon, D. I., Osborne, J. A., Mullins, M. E., Jaraki, O., Michel, T., et al. (1992). S-nitrosylation of proteins with nitric oxide: synthesis and characterization of biologically active compounds. *Proceedings of the National Academy of Sciences of the United States of America, 89*, 444-448.

Stamler, J. S., Vaughan, D. E., & Loscalzo, J. (1989). Synergistic disaggregation of platelets by tissue-type plasminogen activator, prostaglandin E1, and nitroglycerin. *Circulation Research, 65*, 796-804.

Steffen, Y., Gruber, C., Schewe, T., & Sies, H. (2008). Mono-O-methylated flavanols and other flavonoids as inhibitors of endothelial NADPH oxidase. *Archives of Biochemistry and Biophysics, 469*, 209-219.

Steffen, Y., Schewe, T., & Sies, H. (2007). (–)-Epicatechin elevates nitric oxide in endothelial cells via inhibition of NADPH oxidase. *Biochemical and Biophysical Research Communications, 359*, 828-833.

Stone, J. R., & Marletta, M. A. (1994). Soluble guanylate cyclase from bovine lung: activation with nitric oxide and carbon monoxide and spectral characterization of the ferrous and ferric states. *Biochemistry, 33*, 5636-5640.

Sudar, E., Dobutovic, B., Soskic, S., Mandusic, V., Zakula, Z., Misirkic, M., et al. (2010). Regulation of inducible nitric oxide synthase activity/expression in rat hearts from ghrelin treated rats. *Journal of Physiology and Biochemistry, 67*, 195-204.

Suzuki, A., Kagawa, D., Fujii, A., Ochiai, R., & Tokimitsu, I. (2002). Short- and long-term effects of ferulic acid on blood pressure in spontaneously hypertensive rats. *Journal of the American Society of Hypertension, 15*, 351-357.

Suzuki, A., Yamamoto, M., Jokura, H., Fujii, A., Tokimitsu, I., Hase, T., et al., (2007). Ferulic acid restores endothelium-dependent vasodilation in aortas of spontaneously hypertensive rats. *Journal of the American Society of Hypertension, 20*, 508-513.

Szabò, C., Ischiropoulos, H., Radi, R. (2007). Peroxynitrite: biochemistry, pathophysiology and development of therapeutics. *Nature Reviews Drug Discovery, 6*, 662-680.

Tepperman, B. L., Brown, J. F., Korolkiewicz, R. & Whittle, B. J. R. (1994). Nitric oxide synthase activity, viability and cyclic GMP levels in rat colonic epithelial cells: Effect of endotoxin challenge. *Journal of Pharmacology and Experimental Therapeutics, 271*, 1477-1482.

Teuchy, H., & Van Sumere, C. F. (1971). The metabolism of (1-14C) phenylalanine, (3-14C) cinnamic acid and (2-14C) ferulic acid in the rat. Archives *Internationales de Physiologie et de Biochimie, 79*, 589-618.

The Mediterranean Diet Food Pyramid (2017). America's test kitchen, URL https://www.americastestkitchen.com/articles/509-the-mediterranean-diet-food-pyramid-infographic. Achieved 25/08/2017

Thomsen, L. L., Lawton, F. G., Knowles, R. G., Beesley, J. E., Riveros-Moreno, V., & Moncada, S. (1994). Nitric oxide synthase activity in human gynecological cancer. *Cancer Research, 54*, 1352-1354.

Trichopoulou, A. (2004). Traditional Mediterranean diet and longevity in the elderly: a review. *Public Health Nutrition*, 7(7), 943-947.

Trichopoulou, A., Costacou, T., Bamia, C., & Trichopoulos, D. (2003). Adherence to a Mediterranean diet and survival in a Greek population. *The New England Journal of Medicine, 348*, 2599-2608.

Tsui, K-H., Li H-Y., Cheng, J-T., Sung Y-J., Yen, M-S., Edmond Hsieh, S-L., et al. (2015). The role of nitric oxide in the outgrowth of trophoblast cells on human umbilical vein endothelial cells. *Taiwanese Journal of Obstetrics & Gynecology, 54*, 227-231.

Tu, J., Xu, Y., Xu, J., Ling, Y., & Cai, Y. (2016). Chitosan nanoparticles reduce LPS-induced inflammatory reaction viainhibition of NF-κB pathway in Caco-2 cells. *International Journal of Biological Macromolecules, 86*, 848-856.

Tuck, K. L., Hayball, P. J., & Stupans, I. (2002). Structural characterization of the metabolites of hydroxytyrosol, the principal phenolic component in olive oil, in rats. *Journal of Agricultural and Food Chemistry, 50(8)*, 2404-2409.

Tuteja, N., Chandra, M., Tuteja, R., & Misra, M. K. (2004). *Journal of Biomedicine and Biotechnology, 4*, 227-237.

Ullman, T. A., Itzkowitz, S. H. (2011). Intestinal inflammation and cancer. *Gastroenterology, 140*, 1807-1816.

Unno, N., Wang, H., Menconi, M. J., Tytgat, S. H. A. J., Larkin, V., Smith, M., et al. (1997). Inhibition of inducible nitric oxide synthase ameliorates endotoxin-induced gut mucosal barrier dysfunction in rats. *Gastroenterology, 113*, 1246-1257.

Uylaser, V. & Yildiz, G. (2014). The historical development and nutritional importance of olive and olive oil constituted an important part of the Mediterranean diet. *Critical Reviews in Food Science and Nutrition, 54(8)*, 1092-1101.

Van De Walle, J., Hendrickx, A., Romier, B., Larondelle, Y., & Schneider, Y-J. (2010). Inflammatory parameters in Caco-2 cells: Effect of stimuli nature, concentration, combination and cell differentiation. *Toxicology in Vitro, 24*, 1441-1449.

Vanhaesebroeck, B., Leevers, S. J., Panayotou, G., & Waterfield, M. D. (1997) Phosphoinositide 3-kinases: a conserved family of signal transducers. *Trends in Biochemical Sciences, 22*, 267-272.

Van Rymenant, E., Van Camp, J., Pauwels, B., Boydens, C., Vanden Daele, L., Beerens, K., et al. (2017). Ferulic acid-4-*O*-sulfate rather than ferulic acid relaxes arteries and lowers blood pressure in mice. *Journal of Nutritional Biochemistry, 44*, 44-51.

Virag, L., Szabo, E., Gergely, P., Szabo, C. (2003). Peroxynitrite-induced cytotoxicity: mechanism and opportunities for intervention. *Toxicology. Letters, 140-141*, 113-124.

Visioli, F., Bellomo, G., Montedoro, G., & Galli, C. (1995). Low density lipoprotein oxidation is inhibited in vitro by olive oil constituents. *Atherosclerosis, 117*, 25-32.

Visioli, F., Caruso, D., Grande, S., Bosisio, R., Villa, M., Galli, G., et al. (2005). Virgin Olive Oil Study (VOLOS): vasoprotective potential of extra virgin olive oil in mildly dyslipidemic patients. *European Journal of Nutrition, 44(2)*, 121-127.

Visioli, F., Caruso, D., Plasmati, E., Patelli, R., Mulinacci, N., Romani, A., et al. (2001). Hydroxytyrosol, as a component of olive mill waste water, is dose- dependently absorbed and increases the antioxidant capacity of rat plasma. *Free Radical Research, 34(3)*, 301-305.

Visioli, F., & Galli, C. (1994). Oleuropein protects low density lipoprotein from oxidation. *Life Sciences, 55*, 1965-1971.

Visioli, F., & Galli, C. (1998). The effect of minor constituents of olive oil on cardiovascular disease: new findings. *Nutrition Reviews, 56*, 142-147.

Visioli, F., & Galli, C. (2002). Biological properties of olive oil phytochemicals. *Critical Reviews in Food Science and Nutrition, 42(3)*, 209-221.

Visioli, F., Galli, C., Bornet, F. Mattei, A., Patelli, R., Galli, G., et al. (2000). Olive oil phenolics are dose-dependently absorbed in humans. *FEBS Letters, 468*, 159-160.

Vissers, M. N., Zock, P. L., & Katan, M. B. (2004). Bioavailability and antioxidant effects of olive oil in humans: a review. *European Journal of Clinical Nutritrion, 58*, 955-965.

Vissers, M. N., Zock, P. L., Roodenburg, A. J., Leenen, R. & Katan, M. B. (2002). Olive oil phenols are absorbed in humans. *Journal of Nutrition, 132(3)*, 409-417.

Vissers, M. N., Zock, P. L., Wiseman, S. A., Meyboom, S., & Katan, M. B. (2001). Effect of phenol-rich extra virgin olive oil on markers of oxidation in healthy volunteers. *European Journal of Clinical Nutrition, 55(5)*, 334-341.

Vitaglione, P., Savarese, M., Paduano, A., Scalfi, L., Fogliano, V., & Sacchi, R. (2013). Healthy virgin olive oil: a matter of bitterness. *Critical Reviews in Food Science and Nutrition, 55*, 1808-1818.

Walford, G., & Loscalzo, J. (2003). Nitric oxide in vascular biology. *Journal of Thrombosis and Haemostasis, 1*, 2112-2118.

Wang, B., Ouyang, J., Liu, Y., Yang, J., Wei, L., Li, K., et al. (2004). Sodium ferulate inhibits atherosclerogenesis in hyperlipidemia rabbits. *Journal of Cardiovascular Pharmacology, 43*, 549-554.

Wang, B. H., & Ou-Yang, J. P. (2005). Pharmacological actions of sodium ferulate in cardiovascular system. *Cardiovascular Drug Reviews, 23*, 161-172.

Wang, R., Kwon, I. K., Singh, N., Islam, B., Liu, K., Sridhar, S., et al. (2014). Type 2 cGMP-dependent protein kinase regulates homeostasis by blocking c-Jun N-terminal kinase in the colon epithelium. *Cell Death & Differentiation, 21(3)*, 427-437.

Wang, X., Li, X., & Chen, D. (2011). Evaluation of antioxidant activity of isoferulic acid in vitro. *Natural Products Communications, 6*, 1285-1288.

Wang, Y., & Marsden, P. A. (1995). Nitric Oxide Synthases: gene structure and regulation. *Advances in Pharmacology, 34*, 71-90.

Wang, W., Xia, Y., Yang, B., Su, X., Chen, J., Li, W., et al. (2017). Protective effects of tyrosol against LPS-induced acute lung injury via inhibiting NF-κB and AP-1 activation and activating the HO-1/Nrf2 pathways. *Biological and Pharmaceutical Bulletin 40(5)*, 583-593.

Weinbrenner, T., Fitó, M., Farré Albaladejo, M., Saez, G. T., Rijken, P., Tormos, C., et al. (2004). Bioavailability of phenolic compounds from olive oil and oxidative/antioxidant status at postprandial state in healthy humans. *Drugs Under Experimental and Clinical Research, 30*, 207-212.

Wilson, K. T., Vaandrager, A. B., De Vente, J., Musch, M. W., de Jonge, H. R., & Chang, E. B. (1996). Production and localization of cGMP and PGE2 in nitroprusside-stimulated rat colonic ion transport. *American Journal of Physiology*, 270, 832-840.

Witthoft, T., Eckmann, L., Kim, J. M., & Kagnoff, M. F. (1998). Enteroinvasive bacteria directly activate expression of iNOS and NO production in human colon epithelial cells. *American Journal of Physiology, 275(3)*, 564-571.

Wolf, G. (1997). Nitric oxide and nitric oxide synthase: biology, pathology, localization. *Histology and Histopathology, 12*, 251-261.

Wu, J.-L., Shen, M.-M., Yang, S.-X., Wang, X., & Ma, Z.-C. (2015). Inhibitory effect of ferulic acid on neuroinflammation in LPS-activated microglia. *Chinese Pharmacological Bulletin, 31(1)*, 97-102.

Xu, K. Y., Huso, D. L., Dawson, T. M., Bredt, D. S., & Becker, L.C., (1999). Nitric oxide synthase in cardiac sarcoplasmic reticulum. *Proceedings of the National Academy of Sciences of the United States of America, 96*, 657-662.

Yang, T., Wang, L., Sun, R., Chen, H., Zhang, H., Yu, Y., et al. (2016). Hydrogen-rich medium ameliorates lipopolysaccharide-induced barrier dysfunction via rhoa-mdia1 signaling in Caco-2 cells. *Shock, 45(2)*, 228-237.

Yu, Z., Zhang, W., & Kone, B. C. (2002). Signal transducers and activators of transcription 3 (STAT3) inhibits transcription of the inducible nitric oxide synthase gene by interacting with nuclear factor κB. *Biochemical Journal, 11*, 97-105.

Zhang, J., Shattil, S. J., Cunningham, M. C., & Rittenhouse, S. E. (1996). Phosphoinosi-tide 3-kinase gamma and p85/phosphoinositide 3-kinase in platelets. Relative activation by thrombin receptor or beta-phorbol myristate acetate and roles in promoting the ligand-binding function of alphaIIb-beta3 integrin. *Journal of Biological Chemistry, 271*, 6265-6572.

Zhang, J. L., Zhang, G. D., & Zhou, T. H. (2005). Metabolism of ferulic acid in rats. *Journal of Asian Natural Products Research, 7*, 49-58.

Zhao, B., Maa, Y., Xu, Z., Wanga, J., Wanga, F., Wang, D., et al. (2014). Hydroxytyrosol, a natural molecule from olive oil, suppresses the growth of human hepatocellular carcinoma cells via inactivating AKT and nuclear factor-kappa B pathways. *Cancer Letters, 347*, 79-87.

Zhao, J., Suyama, A., Chung, H., Fukuda, T., Tanaka, M., & Matsui, T. (2016). Ferulic acid enhances nitric oxide production through up-regulation of argininosuccinate synthase in inflammatory human endothelial cells. *Life Sciences, 145*, 224-232.

Zhao, Y., Vanhoutte P. M., & Leung, S. W. S. (2015). Vascular nitric oxide: Beyond eNOS. *Journal of Pharmacological Sciences, 129*, 83-94.

Zhao, Z., Egashira, Y., & Sanada, H. (2003a). Digestion and absorption of ferulic acid sugar esters in rat gastrointestinal tract. *Journal of Agricultural and Food Chemistry, 51*, 5534-5539.

Zhao, Z., Egashira, Y., & Sanada, H. (2003b). Ferulic acid sugar esters are recovered in rat plasma and urine mainly as the sulfoglucuronide of ferulic acid. *The Journal of Nutrition, 133*, 1355-1361.

Zhao, Z., Egashira, Y., & Sanada, H. (2004). Ferulic acid is quickly absorbed from rat stomach as the free form and then conjugated mainly in liver. *The Journal of Nutrition, 134*, 3083-3088.

Zhao, Z., Egashira, Y., & Sanada, H. (2005). Phenolic antioxidants richly contained in corn bran are slightly bioavailable in rats. *Journal of Agricultural and Food Chemistry, 53*, 5030-5035.

Zhao, Z. & Moghadasian, M. H. (2008). Chemistry, natural sources, dietary intake and pharmacokinetic properties of ferulic acid: A review. *Food Chemistry, 109*, 691-702.

Zhou, L., & Zhu, D. (2009). Neuronal nitric oxide synthase: Structure, subcellular localization, regulation, and clinical implications. *Nitric Oxide, 20*, 223-230.

Ziche, M., Morbidelli, L., Masini, E., Amerini, S., Granger, H. J., Maggi, C. A., et al. (1994). Nitric oxide mediates angiogenesis in vivo andendothelial cell growth and migration in vitro promoted by substance P. *Journal of Clinical Investigation, 94*, 2036-2044.

Ziche, M., Parenti, A., Ledda, F., Dell'Era, P., Granger, H. J., Maggi, C. A. et al. (1997). Nitric oxide promotes proliferation and plasminogen acti-vator production by coronary venular endothelium through endogen-ous bFGF. *Circulatory Research, 80*, 845-852.