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Modulatory effect of EVOO polyphenols and their metabolites on inflammatory response in Adenocarcinoma Colon Cells (Caco-2) and Human Umbilical Vein EC (HUVEC)

Scientific Disciplinary Sector(s)

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Contents

Abstract

Mediterranean diet is considered a model of healthy nutrition, able to prevent and/or slow down the progression of the most common degenerative diseases, such as Cardiovascular Disease (CVD) and Inflammatory Bowel Disease (IBD), characterized by a chronic inflammation state that alters structure and functionality of the endothelial and intestinal barrier. EVOO, the main lipid source in the Mediterranean diet, is rich in bioactive compounds mainly hydroxytyrosol (HT) and tyrosol (Tyr), that have been shown to possess antimicrobial, antioxidant and anti-inflammatory properties. However, it is well known that free forms of phenolic compounds ingested with food are largely metabolized in the intestine and liver and their concentrations after ingestion are too low to explain the biological effects observed *in vivo* and *in vitro* models. In particular, HT and Tyr undergo glucuronidation and sulfonation, and their sulphate and glucuronide metabolites are the prevalent forms found in human plasma and urine where they reach relevant concentrations compatible with biological activity.

In this context, the aim of the present research project was to evaluate the protective effect of the main phenolic compounds of EVOO, HT and Tyr, and their sulphate and glucuronide metabolites, on the inflammatory response at intestinal level (in Caco-2 cell monolayer) and endothelial level (in HUVEC cell monolayer), using pro-inflammatory stimuli, such as LPS and/or a hyperglycemia (HG) condition. Their modulatory action was evaluated focusing on the alteration of tight junctions (TJ) and the activation of cellular pathways, as mitogen-activated protein kinases (MAPK) and NLRP3 inflammasome, which are linked to chronic inflammatory diseases.

The alteration of epithelial/endothelial barrier in Caco-2 and HUVEC monolayers, treated with LPS or HG alone or together with EVOO phenolic compounds and their sulphate and glucuronide metabolites, was evaluated through cell permeability tests (TEER, FITC-

Dextran permeability assay) and the determination of the disruption and/or relocation of TJ proteins, in relation to redox-sensitive MAPK modulation and activation of the NLRP3 inflammasome.

Obtained data showed that HG and physiopatologically relevant concentration of LPS increase permeability in both Caco-2 and HUVEC monolayers, through the alteration of TJ proteins, occludin, zonulin and JAM-A (Junctional adhesion molecule A), following the activation of pathways involved in the inflammatory process such as p38 and ERK1/2 MAPK and NLRP3 inflammasome. It was also observed that pretreatment with physiologically concentration of HT, Tyr and their sulphated and glucuronidated derivatives induce a protective effect, limiting the alteration of TJ and the activation of MAPK and NLRP3 inflammasome, strengthening the hypothesis that HT and Tyr, as well as their metabolites, may exert a significant role in the maintenance of intestinal and endothelial barrier integrity. Finally, it was observed that HT and Tyr metabolites and their parent compounds exert a comparable effect both in intestinal and EC, by modulating key intracellular signaling pathways involved in the cellular pro-inflammatory response.

1 Introduction

1.1 Intestinal epithelium and vascular endothelium

Tissue functionality is guaranteed by the proper regulation of its barriers, as the endothelium and the epithelium. The endothelium lines the vasculature and ensures tissue supply with nutrients and oxygen. The epithelium forms the first line defense barrier between tissues and the outer environment thus protecting organs from invading harmful agents. Both barriers also play a critical role in the innate immune response to injury and infection **(Garcìa-Ponce A et al, 2016)**.

The gastrointestinal mucosa is a semipermeable barrier allowing the absorption of nutrients and fluids, whole body homeostasis regulation and immune sensing, while limiting the passage of potentially harmful antigens and microorganisms from the intestinal lumen. The mucus layer and the epithelial cell monolayer act as a physical barrier, which togheter with antimicrobial peptides and factors of adaptative immunity, forbades bacterial adhesion and regulate trans-epithelial diffusion of small molecules and bacteria to host systemic circulation. As for the lamina propria and the submucosa, they organize the immune response to face the passage of commensal and pathogenic microbes **(Vancamelbeke M et al, 2017; Odenwald MA et al, 2017)**.

The intestinal epithelium barrier consists of multiple components, mostly contributed by intestinal epithelial cell subsets, a robust junctional complex consisting of tight junctions (TJ) and adherens junctions (AJ), that form a physical barrier, reinforced by the cytoskeleton **(Elias BC et al, 2009)**, as well as secretion by goblet cells of mucus that separates the epithelial layer from the luminal microbiota **(Peterson LW and Artis D, 2014)**. Additionally, gut barrier is fortified by antimicrobial peptides produced by specialized cells like Paneth and diverse mucosal immune system (**Fig 1**), in conferring tolerance against food and microbiota-derived antigens, while preserving the ability to elicit an intense immune response when the barrier is breached **(Hooper LV and Macpherson AJ, 2010; Roy U et al, 2017)**. Among these

components, the TJ constitute the major determinant of the intestinal physical barrier. TJ are formed by the assembly of multiple proteins located close at the apical portion of the lateral membrane of epithelial cells. The TJ structure consists of transmembrane proteins, such as claudin **(Schlingmann B et al, 2015)**, occludin **(Steed E et al, 2009)**, tricellulin **(Ikenouchi J et al, 2005)**, and Junctional Adhesion Molecule-A (JAM-A) **(Ma TY et al, 2018)**, and intracellular plaque proteins, such as zonula occludens (ZO) and cingulin **(Zihni C et al, 2016)**. The interactions between extracellular regions of the transmembrane proteins of adjacent cells regulate the paracellular passage of molecules **(Ikenouchi J et al, 2005)**. These proteins work together to enable cell-cell communication and determine paracellular permeability and their expression and distribution is altered in diseases characterized by increased permeability **(Odenwald MA et al, 2017)**.

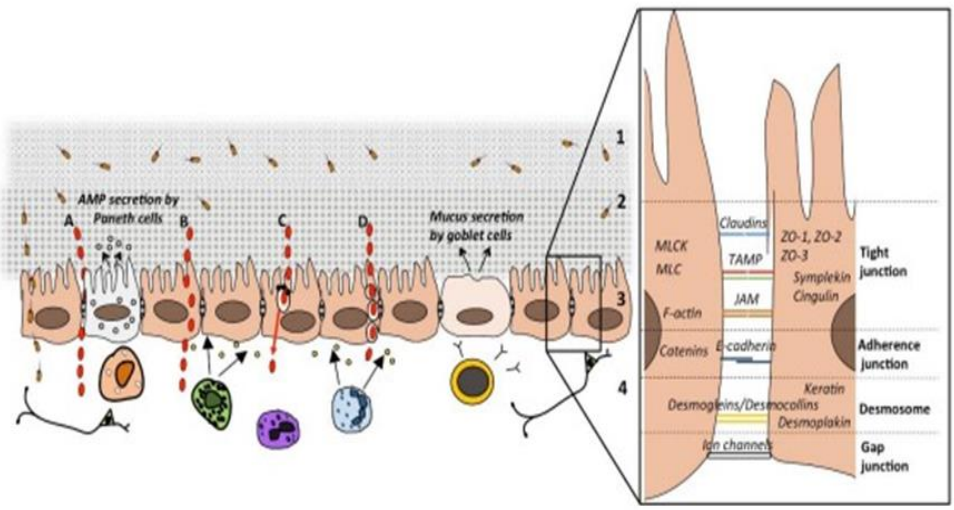


Fig 1. A schematic drawing of the intestinal barrier and passage routes across the epithelium (From **Schoultz I and Keita VA, 2020**)

Intestinal permeability is defined as the non-mediated intestinal passage of medium-sized hydrophilic molecules occurring towards a concentration gradient without the assistance of a carrier system. Tightly regulated gut permeability is indispensable for the maintenance of

intestinal homeostasis, healthy metabolism and immune tolerance. Hence, increased intestinal permeability is a sign of a disturbed intestinal barrier function **(France MM et al, 2017)**.

While the intestinal epithelium plays an important role in dictating permeability, access of bacteria to systemic circulation is a two-way street and the endothelial barrier has a functional contribution **(Ma J and Li H, 2018**).

The vascular endothelium (VE), consisting of monolayers of EC (EC), is located at the interface between the vascular and perivascular compartments and extends over a wide surface area. The VE separates strictly two compartments and regulates the trafficking of ions, solutes, macromolecules and leukocytes across the vessel wall, thus maintaining tissue homeostasis **(Komarova YA et al, 2017; Wettschureck N et al, 2019)**. Additionally, it secretes several vasoactive agents that not only maintain its integrity but also regulate platelet function and vascular smooth muscle tone, and thus actively participate in the regulation of blood pressure. The semipermeable barrier function of VE is dependent on the size of the molecules, and this size-selective nature of the barrier to plasma proteins is a key factor in establishing protein gradients, which is required for fluid balance of tissues **(Komarova YA et al, 2017; Kumar P et al, 2009)**. Therefore, the endothelium plays a pivotal role in controlling blood fluidity, vascular tone, signaling, angiogenesis and transendothelial migration of blood cells **(Poredos P and Jezovnik MK, 2018)**.

The EC, consisting of a single cell layer, covers the interior walls of the vasculature in the body **(Sturtzel C, 2017**). The aligned and connected layer of EC forms a barrier, semi-permeable to water and non-lipophilic particles, that selectively orchestrates passage of solutes differing in size and charge **(Kumar P et al, 2009)**. A number of EC proteins form domains present on the endothelial membranes that contribute to the adhesive structures of the endothelial barrier. Molecules of different sizes are able to cross the EC layer via a variety of passive and active routes. Small molecules are able to traverse via concentration gradients,

whereas larger molecules are transported via receptors, endocytic vesicles or by dysfunctional endothelial junctions, a hallmark of vascular permeability **(Egawa G et al, 2013)**.

Endothelial barrier integrity and vascular permeability are maintained by the equilibrium of competing adhesive and contractile forces generated by adhesive molecules located at cell-cell and cell-matrix contacts and the acto-myosin-based contractile machinery, respectively **(Mehta D et al, 2006)**. EC are tightly interconnected by the interaction of junctional proteins such as VE-cadherin, ZO-1, occludins, and catenins that are linked to the actin cytoskeleton of adjacent cells **(Dejana E et al, 2008)**. Junctions in endothelial and epithelial cells share common features. In both cell types, are present adherens junctions (AJ) and TJ, however, epithelial cells also form desmosomes, which are absent in the endothelium **(Bazzoni G and Dejana E, 2004)**. The main component of endothelial junctions, VE-cadherin, forms homophilic interactions between adjacent EC that are essential for maintenance of the endothelial barrier. In particular, VE-cadherin interacts with catenin and forms an intracellular complex which is essential for junctional stability **(Schulte D et al, 2011)**. EC TJ, are composed of members of the claudin and JAM families, occludin, endothelial cell-selective adhesion molecule (ESAM), and other adhesion molecules **(Dejana E et al, 2013)**. Transmembrane TJ proteins exist in complex with the intracellular scaffold proteins cingulin, paracingulin, and ZO family members **(Zihni C et al, 2016)** and with membrane lipids **(Lee DBN, 2008; Shigetomi K et al, 2018)**. Changes in the actin cytoskeleton dynamics and/or activation state of the EC contractile machinery may affect the stability of cell-cell junctions and barrier function. The loss of this barrier function, results in increased vascular permeability and leakage of blood components, which may finally result in organ dysfunction and life-threatening edema formation **(Wettschureck N et al, 2019; Daniel AE and van Buul JD, 2013)**. Impaired endothelial function is associated with the pathogenesis of a broad spectrum of diseases, including atherosclerosis, other chronic inflammatory diseases, venous thrombosis,

hypertension and malignancies **(Poredos P and Jezovnik MK, 2018; Steyers CM and Miller FJJ, 2014)**.

1.1.1 Tight junctions (TJ)

TJ are multiple protein complexes located at the most apical region of intestinal epithelial cells (**Fig 2**) and EC (**Fig 3**). An important function of TJ is to form a permeability barrier that restricts free diffusion of molecules across the intercellular space and to act as a membrane fence that restricts intermixing of apical and basolateral plasma membrane domains **(Zihni C et al, 2016; Anderson JM and Van Itallie CM, 2009; Shen L et al, 2011)**. The extracellular domains of the transmembrane proteins facilitate cell adhesion and form the selective barrier by homophilic and heterophilic interactions with the adjacent cells **(Gonzalez-Mariscal L et al, 2003)**.

In epithelial cells, junctions are better organized, with TJ and AJ following a well defined spatial distribution along the intercellular cleft. TJ are concentrated at the apical side of the rim, while AJ are located below the TJ. In contrast, in EC, the junctional architecture is less defined and, along the cleft, AJ are intermingled with TJ **(Simionescu M, 2000).** The organization of endothelial junctions varies along the vascular tree in function of organ specific requirements. For instance, postcapillary venules, which allow dynamic trafficking of circulating cells and plasma proteins, display poorly organized TJ. In contrast, the endothelium of large arteries, which tightly controls permeability, has a well developed system of TJ. The development of fully mature endothelial junctions is reached only late in development and, in general, junctions are not completely differentiated in the embryo **(Bazzoni G and Dejana E, 2004)**.

Intestinal TJ regulate epithelial permeability through the dynamic nature of their three-dimensional, multiprotein conformation **(Shen L et al., 2011)**. In particular, the intracellular domains of transmembrane proteins interact with cytosolic scaffold proteins, such as ZO proteins, which in turn anchor the transmembrane proteins to the perijunctional actomyosin ring. Several evidences suggest that the actin cytoskeleton plays a pivotal role in regulating junctional integrity and remodelling under physiological and pathological states. Thus, the

interaction of TJ proteins with the actin cytoskeleton is vital to the maintenance of TJ structure and function **(Garcia MA et al, 2018)**.

The expression of TJ components in endothelium can be controlled by AJ and TJ organization following AJ formation. Some TJ components such as ZO-1 are found in AJ at early stages of junction formation and concentrate in TJ only subsequently when junctions are stabilized. Interestingly, however, AJ are required for TJ assembly but are dispensable for TJ maintenance in epithelial cells **(Dejana E et al, 2009; Dejana E and Giampietro C, 2012)**. In addition, AJ and TJ are interconnected **(Taddei A et al, 2008**), for example ZO proteins crosstalk with AJ **(Tornavaca O et al, 2015)**. VE-cadherin is linked indirectly via its cytoplasmic tail to actin filaments by a complex of proteins including α- and β-catenins, plakoglobin (γ-catenin), p120-catenin, vinculin and α-actinin **(Ebnet K, 2008)**, which are vital for junctional stability and also for the dynamic opening and closing of junctions **(Vestweber D, 2008)**.

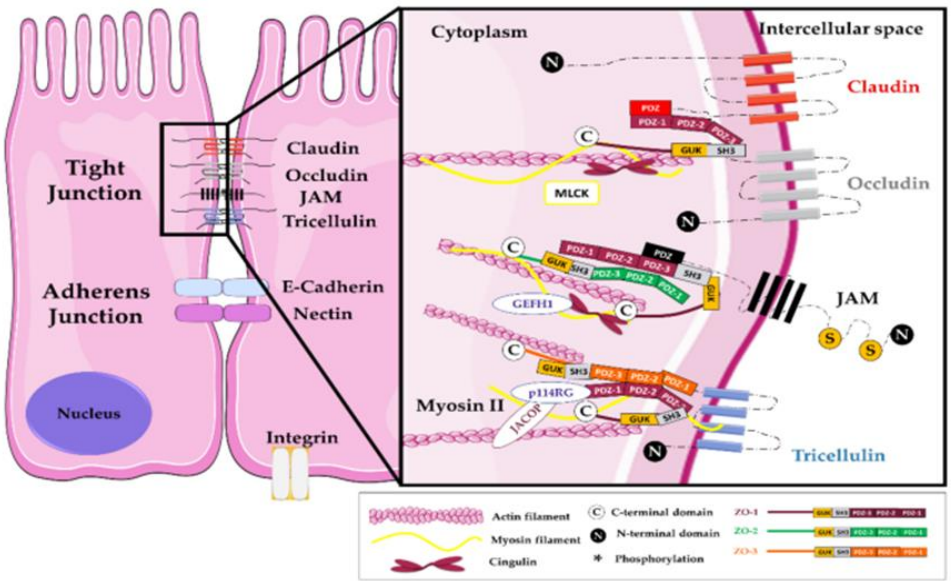


Fig 2. Composition of tight junctions in intestinal epithelial cells (From **Paradis T et al, 2021**).

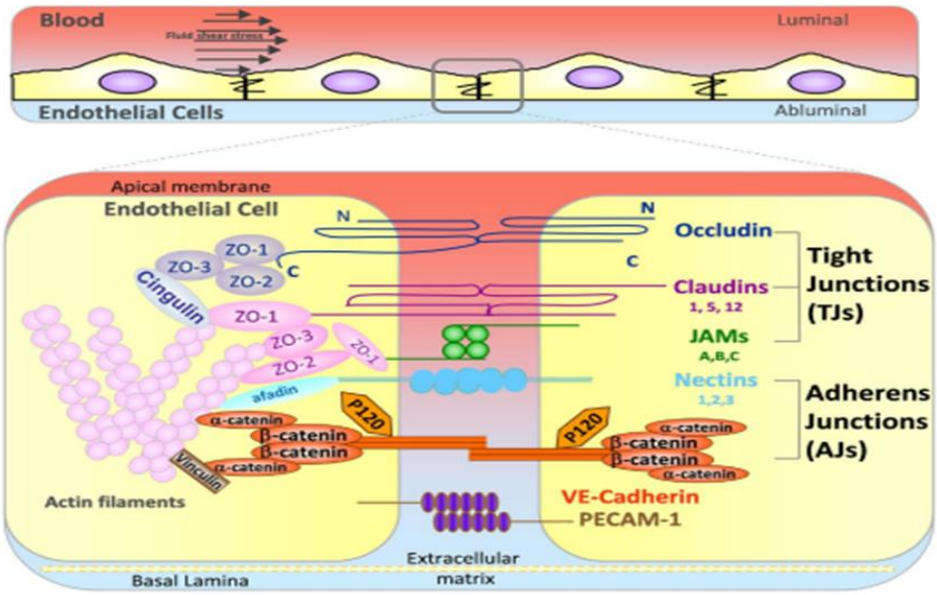


Fig 3. Composition of tight junctions in EC (From **Cerutti C and Ridley AJ, 2017**).

TJ organization and function are regulated by the phosphorylation status of occludin, claudins, and ZO-1. In this context, the maintenance of the TJ proteins to the junctional complex has been correlated to the activation of kinase pathways including PKC and MAPK (ERK, p38, JNK), the calcium/calmodulin-dependent kinase 2 (CaMKK2)–AMP-activated protein kinase (AMPK), as well as Rho and NF-κB pathways **(Paradis T et al, 2021)**. For example, PKA-mediated phosphorylation increases the assembly of claudin-3 at TJ, and decreases the assembly of claudin-16 **(D'Souza T et al, 2005; Ikari A et al, 2006)**. Phosphorylation of Tyr398 and Tyr402 by Src kinase prevents occludin binding to ZO-1, resulting in the destabilization of occludin assembly at the TJ in Caco-2 intestinal epithelial cells *in vitro*, and colonic mucosa *in vivo* **(Rao RK et al, 2002; Basuroy S et al, 2005; Elias BC et al, 2009)**. MAPK signaling pathway is able to modulate TJ paracellular transport by up- or downregulating the expression of several TJ proteins and hence altering the molecular composition within TJ complexes. In epithelia it has been observed that treatment with the cytokine IL-17 or with several growth factors including transforming growth factor β (TGFβ), or the exposure of intestinal cells to bile, activates ERK1/2 with consequent dysfunction of TJ

(González-Mariscal L et al, 2008). Interactions between the TJ and cytoskeleton are important in TJ barrier function, cell proliferation, and integrating the TJ with other junctions in the cell. Actomyosin contraction plays an important role in TJ formation, regulation of TJ dynamics, and TJ-mediated responses of cells to external stimuli **(Rodgers LS and Fanning AS, 2011; Lechuga S et al, 2015)**. Generally, myosin light chain kinase (MLCK) activity, which activates myosin II, regulates barrier function by remodeling TJ structure, specifically through the redistribution of ZO-1 and occludin as shown in Caco-2 intestinal cells *in vitro* **(Shen L et al, 2006)**. In the same way, Rho proteins have been suggested to play an important role in maintaining the association of TJ with the membrane. Indeed, the kinase ROCK, downstream effector of Rho, is reported to regulate specifically TJ via its effects on the F-actin cytoskeleton **(Cong X and Kong W, 2020)**. JAM proteins appear to play an indirect role in TJ strand formation because JAM-A depletion results in increased paracellular permeability and decreased transepithelial resistance **(Laukoetter MG et al, 2007; Vetrano S et al, 2008)**.

It has been identified a wide range of modulators of endothelial barrier function that can promote barrier opening, restore barrier closure, or maintain barrier integrity. Some of these modulators act directly on barrier components, while others act indirectly. For example, the VEGFA and VEGF family acts through three structurally related transmembrane tyrosine kinase receptors, VEGFR1, VEGFR2 and VEGFR3. VEGFR2 is the most abundant receptor of this family on EC and is essential for VEGFA-induced increase in vascular permeability. VEGFR2 regulation of vascular permeability and leakage depends on signaling via SFKs (cytoplasmic tyrosine kinases) and Rho family GTPases **(Zhang F et al, 2018; Heinolainen K et al, 2017)**. In particular, Rho GTPases connect to the actin cytoskeleton and, thereby, communicate with AJ and possibly TJ. A well-studied effect of Rho GTPases in cultured EC is the balance between actin stress fibers, which are thought to pull junctions apart, and cortical actin filaments that stabilize junctions. RhoA activation leads to formation of radial stress fibers

and increased contractility and permeability of endothelial monolayers **(Waschke J et al, 2004 A and B)**. Rac1 is required for endothelial barrier maintenance and stability under resting conditions. Thus, Rac1 activation downstream of inflammatory cytokines and VEGFA promotes reassembly of endothelial junctions **(Marinkovic G et al, 2015; van Buul JD and Timmerman I, 2016)**. In EC endostatin, a 20-kDa fragment of collagen XVIII, reverses VEGF-mediated increase in permeability of the blood retinal barrier (BRB), through activation of p38 MAPK and Erk1/2 **(Campbell M et al, 2006)**. Interestingly, pretreatment of HUVEC cells with the steroid hormone dihydrotestosterone (DHT), and to a lesser extent with estradiol (E2), mitigates the permeability defects associated with brief periods of hypergravity **(Sumanasekera WK et al, 2006; Sumanasekera WK et al, 2007)**. JAM-1, a type I protein of the TJ characterized for exhibiting two extracellular Ig-like domains, appears to constitute a primary signaling component of the ERK pathway **(Naik MU et al, 2003)**. Thus, JAM-1 silencing hinders the migration of HUVEC on vitronectin induced by basic fibroblast growth factor (bFGF), due to the inability of the growth factor to activate ERK. ERK activation by bFGF is fundamental for triggering endothelial cell migration required for sealing injured vasculature **(Naik MU and Naik UP, 2006)**.

1.1.1.1 Occludin

Originally discovered in avian tissues by **Furuse M et al (1993)**, occludin is the first integral TJ protein to be identified in epithelial and endothelial TJ and is exclusively localized at the TJ of these cells **(Hirase T et al, 1997)**. It is a ~65 kDa tetraspan protein with an NH_2- and COOH-terminal cytoplasmic domains and two extracellular loops, the first enriched with tyrosine and glycine residues and the second full of tyrosines **(Schneeberger EE et al, 2004)**. The extracellular domains of occludin are shown to function in localization of occludin in TJ and in regulating the paracellular permeability barrier between cells **(Tash BR et al, 2012)**. The COOH-terminal cytoplasmic domains have been shown to be essential for occludin interactions with ZO-1, subsequently mediating its intracellular trafficking to the plasma membrane TJ site, for signaling functions and for occludin dimerization **(Li Y et al, 2005; Walter JK et al, 2009 A and B)**. Occludin also exhibits a MARVEL (MAL (myelin and lymphocyte) and related proteins for vesicle trafficking and membrane link domain), a four-transmembrane structural motif common among junctional proteins involved in membrane apposition and fusion events **(Cummins PM, 2012)**.

There are two isoforms of occludin that result from alternative mRNA splicing, but have similar tissue distributions **(Muresan Z et al, 2000)**.

Localization of occludin to TJ is regulated by phosphorylation in both epithelial and EC **(Sakakibara A et al, 1997; Chen YS et al, 2007)**. Multiple phosphorylation sites have been identified on tyrosine **(Chen Y et al, 2000)**, serine, and threonine residues. Non-phosphorylated occludin is localized to both the basolateral membrane and in cytoplasmic vesicles, whereas phosphorylated occludin is localized to TJ. Multiple kinases and phosphatases are proposed to regulate occludin phosphorylation states and its localization and function within the TJ **(Sakakibara A et al, 1997)**. For example, protein kinase C (PKC) has long been recognized to affect epithelial and endothelial barriers. The participation of PKC on

TJ disassembly has been studied employing diverse protocols that trigger TJ disruption such as oxidative stress **(Perez LM et al, 2006)**. Signaling by protein kinase A (PKA) has long been known to regulate both the assembly and opening of the paracellular route in epithelial and EC **(Balda MS et al, 1991)**. Such appears to be the case since enhancing the activity of the Ser/Thr protein phosphatase 2A (PP2A) induces dephosphorylation of ZO-1, occludin, and claudin-1, and an increased paracellular permeability, while inhibition of PP2A promotes the phosphorylation of these TJ proteins and accelerates junctional assembly **(Nunbhakdi-Craig V et al, 2002; Seth A et al, 2007)**. So, the important role of occludin for actin organization and barrier integrity in various endothelial and epitelial cell models has also been robustly demonstrated **(Kuwabara H et al, 2001)**.

Interestingly, expression of occludin in the endothelium correlates with the permeability of different segments in the vascular tree. Occludin is expressed at high levels (with a continuous distribution) in brain EC and at much lower levels (with a discontinuous pattern) in EC of non-neural tissues **(Hirase T et al., 1997)**. In addition, **Kevil CG et al (1998)**, examined the expression of occludin in arterial and venous endothelial monolayers. It was observed that occludin in endothelial monolayers was more concentrated in arterial junctions than in venous junctions both *in vivo* and *in vitro***.**

1.1.1.2 Zonula Occludens (ZO)

The ZO proteins were the first TJ-specific proteins identified and 3 ZO proteins, ZO-1 (220 kDa), -2 (160 kDa), and -3 (130 kDa), have been characterized **(Gumbiner B et al, 1991; Willott E et al, 1993)**. According to sequence analysis, these ZO proteins are categorized as members of the membrane-associated guanylate kinase homolog (MAGUK) family **(Ikenouchi J et al, 2005)**. They are multi-domain proteins carrying 3 PDZ domains, a Src homology-3 (SH3) domain and a region of homology to GUK from the side of the N-terminus. The PDZ1 domain of ZO-1 is required for the proper organization of the TJ and associated cytoskeleton. Other ZO-1 domains, including the SH3 domain/U5 motif and PDZ2, are required for proper ZO-1 localization to the apical junction complex, and to establish normal permeability through the recruitment of a continuous circumferential band of other TJ proteins **(Rodgers LS et al, 2013)**. These multi-domain structures provide an intracellular scaffold in the TJ and are required for regulation and maintenance of TJ structure **(Fanning AS et al, 2002)**. *In vitro* binding assays demonstrated that ZO-1 binds directly to occludin. Upon tyrosine phosphorylation of occludin, interactions between all ZO proteins and occludin are reduced. The proline rich C-terminal of ZO-1 and ZO-3 bind F-actin in co-sedimentation assays, while ZO-2 does not bind actin **(Hartsock A and Nelson WJ, 2008)**. These interactions are critical to life, as a deficiency in either ZO-1 or ZO-2 results in embryonic lethality. ZO-1 and ZO-2 bind to the transcription factor ZO-1-associated nucleic acid binding protein (ZONAB) in a cell-density-dependent manner. Depletion of ZO-1 and ZO-2 results in loss of ZONAB from the TJ and its degradation **(Spadaro D et al, 2014)**. Thus, ZO proteins regulate both barrier function and cell proliferation through interactions with different proteins at the TJ **(Garcia MA et al, 2018)**.

The protein ZO-1 was named after its localization at the Zonula Occludens of several epithelial and endothelial cell types **(Stevenson BR et al, 1986)**. ZO-1 distribution, however,

is variable depending on the cell type. In particular, ZO-1 is restricted to the TJ in those epithelia (e.g., in intestine) and endothelia (e.g., in arteries) that form distinct junctional complexes. At variance, ZO-1 localizes to both TJ and AJ in other epithelial cells (e.g., hepatocytes) and in non neural EC, in which TJ are not so well developed. The ZO-1-isoform is expressed in most epithelia, while it is only detectable in EC, Sertoli cells, and slit diaphragms of kidney glomeruli. As these cells have dynamic junctions, absence of the motif might correlate with TJ plasticity **(Bazzoni G and Dejana E, 2004)**.

1.1.1.3 Junctional adhesion molecule (JAM)

Junctional adhesion molecule-A (JAM-A, which was previously called JAM, JAM-1, and F11R) is a member of the immunoglobulin superfamily (JAM-A, -B, and –C) **(Ebnet K et al, 2004)** and is a 32-kDa glycoprotein composed of an extracellular region, a transmembrane segment, and a short cytoplasmic tail. JAM-A localizes to sites of cell contact of endothelial and epithelial cells, and reduces paracellular fluxes, possibly by favoring intercellular adhesion. The molecule was named after its subcellular localization and predicted function in adhesion. In addition, a blocking antibody and a recombinant fragment, inhibit the establishment of Transepithelial Electrical Resistance (TEER), thus further supporting a role for JAM-A in TJ function **(Martin-Padura I et al, 1998)**. The extracellular segment of JAM-A comprises two Ig-like domains, an amino-terminal (VH-type) and a carboxy-terminal (C2-type) fold, respectively. In solution, a recombinant soluble protein, which corresponds to the whole extracellular domain of JAM-A, binds in a homophilic manner, thus suggesting that JAM-A may mediate homotypic cell adhesion **(Bazzoni G et al, 2000; Garrido-Urbani S et al, 2014)**. So, the homophilic interactions involving the JAM members have a role in the formation of TJ and the cell-cell border. In contrast, the heterophilic interactions in cell-cell adhesion, association between leukocytes and epi/EC, platelet activation, and virus recognition **(Kirchner E et al, 2008)**, indicate a function not only as adhesion molecules between cells of the same type but also between distinct types of cells. The conserved residues phenylalanine, leucine and valine at the carboxy terminus of JAM-A represent a consensus motif for binding type II PDZ domains. JAM-A interacts with proteins containing this domain, such as ZO-1, AF-6/ Afadin, etc., interactions that may be functionally relevant for linking JAM-A to the cytoskeleton, because both ZO-1 and AF-6 associate directly with F-actin. More recent evidence supports a direct role for individual members of the complex in the assembly of epithelial TJ **(Bazzoni G and Dejana E, 2004)**. In particular, JAM was shown to be co-

precipitated with ZO-1, which suggests that JAM may indirectly mediate the recruitment of occludin to the TJ via ZO-1 **(Feldman GJ et al, 2005)**.

1.2 Alteration of intestinal and endothelial permeability

Selective permeability is one of the key features of cell membrane to maintain its functionality as active barrier between life and death. Altering the membrane permeability not just leads to cell death, but also potentially leads to impaired cellular functions **(van Meer G et al, 2008)**.

The human intestine allows the absorption of nutrients while also functioning as a barrier, which prevents antigens and pathogens entering the mucosal tissues and potentially causing disease (**Fig 4**). An important component of the intestinal barrier is the intercellular junctional complex, crucial for the maintenance of barrier integrity. TJ are not static barriers but highly dynamic structures that are constantly being remodeled due to interactions with external stimuli, such as food residues and pathogenic and commensal bacteria **(Ulluwishewa D et al, 2011)**. Previous studies have shown that in intestinal tissue expression of occludin is markedly decreased in patients with intestinal permeability disorder, including Crohn's disease, ulcerative colitis, and celiac disease **(Al-Sadi R et al, 2011)**. It has been proposed that the decrease in intestinal occludin expression may be an important mechanism leading to the increase in intestinal epithelial TJ permeability. So, a significant body of evidence indicates that the disruption of TJ and increase in paracellular permeability play a crucial role in the pathogenesis of gastrointestinal disorders, such as inflammatory bowel disease (IBD) **(Laukoetter MG et al, 2007)**. In particular, IBD are characterized by chronic and unpredictable attacks of inflammation of the intestine with defective epithelial cell barrier functioning and an exaggerated immune activity that causes increased paracellular permeation of harmful luminal antigens and increases activation of mucosal immune cells, leading in turn to increased release of inflammatory stimuli, IEC response and further barrier dysfunction **(Bruewer M et al, 2003; Wang F et al, 2006; Al-Sadi RM and Ma TY, 2007)**.

Oxidative stress in intestinal mucosa is relevant in the initiation and progression of barrier dysfunction, due to the enhanced production of reactive oxygen species (ROS) and reactive nitrogen species (RNS), mainly triggered by neutrophils and activated leukocytes, causing lipid peroxidation, protein modification and pro-inflammatory mediators production **(Zhu H and Li YR, 2012)**. This condition, in which there is an increased release of proinflammatory cytokynes, such as tumor necrosis factor (TNF)-α and IFN-γ, induces intestinal damage in IBD, such as changes in TJ structures, apoptosis and enhanced bacterial translocation **(Hering NA et al, 2012)**.

The presence of bacterial components, such as lipopolysaccharides (LPS) is also considered as an important factor both in the initiation and in the reactivation of IBD. LPS, binds to cell surface receptors, which may regulate TLR4/NF-kB signaling pathway, increase inflammation and activate oxidative stress and inflammatory cascade, thus playing a crucial role in intestinal and systemic inflammatory reaction **(He C et al, 2019; Omonijo FA et al, 2019)**. Previous studies have demonstrated that LPS increases TJ permeability and disruption of the intestinal TJ barrier **(Hasegawa T et al, 2021; Siliciano JD and Goodenough DA, 1988; Tang X et al, 2018 A)**. The *in vitro* effects of LPS, IL-1β, TNF-α, IFN-γ, consist in activation of intracellular cascades, leading to an increased transcriptional activity and the secretion of interleukin (IL)-8, IL-6, prostaglandin (PG)-E2 and/or NO, as well as increase of the paracellular permeability through defects in TJ functioning or assembly **(Van De Walle J et al, 2010)**.

The vascular endothelium participates in the vascular barrier, vascular integrity, inflammation regulation, blood coagulation, and maintenance of homeostasis. Usually, the vascular endothelium forms a continuous, semipermeable barrier which regulates the transition of cellular molecules **(Kang Q et al, 2016)**. Endothelial TJ are more sensitive than epithelial TJ to the microenvironment **(Wolburg H and Lippoldt A, 2002)**. Many stimuli

(neurotransmitters, cytokines, toxins, drugs, and nanoparticles) and conditions (such as ischemia, hypoxia, hyperglycemia and the presence of ROS, proteolytic enzymes, and acids) can alter the endothelial barrier through multiple signaling pathways, mainly through the up or downregulation of TJ expression, the mislocalization of TJ molecules and their phosphorylation or dephosphorylation **(González-Mariscal L et al, 2008)**. Once the TJ architecture is changed, the disruption of the endothelial barrier leads to the leakage of fluid, macromolecules, and even cells, thereby affecting vascular homeostasis as well as related organs and tissues **(Rodrigues SF and Granger DN, 2015)**. Dysregulated inflammation and angiogenesis are major factors contributing to a wide array of diseases including cardiovascular diseases (CVD) and cancer **(Rajendran P et al, 2013)**.

At endothelial level, also a hyperglicemic condition (one of the most likely cause of endothelial damage) leads to the overproduction of reactive oxygen species (ROS) in mitochondria **(van den Oever IA et al, 2010**). ROS can modify endothelial function by a variety of mechanisms, such as peroxidation of membrane lipids, activation of NF-κB, and decrease of the availability of NO **(Madamanchi NR et al, 2005)**. Vascular disorders through overexpression of adhesion molecules are thought to play an important role in the pathogenesis of atherosclerosis. Activation of NF-κB induces adhesion molecules, such as VCAM-1 and ICAM-1, and, subsequently, induces an increase in the migration and adhesion of monocytic cells to EC, which are very important events during the inflammatory process. Previous studies have shown that high glucose (HG) activates nuclear factor-κB (NF-κB), one of the transcription factors for proinflammatory genes. NF-κB is present in the cytoplasm as inactive form bound to its inhibitor molecule, inhibitory factor of NF-κB-α (IκB-α). Translocation of NF- κB from the cytoplasm to the nucleus is preceded by the phosphorylation, ubiquitination, and proteolytic degradation of IκB-α **(Lee W et al, 2012)**. It is assumed that HG-induced CAMs expression may depend on activation of NF-κB. Under oxidative stress, EC generate

ROS, such as superoxides and peroxynitrite, leading to low-density lipoprotein (LDL) oxidation. The formation of ROS together with inflammatory factors including chemokines, cytokines, and adhesion molecules has been shown to be increased in atherosclerotic lesions **(Ham D and Skoryna SC, 2004)**.

Inflammatory responses, including inflammatory gene transcription, appear to involve free radicals or oxidative stress, and thus free radical scavengers can suppress inflammatory gene expression. LPS has been considered as a very important damaging factor and plays a pivotal role in the pathogenesis of cardiovascular disease **(Chen W et al, 2020)**. LPS, pathologically released into the circulation of a human host, interacts with specific receptors on the EC surface of all blood vessels **(Lee JW et al, 2008)**. LPS can also cause changing of endothelial cellular functions and morphology, slacking the linkage between EC, resulting in the aggravation of vascular endothelial tissue permeation. LPS can injure vascular EC (VEC) through indirect pathways and cause EC reaction to injury **(Lee W et al, 2014)**. In indirect pathways the immune cells can be stimulated by LPS, through the special LPS receptor CD14 on their membrane, to release many immune mediators (such as interleukins and TNF, etc.) and injure VEC. LPS can directly restrain Human Umbilical Endothelial Vein Cell (HUVEC) mitochondria dehydrogenase and stimulate ERK1/ERK2 and p38 MAPK phosphorylation **(Wang X et al, 2005)**. In addition, LPS can induce the depolymerization of F-actin microfilament and suppress the expression of VE-cadherin (a transmembrane protein) and intracellular nexin of HUVEC. The changes of endothelial barrier and cytoskeleton structure and adhesion between EC would lead to damage of endothelial barrier function **(Flemming S et al, 2015)**. Summing up, LPS acts as endotoxins in hosts, evoking several pathophysiological reactions in the vascular system, such as the breakdown of the barrier function **(Chan YH et al, 2020)**, apoptosis of ECs **(Hotchkiss RS et al, 2002)** and stimulation of coagulation cascades **(Pawlinski R and Mackman N, 2004)** with further progression of organ dysfunctions.

So, a growing body of evidence indicates that a variety of inflammatory factors including LPS, TNF-α, interleukin-1 (IL-1β), and ROS (hyperglycemic condition) are modulators of TJ **(Rao R, 2008)**.

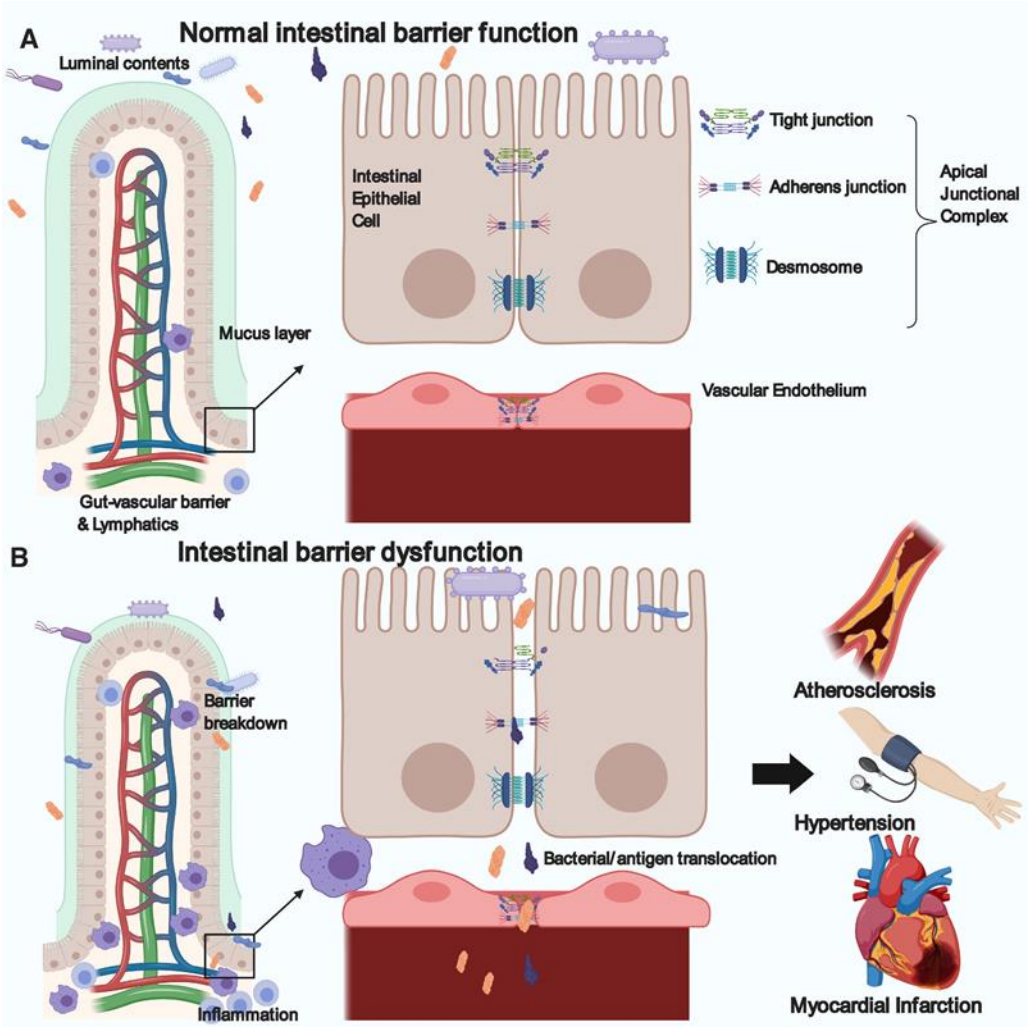


Fig 4. A schematic normal intestinal barrier (A) and alteration of intestinal structure (B) (From **Lewis CV and Taylor RW, 2020**).

1.2.1 Mitogen-Activated Protein Kinases (MAPK)

The signaling pathways involved in TJ regulation and interactions between transmembrane proteins and the actomyosin ring are controlled by several proteins, such as mitogen-activated protein kinases (MAPK). MAPK are a highly conserved family of serine/threonine protein kinases that respond to diverse stimuli (hormones, growth factors, cytokines, environmental stresses), especially in rapid inflammatory signal transduction, leading to the activation of multiple nuclear proteins and transcriptional factors that regulate gene expression, mitosis, differentiation, and apoptosis. The MAPK signaling pathway is activated through sequential steps of phosphorylation, beginning with the activation of MAPK kinase kinase (MAP3K), which phosphorylates and activates MAPK kinases (MAP2K). MAPK Kinases in turn phosphorylate and activate MAPK **(McCain J, 2013)** (**Fig 5**).

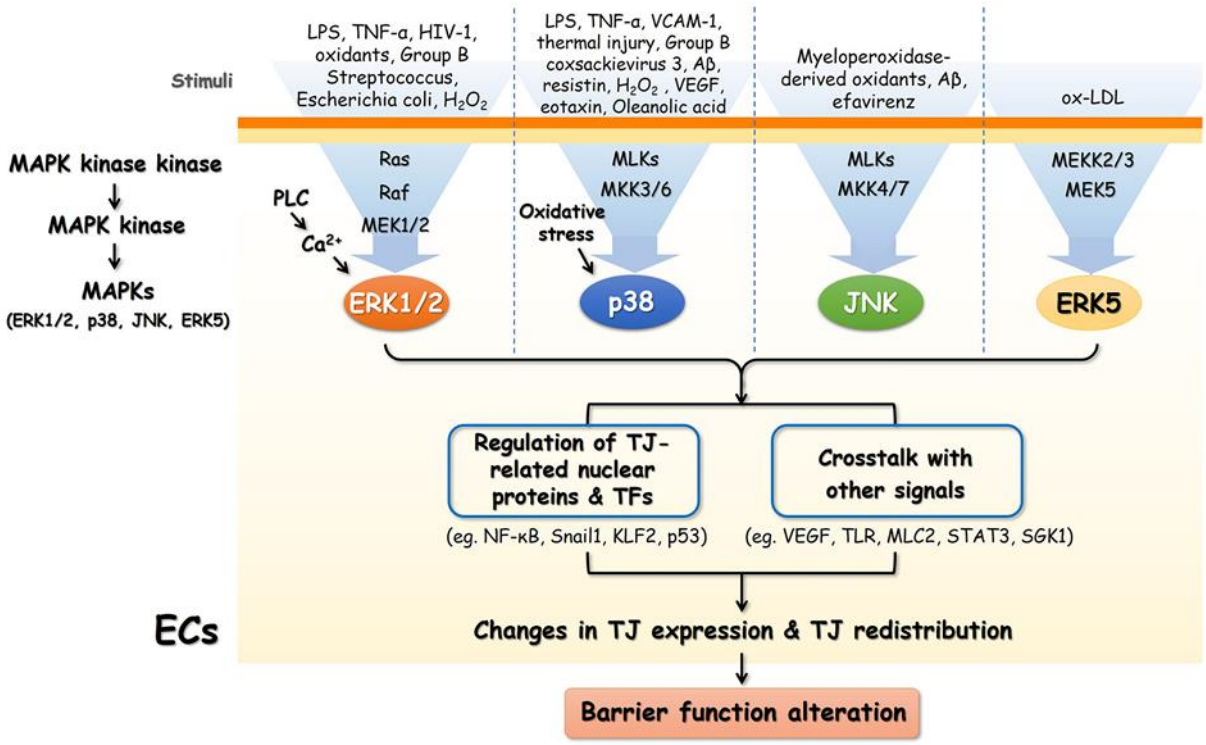


Fig 5. Schematic model of the effects of mitogen-activated protein kinase (MAPK) on the tight junction (TJ) in EC (ECs) (From **Cong X and Kong W, 2019**).

In particular, the mammalian MAPK family consists of extracellular signal-regulated kinase (ERK), p38, and c-Jun NH_2-terminal kinase (JNK; also known as stress-activated protein kinase or SAPK). Each of these enzymes exists in several isoforms: ERK1 to ERK8; p38-α, -β, -γ, and -δ; and JNK1 to JNK3 **(Dhillon AS et al, 2007)**.

MAPK cascade specificity is also mediated through interaction with scaffolding proteins which organize pathways in specific modules through simultaneous binding of several components. Kinase suppressor of Ras-1 (KSR) and MEK partner 1 (MP1) function as scaffold proteins for the ERK signaling pathway, whereas JNK-interacting proteins (JIPs) serve as scaffold proteins for the JNK pathway. β-Arrestin 2 acts as a scaffold protein for both the ERK and JNK signaling pathway **(Kyriakis JM and Avruch J, 2012; Whitmarsh AJ, 2006)**. The p38 and JNK signaling pathways are activated by proinflammatory cytokines such as tumor necrosis factor (TNF)-α and interleukin (IL)-1β or in response to cellular stresses such as genotoxic, osmotic, hypoxic, or oxidative stress **(Plotnikov A et al., 2011)**.

ERK 1/2, p38, and JNK, play a key role in modulating the actin-myosin cytoskeleton and regulating vascular and intestinal permeability. This major MAPK subfamilies, play an important role in human diseases, including CVD **(Hoefen RJ and Berk BC, 2002; Muslin AJ, 2008)**, IBD **(Waetzig GH et al, 2002; Scaldaferri F et al, 2009)** and cancer **(Bradham C and McClay DR, 2006)**.

For example, LPS can directly restrain HUVEC mitochondria dehydrogenase and stimulate ERK1/2 and p38 phosphorylation **(Wang X et al, 2005)**. Activated MAPK can then bind to and stimulate other kinase targets, translocate to the nucleus and activate the transcription of pro-inflammatory genes, such as Nf-κB, strongly activated in patients with IBD **(Atreya I et al, 2008)**. **Sun P et al (2013)** hypothesize that MAPK/NF-κB signaling was involved in the activation of the cholinergic anti-inflammatory pathway in IBD. Furthermore,

Kim SW et al (2011) and **Kim MH et al (2018)** demonstrated that high concentrations of glucose increased phosphorylation of p38 and ERK pathways in HUVEC.

The main regulators of MAPK are specific phosphatases belonging to various families, including PP2C Ser/Thr phosphatases, Tyr phosphatases (PTPs) or dual specificity phosphatases (DSPs) such as the MAPK phosphatase (MKP) subgroup. MAPK phosphatases (MKPs) are a family of dual-specificity phosphatases (DUSPs) that can recognize and inactivate MAPK. EC express several MKPs, including MKP-3 **(Rossig L et al, 2002)**, which is also termed DUSP6 and predominantly localized in the cytoplasm and dephosphorylates not only ERK1/2 but also p38 **(Zhang YY et al, 2011)** and JNK **(Ndong C et al, 2014)**. Although MKP-3 has been shown to attenuate endothelial inflammation **(Whetzel AM et al, 2009; Leng YP et al, 2018)**. **Hsu SF et al (2018)** recently suggested that MKP-3 promotes inflammation through inducible expression of intercellular adhesion molecule-1 (ICAM-1) in TNF-α stimulated EC.

Previous studies have indicated that the enhanced expression of adhesion molecules on EC is associated with the activation of pro-inflammatory transcriptional programs, such as NF-κB **(Liao JK, 2013)** and MAPK pathway **(Hommes DW et al, 2003)**. Inhibition of both ERK1/2 and p38 activation can reduce LPS-induced expression of adhesion molecules in EC **(Xu Y et al, 2013)**. ROS induced by inflammatory stimuli also regulate NF-κB activation and the consequential elevation of adhesion molecules **(Gloire G et al, 2006)**.

In general, dephosphorylation of MAPK inactivates their function in many metabolic, developmental or adaptive responses **(Vilela B et al, 2010)**. Activation of MAPK by various cytokines and growth factors is important in modulating cellular responses **(Hagemann C and Blank JL, 2001; Bogoyevitch MA and Court NW, 2004)** and the role of MAPK signaling has been of interest in TJ regulation **(Oshima T et al, 2007; Schneeberger EE and Lynch RD, 2004)**.

Therefore, there is a clear agreement among all the studies in endothelial and intestinal monolayers that activation of MAPK, ERK1/2, p38 and JNK1/2, is involved in the disruption of TJ and resulting barrier dysfunction, so the mitogenic effect of MAPK activity is logically opposed to TJ formation **(Oshima T. et al., 2007)**.

1.2.1.1 p38

The p38 MAPK, originally referred as cytokine-suppressive anti-inflammatory drug binding protein (CSBP), was first discovered in 1994 as 38 kDa protein kinase involved in the regulation of inflammatory cytokine biosynthesis **(Cuenda A and Rousseau S, 2007)**. p38 cascade is known to be activated by various pro-inflammatory and stressful stimuli and thus plays an essential role in cellular responses including inflammation, cell proliferation, differentiation, apoptosis, and invasion **(Ono K and Han J, 2000; Pearson G et al, 2001)**.

There are four p38 MAP kinases in mammals: α, β, γ and δ. Among all p38 isoforms, p38α is the best characterised and is expressed in most cell types. The p38 MAPK are strongly activated *in vivo* by environmental stresses and inflammatory cytokines, and less by serum and growth factors. Together with the JNK family, p38 are also known as Stress-Activated Protein Kinases (SAPKs). The canonical activation of p38 occurs via dual phosphorylation by MKK3 and MKK6 (also called SKK3) that are highly selective for p38 and do not activate JNK or ERK1/2 **(Cuenda A and Rousseau S, 2007)**. Some p38α and p38β physiological substrates have been shown to be transcription factors, other protein kinases which in turn phosphorylate transcription factors, cytoskeletal proteins, and translational machinery components, and other proteins such as metabolic enzyme, glycogen synthase or cytosolic phospholipase A2 **(Kuma Y et al, 2004; Yong HY et al, 2009)**.

Inflammatory signaling via p38 may also be an important regulator of intestinal permeability. p38 and JNK show increased phosphorylation level in the inflamed tissue from IBD patients, and some clinical studies have been carried out to cure IBD or other autoimmune diseases with small molecule inhibitors of MAPK **(Mitsuyama K et al, 2006; Waetzig GH et al, 2002)**. *In vitro* studies have shown that pro-inflammatory mediators increase phosphorylation of intestinal p38, with pharmacologic inhibition of p38 activation improving intestinal barrier integrity **(Wang Q et al, 2008)**.

p38 regulates stress responses, inflammation and apoptosis, and it has been implicated in the pathogenesis of endothelial dysfunction and atherosclerosis **(Papaconstantinou J, 2019)**. Endothelial function is impaired in hyperglycaemia **(Jansen F et al, 2013)** and p38 activation has been described in the context of progression and complications of diabetes **(Carlson CJ et al, 2003)**. **Liu Z and Cao W (2009)** reported in patients with type 2 diabetes mellitus, hyperglycemia, chronic inflammation, high plasma levels of FFAs and over-activation of the cardiovascular RAS, all conditions that characterize the pathological status and where p38 results to be activated. Chronic activation of p38 *in vivo* could lead to decreased numbers of endothelial progenitor cells, impair angiogenesis and vascular repair, and facilitate the development of atherosclerotic diseases. **Zheng HT et al (2008)** show that p38 is important for HG and high insulin-induced expression of adhesion molecules in EC.

1.2.1.2 ERK

MAPK ERKs are a group of serine/threonine kinases, involved in the *Ras-Raf-MEK-ERK* signaling pathway **(Yang S and Liu G, 2017)** and expressed to various extent in all tissues **(Chen Z et al, 2001)**, where they regulate gene expression, mitosis, differentiation, cell survival and apoptosis **(Yoon S and Seger R, 2006)**. The ERK family consists of ERK 1, 2, 3, 5 and 6 **(Plotnikov A et al, 2011)**. ERK1 and ERK2 are two important members of the ERK pathway, with molecular weights of 44 and 42 kDa, respectively **(Yang S and Liu G, 2017)**. They are strongly activated by growth factors, serum, and phorbol esters and to a lesser extent by ligands of the heterotrimeric G protein-coupled receptors, cytokines, osmotic stress, and microtubule disorganization. Activated ERK1 and ERK2 phosphorylate numerous substrates in all cellular compartments, including various membrane proteins, nuclear substrates, cytoskeletal proteins and several MKs **(Dhillon AS et al, 2007)**. Excessive activation of upstream proteins and kinases in the ERK pathway has been shown to induce various diseases, including cancer, inflammation, developmental and neurological disorders **(Yoshizumi M et al, 2012; Wagner EF and Nebreda AR, 2009; Gupta J and Nebreda AR, 2015)**.

A study showed that activation of PKC disrupts TJ by a MAPK-dependent mechanism **(Wang Y et al, 2004)**, and constitutively active Ras **(Mullin JM et al, 2005)** or constitutively active Raf-1 **(Li D and Mrsny RJ, 2000)** induce ERK activation and disruption of epithelial TJ.

Varma S et al (2002) showed that the ERK1/2 signalling pathway was important in the regulation of baseline permeability and cGMP-induced hyperpermeability, which was inhibited by a selective p44/42-MAPK inhibitor, AG126, in HUVEC. ERK signaling activation and ERK phosphorylation was speculated to be involved in the regulation of cell junction integrity and the increase in permeability observed in HUVEC monolayers upon exposure of fine powder PM 2.5 **(Long YM et al, 2020)**.

1.2.2 NLRP3 Inflammasome

The innate immune system is the first line of host defense and the engagement of germline-encoded pattern-recognition receptors (PRRs) activates it in response to harmful stimuli, such as invading pathogens, dead cells, or environmental irritants **(Takeuchi O and Akira S, 2010)**. PRRs recognize the presence of unique microbial components, called pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs), which are generated by endogenous stress, and trigger downstream inflammatory pathways to eliminate microbial infection and repair damaged tissues **(Franchi L et al, 2009)**. The inflammasome is a multimolecular complex which functions as an intracellular sensor (a PRR) for signals from host, environment and pathogens, and is generally triggered by either exogenous PAMPs or DAMPs **(Gao YL et al, 2018)**. There are five members of PRRs that have been confirmed to form inflammasomes: NLRP1, NLRP3, and NLRC4, members of the NLR family, nucleotide-binding oligomerization domain (NOD), leucine-rich repeat (LRR)-containing proteins, as well as absent-in-melanoma 2 (AIM2) and pyrin. **(Sharma D and Kanneganti TD, 2016; Lamkanfi M and Dixit VM, 2014)**. In the case of some of these PRRs, a bipartite adaptor protein, which is known as apoptosis-associated speck-like protein containing a caspase-recruitment domain (ASC), facilitates the recruitment of pro-caspase-1 to the inflammasome complex **(Fernandes-Alnemri T et al, 2007)**. In addition, other members of PRRs, such as NLRP2, NLRP6, NLRP7, NLRP12, and IFI16, were also reported to form inflammasomes, but do not need the adapter protein ASC **(Sharma D and Kanneganti TD, 2016).**

Among all the NLR inflammasomes, NLRP3 inflammasome is the best characterized and most extensively studied canonical inflammasome, acting as a platform that recruits apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) and promotes autocatalytic activation of caspase-1 as well as subsequent maturation of IL-1β and IL-18

(Lamkanfi M and Kanneganti TD, 2010). In particular, once being activated, NLRP3 recruits and cleaves pro-caspase-1 into its active forms (p20 and p10 subunit), in turn processing pro-IL-1β and pro-IL-18 into their mature forms **(Schroder K and Tschopp J, 2010)**. This pathway is currently designated as "canonical NLRP3 inflammasome activation". In addition to processing proinflammatory cytokines, active caspase-1 also mediates proteolytic cleavage of GSDMD (gasdermin D) and induces a regulated cell death called pyroptosis. Cell death by pyroptosis is accompanied by membrane pore formation, fluid influx, cell swelling, cell lysis and eventually plasma membrane rupture and leakage of cellular contents **(Bergsbaken T et al, 2009)**. Inflammasome activation causes a very potent self-amplifying response, which might cause damage to the host if not properly controlled. Recent studies have revealed that NLRP3-related pyroptosis plays an important role in intestinal diseases, particularly in DSS-induced colitis and inflammatory bowel diseases **(Cui S et al, 2020; Chen X et al, 2019)**.

1.2.2.1 Activation and regulation of NLRP3 inflammasome

Canonical NLRP3 inflammasome activation requires two parallel and independent steps: transcription and oligomerization **(Latz E et al, 2013)**. The first step is regulated by innate immune signaling, mediated primarily by toll-like receptor (TLR)-adaptor molecules myeloid differentiation primary response 88 (MyD88) and/or cytokine receptors, such as the TNF receptor, which, in turn, activate pro-IL-1β and NLRP3 transcription via NF-κB activation **(Bauernfeind FG et al, 2009)**. The second step results in NLRP3 inflammasome oligomerization, caspase-1 activation and IL-1β and IL-18 processing and release **(Faustin B et al, 2007)**. Various cellular insults can provide the second signal for the NLRP3 inflammasome, resulting in caspase-1 cleavage and cytokine release. These include stimuli associated with infections **(Hornung V et al, 2008; Zhong Z et al, 2013)**, ROS, agents and conditions triggering mitochondrial dysfunction. Metabolic stress, inhibitors of respiratory chain complexes **(Zhou R et al, 2011)** can also promote NLRP3 inflammasome oligomerization and activation by initiating assembly of a multiprotein complex consisting of NLRP3, the adaptor protein ASC, and pro-caspase-1. These highly diverse activation signals seem to engage several pathways to activate NLRP3 **(Elliott EI et al, 2015)** (**Fig 6**).

Subsequent studies revealed that intracellular LPS can activate caspase-11 in mice (caspase-4/5 in humans) leading to the non-canonical activation of the inflammasome, independent of TLR4 **(Kayagaki N et al, 2013; Hagar JA et al, 2013)**. Caspase-11 is subsequently identified as the direct sensor of intracellular LPS and can be activated directly by binding of cytosolic LPS **(Shi J et al, 2014)**. Both caspase-1 (released via the canonical activation of inflammasome) and caspase-11 can cleave the gasdermin D (GSDMD) to generate its N-terminal fragment (GSDMD-NT) which executes pyroptosis through forming pores on the plasma membrane **(Kayagaki N et al., 2015)**.

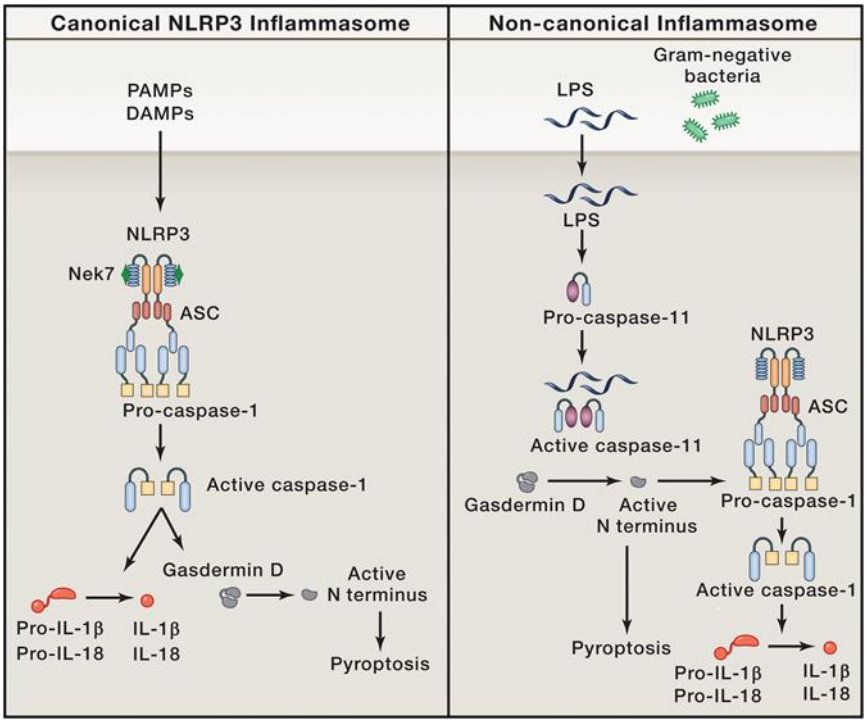


Fig 6. NLRP3 Inflammasome activation: canonical and non-canonical modes of activation (From **Rathinam VAK and Fitzgerald KA, 2016**).

1.2.2.2 Role of NLRP3 in intestinal and endothetial diseases

A growing body of evidence highlights the relevance of the NLRP3 inflammasome in the pathophysiology of several autoinflammatory syndromes, as well as metabolic and/or inflammatory disorders (such as, obesity, atherosclerosis, type 2 diabetes, gout, and intestinal inflammation) **(De Nardo D and Latz E, 2011; Broderick L et al, 2015; Elinav E et al, 2013)**.

In the setting of intestinal microenvironment, NLRP3 inflammasome plays a pivotal role both in regulating the integrity of intestinal homeostasis and in shaping innate immune responses during bowel inflammation **(Elinav E et al, 2013)**. Scientific evidence has demonstrated that NLRP3 over-activation during bowel inflammation could be associated with a breakdown of enteric immune balance, suggesting the involvement of NLRP3 in the pathogenesis of various immune-mediated diseases as IBD **(Pellegrini C et al, 2017)**. Dysbiosis, alterations of intestinal epithelial barrier and uncontrolled immune responses to pathogenic stimuli represent the main factors implicated in the pathogenesis of bowel inflammation and thus IBD **(Camilleri M et al, 2012)**.

Additionally, the over-activation of NLRP3 inflammasome participates in abnormal expressions of TJ proteins in inflammatory gut disorders **(Feng Y et al, 2019; Zhuang Y et al, 2015; Lei-Leston AC et al, 2017)**. Several lines of preclinical evidence have unraveled a dual role of NLRP3 inflammasome in the pathogenesis of bowel inflammation. In particular, some studies showed a regulatory and reparative role of NLRP3 in the maintenance of immune tolerance and epithelial barrier integrity **(Hirota SA et al, 2011)**. Conversely, others reported that the overactivation of NLRP3 inflammasome during intestinal inflammation is associated with a breakdown of intestinal immune balance, with consequent detrimental effects to the host **(Zhang J et al, 2014)**. For example, **Zaki MH et al (2010)** observed that the induction of colitis by dextran sodium sulfate (DSS) in NLRP3-/- mice was associated with disruption of

the intestinal epithelial barrier and increased mucosal permeability compared to wild-type DSS mice (WT), resulting in bacterial translocation into the mucosa and systemic dissemination. This detrimental effect on the intestinal epithelial barrier was due to a decrease in the release of the inflammasome-dependent cytokine IL-18, a crucial mediator in colonic mucosal barrier repair, and a decrease in the anti-inflammatory cytokine IL-10 and protective growth factor expression, thus suggesting the ability of NLRP3 to regulate the production of pro- and anti-inflammatory mediators in presence of intestinal inflammation **(Hirota SA et al, 2011)**.

Several pathogenic factors including hyperglycemia, hyperlipemia, proinflammatory agents and oxidative stress have been identified as risk factors of endothelial dysfunction **(Leng B et al, 2019)**. IL-1β is a potent proinflammatory cytokine, related to insulin resistance, that has a key proatherogenic effect in vascular diseases **(Grebe A et al, 2018)**. NLRP3 inflammasome activation is involved in several cardiovascular risk factors, including insulin resistance, obesity, diabetes **(Legrand-Poels S et al, 2014)**, metabolic diseases **(Goldberg EL and Dixit VD, 2015)** and atherosclerosis **(Duewell P et al, 2010; Liu W et al, 2014)**.

Atherosclerosis, as the major cause of CVD, is the primary cause of death worldwide for its high morbidity and mortality **(Ladapo JA et al, 2015)**. EC dysfunction, the priming event in the development of atherosclerosis, is a robust indicator of CVD risk **(Lusis AJ, 2000)**. Previous studies reported that inappropriate cell apoptosis, proliferation, migration, and abnormal expression of adhesion molecule proteins are all manifestations of EC dysfunction **(Yao X et al, 2018)**. Thence, inhibition of endothelial cell dysfunction has a protective effect against atherosclerosis. Recent studies have indicated that atherosclerosis is closely related to innate immunity, in which NLRP3 inflammasomes plays an important role by activating cytokine and chemokines maturation and secretion and cell death in response to endogenous and exogenous danger signals **(Abderrazak A et al, 2015)**. In addition, IL-1β maturation and secretion is recognized as another major response of NLRP3 inflammasome activation and

plays a critical role in the initiation and progression of atherosclerosis **(Pavillard LE et al, 2018)**. In particular, IL-1β is the major proinflammatory cytokine that induces the generation of other inflammatory mediators, thus instigating a self-enlarging cytokine network **(Arend WP et al, 2008)**. NLRP3 inflammasome is a platform for IL-1β production and links inflammation and metabolic diseases **(De Nardo D and Latz E, 2011)**. Based on these references, the inhibition of NLRP3 inflammasome activation could contribute to attenuate pyroptosis and endothelial cell dysfunction.

It is notable that ROS mediates NLRP3 inflammasome activation and contributes to hemodynamic-induced endothelial inflammation and atherosclerosis **(Xiao H et al, 2013; Schroder K and Tschopp J, 2010)**. Increased oxidative stress has been detected in diabetic model mice and diabetes patients. Hyperglycemia drives ROS production by mitochondrial electron transport chain, glucose autoxidation and NADPH oxidase **(Giacco F and Brownlee M, 2010)**. Moreover, NLRP3 inflammasome could be activated by HG in several types of cells **(Wan Z et al, 2019)**.

A sustained HG environment promotes oxidative stress, apoptosis, and inflammatory factors expression, which results in the dysfunction of ECs. Previous studies have shown that suppression of the NF-κB and NLRP3 inflammasome pathways ameliorate HG-induced inflammatory responses and EC dysfunction. Increased evidence has been presented to indicate that TLR4 is implicated in diabetes mellitus progression via activation of the NF-κB and NLRP3 inflammasome pathways. Therefore, it is believed that downregulation of TLR4 will attenuate HG induced inflammatory responses and EC dysfunction in diabetes mellitus **(Cheng J et al, 2019)**.

1.3 Polyphenols in the Mediterranean diet and EVOO

It is well established that diet, among other external influencing factors, has a key role in the prevention and management of different diseases. The health benefits of dietary patterns are derived from the nutritional and non-nutritional bioactive compounds present, which exert a direct effect on the individual (firstly on the intestinal epithelial barrier and then on distant tissues), on the microbiota (composition and functionality), and on the immune system (the intestinal and systemic immunity). Of the different occidental diets, the Mediterranean diet (MD) is the one with more scientific evidence suggesting a positive impact on health **(Pérez-Cano FJ, 2022)**. This dietary pattern is characterized by a high intake of fruits and vegetables, whole grains, legumes, nuts, fish, white meat and olive oil. It also includes moderate consumption of fermented dairy products, low intake of red meat and drinking wine with moderation during meals. Nutritionally, this diet is low in saturated fats and animal protein, high in bioactive compounds, fibers and monounsaturated fats (MUFA), and exhibits an adequate omega-6/omega-3 fatty acid balance **(Pérez-Cano FJ, 2022)**.

The study PREDIMED (observational cohort studies and a secondary prevention trials that show an inverse association between adherence to the Mediterranean diet and cardiovascular risk) showed that the intervened groups that had a higher intake of total polyphenols presented a reduction of 46% in the CVD risk, when compared to those with lower intake of polyphenols **(Tresserra-Rimbau A et al, 2014)**. The PREDIMED trial demonstrated that MD intervention supplemented with EVOO results in the reduction of CVD risk, reduction of atrial fibrillation, prevention of diabetes and metabolic syndrome, reduction of diastolic blood pressure, higher protection against breast cancer and lower prevalence of non-alcoholic fatty liver disease (**Sánchez-Villegas A and Sánchez-Tainta A, 2018; Storniolo CE et al, 2017; Pintó X et al, 2019; Mourouti N and Panagiotakos DB, 2016)**, with a downregulation of inflammatory biomarkers **(Estruch R, 2010)**. In particular, an observational study based on

the PREDIMED cohort indicated that consumptions of 10 g of EVOO/day were related to CVD risk diminutions up to 10 % **(Guasch-Ferré M et al, 2014)**. In another meta-analysis of randomized controlled trials including cases of stroke and cases of coronary heart disease (CHD), it was reported that for each increase of 25 g of olive oil intake (rich in HT and Tyr, oleocanthal, and resveratrol), stroke and CHD risks were reduced by 26% and 4%, respectively **(Martínez-González MA et al, 2014)**.

The main bioactive compounds, which explain the health benefits of this dietary pattern, are antioxidants, fibers, monounsaturated and omega-3 fatty acids, phytosterols and probiotics **(Urquiaga I et al, 2017)**. MD provides a high amount of antioxidants of vegetable origin, among them, vitamin C, vitamin E, β-carotene, glutathione, lycopene and polyphenols, which help to reduce oxidative damage at cellular and systemic level and, therefore, to reduce the incidence of chronic diseases, such as cardiovascular disease, metabolic sindrome, carcer, etc. **(Halliwell B, 1997; Urquiaga I et al, 2010)**. Polyphenols can exert a direct antioxidant effect, as well as participate in the cascades of intracellular signaling, through their interaction with cellular receptors or proteins, modulating in this way gene expression and determining beneficial physiological responses **(Galleano M et al, 2010)**.

The protective effects of polyphenols on CVD have been attributed to the improvement in the endothelial function, to the reduction of the arterial pressure and to the improvement of the lipid profile **(Grassi D et al, 2010)**. Studies *in vitro* show an increase in endothelial nitric oxide synthase (eNOS) expression and activity, and an inhibition of endothelial NADPH oxidase in response to polyphenols, which could explain, in part, their modulating effect on the endothelial function **(Sies H, 2010; Leikert JF et al, 2002)**. In the upper gastrointestinal tract, polyphenols, like other antioxidants, would act reducing hydroperoxides from ingested food and complexing transition metals, thus helping to counteract the postprandial oxidative stress **(Sies H et al, 2005)**. Additionally, polyphenols are partially metabolized by colonic microbial

flora, generating bioactive mediators beneficial to human health **(Davis CD and Milner JA, 2009)**.

EVOO is an essential food of the MD and countries in the Mediterranean area like Spain, Italy, and Greece represent the most important producers worldwide **(Kalogiouri NP et al, 2018)**. As documented by several studies published over the past decades, a large part of the beneficial effects of MD on human health promotion can be ascribed to EVOO. In fact, consumption of EVOO is able to reduce lipid and DNA oxidation, ameliorate lipid profile and insulin-resistance, endothelial dysfunction, inflammation, and to lower blood pressure in hypertensive patients **(Violi F et al, 2015; Buckland G and Gonzalez CA, 2015)**. The quality and the organoleptic properties of EVOO depend on different factors such as cultivar, geographic origin, climatic conditions, agronomic and processing techniques that are able to modify fatty acids composition and bioactive compounds concentration. Nutritional and anti-oxidant properties of EVOO are related to the presence and concentration of tocopherols, carotenoids and phenolic compounds which are of great importance in human health **(Ranalli F et al, 2013)**.

Regarding its composition, EVOO is mainly composed of triglycerides (97–99%) and minor compounds (1–3%), which are the principal responsible for its biological properties and sensory attributes. It has a high content of MUFA (65–83%), especially oleic acid, and some polyunsaturated fatty acids (PUFA) such as linoleic acid, with a moderate ω6/ω3 ratio; this lipid profile has been recognised as anti- thrombotic and regulators of blood pressure and linked to protective effects on coronary, autoimmune and inflammatory disorders **(Mariotti M and Peri C, 2014; Sánchez-Villegas A and Sánchez-Tainta A, 2018; Lombardo L et al, 2018)**.

1.3.1 EVOO polyphenols and their bioavailability

Phenolic compounds, commonly called polyphenols, have been largely reported in EVOO composition, with more than 30 different compounds identified **(Kalogeropoulos N And Kaliora AC, 2015; Segura-Carretero A et al, 2010)**, such as phenylethanoids (hydroxytyrosol (HT) and tyrosol (Tyr)), cinnamic (caffeic acid and p-coumaric acid) and benzoic (vanillic acid) acids, flavones (apigenin and luteolin), and secoiridoids (oleuropein and ligtroside derivatives) **(Bianco A et al, 2004; Bendini A et al, 2007) (Fig 7)**. Traditionally, the beneficial properties of EVOO have been attributed to its high MUFA content; however, cumulative evidence has shown that the minor components of EVOO, as phenolic compounds and other compounds with antioxidant/antinflammatory characteristics, may also contribute to the healthy features of EVOO **(Tripoli E et al, 2005)**. These components make up only 1–2% of EVOO, but they are completely absent in other types of oils derived from seeds or fruits **(Yubero-Serrano EM et al, 2019)**.

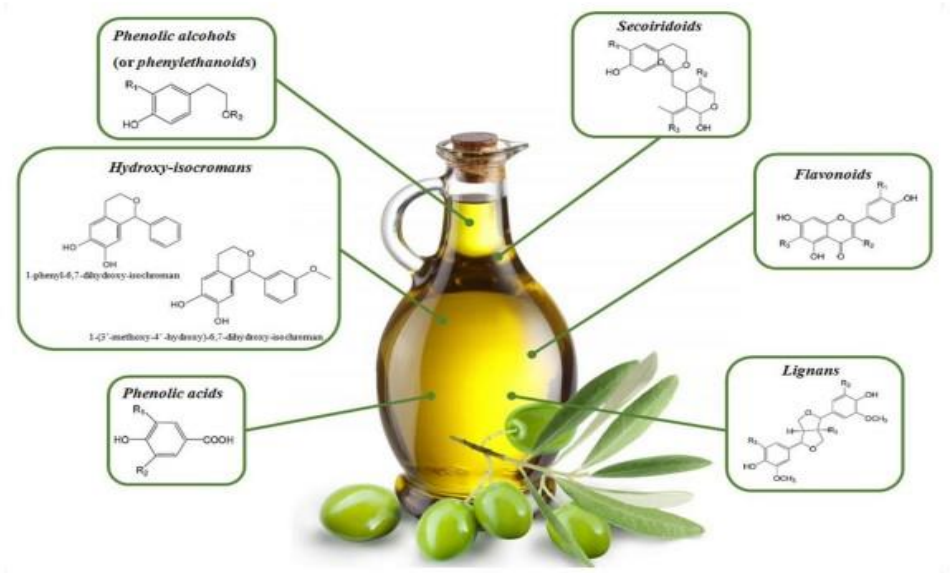


Fig 7. The main phenolic compounds in EVOO (From **Finicelli M et al, 2021**).

Most of the effects of the phenolic compounds are due to their *in vivo* bioavailability **(Manna C et al, 2009)**. After ingestion, EVOO polyphenols can be partially modified in the acidic environment of the stomach. Aglycone secoiridoids are subject to a time-dependent hydrolysis in the acidic gastric environment, leading to an approximate 5-fold increase in the amount of free HT and 3-fold increase in free Tyr, after only 30 min **(Corona G et al, 2006)**. Food phenolics absorption is determined primarily by their chemical structure, which depends on several factors such as the degree of glycosylation, acylation, their basic structure, conjugation with other phenolics, molecular size, degree of polymerization, and solubility **(Karakaya S, 2004)**. Besides, different polarities of the various phenolics have been postulated to play a key role in the absorption of these compounds. A study by **Visioli F et al (2000)**, has revealed that after ingestion, EVOO phenolics such as HT and Tyr are dose-dependently absorbed in humans. Other clinical and animal studies have provided evidence that phenolic compounds exert their biological effects in a dose-dependent manner **(de la Torre R, 2008)**. A human study conducted in normal and ileostomy subjects fed with EVOO polyphenols has indicated that the major site for the absorption of EVOO polyphenols is the small intestine **(Vissers MN et al, 2002)**.

In the process of crossing epithelial cells of the gastrointestinal tract, phenolic compounds from EVOO undergo to a classic phase I/II biotransformation, and an extensive first-pass metabolism may already occur at this level. Conjugation is characterised by the presence of conjugating enzymes consisting of many superfamilies of enzymes including sulphotransferases (SULT), UDP glucuronosyltransferases (UGT), catechol-O-methyltransferase (COMT) and glutathione transferases (GTs) (**Serreli G and Deiana M, 2019)**. The metabolic pathway of each compound depends on their affinity towards each phase II metabolic enzyme, as well as on the quantity of expression and location of the enzyme **(Boronat A et al, 2021)**. For instance, hydroxycinnamic acids are essentially sulphated (mainly

by SULT1A1) and, to a lesser extent, glucuronidated (by UGT1A9) **(Wong CC et al, 2010)**, whereas flavonoids are both sulfated and glucuronidated, but the rates of glucuronidation are faster than those of sulfation **(Tang L et al, 2012)**. The simple phenol HT can be both sulphated and glucuronidated, glucuronides being more predominant at lower doses, and sulphates being the major metabolites found following high doses **(Kotronoulas A et al, 2013)**.

Once metabolised, polyphenols can reach the liver where they can undergo further metabolisation by phase I (led by the cytochrome P450 (CYP450) superfamily of microsomal enzymes) or phase II metabolism, and then excreted into the bile to go back to the intestinal tract **(Kawabata K et al, 2019)**. Furthermore, polyphenols can also reach the colon at relevant concentrations, where a significant number of bacteria participate in their metabolism, in particular, as regards dietary polyphenols that are not absorbed and hydrophilic polyphenol conjugates (sulphates and glucuronides) that are excreted by the intestine and liver **(Liu Z and Hu M, 2007)**.

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 the attained concentrations after their ingestion, which are too low to explain the observed biological activities in *in vitro* and *in vivo* models at higher doses/concentrations **(Serreli G and Deiana M, 2018; Vissers MN et al, 2004)**.

As regards the metabolic pathways of EVOO phenols, previous studies have revealed that they are excreted in the urine, mainly as glucuronide conjugates and, to a lesser extent, sulphates **(Atzeri A et al, 2016; de la Torre R, 2008; Caruso D et al, 2001)**. It has been shown that after ingestion, a large number of metabolites are produced from the phenolics HT, oleuropein aglycone and oleocanthal, indicating significant postabsorption metabolism of these compounds.

Conversely, the lowest number of metabolites originated from Tyr, luteolin, apigenin and acetoxypinoresinol, suggest that these compounds might have been excreted in faeces, destroyed in the gastrointestinal tract, excreted through another metabolic pathway or poorly absorbed **(Garcia-Villalba R et al, 2010)**. In this case, it has been proposed that these compounds may exert local antioxidant activities in the gastrointestinal tract and this suggestion is supported by research demonstrating the free radical scavenger activity of EVOO phenolics in both the faecal matrix and intestinal epithelial cells **(de la Torre R, 2008)**.

1.3.2 Biological activity of EVOO polyphenols

EVOO is known for having a high content of antioxidant compounds with protective properties (**Fig 8**) against free radicals. Therefore, it has been pointed out that its high consumption is related to a generally low risk of suffering colon, breast or skin cancer as well as beneficial effects on aging and coronary diseases **(Foscolou A et al, 2018)**. The preventive role of EVOO polyphenols against CVD was also documented in a meta-analysis of controlled trials that evaluated the effect of low versus high polyphenols EVOO on markers of CVD risk. In this study, EVOO consumption ranged from 25–75 mL/day. High polyphenols EVOO significantly reduced the CVD-risk markers malondialdehyde, oxidized LDL, total cholesterol, and some inflammatory indicators like C-reactive protein (CRP) or interleukin-6 (IL-6) **(George ES et al, 2019)**.

High polyphenols EVOO has also been proposed as a preventing tool of Alzheimer's and other neurodegenerative diseases **(Román GC et al, 2019)** as anti-inflammatory **(Casas R et al, 2017)** and immune-stimulating **(Mariotti M and Peri C, 2014)**. In particular, a recent meta-analysis of randomized controlled trials, showed a significant reduction of IL-6 and CRP levels, when EVOO was consumed as a supplementary or natural intake **(Schwingshackl L et al, 2015)**. The adherence to a high polyphenols EVOO breakfast decreased the postprandial inflammatory response, reducing the levels of plasma lipoproteins in patients with metabolic syndrome **(Camargo A et al, 2014)**. The higher polyphenol content of EVOO may mediate the mentioned favorable effect as it has demonstrated anti-inflammatory effects *in vitro* **(Rosignoli P et al, 2013)**. The anti-inflammatory effect of phenolic compounds-enriched EVOO has also been reported in the adipose tissue in mice, with anti-atherosclerotic effects **(Luque-Sierra A et al, 2018)**. Due to these mentioned capacities, EVOO has also been proposed as a potential therapeutic product, reducing inflammation in inflammatory bowel diseases, including ulcerative colitis and Crohn's disease, both related to chronic inflammation

of the intestinal mucosa **(Limketkai BN et al, 2018; Cabré E et al, 2019)**. Besides, both *in vitro* and *in vivo* studies outline that the anti-inflammatory activity of EVOO provides neuroprotective effects that could prevent cognitive decline and, therefore, the development of Alzheimer's disease or elderly dementia **(Román GC et al, 2019; Klimova B et al, 2019)**.

Additionally, EVOO consumption has shown positive effects on gut microbiota **(Gavahian M et al, 2019)**. Much of the health benefits of EVOO consumption are attributed to the metabolism of the phenolic compounds carried out by the gut microbiota **(Romani A et al, 2019)**. A recent meta-analysis of randomized controlled trials supports the prebiotic action of polyphenols, capable of modulating and improving intestinal microbe populations, which affects CVD and colorectal cancer markers **(Moorthy M et al, 2020)**. Furthermore, another randomized controlled trial showed that the ingestion of EVOO enriched with phenolic compounds decreases the serum level of oxidized LDL in hypercholesterolemic participants as well as increases the presence of *Bifidobacterium spp* in feces. The possible modulation of gut microbiota by EVOO and its role in cancer prevention, especially colorectal cancer, has also been suggested **(Borzì AM et al, 2018)**.

The contribution of the phenolic components to the beneficial properties of EVOO are mainly due to their antioxidant activity.

Oliveras-López MJ et al (2008), investigated the mechanisms by which polyphenols from EVOO impart protective and beneficial effects as antioxidants in Oncins France 1 (OF1) mice. They found that the ratio of reduced:oxidized GSH and the antioxidant capacity in plasma was greater in animals fed with EVOO than those fed with sunflower oil or control group. Furthermore, these animals presented an up-regulation of CAT and GTPX activities in the islet of Langerhans. These authors postulated that HT plasma levels, higher in animals fed with EVOO, might support the key role of this polyphenol in these antioxidant mechanisms. The results indicated that, in metabolic tissues, protection by EVOO against oxidative stress occurs

primarily through a direct antioxidant effect as well as through an indirect mechanism that involves greater expression and activity of cellular antioxidant enzymes.

In another study, **Bogani et al (2007)** demonstrated that EVOO is capable of reducing the post prandial events associated with inflammation and oxidative stress, as well as to increase serum antioxidant capacity in normolipemic, healthy subjects fed with a fat-rich meal (consisting in a different kind of oil (EVOO, OO or corn oil). EVOO (but not OO or corn oil) decreased the levels of thromboxane B2 (TXB2) and leukotriene B4 (LTB4) and oxidative stress markers (urinary hydrogen peroxide level and serum antioxidant capacity).

Our group evaluated the effect of the phenolic fraction extracted from a cultivar of olives (*Tonda di Cagliari*) in the protection of Caco-2 cells from oxidative stress, demonstrating that the main components, HT, oleuropein, and verbascoside inhibited ROS generation and reduced membrane oxidative damage **(Serreli G et al, 2017)**. A pilot study on healthy subjects showed that administration of 12 g of table olives of the Nocellara del Belice variety modulated systemic inflammation reducing IL-6 and MDA levels **(Czerska M et al, 2015)**. Based on all the mentioned evidence, the EFSA (European Food Safety Authority) has approved a claim which states that polyphenols protect against lipid peroxidation at a minimal dose of 5 mg/kg/day, equivalent to the consumption of 23 g of EVOO **(EFSA Panel on Dietetic Products, Nutrition and Allergies, 2011)**. Specifically, the phenolic compounds bind to LDL particles and protect them against oxidation. High level of oxidized LDL in plasma is considered a strong predictor of CVD and has been widely associated with metabolic diseases, such as obesity, metabolic syndrome, and type 2 diabetes **(Njajou OT et al, 2009; de la Torre-Carbot K et al, 2010)**.

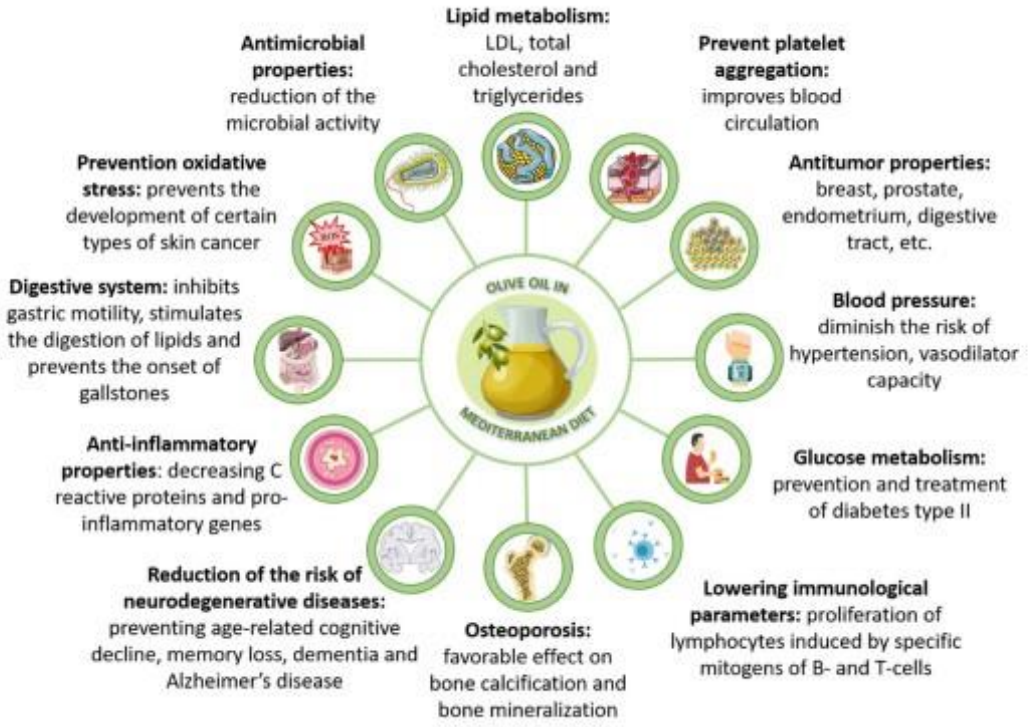


Fig 8. Scheme of the beneficial and healthy effects of the EVOO inclusion in the Mediterranean diet (From **Jimenez-Lopez C et al, 2020**).

Among the phenolic compounds characterized in EVOO, secoiridoid derivatives such as oleuropein, oleocanthal and oleacein, and simple phenols HT and Tyr, have largely been studied for their multiple protective effects against CVD, cancer, and infections **(Karkovic Markovic A et al, 2019)**.

Oleuropein exerts potent antioxidant activity with a strong evidence of free radical-scavenging activity *in vitro* and *ex vivo* **(Hassen I et al, 2015; Fabiani R et al, 2008)**. Many of recognized health-benefiting effects of oleuropein can be attributed to its anti-inflammatory activity. Probably the first evidence of its anti-inflammatory effect in human cells was a decrease of IL-1β concentration in human whole blood culture, upon the addition of oleuropein **(Miles EA et al, 2005)**. An *in vivo* study on an experimentally induced acute colitis in mice showed decreased production of IL-1β and IL-6, NO and TNF-α, and expression of iNOS, COX-2, and MMP-9 after oral administration of oleuropein, principally through reduction of

NF-κB activation **(Giner E et al, 2011)**. The antioxidant and anti-inflammatory effects of oleuropein are considered to be at the basis of its pharmacological activities such as anticancer, cardioprotective, neuroprotective, gastroprotective, hepatoprotective, antidiabetic, antiobesity, radioprotective and others **(Hassen I et al, 2015)**.

Among all of them, oleuropein is considered to have the greatest potency as an anti-atherosclerotic agent. Oleuropein and HT were shown to inhibit the endothelial activation and monocytoid cell adhesion within the concentration range expected after nutritional intake from MD **(Dell'Agli M et al, 2006)**. Those effects are due to their prominent antioxidant and anti-inflammatory activity, and represent possible molecular mechanisms by which oleuropein and other natural polyphenols may prevent early atherogenesis. Furthermore, it was shown that oleuropein exerts cardioprotection in cholesterol-fed animals through activation of intracellular signaling, reduces the circulating cholesterol and LDL levels and increases myocardial ATP content **(Andreadou I et al, 2015)**. Oleuropein reduced the release of oxidized glutathione, a sensitive marker of oxidative stress, in post-ischemic oxidative burst **(Manna C et al, 2004)**. Recent study suggested protective effect of oleuropein on myocardial ischemia/reperfusion in neonatal rat cardiomyocytes due to inhibition of apoptosis **(Zhao Q et al, 2017)**. In addition, it was shown that oleuropein exerts cardioprotection in cholesterol-fed animals through activation of intracellular signaling, reduces the circulating cholesterol and LDL levels and increases myocardial ATP content **(Andreadou I et al, 2015)**.

Oleocanthal, a derivative of Tyr, was shown *in vitro* to inhibit inflammatory enzymes (such as COX-2) **(Beauchamp GK et al, 2005)** and to attenuate inflammatory mediators such as iNOS which plays a role in the pathogenesis of joint degenerative disease **(Iacono A et al, 2010)**. Also, inhibiton of platelet aggregation by EVOO was shown to be in correlation with the amount of oleocanthal **(Abuznait AH et al, 2013)**. Studies conducted both *in vivo* and *in vitro* have revealed the great potential of oleocanthal in counteracting amyloid aggregation and

toxicity, recognizing oleocanthal as a potent pharmacological agent in the treatment of neurodegenerative diseases **(Abuznait AH et al, 2013; Parkinson L et al, 2014)**. Moreover, oleocanthal exerts antimicrobial activity against *Escherichia coli*, *Salmonella enterica*, *Listeria monocytogenes*, *Helicobacter pylori*, *Staphylococcus aureus* and *Enterococcus faecalis* **(Pang KL and Chin KY, 2018)**.

Oleacein, the dialdehydic form of decarboxymethyl elenolic acid linked to HT, has been proven to possess antioxidant and anti-inflammatory activity. Firstly, it was recognized that oleacein acts as free radical scavenger **(Czerwinska M et al, 2012)**. Later it was shown that its antioxidant activity is associated with effects on the function of human neutrophils **(Naruszewicz M et al, 2015)**. Apart from antioxidant activity, oleacein exhibited some effects on inflammatory mediators. In particular, it was demonstrated that oleacein enhanced the anti-inflammatory activity of human macrophages by increasing CD163 receptor expression **(Filipek A et al, 2015)**. It also reduces the release of elastase, MMP-9 and interleukin IL-8. Moreover, it prevents the increase of CD11b/18 expression on the surface of neutrophils as well as inhibits adhesion molecules expression of VCAM-1, ICAM-1 and E-selectin, and consequently diminishes neutrophils and monocyte adhesion to human vascular EC **(Naruszewicz M et al 2015)**. It has an endothelium-dependent vasorelaxant effect mediated by an enhanced NO production, probably through a redox mechanism within EC and, at higher concentrations, an endothelium-independent vasorelaxant effect. At plasmatic concentrations, it could modulate vascular tone *in vivo* **(Segade M et al, 2016)**. It was shown that oleacein is more effective inhibitor of enzyme 5-lipoxygenase than HT and oleocanthal **(Naruszewicz M et al, 2015)**.

1.3.3 HT and Tyr

HT and Tyr are two bioactive phenolic acids abundant in EVOO, present predominantly as secoiridoid derivatives, such as oleuropein and ligstroside aglycone derivatives, that after ingestion are idrolised in the acidic gastric environment and release free HT and Tyr **(Corona G et al, 2009 A and B)**. In particular, once ingested with the diet, an absorption of 40%–95% of HT and Tyr occurs in humans **(Vissers MN et al, 2002)**. The chemical formula of HT is $C_8H_{10}O_3$ and is identical to Tyr except for an extra hydroxyl group in meta-position in the aromatic ring (**Fig 9**).

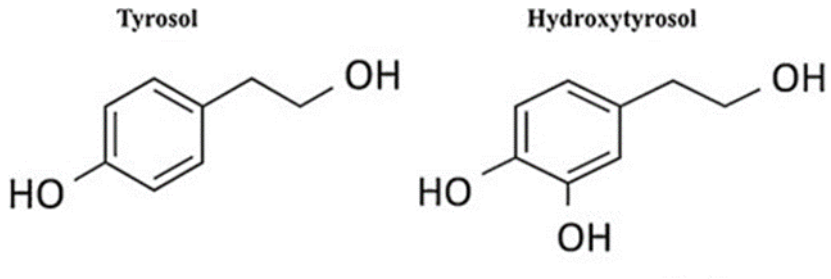


Fig 9. Chemical structure of Tyr and HT (From **Bertelli M et al, 2019**).

In an *in vitro* study using differentiated Caco-2 cell monolayers, it was suggested that HT is quantitatively absorbed at intestinal level via a passive diffusion mechanism **(Manna C et al, 2000)** with an efficiency that oscillates from 75% up to 100% **(Robles-Almazan M et al, 2018)**. Following ingestion of EVOO, the levels of HT and Tyr increase rapidly achieving a peak of concentration at approximately 1 h in human plasma and around 2 h in urine **(Miro-Casas E et al, 2001 B; Miro-Casas E et al, 2003)**, which supports the evidence that the small intestine is the major site of absorption for these compounds. The analysis of human plasma and urine has also demonstrated that both HT and Tyr are dose-dependently absorbed and the main metabolites found in this study are primarily to O-glucuronidated conjugates **(Visioli F et al, 2000; Caruso D et al, 2001; Miro Casas E et al, 2003)**. Taking into account the structure

and hydrophilic properties of HT molecule and its intense metabolism and transformations, its conjugated metabolites are mainly excreted by the kidneys **(Karkovic Markovic A et al, 2019)**. The absorption of HT is rapid and the time required for the complete elimination from the body, both for HT and its metabolites, is approximately 6 h in humans **(Rodríguez-Morató J et al, 2016)** and around 4 h in rats **(Domínguez-Perles R et al, 2017)** after EVOO ingestion and returning to basal values at 12–24 h **(Miro Casas E et al, 2001 B)**.

The results of one study carried out in rats indicate that Tyr is rapidly absorbed and excreted via the kidney within 8 h after oral administration **(Lee DH et al, 2016)**.

The poor bioavailability of HT and Tyr observed in different studies related to the low or null concentration of circulating HT and Tyr is due to the subsequent extensive first-pass phase-I and phase-II metabolism in the gut and liver **(Lopez de las Hazas MC et al, 2018) (Fig 10)**. In particular, both HT and Tyr undergo substantial metabolism involving phase II transformations to produce their glucuronide and sulphate-conjugated forms formed by the respective action of UDPGT and SULT. Due to the high metabolism of parental forms, metabolic products such as glucuronidated and sulphated conjugates are the predominant bioavailable forms **(Corona G, 2009; Serreli G and Deiana M, 2019)**. Sulphation in the liver appears to be the major metabolic pathway of Tyr **(Hassen I et al, 2015)**. HT glucuronides are the main metabolites recovered in urine after EVOO ingestion **(Visioli F et al, 2000; Vissers MN et al, 2002)** whereas sulphates are the main metabolites recovered after pure HT use **(Rubió L et al, 2014; Khymenets O et al, 2010)**. These differences came from the administered dose of free HT. Indeed, sulphatation is a high affinity-low capacity pathway while glucuronidation is a low affinity-high capacity pathway. Thus, at low dose, sulphatation dominates but, at higher doses, glucuronidation becomes the major route. The glucuronides formed through this pathway are considered to be better antioxidants than free HT and sulphate metabolites **(Tuck KL et al, 2002)**.

It is noteworthy that, after its intake, HT also undergoes O-methylation (a minor metabolic pathway) by the action of COMT, and both homovanillic acid and homovanillyl alcohol have been detected in human and animal plasma and urine after the oral administration of either EVOO or pure HT **(D'Angelo S et al, 2001; Tuck KL et al, 2001; Tuck KL et al, 2002; Miro-Casas E et al, 2003)**. Tyr, lacking an ortho-diphenolic structure, cannot be methylated by COMT **(Mateos R et al., 2005)**. It seems that under alkaline conditions in the human lumen, HT can also be a substrate of enzyme acyltransferase which catalyses the transfer of acetyl group from acetyl-CoA to HT and forms metabolite HT-1 acetate. This metabolite can be biotransformed into HT-1 acetate-4′ -O-sulphate by enzyme SULT **(Lopez de las Hazas MC et al, 2018; Rubió L et al, 2012)**. The metabolite N-acetyl-5-S-cysteinyl HT starts from the autoxidation of HT to HT quinone. In the further reaction with glutathione (GSH), it produces the conjugate, which is cleaved to the final metabolite with the enzymes γ-glutamyl transpeptidase (GGT) and N-acetyl transferase (NAT) **(Karkovic Markovic A et al, 2019)**. The mercapturate conjugate of HT is formed in a dose-dependent manner **(Kotronoulas A et al, 2013)**. In the intestine and cecum, HT is thoroughly transformed by microbiota into phenylacetic acid (PA) and its derivatives; however, the catabolism of HT-Ac produces phenylpropionic (PP) derivatives. Then, these colonic metabolites could be absorbed and subsequently transformed into a phase-II metabolites **(Lopez de las Hazas MC et al, 2017; Mosele J et al, 2015**).

It is important to mention that despite the lower concentration of Tyr and its precursors in EVOO compared to HT, it was shown that the absorbed Tyr could be interconverted into HT in liver microsomes by the activity of CYP2A6 and CYP2D6, so Tyr could be a precursor of HT **(Lopez de las Hazas MC et al, 2018; Rodríguez-Morató J et al, 2017)**. Once absorbed, HT and Tyr together with their metabolites are widely distributed in the entire organism **(Serreli G and Deiana M, 2018)**. The biliary route redirects HT metabolites from the liver

back into the duodenum where they can be transformed and reabsorbed. Thus, this enterohepatic recycling may lead to a longer presence of HT and metabolites within the body **(Lopez de las Hazas MC et al, 2018)**. Around 5% of total HT is excreted by faeces 5 h after an injection of HT **(Robles-Almazan M et al, 2018)**.

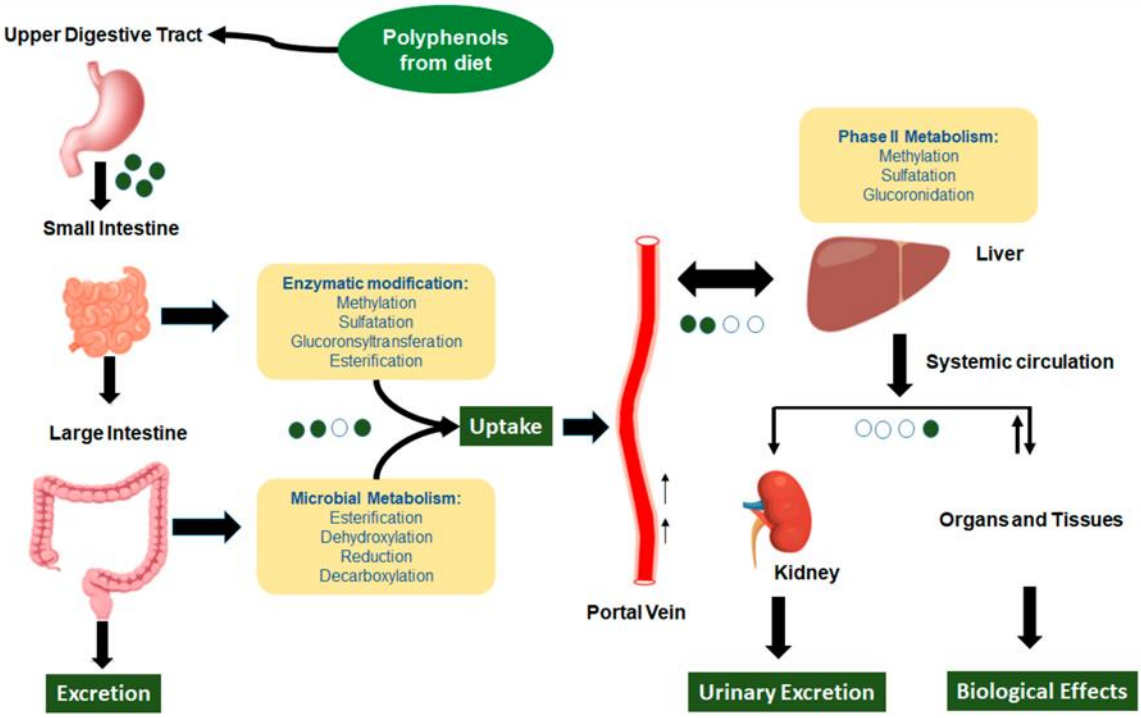


Fig 10. Metabolism of dietary polyphenols (From **Cipolletti M et al, 2018**).

In a study where rats were given a refined olive oil enriched with HT (1, 10, and 100 mg/kg), it was demonstrated that HT and its metabolites (HT glucuronide and sulphate) as well as homovanillyl alcohol, accumulated in a dose-dependent manner mainly in the liver, kidney, and brain **(Lopez de las Hazas MC et al, 2015)**. Previously, it has been shown that HT **(Serra A et al, 2013)** and Tyr **(Bu Y et al, 2007)**, as well as HT sulfate and HT acetate sulfate **(Serra A et al, 2013)**, may cross the blood brain barrier and go through brain uptake in rats. Moreover, still in rats, it was shown an extensive and rapid uptake of these compounds by several organs including heart, lungs, and skeletal muscle **(D'Angelo S et al, 2001)**. In most of the *in vitro* studies the presence of methyl/sulphate/glucuronide functional groups did not seem to inhibit

the biological activity **(Serreli G and Deiana M, 2018; Peyrol J et al, 2018; Atzeri A et al, 2016)**.

1.3.3.1 Biological activity of HT and Tyr

Among the EVOO phenolic compounds, HT is considered one of the most abundant and representative phenols; in addition, several studies have demonstrated that it has cardioprotective, antiinflammatory, antiplatelet aggregation effects, antimicrobial and anticancer activities **(Granados-Principal S et al, 2010)** (**Fig 11**).

HT has demonstrated a strong antioxidant effect acting as a powerful scavenger of free radicals including the superoxide anion, hydrogen peroxide, hypochlorous acid, etc. **(Granados-Principal S et al, 2010)**. It has also shown a chelating effect on metals such as iron, inhibiting its activity as a catalyst for the formation of ROS **(Visioli F et al, 2002; Andrikopoulos NK et al, 2002)**. In particular, a strong free-radical scavenging action has been demonstrated also by using metal-independent oxidative systems **(Aruoma OI et al, 1998)** or measuring stable free radicals, such as 2,2-diphe-1-picrylhydrazyl (DPPH) **(Visioli F and Galli C, 1998; Carrasco-Pancorbo A et al, 2005)**.

This feature is the main factor responsible for its antioxidant effect in atherosclerosis, characterized by the cytotoxic and proinflammatory effect of oxidized LDL in the vascular endothelium **(Raederstorff D, 2009)**. In fact, HT has been proposed to have therapeutic potential for the treatment of atherosclerosis. LDL cholesterol oxidation is one of the key steps in the initiation of atherosclerosis. In this context, HT proved to be effective in preventing lipid peroxidation and protecting LDL from oxidation **(Rietjens SJ et al, 2007 B)** and it also exerts beneficial effects on high-density lipoprotein (HDL) **(Berrougui H et al, 2015)**. HDL play a central role in reverse cholesterol transport, they remove excess cholesterol from peripheral cells (cholesterol efflux capacity), which predicts coronary event incidence and is inversely related to the development of early atherosclerosis. An increase in HT metabolites (HT-sulphate, as well as homovanillic acid sulphate and glucuronate) bound to the HDL was observed. These compounds could then exert a local antioxidant protection in HDL and might

prevent oxidative modifications of the apolipoprotein A-I (ApoA-I), the main HDL protein involved in cholesterol efflux capacity, and of other HDL proteins. Furtherly, it was shown that HT enhances the expression of cholesterol efflux related genes **(Fernández-Ávila C et al, 2014)**. **Carluccio MA et al (2003)**, also described the inhibition of endothelial activation, an early step in atherogenesis, by oleuropein and HT, both able to reduce LPS-stimulated expression of vascular adhesion molecule-1 (VCAM-1) in human VEC by inhibition of its mRNA levels, thus decreasing monocyte cell adhesion to EC. Another point of interest was to investigate the protective effects of HT against the oxidized cholesterol. Exposure of Caco-2 cells to oxidised cholesterol led to ROS production and oxidative damage **(Atzeri A et al, 2016)**. Cells pretreated with HT significantly inhibited MDA increase, reduced ROS production and the increase of glutathione peroxidase activity, while preserving GSH levels and cell viability. Sulphated metabolites of HT also provided similar protection suggesting that metabolites derived from a conjugation process could contribute to the protective action exerted by HT.

Several studies also demonstrated that HT exhibits antiinflammatory activities *in vivo* and *in vitro.* It has shown a relevant anti-inflammatory action through the attenuation of pro-inflammatory agents iNOS, COX-2, and TNF-α in LPS-challenged human monocytic Tamm-Horsfall protein (THP)-1 cells and in animal models of inflammation, where reduced TNF-α and IL-1β expression has been found **(Parkinson L and Cicerale S, 2016)**. HT can alleviate the inflammatory response of flavin containing monooxygenase 3, the enzyme that may drive the development of atherosclerosis, in cells treated with highly toxic air pollutant acrolein **(Wu X et al, 2018)**. HT is also a potential mitochondria-targeting antioxidant in the inflamed endothelium. The pretreatment of EC with HT suppresses inflammatory angiogenesis, reduces mitochondrial superoxide production and lipid peroxidation and increases superoxide dismutase (SOD) activity **(Calabriso N et al, 2018)**. *In vivo* evidence confirmed that HT

administration prevented LPS-induced effects and improved the antioxidant power of plasma **(Fuccelli R et al, 2018)**. A clinical trial showed that regular intake of 15 mg/day of HT changed body composition parameters and modulated the antioxidant profile, the expression of inflammation and oxidative stress-related genes, exerting a protective effect on the endothelial dysfunction commonly present in atherosclerosis **(Colica C et al, 2017)**. HT prevents early inflammatory events responsible for the onset of insulin resistance and steatosis, by reducing the hepatic inflammation and nitrosative/oxidative stress and restoring glucose homeostasis and intestinal barrier integrity **(Pirozzi C et al, 2016)**. It may be advantageous in rheumatoid arthritis, autoimmune disease characterized by chronic inflammation, with significant impact not only on chronic inflammation but also on the acute inflammatory process. Protective effects could be related to the inhibition of MAPK and NF-κB signalling pathways **(Rosillo MA et al, 2017)**.

Postprandial lipemia is a risk factor for atherosclerosis development and it was reported that HT improves blood lipids profile, due to its ability to lower serum total cholesterol (TC), triglycerides (TG), and LDL levels **(Gonzalez-Santiago M et al, 2006)**. HT and HT-acetate (HT-Ac) were proven to prevent platelet aggregation, one of the factors involved in thrombotic processes, with effects similar to acetylsalicylic acid **(Gonzalez-Correa JA et al, 2008)**. It was also shown that HT has cardioprotective activity by down-regulating proteins related to proliferation and migration of EC and occlusion of blood vessels in aorta and proteins related to heart failure in heart tissue **(Catalan U et al, 2016)**. Furthermore, HT has shown to depress radical NO levels, promoting aortic relaxation and enhancing protection of vascular endothelium in isolated rat aorta **(Rietjens SJ et al, 2007 A)**.

Besides, HT has provided an additional action mechanism to prevent oxidative stress damage through 1) modulation of signalling pathways involved in antioxidant/detoxifying enzymes regulation; 2) increasing the expression and the activity of GSH-related enzymes such

as GSHPX, GSR and GSTS; 3) inducing the activation of the nuclear factor-E2-related factor 2 (Nrf2) which is translocated to the nucleus and dimerizes with small Maf proteins to form a transactivation complex that binds to the antioxidant responsive element (ARE), coordinating the expression of many genes involved in combating oxidative stress and toxicity in a broad range of tissues and cell types **(Martín MA et al, 2010; Goya L et al, 2007)**.

A vast number of *in vitro* and *in vivo* studies have shown significant anticancer effects of HT against the genotoxic damage caused by free radicals in the DNA of multiple human cell lines, with different mechanisms of action being proposed **(Rodriguez-Morato J et al, 2016; Rigacci S and Stefani M, 2016; Fabiani R et al, 2012; Corona G et al, 2006)**. HT has shown protective effects against induced oxidative stress by scavenging several free radical species in different cell lines, such as Caco-2 cells **(Deiana M et al, 2010)**, melanoma cells **(D'Angelo S et al, 2005)** and human hepatoma HepG2 cells **(Goya L et al, 2007)**. Much of the research was focused on colon cancer, the third most common cancer worldwide, with rising incidence and mortality in developing countries. HT reaches significant levels in large intestine due to the gastric hydrolysis and colonic fermentation of secoiridoids present in EVOO **(Corona G et al, 2006)** and, together with its active metabolites, it is likely to be a major candidate for the noticed biological activity of EVOO polyphenols on human colon adenocarcinoma cells **(Corona G et al, 2009 B; Lopez de las Hazas MC et al, 2017)**. The ability of EVOO polyphenols to inhibit Caco-2 cell proliferation has been associated with their potential to induce a G2/M cell cycle arrest via the inhibition of p38 and CREB activity and downstream COX-2 expression **(Corona G et al, 2007)**. Antiproliferative and proapoptotic effect of HT via activation of caspase signaling was shown in colon cancer cells Caco-2 and HT-29 (**Lopez de las Hazas MC et al, 2017)**. Inhibition of the tumor growth and angiogenesis was demonstrated *in vivo*, by suppression of the activation of protein kinase B (Akt) and NF-κB pathways **(Zhao B et al, 2014)**.

The neuroprotective effects of HT were studied in a great number of *in vitro* and *ex vivo* studies. Alzheimer's disease, an amyloid disease characterized by the deposition of typically aggregated protein/peptides in tissues that are associated with brain degeneration and progressive cognitive impairment. In an *in vitro* Alzheimer disease model, HT protected neuronal cells against amyloid-β induced toxicity and prevented tau fibrillization **(Rodriguez-Morato J et al, 2015)**. HT was an effective inhibitor of hen egg white lysozyme aggregation, thus suggesting possible future applications of this natural compound for prevention or treatment of amyloid diseases **(Orsini F et al, 2018)**. HT is also considered as a promising compound for Parkinson's disease medication, as it inhibits both enzymatic and spontaneous oxidation of endogenous dopamine, mitigates the increase in spontaneous oxidation during MAO inhibition, has a protective effect against dopamine and 6-hydroxydopamine (6-OHDA)-induced dopaminergic cell death and counteracts α-synuclein-induced toxicity **(Goldstein DS et al, 2016; Hornedo-Ortega R et al, 2018)**.

HT demonstrates hypoglycemic activity in various diabetic animal models by influencing the major biochemical processes leading to diabetic vasculopathy. In particular, in a study aiming to evaluate the influence of HT on cardiovascular biomarkers and arterial wall morphometric parameters in diabetic rats with streptozotocin and smooth muscle cells, it was shown that HT significantly reduced both oxidative and nitrosative stress, oxLDL concentration, VCAM-1 and inflammatory mediators, platelet aggregation and thromboxane B2 production, influencing the major biochemical processes leading to diabetic vasculopathy, and reduced cell proliferation in the vascular wall in this experimental model **(Lopez-Villodre JA et al, 2016)**. It was also shown that HT could effectively prevent diabetic neuropathy, significantly reducing the exacerbated oxidative stress in diabetic rats, acting primarily on lipid peroxidation rather than on the antioxidant glutathione system **(Reyes JJ et al, 2017)**.

HT exerts a protective effect on the liver. In particular, it was able to participate in the activation of different signaling pathways in hepatocytes, involved in the prevention of inflammation, oxidative stress, endoplasmic reticulum stress, mitochondrial dysfunction and insulin resistance, enabling the prevention o resolution of liver damage, by activating Nfr2, thereby inducing cellular antioxidant response, inactivating NF-κB, thus avoiding cellular inflammatory response and inhibiting p-ERK pathway, preventing endoplasmic reticulum stress, autophagy and the lipogenic response **(Soto-Alarcon SA et al, 2018)**.

Tyr was shown to be an effective cellular antioxidant, probably due to intracellular accumulation, in spite of its weak antioxidant activity **(Di Benedetto R et al, 2007)**. Tyr is a rather stable compound and therefore, when compared with other polyphenols, much less subject to autooxidation. In the presence of oxidized LDL, when autoxidation phenomena had already started, Tyr maintained an unchanged antioxidant activity, while other, more active natural flavonoids, showed a drastic reduction of their antioxidant activity and sometimes became even pro-oxidants **(Perona JS et al, 2006)**.

Many studies have shown that Tyr can also modulate inflammation. The anti-inflammatory properties of Tyr have been studied *in vitro* on cell lines and *in vivo* in models of inflammation **(Lambertsen KL et al, 2012)**. The effects of Tyr on the modulation of the upstream pathways regulating LPS-induced inflammatory response were recently investigated in Caco-2 cells. Treatment of cells with LPS led to stimulation of iNOS with the involvement of NF-κB activation through the inhibitory subunit IκBα phosphorylation and subsequent degradation induced by Akt or MAPK. In these experiments, Tyr and its metabolites acted as inhibitors of iNOS expression and IκBα degradation and as modulators of p38 and ERK 1/2 **(Serreli G et al, 2019)**. It was also shown that Tyr is effective in reducing oxidative damage of L6 muscle cells by inhibiting H_2O_2-induced cell death, in part through regulation of ERK, JNK and p38 and by increasing ATP production **(Lee KM et al, 2018)**. **Turner R et al (2005)**

revealed that Tyr induced a significant reduction in the secretion of intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 in HUVEC measured 24 h after activation with TNF-α. Tyr was also shown to exert its beneficial effects against hypertension, atherosclerosis, coronary heart disease, chronic heart failure, insulin resistance and obesity by modulating cluster of differentiation 14 (CD14) up-regulation and inhibiting inflammation **(Chang CY et al, 2019)**. Tyr reduces oxidative modifications of HDL and maintain the physicochemical properties of HDL, improving their functionality, especially the capacity to promote cholesterol efflux **(Berrougui H et al, 2015)**. It also possesses antiatherogenic activity due to the inhibition of leukotriene B4 production that affects endothelial function **(Perona JS et al, 2006)**. Furtherly, it was shown that Tyr induces myocardial protection against ischemia related stress and could be thus considered as anti-aging therapy **(Samuel SM et al, 2008)**. In acute cerebral ischemia-reperfusion Tyr reduces the blood viscosity and the intensity of oxidative stress in the brain tissue **(Osipenko AN et al, 2017)**. Tyr treatment ameliorated hyperglycemia by regulating key enzymes of carbohydrate metabolism in streptozotocin-induced diabetic rats. Tyr may also play an important role in the treatment of diabete mellitus (DM), as it exerts anti-inflammatory effects on the liver and pancreas of streptozotocin-induced diabetic rats **(Chandramohan R and Pari L, 2016)**, and inhibits endoplasmatic reticulum stress-induced apoptosis in pancreatic β-cell **(Lee H et al, 2016)**. Tyr also exerts beneficial effect in Nonalcoholic Fatty Liver Disease (NAFLD), where oxidative stress is one of the important factors responsible for the development and progression of the disease **(Sarna LK et al, 2016)**. Also, it possesses potent activity against several strains of bacteria responsible for intestinal and respiratory infections *in vitro* **(Medina E et al, 2006)**. Antibacterial properties of Tyr can be linked to the binding and inhibition of bacterial ATP synthase **(Amini A et al, 2017)**. Tyr prevented osteopenia by increasing bone formation, probably because of its

antioxidant properties **(Puel C et al, 2008)**. Furthermore, Tyr showed antigenotoxic activity and could prevent apoptosis in keratinocyte **(Salucci S et al, 2015)**.

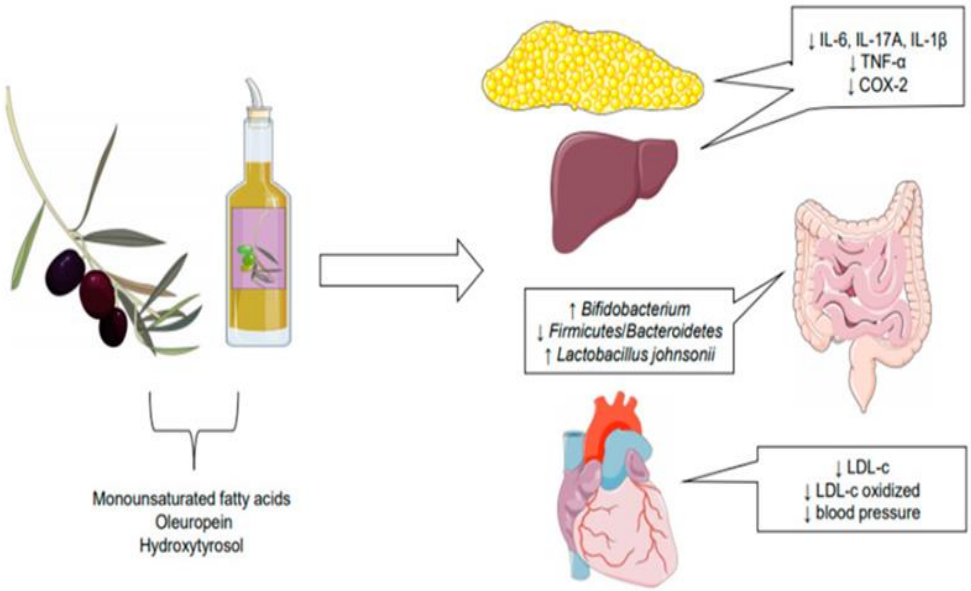


Fig 11. Effects of monounsaturated fatty acids, hydroxytyrosol, and oleiropein on cardiovascular diseases, inflammation, and gut microbiota composition (From **Marcelino G et al, 2019**).

1.3.3.2 HT and Tyr Glucuronide and Sulphate

As previously stated, the concentrations of HT and Tyr themselves in biological fluids are extremely low compared to their metabolites **(Gonzalez-Santiago M et al, 2010; Kotronoulas A et al, 2013; Miro-Casas E et al, 2001 A and B, 2003; Pastor A et al, 2016)** (**Fig 12**).

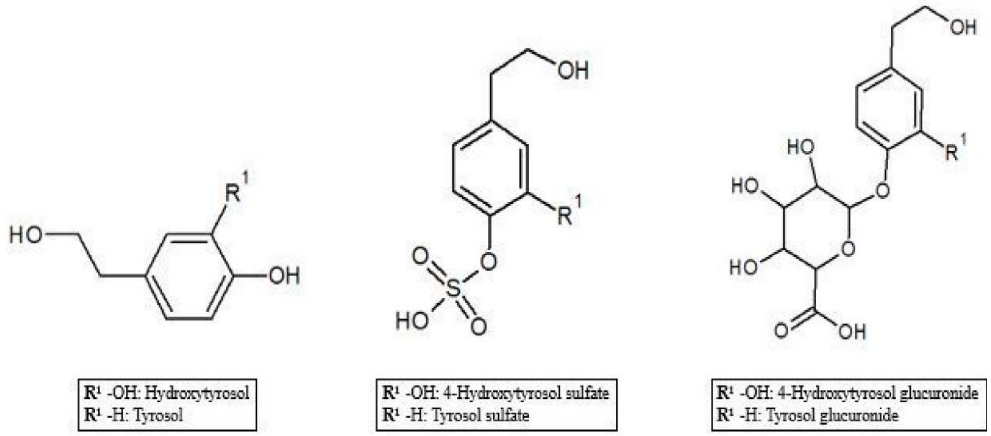


Fig 12. HT, Tyr and their sulphate and glucuronide metabolites (From **Serreli G and Deiana M, 2018**).

Therefore, there is good reason to take into consideration the participation of metabolites, along with their parent compounds, with respect to the beneficial effects reported for dietary consumed HT and Tyr. Indeed, several research groups pointed out that sulphated and glucuronidated moieties can be deconjugated once they get intracellular environment, thus actually acting as free parent compounds and not as metabolites **(Rubió L et al, 2014)**. For example, **Peyrol J et al (2018)** showed that HT glucuronide exerted the same antioxidant activity of HT in EC: however, this was due to the intracellular β-glucuronidase action which favored deconjugation, leading to the formation of free HT. **Deiana M 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. In a different study, HT

glucuronides were shown to protect red blood cells (RBC) from *in vitro* H_2O_2-induced oxidative injury at low concentrations **(Paiva-Martins F et al, 2013)**. At higher concentrations, however, the protective effect of HT glucuronides did not increase. 3-O- and 4-O-glucuronides of HT were demonstrated *in vitro* to prevent chemically induced ER stress (a process mediated by the unfolded protein response and relevant for atherosclerosis development) **(Giordano E et al, 2015)**. It is noteworthy that the mechanisms involved in this effect do not match those involved for HT, thus stressing the concept that biological activities elicited by metabolites do not necessarily mimic those of the parent compound. **Tuck KL et al (2002)** observed that the most potent scavenger was HT-3-O-glucuronide followed by parent HT and its methylated metabolites homovanillic acid (HVA) and homovanillyl alcohol (HVAlc), in Caco-2 cells and in rats.

Tyr 4-O-sulphate could protect from cell death induced by oxidized cholesterol and the biological effect of parent compounds can be retained in the metabolites **(Lee DH et al, 2016; Muriana FJG et al, 2017)**. **López de las Hazas MC et at (2018)** have recently investigated the modulation of brain functions by HT, HT sulfate and HT acetate sulfate, focusing on their protective effects against oxidative stress at physiological concentrations (10 µM) in neuroblastoma (SH-SY5Y) and dopaminergic (LUHMES) neuronal cells. This aspect is biologically relevant because of the proven accumulation of HT and its metabolites in the brain, where they may reach concentrations useful to achieve positive effects. **Fernandes S et al (2020)** demonstrated that HT metabolites present as phase II sulphated conjugates are actually able to protect red blood cells (RBC) from oxidative injury by the radical initiator 2,2'-azo-bis(2-amidinopropane) dihydrochloride (AAPH) and are likely to contribute to the antiatherosclerosis properties of EVOO. **Atzeri A et al (2016)** observed that HT and Tyr sulphate, entered intestinal Caco-2 cells from 30 min of incubation and underwent an extensive metabolization, giving rise to a pool of metabolites, mainly sulphate and methyl-sulfate HT

and Tyr. Thus, it is conceivable that, though deconjugated before entering the cells, HT and Tyr metabolites can be reformed inside the cell environment so acting as conjugated forms together with HT and Tyr free forms. Evaluating the antioxidant effect of HT and Tyr sulphate metabolites in intestinal cells (Caco-2) it was found that they displayed an efficiency comparable to that of the parent compounds. Moreover, HT and Tyr sulphates showed anti-inflammatory and antioxidant activities in HUVEC, preventing the rise of ROS, the depletion of GSH, and the down-regulation of glutathione peroxidase 1, glutamate-cysteine ligase catalytic subunit, and heme oxygenase-1 genes**.** Tyr sulphate 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 EC **(Muriana FJG et al, 2017; López SM et al, 2017)**. Beyond direct antioxidant mechanisms along with the modulation of oxidative status, the non antioxidant activities of metabolites are also of great interest since such events have been reported in several dietary intervention studies **(Covas MI et al, 2006; Marrugat J et al, 2004)**. Among them, impact on gene expression should be considered as it has been shown to be dose-dependently modulated by phenolic compounds **(Martín-Peláez S et al, 2017)**.

Studies from **Catalan U et al (2015 and 2016)** examined a set of phase II HT metabolites, generated in the course of Caco-2 cell metabolism and purified as a complex fraction for their biological activity. The co-incubation of HT and the set of Caco-2 cell derived metabolites with human aortic endothelial cells (HAEC) indicate that both treatments significantly reduced the TNF-α induced secretion of cell adhesion molecules, such as E-selectin, P-selectin, ICAM-1 and VCAM-1. However, only the HT metabolite fraction reduced further excretion of MCP-1 chemokine. On the basis of these results, the authors suggest that phase II metabolites, along with parent HT, might exert a protection in endothelial dysfunction, a key factor in the pathogenesis of atherosclerosis, via modulation of cellular signaling.

Serreli G et al (2019) shown that HT, Tyr, and their sulphated and glucuronidated metabolites were able to prevent a pathological NO overproduction in Caco-2 treated with LPS (1 μM), acting as inhibitors of iNOS expression and NF-κB activation through the modulation of p38 and ERK1/2 MAPK. In particular, all the tested metabolites inhibited NO release induced by LPS, thus significantly contributing to the protective activity exerted by their parent compounds against inflammation. In another *in vitro* study with HAEC, **Serreli G et al (2021 A)** observed that the conjugates metabolites of HT and Tyr, at physiologically relevant concentrations (1 μM), enhanced NO and cGMP concentration, inhibiting its depletion caused by superoxide overproduction. In addition, some of them enhanced the activation of Akt and eNOS. All tested compounds contributed to the maintenance of nitric oxide (NO) balance in the presence of a pathological relevant stressor.

Begines P et al (2019) developed a chemoenzymatic method using the arylsulfotransferase from *Desulfitobacterium hafniense* for the sulfation of the natural EVOO Tyr, HT, and of their monoacetylated derivatives in rat liver microsomes, damaged by tert-butylhydroperoxide (TBH), for evaluating radical scavenging and antilipoperoxidant activity and as reported, the sulphation diminished the radical scavenging properties of the prepared compounds.

2 Aim of the study

Barrier function has a key importance in tissue, organ and organism homeostasis. A leaky epithelial and/or endothelial barrier, often due to TJ alteration, is a common trait of several diseases **(Mullin JM et al, 2005)**.

Intestinal epithelial cells are the key components of the epithelial barrier, which form a physical barrier to prevent the diffusion of pathogens, toxins and allergens from the lumen into the circulatory system **(Noda S et al, 2012)**. Disruption of the intestinal barrier may increase permeability, disturb immune homeostasis, increase inflammation, and further lead to intestinal diseases such as IBD **(Fries W et al, 2013)**. TJ seal the paracellular space between epithelial cells and regulate the movement of fluid and macromolecules between the bloodstream and the intestinal lumen, and are critical to the maintenance of the integrity of the intestinal epithelial barrier **(Shao Y et al, 2017)** and thus to the prevention and treatment of IBD **(Martini E et al, 2017)**.

EC form a semiselective barrier to separate blood flow from peripheral organs and tissues, playing crucial roles in the homeostasis of the vascular system **(Furuse M, 2010)**. In particular, in EC, TJ control endothelial paracellular permeability by regulating the diffusion of fluids, ions, and small plasma proteins, and the penetration of cells such as leukocytes, neutrophils, and lymphocytes **(Dejana E and Giampietro C, 2012)**. TJ are highly dynamic structures that respond to multiple external stimuli and pathological conditions, so alterations in their expression, distribution, and structure may lead to many related vascular diseases, such as atherosclerosis, closely associated with the pro-inflammatory activation of EC and the disruption of the endothelial barrier by reduction of TJ expression **(Funk SD et al, 2012)**.

Among the most common agents shown to affect barrier function, LPS, the major cell wall constituent of Gram-negative bacteria **(Bian Y et al, 2019)**, is known to disrupt both the intestinal **(Hasegawa T et al, 2021)** and endothelial barrier **(Sheth P et al, 2007)**, increasing TJ permability, thus causing intestinal and sistemic inflammatory reaction and tissue damage

(Omonijo FA et al, 2019). In addition, a growing body of evidence has unveiled a robust relationship between a HG condition and vascular complications, by an accelerated formation of ROS, nitrosylated species (RNS), and advanced glycation end products (AGE), which eventually lead to endothelial dysfunction and microvascular and macrovascular disorders **(Rezabakhsh A et al, 2017)**.

In this contest, epidemiological studies have shown that the MD is associated with reduced risk of developing degenerative diseases, as CVD **(Ruiz-Canela M and Martínez-González MA, 2011)** and IBD **(Vrdoljak J et al, 2022)**. These health-benefits are partially attributed to EVOO, the main lipid source in the MD **(Gouvinhas I et al, 2017)**, whose beneficial effects result from the microconstituents, including secoiridoid glycosides, phenolics, and flavonoids **(Tripoli E et al, 2005)**.

EVOO has been reported to possess several biological activities and health effects which have not been observed in other vegetable oils **(Chin KY and Ima-Nirwana S, 2016; Oliveras-López MJ et al, 2013)**. EVOO contains the highest level of olive microconstituents like phenolic compounds **(Gorzynik-Debicka M et al, 2018)**, which are responsible for these biological activities. HT and Tyr are the most abundant phenolic alcohols in EVOO and, together with oleuropein, the most biolocally active as shown *in vitro* and *in vivo* models **(Hassen I et al, 2015; Al-Zamely HAN and Al-Tamemi ZSM, 2018)**.

The study of their bioavailability and metabolism is a crucial task with very important implications for human health. HT and Tyr are readily absorbed in the small intestine, but have low bioavailability due to the first pass metabolism and the formation of sulphate and glucuronide conjugates **(Rubió L et al, 2012; García-Villalba R et al, 2010)**. However, polyphenols that are not absorbed in the small intestine arrive in the colon, where the gut microbiota can metabolize them **(Corona G et al, 2006)**. Metabolic phase II reactions cause the conjugation of polyphenols (especially HT) with glucuronide and sulphate groups, resulting

in a high presence of glucuronidated and sulphated HT and Tyr forms in plasma and urine **(Galmes S et al, 2021)**. Metabolic transformation is very relevant, to the extent that polyphenols in their free form are deemed undetectable in biological matrices. It is not surprising 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 MN et al, 2004)**. Recent studies demonstated that glucuronide and sulphate conjugates of polyphenols are biologically active **(Serreli G et al, 2021 A)**. For this reason, greater attention is also being paid to the study of the biological activity of HT and Tyr metabolites in human health.

In this context, the aim of our research project was to evaluate the protective effect of the main phenolic compounds of EVOO, HT, Tyr and their sulphate and glucuronide metabolites, on the inflammatory response in Adenocarcinoma Colon Cell line (Caco-2) and in Human Umbilical Vein Endothelial Cell (HUVEC), using pro-inflammatory stimuli, such as LPS and/or a hyperglycemia condition focusing on the mechanisms of action in relation to TJ modulation and the activation of cellular pathways, as MAPK and NLRP3 inflammasome, which are linked to chronic inflammatory diseases.

Differentiated Caco-2 (about 21 days after plating) express microvilli, basolateral membranes separated by TJ, intestine-specific antioxidant enzymes and possess absorptive properties similar to those of normal intestine, thus they are commonly used as a model of the intestinal epithelium. HUVEC are used as a model of vascular endothelium. Isolated from the vein of the umbilical cord, they are used for physiological and pharmacological investigations, such as macromolecules transport, blood coagulation, angiogenesis, and fibrinolysis.

A pathophysiological concentration of LPS (10 µg/ml) was used as pro-inflammatory stimulus both in Caco-2 and in HUVEC, as it has been reported to be able to elicit strong immune responses and inflammation *in vivo* and *in vitro* **(Lee W et al, 2012; Bian Y et al,**

2020). LPS acts through the interaction with TLR4 receptor, that in turn activates cellular pathways, in which MAPK are involved and promote the transcription of pro-inflammatory genes. A HG concentration (30 mM) has also been used as pro-inflammatory stimulus in HUVEC because a hyperglycemia condition is a main common metabolic disorder that could induce endothelial apoptosis, dysfunction and inflammation, resulting in endothelial injury **(Leng B et al, 2019)**. In response to HG, the synthesis of diacylglycerol is promoted, protein kinase C is activated, and the level of advanced glycation end products is increased, which further induces ROS and leads to endothelial cell dysfunction. A growing body of evidence indicates that HG impairs the EC radical scavenging function and modulate adhesion molecule expression **(Kim MH et al, 2018)**.

The first part of the project was focused on the pro-inflammatory effect of LPS on Caco-2 cells, monitoring the variation of the monolayer permeability, through the measurement of the transepithelial electrical resistance (TEER), and the alteration of TJ proteins occludin, ZO-1 and JAM-A, in relation to MAPK p38 and ERK 1/2 activation. Furthermore, we investigated the modulatory action of the phenolic compounds and their metabolites on the expression of the NLRP3 inflammasome.

The second part of the project was focused on the modulation by EVOO phenols and metabolites of the inflammatory response on HUVEC cells induced by both LPS and HG, monitoring ROS generation (enhanced by hyperglycemia), alteration of TJ proteins JAM, occludin, and zonulin, and the production of inflammatory mediators such as IL-1β and IL-6. The pro-inflammatory response was evaluated in relation to signaling pathways linked to MAPK p38 and ERK1/2 and NF-κB modulation and NLRP3 inflammasome expression.

3 Materials and methods

3.1 Reagents and chemicals

Bovin Serum Albumin, 2,5-Diphenyltetrazolium Bromide (MTT), 2,7-diclorofluoresceindiacetate, fluorescein isothiocyanate-dextran (wt 4,000), dimetyl-sulfoxide (DMSO), Bradford reagent, CellLytic-M, lipopolysaccharide from Escherichia coli, d-glucose, hydroxytyrosol, tyrosol, NaCl, K_2HPO_4, KH_2PO_4, $MgSO_4x7H_2O$, $CaCl_2x6H_2O$, $NaHCO_3$, Tween 80 and all solvents of analytical grade were purchased from Sigma Aldrich (Milano, Italy). Tyrosol glucuronide, tyrosol sulfate sodium salt, 3'- hydroxytyrosol 3'-glucuronide, hydroxytyrosol 3-sulfate sodium salt, were obtained from LGC standards (Sesto San Giovanni, Italy). Phosphatase and Protease Inhibitor Cocktail, nitrocellulose membranes, gels and all material for electrophoresis and immunoblotting, SYBR Green qPCR Master Mixes, High Capacity cDNA Reverse Transcription kit were purchased from ThermoFisher Scientific (Massachusetts, United States). RNeasy Mini Kit were purchased from Qiagen (Germany).

3.2 Human Colon Adenocarcinoma Cell line (Caco-2)

3.2.1 Materials for Caco-2 cells

Caco-2 cell line was purchased from ECACC (Salisbury, UK). Fetal Bovine serum (FBS), Dulbecco's Modified Eagle's Medium Low Glucose w/Lglutamine, Dulbecco's Phosphate Buffered Saline with $MgCl_2$ and $CaCl_2$, Dulbecco's Phosphate Buffered Saline without $CaCl_2$ and $MgCl_2$, penicillin/streptomycin 1X, Trypsin/EDTA were purchased from Euroclone (Milan, Italy). Transwell inserts were obtained from Corning Costar Corp. (New York, N.Y., USA).

3.2.2 Maintenance of Caco-2 cell cultures

Caco-2 cells were maintained in T75 flasks until their confluence reached the 80%, in D-MEM supplemented with 10% FBS, 1% antibiotic/antimycotic solution (100 U/ml penicillin, 100 mg/ml streptomycin) at 37°C in a 5% CO_2 humidified atmosphere. At passage 45-60, cells were removed from flasks by adding a trypsin solution at 1% and incubating at 37°C for 5-10 minutes; Caco-2 were collected, centrifuged, counted in a Burker chamber and then seeded into 6 well, 96 well or transwell plates at a concentration of 5×10^4/mL for different experiments. Cells were incubated for 14-21 days and used when fully differentiated (14-21d postseeding), replacing the medium twice a week.

3.2.3 Determination of transepithelial electrical resistance (TEER)

Differentiated Caco-2 cells plated in transwell inserts (polycarbonate membrane, 0.4 μm pore size) were used to determine the TEER as described by **Serreli et al (2017)** in a concentration of $5x10^4$ cells/mL in 500 μL of growth media. Briefly, cells in inserts with TEER values >300 Ω/cm2 were pretreated for 30 minutes with Tyr, HT and their sulphate and glucuronide metabolites dissolved in MeOH (1 μM). Then, LPS (10 μg/mL) was added and cells were incubated for 24 h. TEER was monitored at different times and reported as percentage of the corresponding TEER value at time zero (T = 0).

3.2.4 Western blotting for TJ, MAPK and NLRP3 Inflammasome detection

Caco-2 cells in 6-well plates ($5x10^4$ cells/mL in 2 mL of growth media), were treated with only LPS (10 μg/mL) for different times (1 h, 2 h, 3 h, 4 h, 5 h, 6 h, 18 h and 24 h), or were pre-treated with Tyr, HT and their sulphated and glucuronidated metabolites (1 μM) for 24 h before adding the LPS (10 μg/mL) for 3 h for MAPK and NLRP3 and 4 h for TJ proteins. The medium was then removed and 180 μL lysis buffer (supplemented with phosphatase and

protease inhibitor) was added. The cell lysate was placed in Eppendorf tubes, centrifuged at 12500 rpm for 7 minutes and the supernatant recovered. Protein concentration was determined through Bradford protocol **(Bradford MM, 1976)**. Denatured proteins (20–50 μg depending on the protein) were separated using 4-12%, 10% and 4-20% polyacrylamide gel, then transferred into nitrocellulose membrane where they were blocked with 25 mL of a TBS (Tris/HCl, pH 7.5, 100 mM NaCl) and 4% milk solution for 40 minutes. After washing with TBS solution, membranes where incubated overnight, at 4°C, with primary polyclonal antibodies, anti-ERK1/2, anti-phospho ERK1/2, anti-phospho p38, anti-p38 (Cell Signaling Technology, Danvers, Massachussets, USA) anti-β actin, anti-JAM-A, anti-ZO-1, anti-occludin, anti-GADPH (Abcam, Cambridge, UK), anti-NLRP3 (Sigma Aldrich, Milan, Italy) and then washed twice with TTBS (TBS with Tween 20 0.5%) before adding the secondary rabbit and/or mouse antibody IgG peroxidase-conjugated (Sigma Aldrich, Milan, Italy). Both primary and secondary antibody were prepare adding an aliquot of the original solution in 10 mL of TTBS solution with 1% of milk (dilution 1:1000 v/v). Membranes were washed twice with TTBS and one time with TBS, exposed to Clarity™Western-ECL (Bio-Rad) reagents (4-5 minutes) and observed through ChemiDoc™MT System. Analysis of the imagine obtain from ChemiDoc where analyzed using Quantity One (Biorad, Hemel Hempstead UK) software in order to determine the molecular weight of the protein bands, through the comparison with bands obtained by separation of a marker run together with proteins.

3.3 Human umbilical vein EC (HUVEC)

3.3.1 Materials for HUVEC cells

The HUVEC cell line were obtained from Lonza (Basel, Switzerland). The medium EBM-2 with 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).

3.3.2 Maintenance of HUVEC cell culture

HUVEC 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 ReagentPack Subculture Reagents washing with HEPES, adding trypsin and neutralizing with TNS, and then seeded into 96-well, 6-well and transwell plates at a concentration of 5×10^4/mL or $7{,}5x10^4$/mL for different experiments. Cells were cultured for 5-7 days, replacing the medium twice a week.

3.3.3 Fict-Dextran Permeability assay

HUVECs (1×10^5 cells/mL) were seeded on transwell filters (0.4-um pore size, Costar, New York, USA) in 12-well dishes and grown until they reached confluence. The cells were incubated with both only LPS (10 µg/mL) and only HG (30 mM, obtained adding 45 µL/mL of low glucose medium 5 mM from a glucose solution 100 mg/mL) for 2 h, 4 h, 6 h and 18 h, and pretreated with phenolic compounds and their metabolites (1 µM) for 2 h. After treatment, the medium was replaced with Fluorescein isothiocyanate (FITC)-dextran solution in the upper chamber at a final concentration of 1 mg/mL. After 1 h of incubation at 37° C, paracellular flux was assessed by taking 100 µL aliquots from both chambers to measure real-time changes of permeability across endothelial cell monolayers **(Zhang X. et al., 2013)**. Fluorescence was

measured in those samples using a fluorescence plate reader at emission wavelengths of 485 and 530 nm, respectively. The concentration of basal permeable FITC-Dextrans was calculated compared to control samples and each sample was performed in three duplications.

3.3.4 2',7'-Diclorofluorescein-diacetate assay (2,7-DCFH-DA)

HUVEC were seeded in 96-well culture plates and once to confluent were pre-treated with Tyr, HT and their sulphated and glucuronidated metabolites (1 μM) or an equivalent volume of MeOH for the controls, and incubated for 24 h. After that, the medium was removed and 100 μL of HG solution (30 mM in PBS) was added and left for 2 h at 37°C. The medium was then aspirated, 100 μL of 2',7'-DCFH-DA solution (10 mL of PBS + 10 μL of DCFH 10 mM) were added in each well and left for 20-30 minutes in the dark **(Dinicola S et al, 2013)**. The fluorescence of 2',7'-DCFH-DA was determined using a micro plate reader (Infinite 200, Tecan, Salzburg, Austria) by recording the emission signal at 525 nm after excitation signal at 488 nm in a time range of 2 h. The data are shown as mean ± SD from 3 separate experiments with 6 replicates per experiment.

3.3.5 Western blotting for TJ, MAPK and NLRP3 Inflammasome detection

HUVEC cells in 6-well plates ($5x10^4$ or $7,5x10^4$ cells/mL in 2 mL of growth media), were treated with only LPS (10 μg/mL) or HG (30 mM, obtained adding 45 μL/mL of low glucose medium 5 mM from a glucose solution) for different times (1 h, 2 h, 3 h, 4 h, 5 h, 6 h, 18 h and 24 h), or were pre-treated with Tyr, HT and their sulphated and glucuronidated metabolites (1 μM) for 24 h before adding the LPS (10 μg/mL) or HG (30 mM) for 3 h. The medium was then removed and 180 μL lysis buffer (supplemented with phosphatase and protease inhibitor) was added. Protein concentration was determined through Bradford **(Bradford, 1976)**. Denatured proteins (50–70 μg) were separated using 4-12%, 10% and 4-

20% polyacrylamide gel, then transferred into nitrocellulose membrane where they were blocked with 25 mL of a TBS and 4% milk solution for 40 minutes. After washing with TBS solution, membranes where incubated over-night, at 4°C, with primary polyclonal antibodies, anti-ERK1/2, anti-phospho ERK1/2, anti-phospho p38, anti-p38 (Cell Signaling Technology, Danvers, Massachussets, USA), anti-β actin, anti-GADPH, anti-JAM-A, anti-ZO-1, anti-Occludin (Abcam, Cambridge, UK), anti-NLRP3 (Sigma Aldrich, Milan, Italy) and then washed two times with TTBS (TBS with Tween 20 0.5%) before adding the secondary rabbit and/or mouse antibody IgG peroxidase-conjugated (Sigma Aldrich, Milan, Italy). Both primary and secondary antibody were prepare adding an aliquot of the original solution in 10 mL of TTBS solution with 1% of milk (dilution 1:1000 v/v). Membranes were washed twice with TTBS and one time with TBS, exposed to Clarity™Western-ECL reagents (4-5 minutes) and observed through ChemiDoc™MT System. The bands were visualized by using ChemiDoc instrument and Quantity One software in order to determine the molecular weight of the protein bands, through the comparison with bands obtained by separation of a marker run together with proteins.

3.3.6 Reverse transcription-polymerase chain reaction (RT-PCR)

Confluent HUVEC cells in 6-well plates ($5x10^4$ cells/mL in 2 mL of growth media), were pre-treated with Tyr, HT and their sulphated and glucuronidated metabolites (1 μM) for 24 h before adding HG (30 mM, obtained adding 45 μL/mL of low glucose medium 5 mM from a glucose solution) for 2 h and 6 h. The medium was then removed and 200 μL of free RNAse was added and supernatant collected at -20°C. Total RNA was extracted from the HUVECs with RNeasy Mini Kit (Qiagen, Germany). The High-Capacity cDNA Reverse Transcription kit (Thermo Fischer Scientific) was used to reverse transcribe RNA to cDNA through the thermocycler (Mastercycler gradient, Eppendorf) according to the manufacturer's instructions.

The conditions of thermal cycling were as follow: step 1: 25 °C for 10 min; step 2: 37 °C for 120 min; step 3: 85°C for 5 min and final step at 4°C. The relative expression level of genes was analyzed using SYBR Green qPCR Master Mixes (Thermo Fisher Scientific) and the sequences of PCR primers used were as follows: IL-1β (Forward and Reverse), IL-6 (Forward and Reverse), α-NFκBIA (Forward and Reverse) and β-actin (Forward and Reverse) (Sigma-Aldrich, Milan, Italy). β-actin was used as an internal control using 2-ΔΔCT. Gene expression was measured by a real-time polymerase chain reaction with a QuantStudio™ 12K Flex Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Results were obtained with the Expression Suite Software v1.0.3 (Applied Biosystems, Foster City, CA, USA)

3.4 Statistical analysis

The statistical analysis was performed using the average ± standard deviations for each of the groups in all the experiments (each experiment was performed at least 3 times); significative differences were found through the software GraphPad Prism 5 (GraphPad software, San Diego, CA, USA), using the analysis of variance "one-way ANOVA" and post-hoc Tukey's test.

4 Results

4.1 LPS-induced alteration of Caco-2 cell monolayer permeability and protective effect exerted by Tyr, HT and their sulphated and glucuronidated metabolites

4.1.1 Determination of transepithelial electrical resistance (TEER)

The damage caused by LPS on the intestinal epithelium was evaluated *in vitro* in Caco-2 cell monolayers, as alteration of permeability, measured as transepithelial electrical resistance (TEER). The cells were pretreated with Tyr, tyrosol sulphate (Tyr-sulf), tyrosol glucuronide (Tyr-gluc), HT, hydroxytyrosol 3-sulfate (HT-sulf) and 3'- hydroxytyrosol 3'-glucuronide (HT-gluc) at a concentration of 1 µM, reported in literature as representative physiological concentration which may be bioavailable following dietary intake, and treated LPS (10 µg/mL) for 24 h. TEER value was detected at different time until 24 h of treatment.

Fig 14 shows the comparison among untreated Caco-2 cells (CTRL), cells treated with LPS and cells treated with phenolic compounds and their metabolites. Treatment with LPS led to a significant decrease in TEER value with a major decrease ranging from 35 to 40%, at 3-4 h. Pre-treatment with the phenolic compounds limited the increase in permeability compared to cells treated with LPS. This significative protection, about 20-25% from 1 h up to 18 h of incubation, against TEER decrease was observed almost equally for all tested phenolic compounds, with no significant differences in the protective action ($p > 0,05$).

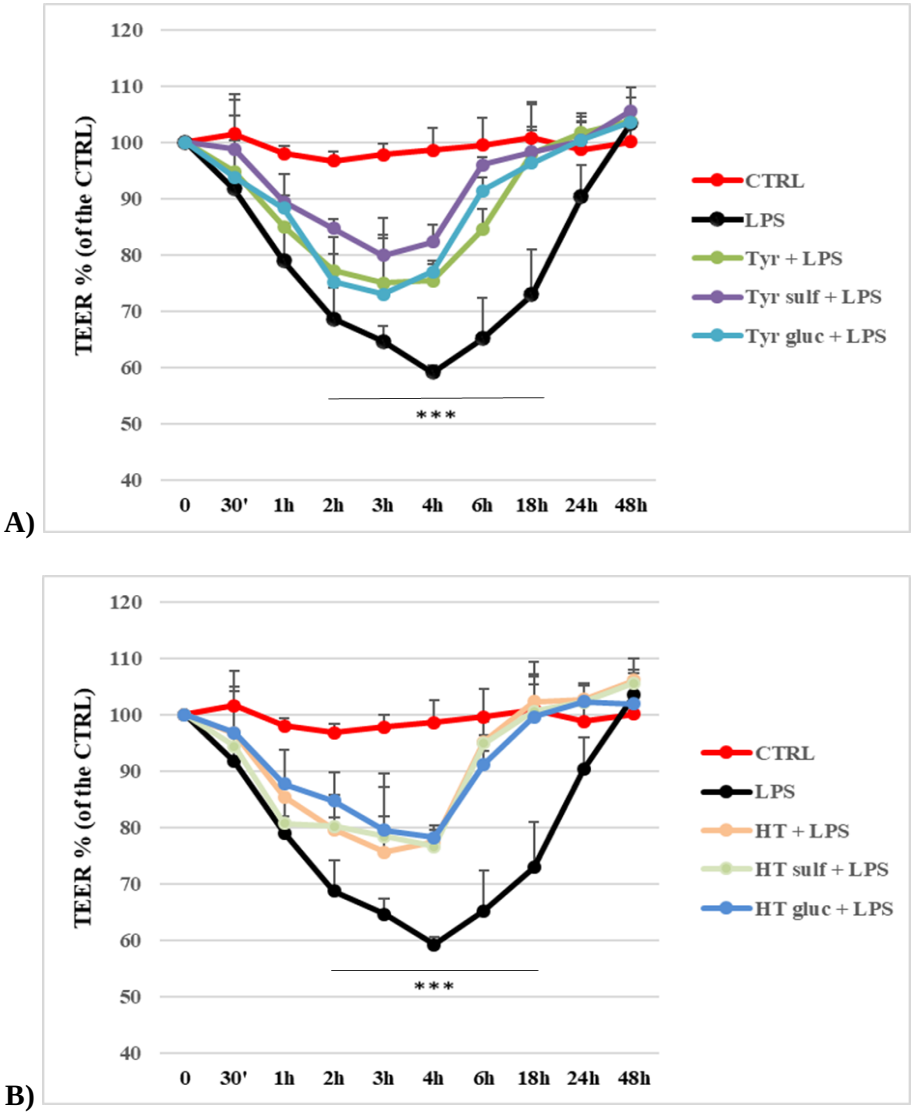


Fig 14. TEER value in Caco-2 cell monolayers pretreated with Tyr and its metabolites (A) and HT and its metabolites (B) (1 µM) and incubated with LPS (10 µg/mL) for 24h, and Caco-2 cells without any treatment (CTRL) at different incubation times. Values are shown as percentage of TEER decrease respect to the CTRL. *** = $p < 0,001$ LPS vs CTRL; $p< 0,05$ Compounds vs LPS; $p> 0,05$ Compound vs Compound (n=6).

4.1.2 Protective effect exerted by Tyr, HT and their sulphated and glucuronidated metabolites against alteration of occludin, ZO-1 and JAM-A levels, in Caco-2 cell monolayers treated with LPS

In order to investigate the mechanism underlying the alteration of permeability induced by LPS on Caco-2 cell monolayers, we evaluated, through Western blotting analyses, the modulation of TJ proteins occludin, ZO-1 and JAM-A with time. The cells were treated with LPS (10 µg/mL), for 2, 3, 4, 5, 6, 18 and 24 h. The ability of the phenolic compounds to modulate LPS alteration of TJ level was then evaluated. In particular, cells were pre-treated with Tyr, Tyr-sulf, Tyr-gluc, HT, HT-sulf and HT-gluc and then incubated with LPS (10 µg/mL) for 4 h.

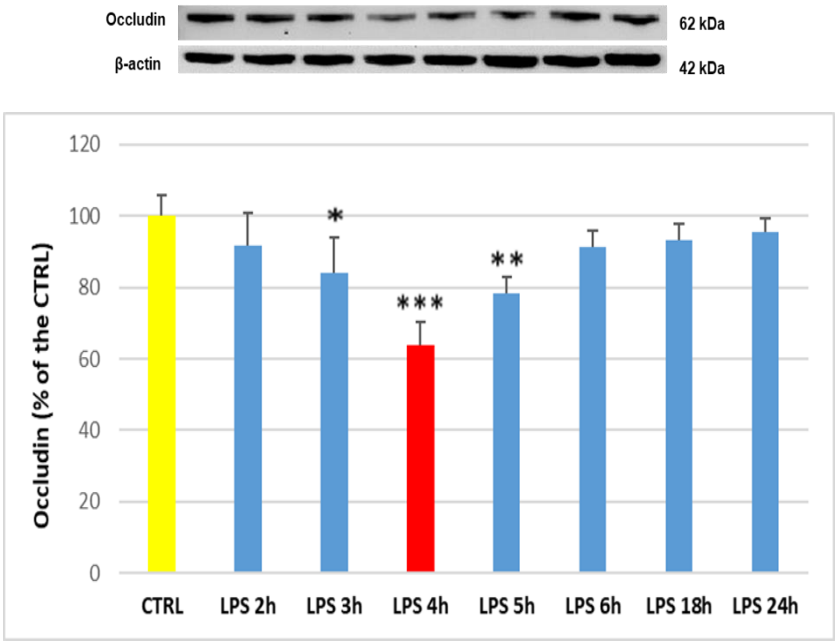


Fig 15. Occludin/β-actin ratio measured in Caco-2 cells not treated (CTRL) or treated with LPS 10 µg/mL at different incubation times. Data are reported as percentage compared to CTRL for each time. * = p< 0,05; ** = p < 0,01; *** = p< 0,001 LPS vs CTRL (n=3).

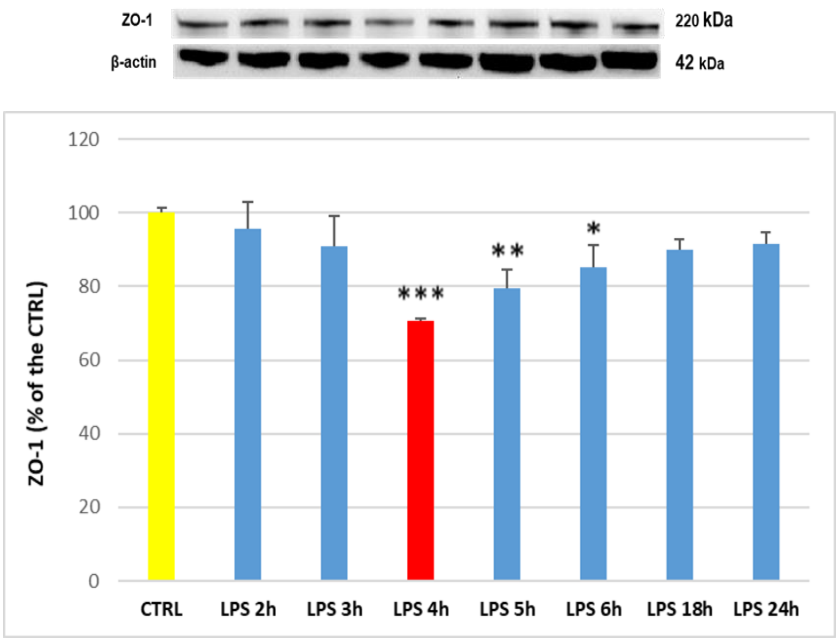


Fig 16. ZO-1/β-actin ratio measured in Caco-2 cells not treated (CTRL) or treated with of LPS 10 µg/mL at different incubation times. Data are reported as percentage compared to CTRL for each time. * = p< 0,05; ** = p < 0,01; *** = p< 0,001 CTRL vs LPS (n=3).

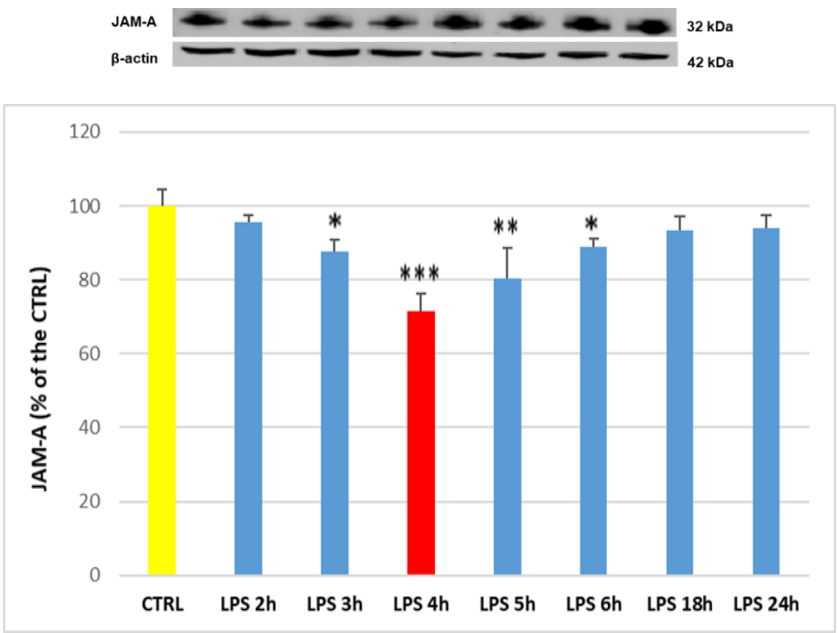


Fig 17. JAM-A/β-actin ratio measured in Caco-2 cells not treated (CTRL) or treated with of LPS 10 µg/mL at different incubation times. Data are reported as percentage compared to CTRL for each time. * = p< 0,05; ** = p < 0,01; *** = p< 0,001 CTRL vs LPS (n=3).

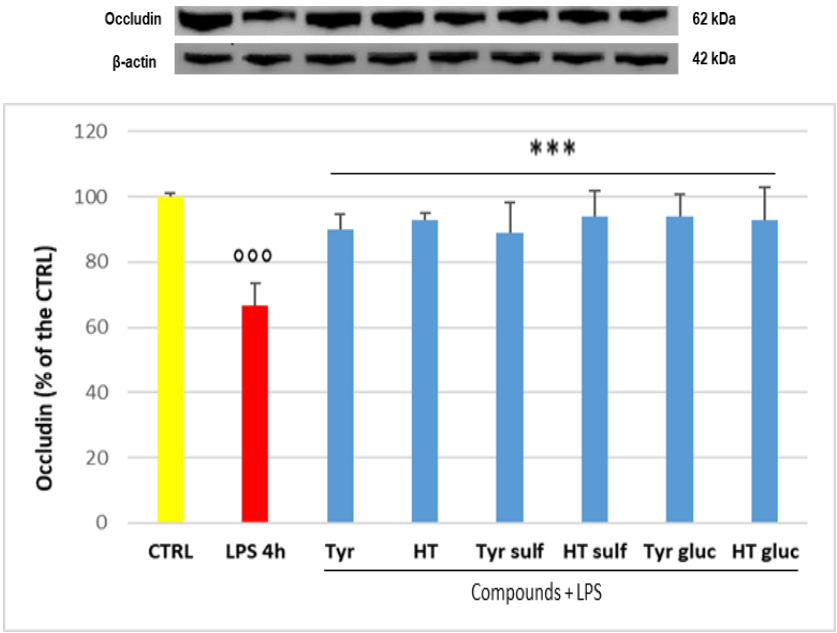


Fig 18. Occludin/β-actin ratio measured in Caco-2 cells not treated (CTRL), pretreated with Tyr, HT, Tyr sulf, HT sulf, Tyr gluc, HT gluc (1µM) and treated with LPS (10 µg/mL) for 4h. Data are reported as percentage compared to CTRL for each sample. °°° = p< 0,001 LPS vs CTRL; *** = p< 0,001 Compounds vs LPS; p> 0,05 Compound vs Compound (n=3).

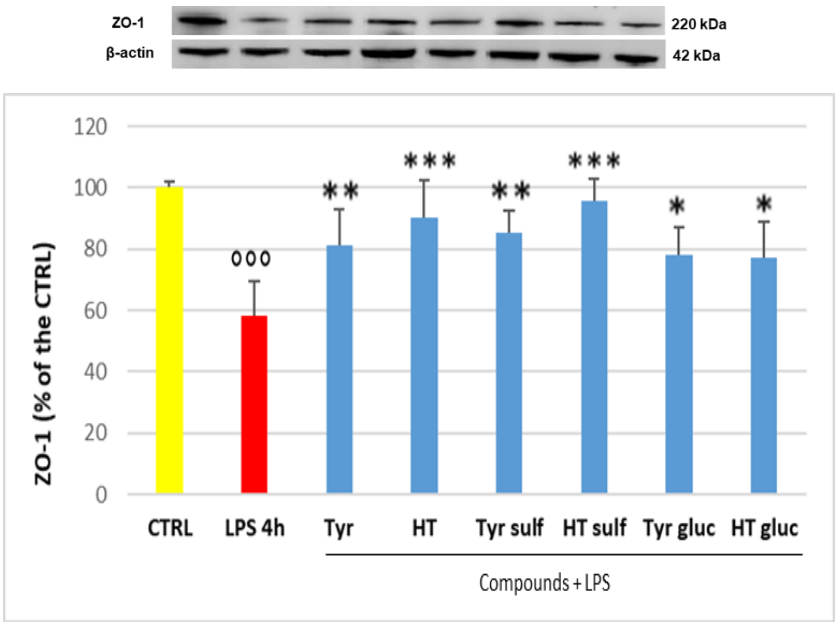


Fig 19. ZO-1/β-actin ratio measured in Caco-2 cells not treated (CTRL), pretreated with Tyr, HT, Tyr sulf, HT sulf, Tyr gluc, HT gluc (1µM) and treated with LPS (10 µg/mL) for 4h. Data are reported as percentage compared to CTRL for each sample. °°° = p< 0,001 LPS vs CTRL; * = p< 0,05; ** = p< 0,01 LPS vs Compounds; *** = p< 0,001 LPS vs Compounds; p> 0,05 Compound vs Compound (n=3).

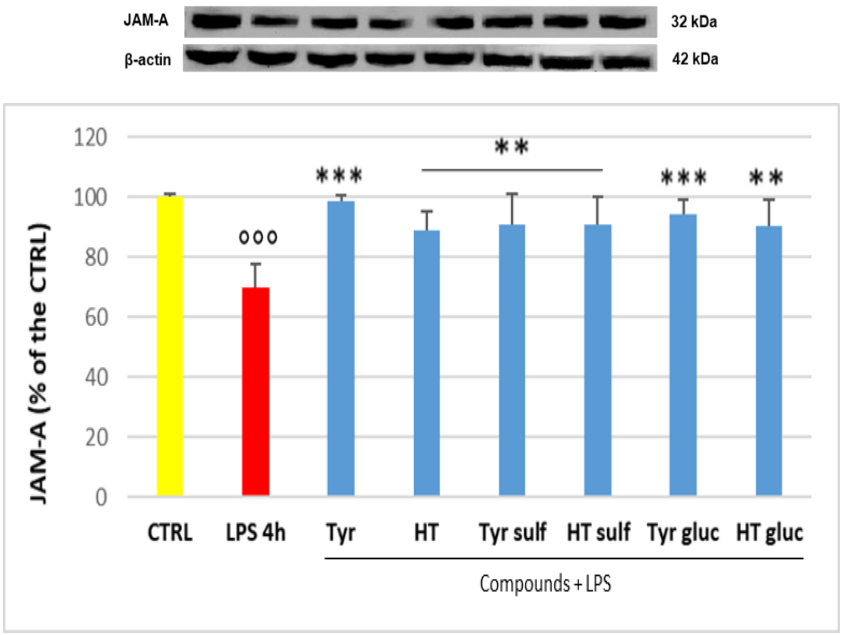


Fig 20. JAM-A/β-actin ratio measured in Caco-2 cells not treated (CTRL), pretreated with Tyr, HT, Tyr sulf, HT sulf, Tyr gluc, HT gluc (1µM) and treated with LPS (10 µg/mL) for 4h. Data are reported as percentage compared to CTRL for each sample. CTRL is the sample with only medium. °°° = p< 0,001 LPS vs CTRL; ** = p< 0,01, *** = p< 0,001 Compounds vs LPS; p> 0,05 Compound vs Compound (n=3).

Treatment with LPS led to a significant decrease in all three TJ proteins level compared to CTRL (100%), as shown in fig 15, 16, 17, reflecting the time-course of the permeability variation detected in Caco-2 cell monolayers as TEER decrease. The lowest level of TJ proteins was observed at 4 h of incubation with LPS. Occludin decreased about 35-40%, ZO-1 and JAM-A about 20-25% respect to the CTRL. At 24 h the level of all the TJ came back to CTRL value.

Fig 18, 19 and 20 show the protective action against LPS-induced decrease of occludin JAM-A and ZO-1, exerted by the pretreatment with EVOO phenolic compounds and their metabolites (1 µM) on Caco-2 cells before the addition of LPS (10 µg/mL). Cells were incubated for 4 h, time in which LPS induced the greatest reduction in the protein levels of all three TJ. All the tested compounds showed a significant protective effect and were able to preserve TJ's level in Caco-2 treated with LPS. Parent compounds and metabolites exerted a comparable efficacy, with no significant difference (p> 0,05) in the exerted protection.

4.1.3 Modulation of p38 and ERK1/2 exerted by phenolic compounds in Caco-2 cell monolayers treated with LPS

In order to investigate the molecular mechanism underlying the distruption of TJ induced by LPS on Caco-2 cell monolayers, we focused on the activation of cellular pathways linked to TJ regulation, as MAPK. We evaluated the modulation of the phosphorylation state of MAPK p38 and ERK1/2 with time, in cells treated with only LPS (10 μg/mL), for 1, 2, 3, 4 and 6 h. In addition, the ability of the tested phenolic compounds to inhibit LPS-induced activation of MAPK, was evaluated through Western blotting, focusing on the modulation of p38 and ERK 1/2, in Caco-2 cells pretreated with HT, Tyr and their glucuronidated and sulphated metabolites and treated with LPS for 3 h (choice following phosphorylation results). Values are reported as ratio between the level of phosphorylated protein and its total amount.

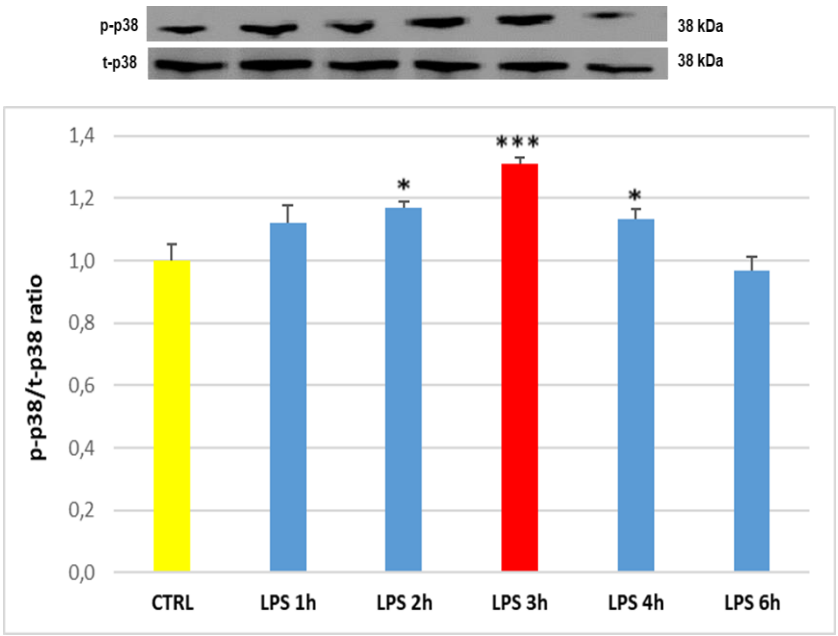


Fig 21. p-p38/t-p38 ratio measured in Caco-2 cells not treated (CTRL) or treated with LPS 10 µg/mL at different incubation times. * = p< 0,05; *** = p< 0,001 LPS vs CTRL (n=3).

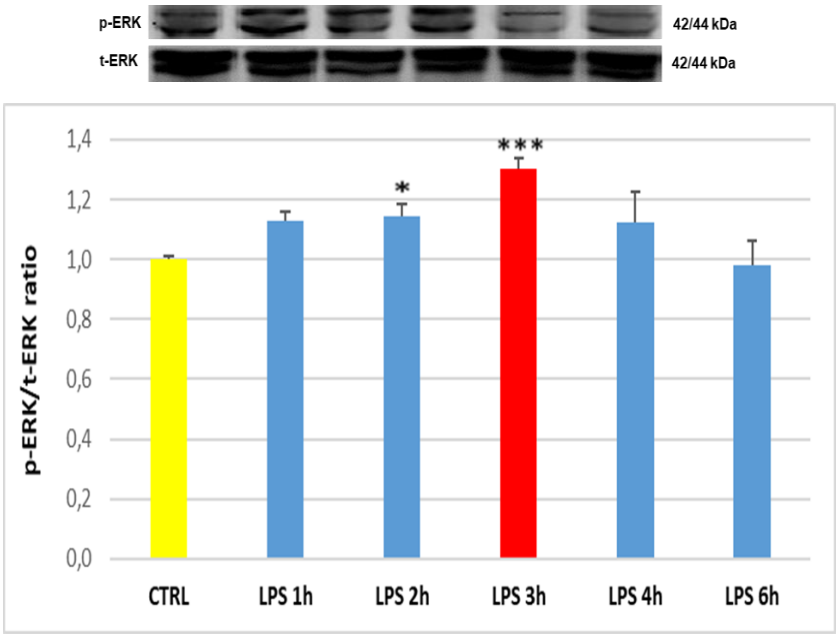


Fig 22. p-ERK/t-ERK ratio measured in Caco-2 cells not treated (CTRL) or treated with LPS 10 µg/mL at different incubation times. * = p< 0,05; *** = p< 0,001 LPS vs CTRL (n=3).

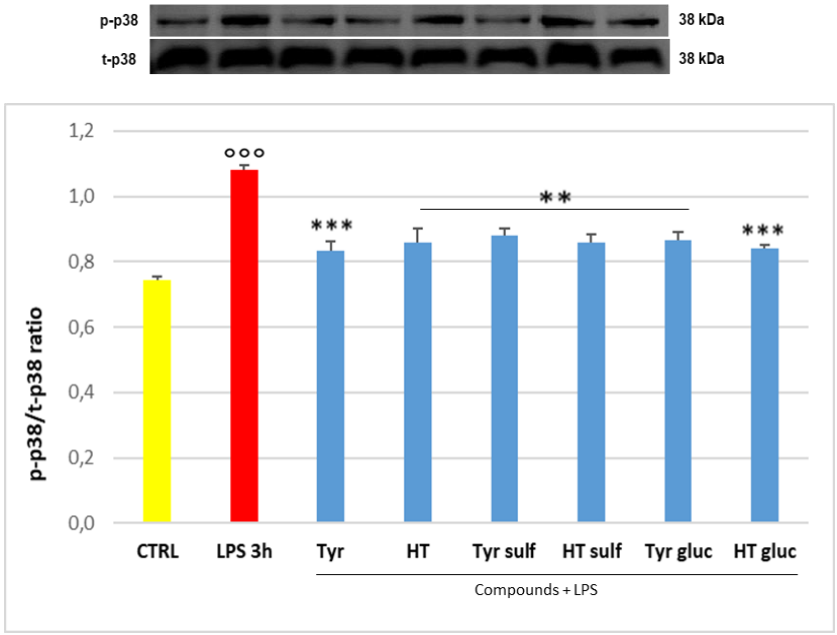


Fig 23. p-p38/t-p38 ratio measured in Caco-2 cells not treated (CTRL), pretreated with Tyr, HT, Tyr sulf, HT sulf, Tyr gluc, HT gluc (1 µM) and treated with LPS (10 µg/mL) for 3 h. °°° = p< 0,001 LPS vs Compounds; ** = p< 0,01; *** = p< 0,001 Compounds vs LPS; p> 0,05 Compound vs Compound (n=3).

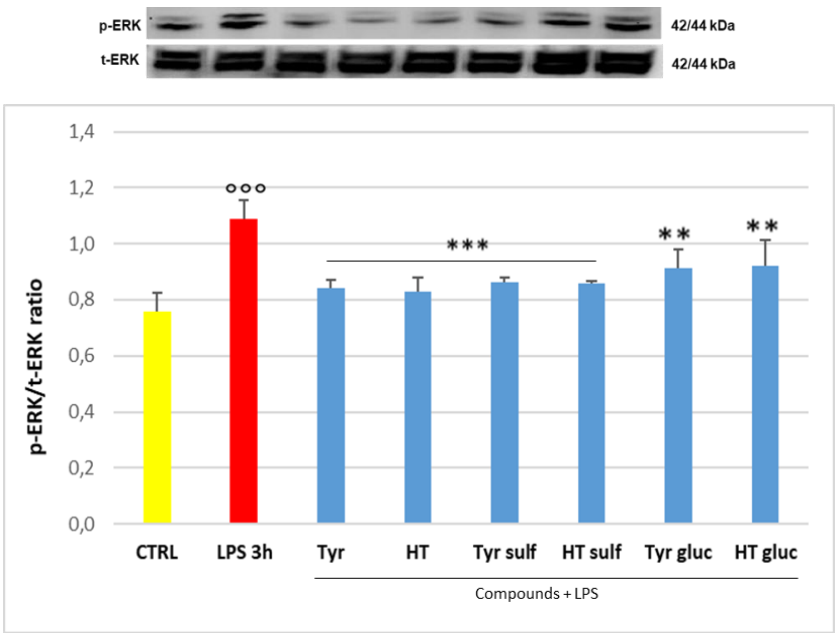


Fig 24. p-ERK/t-ERK ratio measured in Caco-2 cells not treated (CTRL), pretreated with Tyr, HT, Tyr sulf, HT sulf, Tyr gluc, HT gluc (1µM) and treated with LPS (10 µg/mL) for 3 h. °°° = p< 0,001 LPS vs CTRL; ** = p< 0,01; *** = p< 0,001 Compounds vs LPS; p> 0,05 Compound vs Compound (n=3).

Fig 21 and Fig 22 show the differences in MAPK phosphorylation state among untreated samples (CTRL) and the ones treated with LPS for 1, 2, 3, 4, and 6 h. LPS treatment stimulated Caco-2 cells to activate p38 and ERK through their phosphorylation, which occurred mostly at 3 h when the level of p-p38 and p-ERK were significantly higher (25-30%) in samples with LPS compared to CTRL (100%). At 6 h the levels of both MAPK were similar to the CTRL.

Fig 23 and 24 show the protective effect against LPS-induced increase of p38 and ERK phosphorylation, exerted by pretreatmet with the phenolic compounds and their metabolites (1 µM) on Caco-2 cells. LPS determined a significant phosphorylation of p38 and ERK after 3 h of incubation (time in which the highest activation of both MAPK by LPS was observed), that was about 25-30% higher compared to CTRL (100%). Pretreatment with parent compounds and metabolites significantly limited the phosphorylation of the proteins and the action was similar for all tested compounds, with no significant differences ($p > 0,05$).

4.1.4 Protective effect exerted by Tyr, HT and their sulphated and glucuronidated metabolites against activation of NLRP3 inflammasome in Caco-2 cell monolayers treated with LPS

In the cellular inflammatory response at intestinal level, an over-activation of NLRP3 inflammasome complex seems to be linked to TJ alteration. We investigated the modulation of NLRP3 inflammasome protein levels induced by LPS on Caco-2 cell monolayers with time, through Western blotting. The cells were treated with LPS (10 µg/mL), for 1, 2, 3, 4, 6, 18 and 24 h. The ability of the phenolic compounds to modulate LPS-induced increase of NLRP3 protein was evaluated, in Caco-2 cells pretreated with HT, Tyr and their glucuronidated and sulphated metabolites (1 µM) and treated with LPS (10 µg/mL) for 3 h, time in which, as previously shown, the highest increase in NLRP3 protein levels was observed.

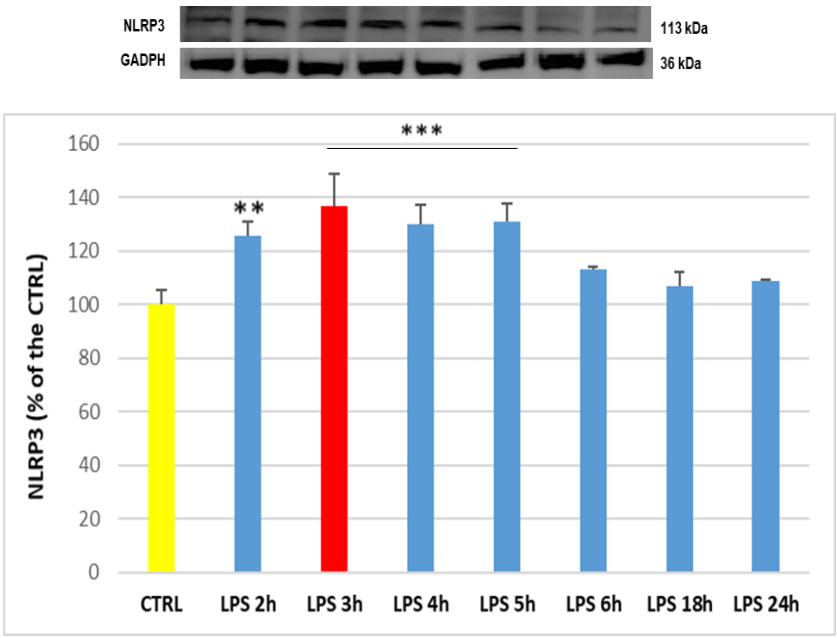


Fig 25. NLRP3/GADPH ratio measured in Caco-2 cells not treated (CTRL) or treated with LPS 10 µg/mL at different incubation times. Data are reported as percentage compared to CTRL for each time. ** = p < 0,01; *** = p< 0,001 LPS vs CTRL (n=3).

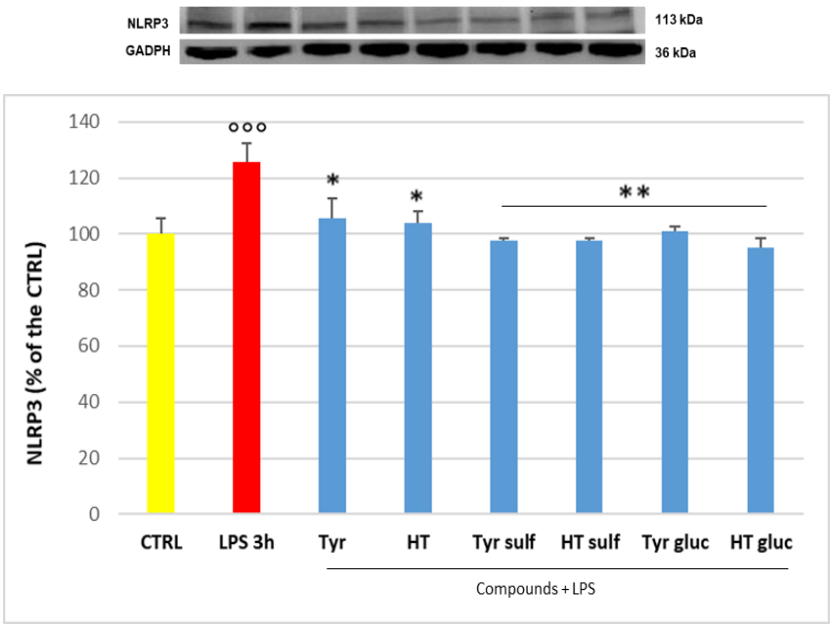


Fig 26. NLRP3/GADPH ratio measured in Caco-2 cells not treated (CTRL), pretreated with Tyr, HT, Tyr sulf, HT sulf, Tyr gluc, HT gluc (1 µM) and treated with LPS (10 µg/mL) for 3 h. Data are reported as percentage compared to CTRL for each sample. °°° = p< 0,001 LPS vs CTRL; * = p< 0,05; ** = p< 0,01 Compounds vs LPS; p> 0,05 Compounds vs Compounds (n= 3).

LPS treatment determined in Caco-2 a significant increase of NLRP3 protein levels compared to CTRL (100%), as shown in Fig 25. NLRP3 levels increased from 2 h of incubation, reached its maximum level (about 35%) between 3 h and 5 h and went back to CTRL values after 6 h.

As it can be observed in Fig 26, LPS induced a significant increase (about 25-30%) of NLRP3, while pretreatment with all tested phenolic compounds limited NLRP3 rise compared to samples treated with only LPS. This protective effect was observed almost equally for all tested compounds (p> 0,05).

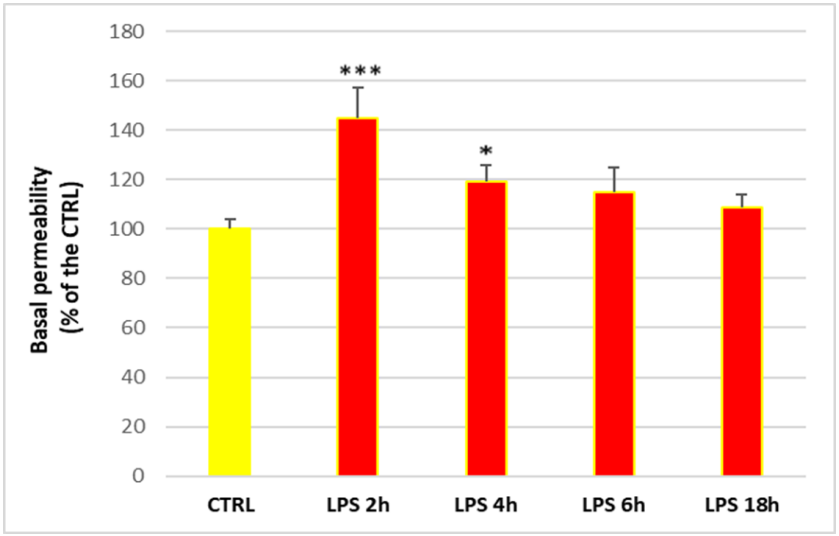
4.2 LPS-induced alteration of HUVEC cell monolayer permeability and protective effect exerted by Tyr, HT and their sulphated and glucuronidated metabolites

4.2.1 FITC-Dextran Permeability assay

The damage caused by LPS on the endothelial barrier was evaluated *in vitro* in HUVEC cell monolayers, as alteration of permeability, by measurement of FITC-Dextran, a typical molecule that is transported via the paracellular route through cell TJ. Cells were treated with LPS (10 µg/mL) and incubated for 2 h, 4 h, 6 h and 18 h, at the end of which the basal permeability of FITC-Dextran was measured spectrophometrically. Then, in the same way, we measured the ability of the phenolic compounds to limit the alteration of endothelial permeability induced by LPS, pretreating the cells with Tyr, HT and their sulphated and glucuronidated metabolites (1 µM) and with LPS for 2 h.

Fig 27A shows the changes in endothelial cell permeability induced by LPS treatment from 2 h to 18 h. LPS significantly increased the basal permeability at 2 h of incubation, about 40-45% respect the untreated cells (CTRL), highlighting the role of LPS in the alteration of endothelial TJ. However, after 4 h of incubation the basal permeability of FITC-Dextran started to decrease and at 18 h of incubation it was similar to CTRL values. Data reported in fig 27B show how pretreatment with all tested phenolic compounds limited the rise of basal FITC-Dextran permeability compared to samples treated with only LPS for 2 h. This protective effect was observed almost equally for all tested compounds ($p > 0,05$).

A)



B)

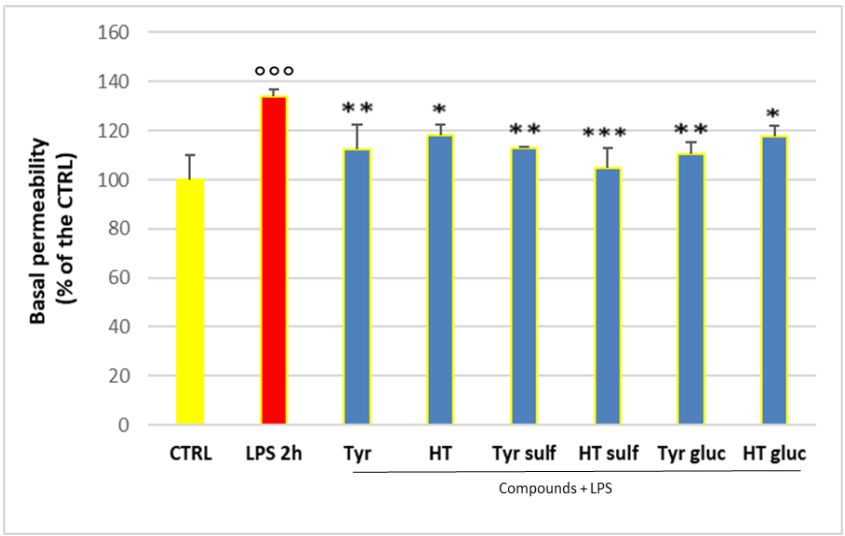


Fig 27. **A**) FITC-Dextran basal permeability measured in HUVEC cells not treated (CTRL) or treated with LPS 10 µg/mL at 2 h, 4 h, 6 h and 18 h. Data are reported as percentage compared to CTRL for each time. * = p< 0,05; *** = p < 0,001 LPS vs CTRL (n=3). **B**) FITC-Dextran basal permeability measured in HUVEC cells not treated (CTRL) and pretreated with Tyr, HT, Tyr sulf, HT sulf, Tyr gluc, HT gluc (1 µM) and treated with LPS (10 µg/mL) for 2h. Data are reported as percentage compared to CTRL for each sample. °°° = p< 0,001 LPS vs CTRL; ** = p< 0,01; *** = p< 0,001 Compounds vs LPS; p> 0,05 Compound vs Compound (n= 3).

4.2.2 Protective effect exerted by Tyr, HT and their sulphated and glucuronidated metabolites against alteration of Occludin, ZO-1 and JAM-A in HUVEC cell monolayers treated with LPS

In order to investigate the mechanism of action by which LPS determined a significant alteration of permeability in HUVEC cell monolayers, we examined the modulation of TJ, focusing on Occludin, ZO-1 and JAM-A with time, through Western blotting. Cells were treated with LPS (10 µg/mL), for 1, 2, 3, 4, 6, 18 and 24 h. The protective effect of the phenolic compounds in LPS-induced TJ disruption was then evaluated, pre-treateding cells with Tyr, Tyr-sulf, Tyr-gluc, HT, HT-sulf and HT-gluc and incubating with LPS (10 µg/mL) for 3 h, time in which LPS induced the greatest reduction in the protein levels of all three TJ.

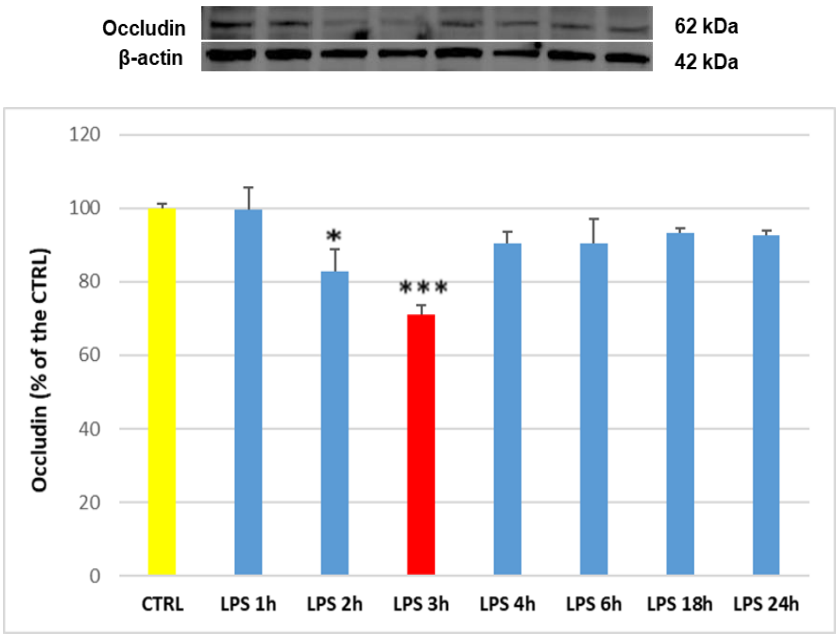


Fig 28. Occludin/β-actin ratio measured in HUVEC cells not treated (CTRL) or treated with of LPS 10 µg/mL at different incubation times. Data are reported as percentage compared to CTRL for each time. * = p< 0,05; *** = p < 0,001 LPS vs CTRL; (n=3).

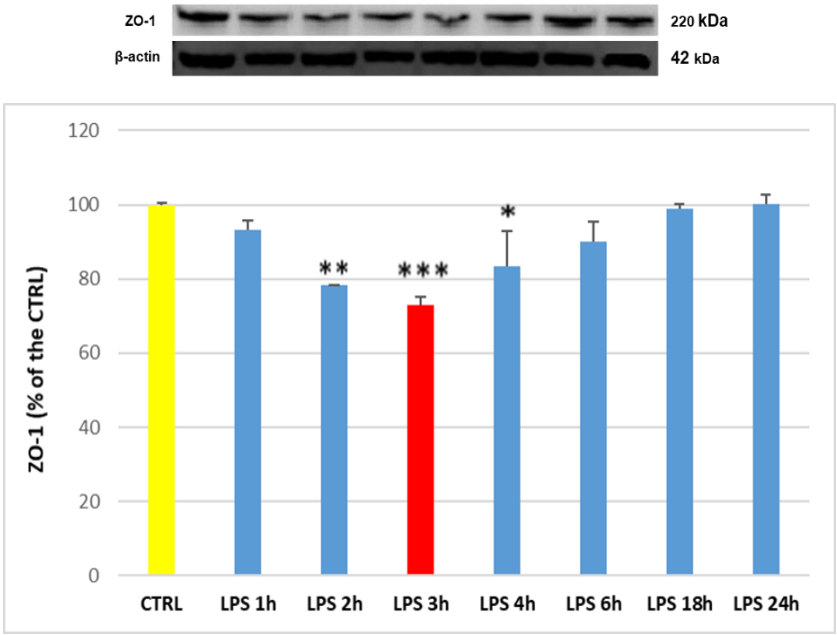


Fig 29. ZO-1/β-actin ratio measured in HUVEC cells not treated (CTRL) or treated with of LPS 10 µg/mL at different incubation times. Data are reported as percentage compared to CTRL for each time. * = p< 0,05; ** = p < 0,01; *** = p< 0,001 LPS vs CTRL (n=3).

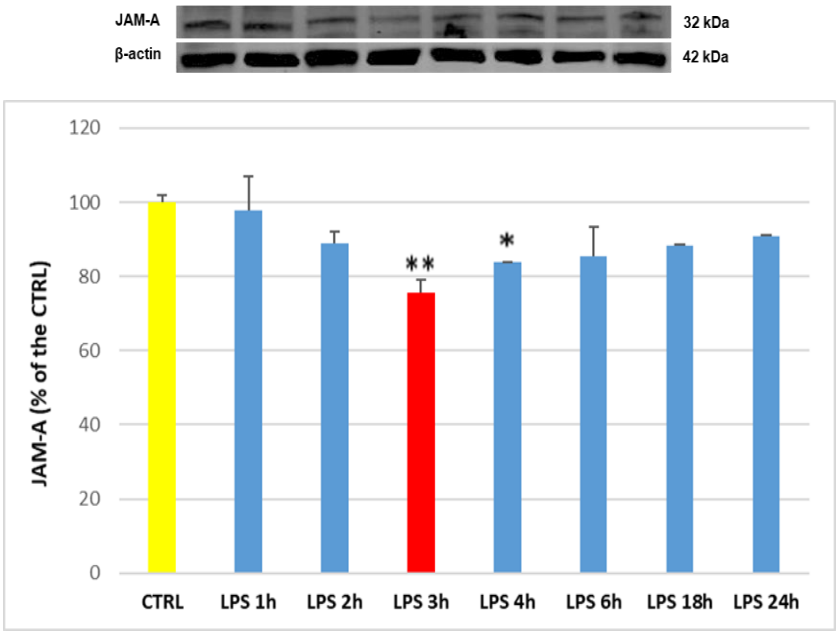


Fig 30. JAM-A/β-actin ratio measured in HUVEC cells not treated (CTRL) or treated with of LPS 10 µg/mL at different incubation times. Data are reported as percentage compared to CTRL for each time. * = p< 0,05; ** = p < 0,01 LPS vs CTRL (n=3).

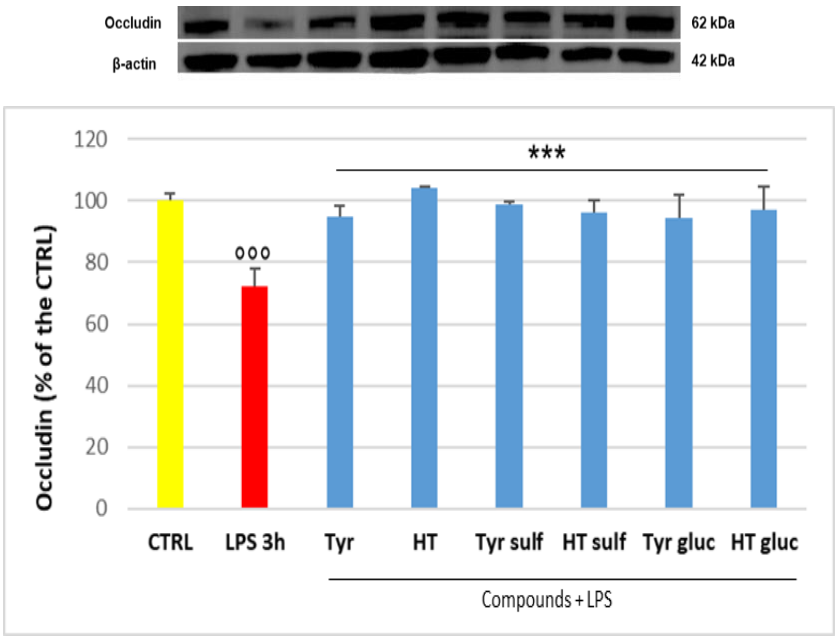


Fig 31. Occludin/β-actin ratio measured in HUVEC cells not treated (CTRL), pretreated with Tyr, HT, Tyr sulf, HT sulf, Tyr gluc, HT gluc (1µM) and treated with LPS (10 µg/mL) for 3 h. Data are reported as percentage compared to CTRL for each sample. °°° = p< 0,001 LPS vs CTRL; *** = p< 0,001 Compounds vs LPS; p> 0,05 Compound vs Compound (n= 3).

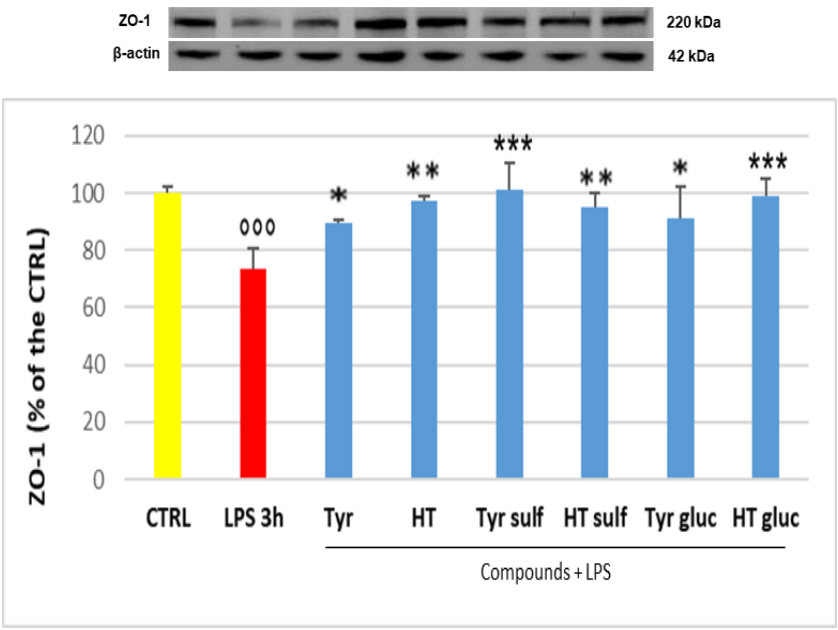


Fig 32. ZO-1/β-actin ratio measured in HUVEC cells not treated (CTRL), pretreated with Tyr, HT, Tyr sulf, HT sulf, Tyr gluc, HT gluc (1µM) and treated with LPS (10 µg/mL) for 3 h. Data are reported as percentage compared to CTRL for each sample. °°° = p< 0,001 LPS vs CTRL; * = p< 0,05; ** = p< 0,01; *** = p< 0,001 Compounds vs LPS; p> 0,05 Compound vs Compound (n= 3).

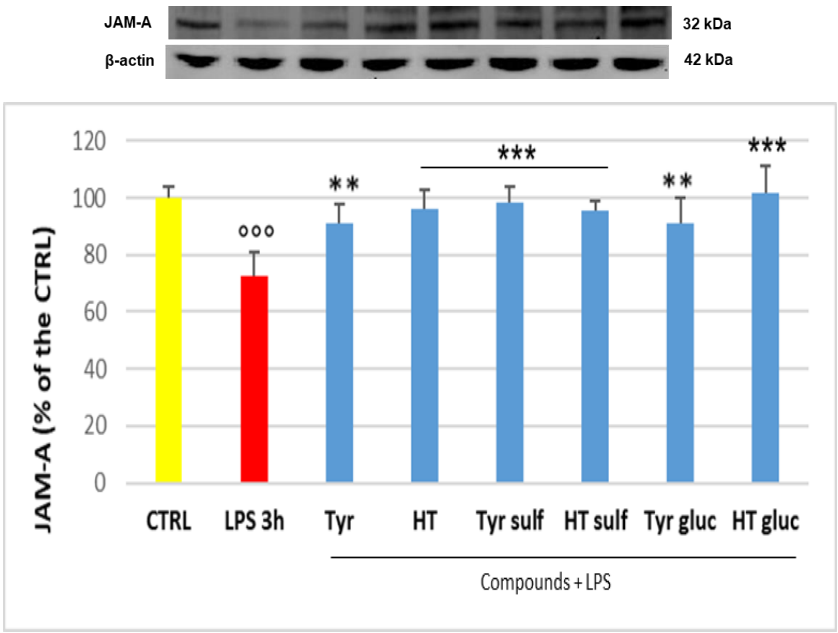


Fig 33. JAM-A/β-actin ratio measured in HUVEC cells not treated (CTRL), pretreated with Tyr, HT, Tyr sulf, HT sulf, Tyr gluc, HT gluc (1µM) and treated with LPS (10 µg/mL) for 3h. Data are reported as percentage compared to CTRL for each sample. °°° = p< 0,001 LPS vs CTRL; ** = p< 0,01; *** = p< 0,001 Compounds vs LPS; p> 0,05 Compound vs Compound (n= 3).

Fig 28, 29 and 30 show the effect of LPS on TJ integrity in HUVEC cell monolayers. The treatment with LPS led to a decrease in all three TJ proteins level compared to CTRL (100%), with direct alteration of membrane permeability. The highest decrease was observed at 3 h of incubation, with occludin and ZO-1 decreased of about 25-30% respect to the CTRL and JAM of about 22-25%. At 24 h all analyzed TJ proteins reached the CTRL values.

Data obtained after pre-treatment with the compounds and the metabolites are reported in Fig 31, 32 and 33. After 3 h of incubation LPS was able to cause a decrease in TJ protein levels (about 25-30% compared to CTRL (100%)), while pretreatment with Tyr, HT and their sulphated and glucuronidated metabolites preserved TJ levels. Results are similar for all tested phenolic compounds with no significant differences (p> 0,05) in their protective action.

4.2.3 Modulation of p38 and ERK 1/2 exerted by phenolic compounds in HUVEC cell monolayers treated with LPS

In order to study the molecular mechanism underlying the distruption of endothelial TJ induced by LPS, we evaluated its capacity to activate MAPK signaling pathway, focusing on the phosphorylation state of p38 and ERK1/2. Cells were treated with LPS (10 µg/mL), for 1, 2, 3, 4 and 6 h. The protective effect of EVOO phenolic compounds and their metabolites was then analyzed in HUVEC cells pretreated with HT, Tyr and their glucuronidated and sulphated metabolites (1 µM) and LPS (10 µg/mL) for 3 h, time in which increased activation of both MAPK by LPS was previously observed.

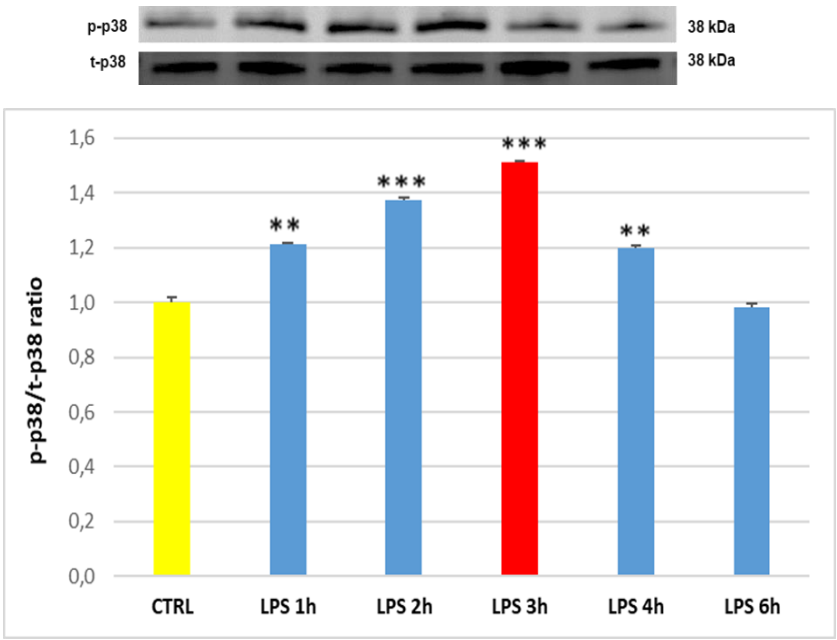


Fig 34. p-p38/t-p38 ratio measured in HUVEC cells not treated (CTRL) or treated with LPS 10 µg/mL at different incubation times. ** = p < 0,01; *** = p< 0,001 LPS vs CTRL (n=3).

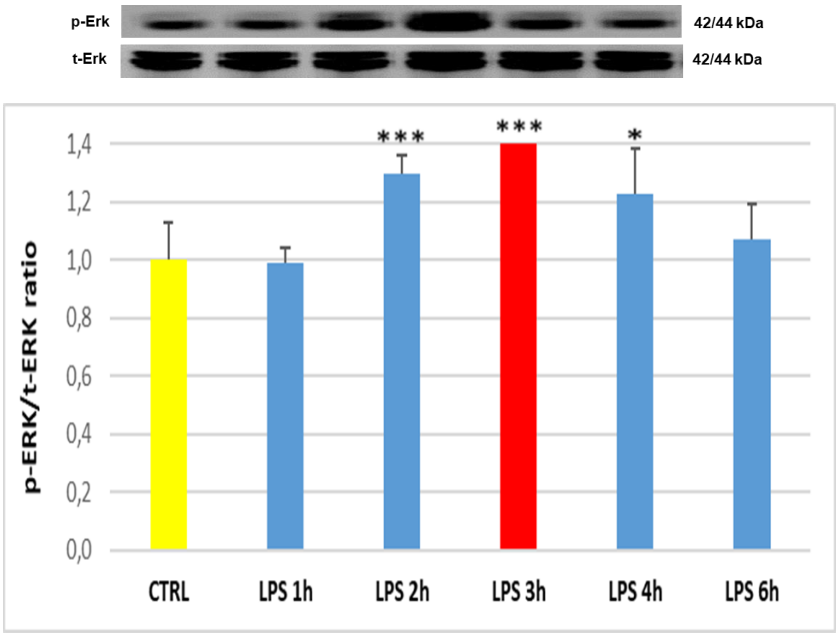


Fig 35. p-ERK/t-ERK ratio measured in HUVEC cells not treated (CTRL) or treated with LPS 10 µg/mL at different incubation times. * = p< 0,05; *** = p< 0,001 LPS vs CTRL (n=3).

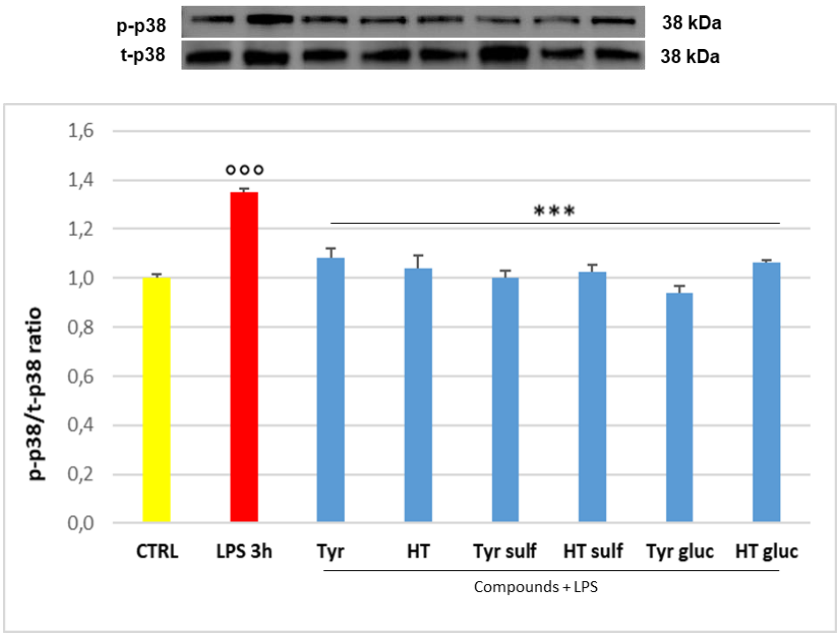


Fig 36. p-p38/t-p38 ratio measured in HUVEC cells not treated (CTRL), pretreated with Tyr, HT, Tyr sulf, HT sulf, Tyr gluc, HT gluc (1µM) and treated with LPS (10 µg/mL) for 3 h. °°° = p< 0,001 LPS vs CTRL; *** = p< 0,001 Compounds vs LPS; p> 0,05 Compound vs Compound (n= 3).

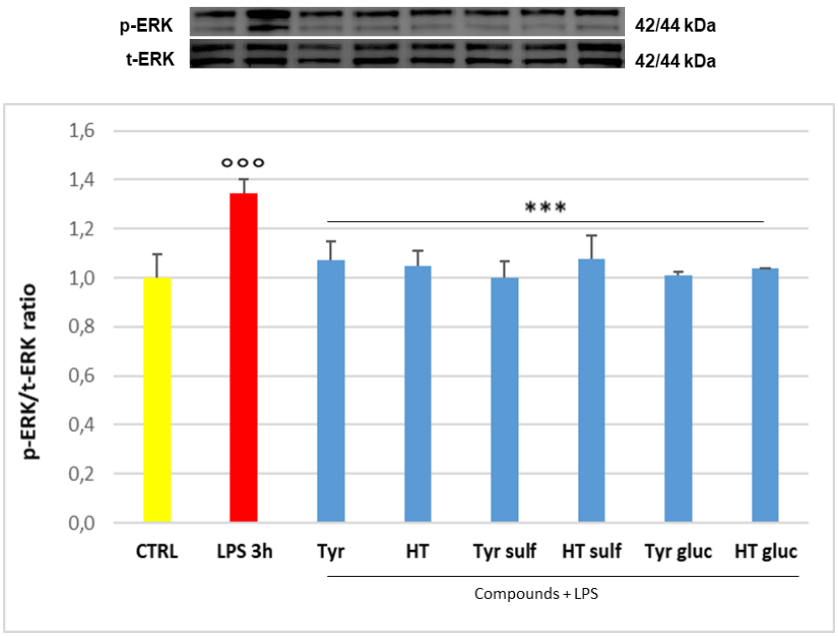


Fig 37. p-ERK/t-ERK ratio measured in HUVEC cells not treated (CTRL), pretreated with Tyr, HT, Tyr sulf, HT sulf, Tyr gluc, HT gluc (1µM) and treated with LPS (10 µg/mL) for 3h. °°° = p< 0,001 LPS vs CTRL; *** = p< 0,001 Compounds vs LPS; p> 0,05 Compound vs Compound (n= 3).

Phosphorylation level of p38 and ERK 1/2 detected in HUVEC cells treated with LPS, at 1-6 h of incubation is reported in Fig 34 and 35. LPS induced a significant phosphorylation of both proteins respect to the CTRL and at 3 h of incubation the activation reached the higher values (about 50% for p-p38 and about 40% for p-ERK). At 6 h the level of both MAPK was similar to the CTRL.

Fig 36 and 37 show the phosphorylation of p38 and ERK1/2 induced by LPS and the effect exerted by phenolic compounds and their metabolites on HUVEC cells. LPS was able to significantly enhance the levels of p-p38 and p-ERK1/2 compared to not treated samples (CTRL) after 3 h of incubation, when the ratio p-p38/t-p38 and p-ERK1/2/t-ERK1/2 were about 35-40% higher in LPS samples, compared to the CTRL (100%). Pretreatment with compounds and metabolites significantly limited the phosphorylation of both kinases respect to the cells treated with LPS only and the efficacy was similar for all tested phenolic compounds ($p > 0,05$).

4.2.4 Protective effect exerted by Tyr, HT and their sulphated and glucuronidated metabolites against activation of NLRP3 inflammasome in HUVEC cell monolayers treated with LPS

NLRP3 inflammasome plays a fundamental role in the inflammatory response and in the mechanism of membrane permeability alteration also at endothelial level; therefore, we investigated its modulation by LPS on HUVEC cell monolayers with time, through western blotting. Cells were treated with LPS (10 µg/mL), for 1, 2, 3, 4, 6, 18 and 24 h. The ability of Tyr, HT and their metabolites to modulate LPS effect was evaluated in HUVEC pretreated with the phenolic compounds (1 µM) and with LPS (10 µg/mL) for 3 h.

As shown in Fig 38, LPS determined in HUVEC a significant increase of NLRP3 protein levels which occurred mostly at 2 h and 3 h (about 25-30%), compared to CTRL (100%). At 24 h the NLRP3 protein levels reached the CTRL values.

LPS treatment for 3 h, as previously shown, induced a significant increase (about 30%) of NLRP3, while pretreatment with all phenolic compounds inhibited this rise compared to sample treated with only LPS (Fig 39). This protective effect was observed almost equally for all tested phenolic compounds, with no significant differences ($p > 0,05$).

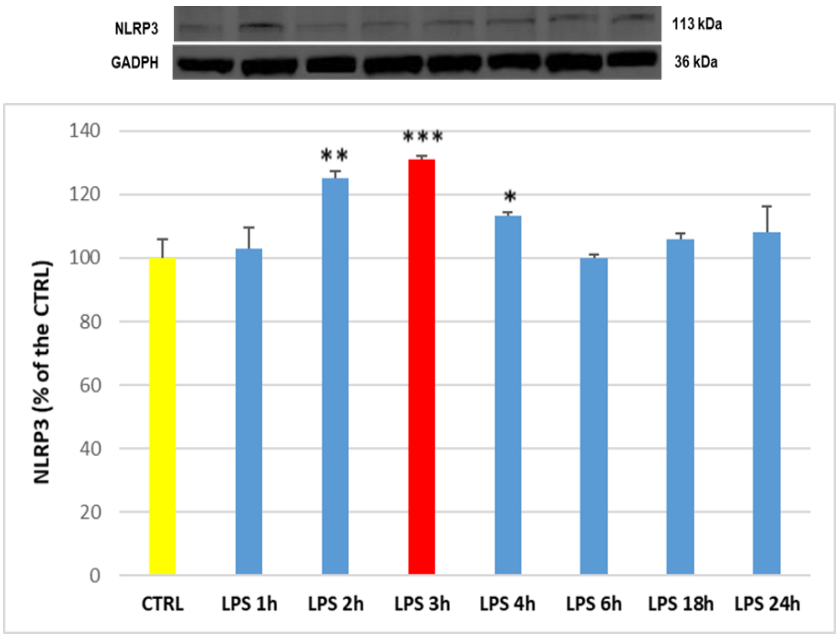


Fig 38. NLRP3/GADPH ratio measured in HUVEC cells not treated (CTRL) or treated with LPS 10 µg/mL at different incubation times. Data are reported as percentage compared to CTRL for each time. * = p< 0,05; ** = p < 0,01; *** = p< 0,001 LPS vs CTRL (n=3).

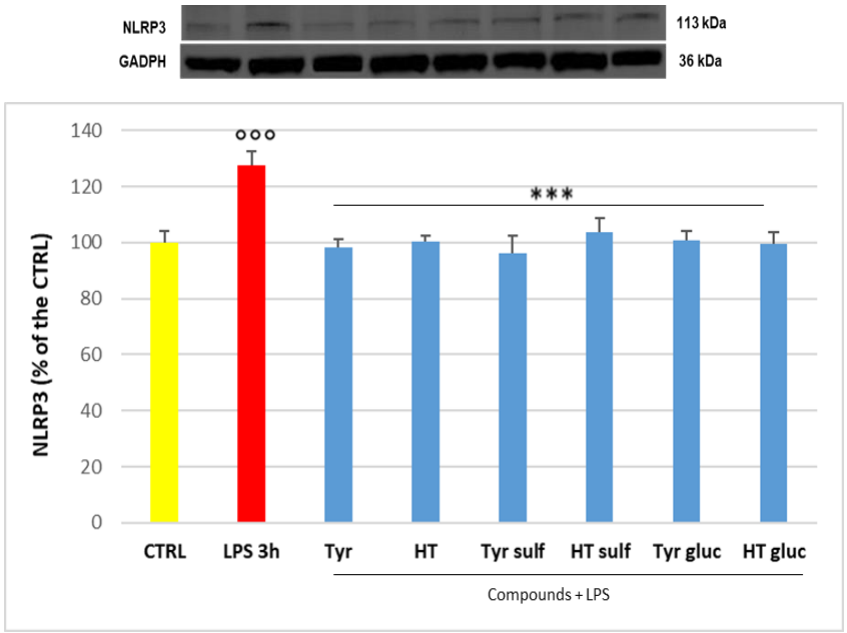


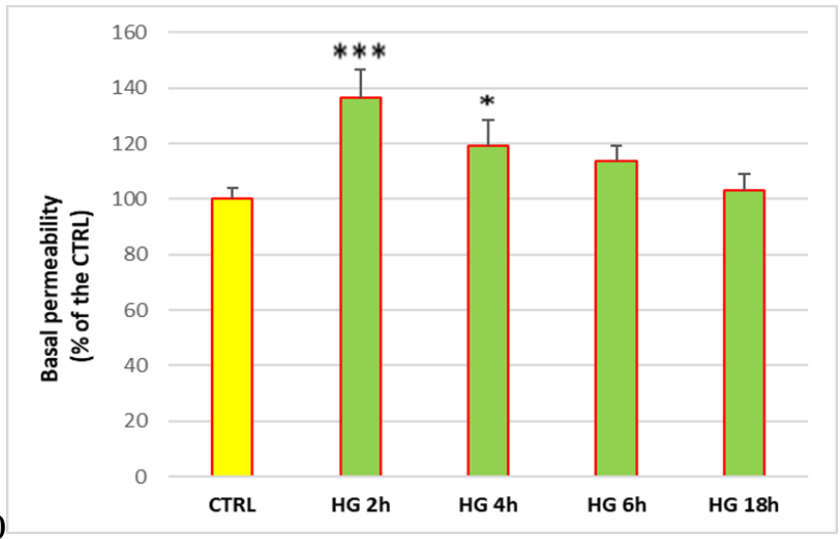
Fig 39. NLRP3/GADPH ratio measured in HUVEC cells not treated (CTRL) and pretreated with Tyr, HT, Tyr sulf, HT sulf, Tyr gluc, HT gluc (1µM) and treated with LPS (10 µg/mL) for 3 h. °°° = p< 0,001 LPS vs CTRL; *** = p< 0,001 Compounds vs LPS; p> 0,05 Compound vs Compound (n= 3).

4.3 HG-induced alteration of HUVEC cell monolayer permeability and protective effect exerted by Tyr, HT and their sulphated and glucuronidated metabolites

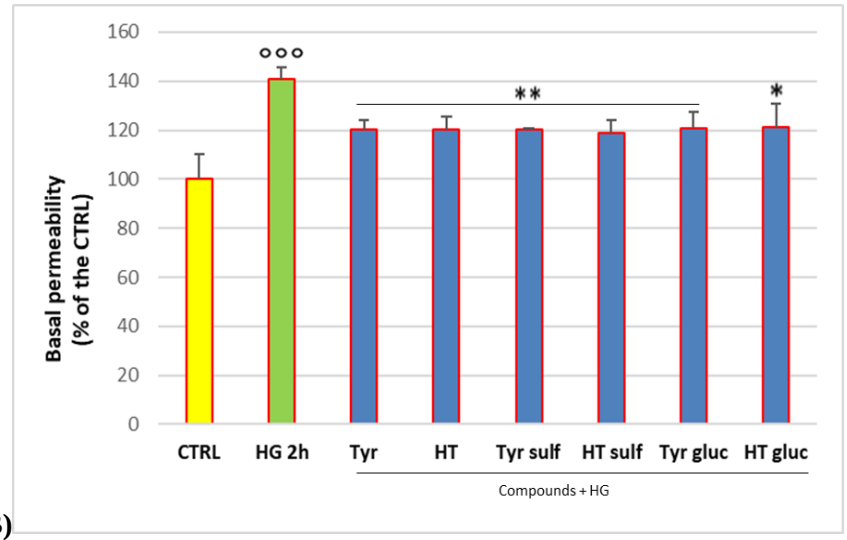
4.3.1 FITC-Dextran Permeability assay

The capacity of HG to induce alteration of endothelial permeability was evaluated *in vitro* in HUVEC cell monolayers, by measurement of FITC–dextran flux. Cells were treated with HG (30 mM) and incubated for 2 h, 4 h, 6 h and 18 h, at the end of which the basal permeability of Fitc-Dextran was measured spectrophotometrically. In addition, the ability of the phenolic compounds tested to limit the alteration of endothelial permeability induced by HG was detected, measuring FITC-Dextran flux in HUVEC cells pretreated with Tyr, HT and their glucuronidated and sulphated metabolites (1 µM) and treated with HG for 2 h.

The changes in HUVEC cell monolayers permeability induced by HG treatment are shown in Fig 40 A, where a significant increase of permeability, about 35-40% respect to the untreated cells (CTRL), can be observed at 2 h of treatment. The permeability of the monolayer decreases with time until it reaches the level of the CTRL at 18 h of incubation with HG. Fig 40 B shows the change in endothelial permeability induced by HG treatment in HUVEC cells pretreated with the tested phenolic compounds and their metabolites for 2 h, where FITC-Dextran basal permeability increases about 40% respect to the normal glucose sample (CTRL). Phenolic compounds counteracted this rise, exerting a protective action on the endothelial monolayer, which was almost equally for all tested compounds ($p > 0,05$).



A)



B)

Fig 40. A) FITC-Dextran basal permeability measured in HUVEC cells not treated (CTRL) or treated with HG 30 mM at 2 h, 4 h, 6 h and 18 h. Data are reported as percentage compared to CTRL for each time. CTRL is the sample with low glucose medium (5 mM). * = p< 0,05; *** = p < 0.001 HG vs CTRL; (n=3). **B**) FITC-Dextran basal permeability measured in HUVEC cells not treated (CTRL) and pretreated with Tyr, HT, Tyr sulf, HT sulf, Tyr gluc, HT gluc (1µM) and treated with HG 30 mM for 2 h. Data are reported as percentage compared to CTRL for each sample. CTRL samples come from cells treated with low glucose medium (5 mM). °°° = p< 0,001 HG vs CTRL; ** = p< 0,01; *** = p< 0,001 Compounds vs HG; p> 0,05 Compound vs Compound (n= 3).

4.3.2 Determination of intracellular ROS level in HUVEC cell monolayers treated with HG and pretreated with Tyr, HT and their sulphated and glucuronidated metabolites.

In order to evaluate the effect of HG-treatment and phenolic compounds and their sulphated and glucuronidated metabolites on cellular redox status, we measured ROS generation in HUVEC cells, treated with HG (30 mM) and pretreated with phenolic compounds and their metabolites (1 μM) with time, taking readings at intervals of 5 min for 120 min, using 2',7'-DCFH-DA assay.

Treatment with HG resulted in a significant increase of ROS production starting from 30 min of incubation (about 40%) and proceeding with a time-dependent increase (50% at 1 h of incubation and 60% at 2 h of incubation) in comparison with the CTRL, as shown in Fig 41. Pretreatment with the phenolic compounds and their sulphated and glucuronidated metabolites counteracted HG-induced alteration of cellular redox status, inhibiting ROS production and notably, all compounds prevented ROS formation in a similar way ($p > 0,05$).

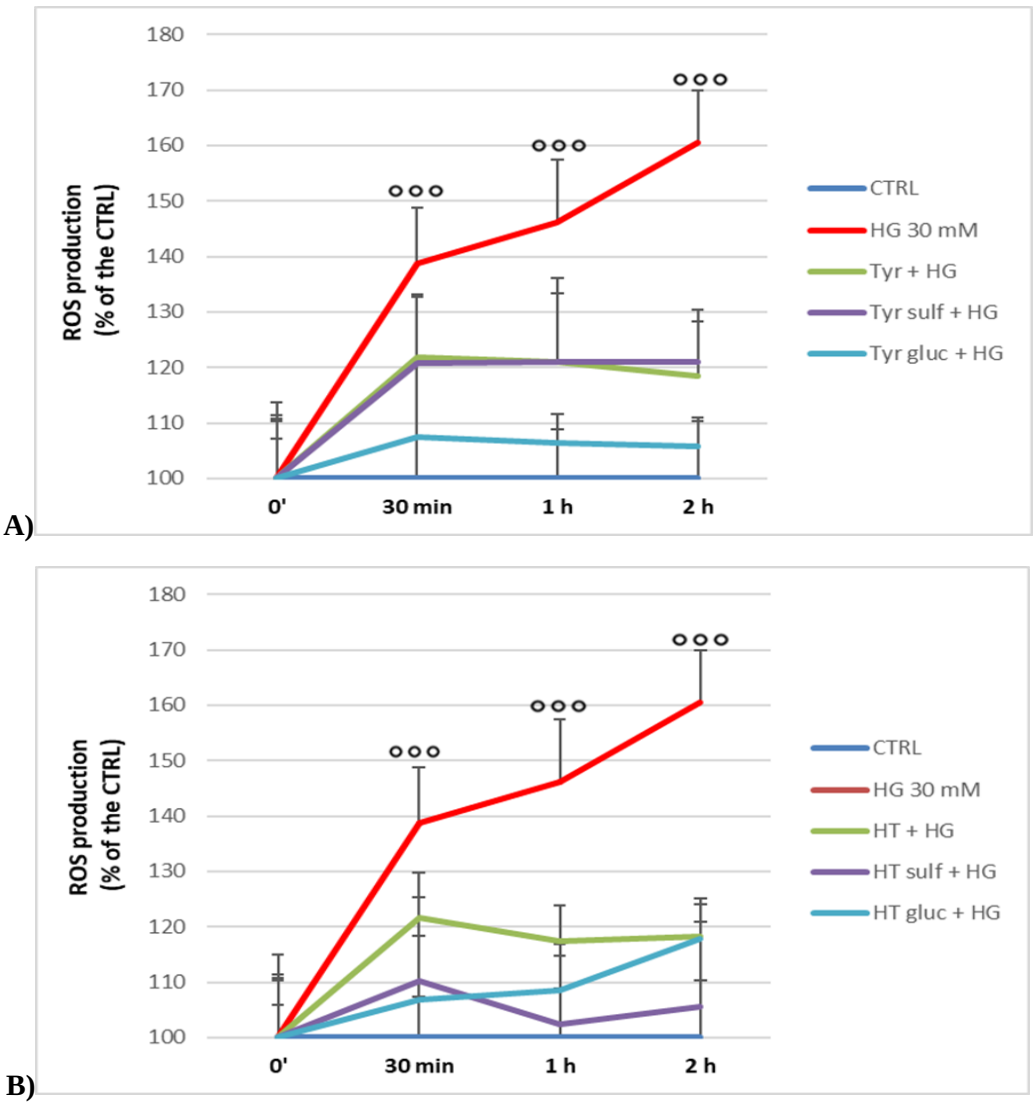


Fig 41. ROS generation in HUVEC cell monolayers treated with HG 30 mM and pretreated with Tyr and its metabolites (A) and HT and its metabolites (B) (1 µM), and HUVEC cells without any treatment (CTRL) with time. Values are shown as percentage of ROS production increase respect to the CTRL. CTRL samples come from cells treated with low glucose medium (5 mM). °°° = p< 0,001 HG vs CTRL; p < 0,001 for all Compounds vs HG; p> 0,05 Compound vs Compound (n= 6).

4.3.3 Protective effect exerted by Tyr, HT and their sulphated and glucuronidated metabolites against alteration of occludin, ZO-1 and JAM-A levels in HUVEC cell monolayers treated with HG

We investigated HG-induced alteration in HUVEC cell monolayers, focusing on the modulation of TJ proteins occludin, ZO-1 and JAM-A with time, through Western blotting. Cells were treated with HG (30 mM), for 1, 2, 3, 4, 6, 18 and 24 h, and the protective effect of phenolic compounds was determined pretreating cells with Tyr, Tyr-sulf, Tyr-gluc, HT, HT-sulf and HT-gluc and incubating cultures with HG (30 mM) for 3 h, time that induced the higest TJ decrease.

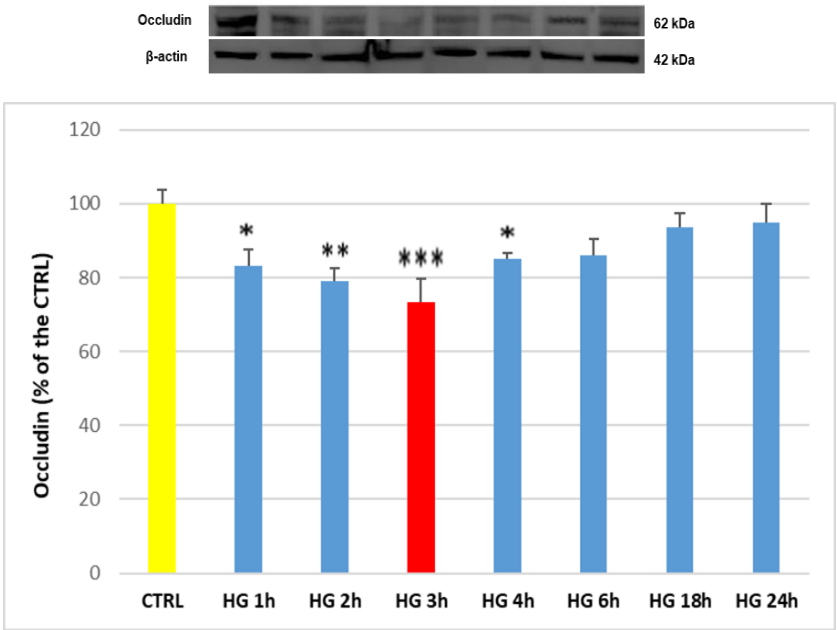


Fig 42. Occludin/β-actin ratio measured in HUVEC cells not treated (CTRL) or treated with HG 30 mM at different incubation times. Data are reported as percentage compared to CTRL for each time. CTRL samples come from cells treated with low glucose medium (5 mM). * = p< 0,05; ** = p< 0,01; *** = p < 0,001 HG vs CTRL; (n=3).

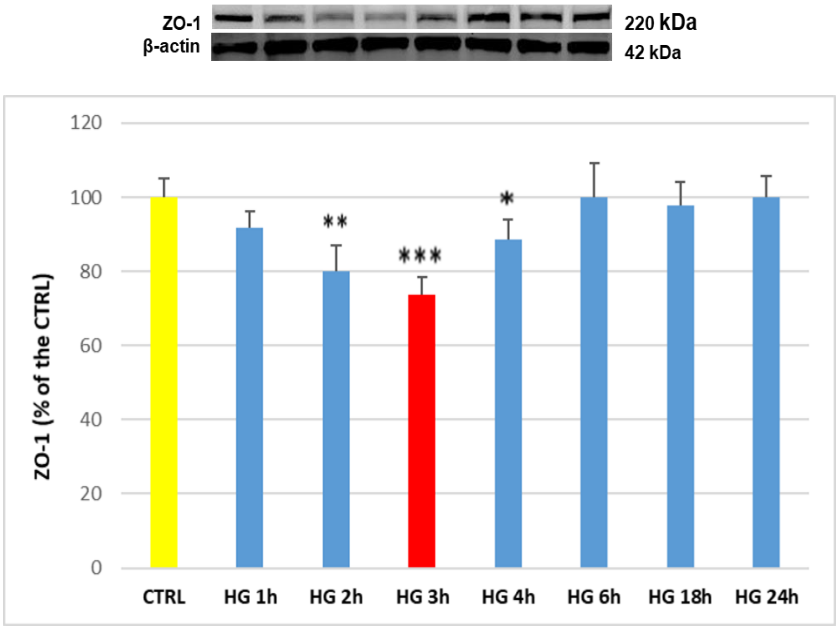


Fig 43. ZO-1/β-actin ratio measured in HUVEC cells not treated (CTRL) or treated with HG 30 mM at different incubation times. Data are reported as percentage compared to CTRL for each time. CTRL samples come from cells treated with low glucose medium (5 mM). * = p< 0,05; ** = p < 0,01; *** = p< 0,001 HG vs CTRL (n=3).

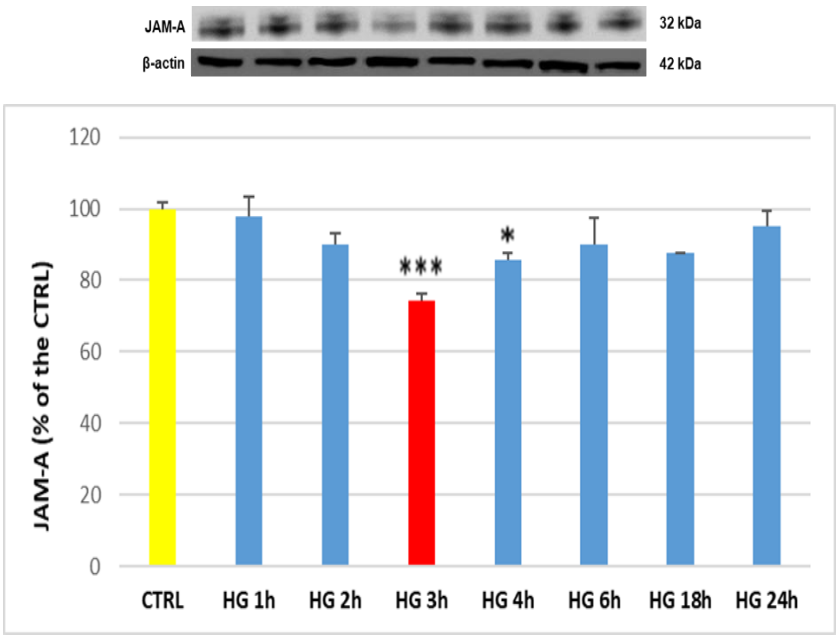


Fig 44. JAM-A/β-actin ratio measured in HUVEC cells not treated (CTRL) or treated with HG 30 mM at different incubation times. Data are reported as percentage compared to CTRL for each time. CTRL samples come from cells treated with low glucose medium (5 mM). * = p< 0,05; *** = p < 0,001 HG vs CTRL (n=3).

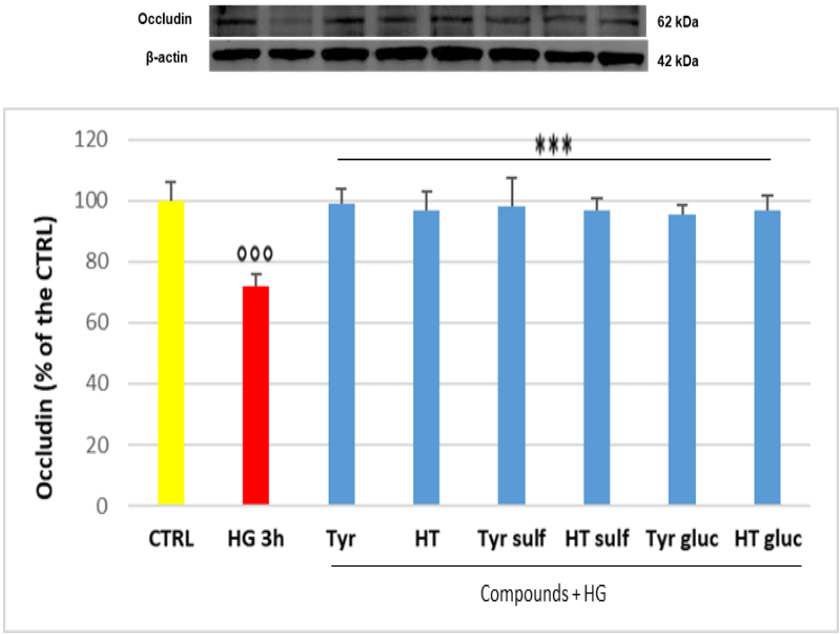


Fig 45. Occludin/β-actin ratio measured in HUVEC cells not treated (CTRL), pretreated with Tyr, HT, Tyr sulf, HT sulf, Tyr gluc, HT gluc (1µM) and treated with HG (30 mM) for 3h. Data are reported as percentage compared to CTRL for each sample. CTRL samples come from cells treated with low glucose medium (5 mM). °°° = p< 0,001 HG vs CTRL; *** = p<0,001 Compounds vs HG; p> 0,05 Compound vs Compound (n= 3).

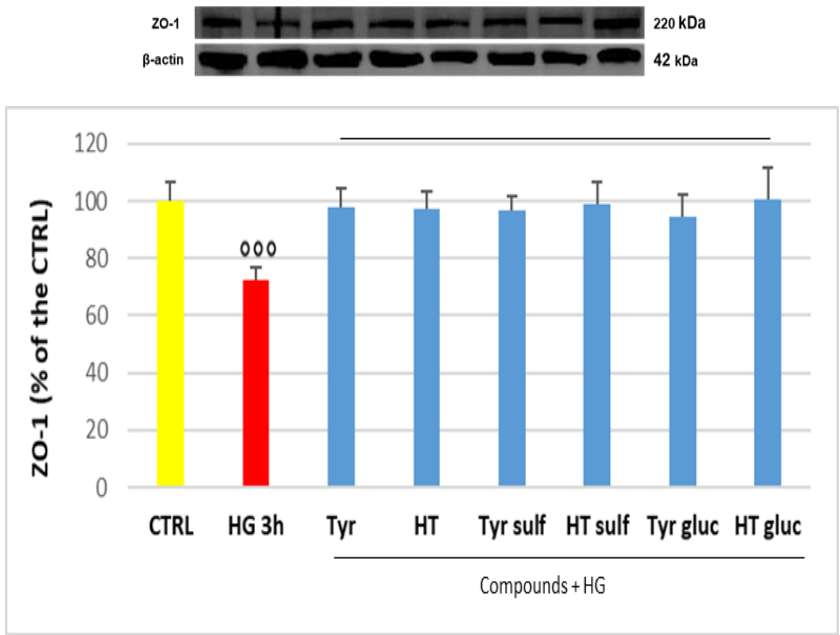


Fig 46. ZO-1/β-actin ratio measured in HUVEC cells not treated (CTRL), pretreated with Tyr, HT, Tyr sulf, HT sulf, Tyr gluc, HT gluc (1µM) and treated with HG (30 mM) for 3h. Data are reported as percentage compared to CTRL for each sample. CTRL is the sample with low glucose medium (5 mM). °°° = p< 0,001 HG vs CTRL; *** = p<0,001 Compounds vs HG; p> 0,05 Compound vs Compound (n= 3).

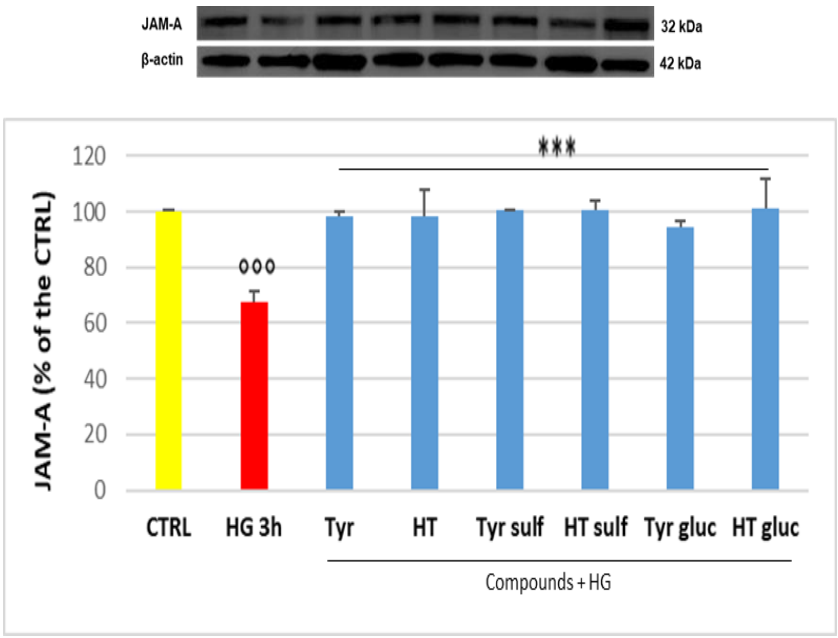


Fig 47. JAM-A/β-actin ratio measured in HUVEC cells not treated (CTRL), pretreated with Tyr, HT, Tyr sulf, HT sulf, Tyr gluc, HT gluc (1µM) and treated with HG (30 mM) for 3h. Data are reported as percentage compared to CTRL for each sample. CTRL samples come from cells treated with low glucose medium (5 mM). °°° = p< 0,001 HG vs CTRL; *** = p<0,001 Compounds vs HG; p> 0,05 Compound vs Compound (n= 3).

As shown in Fig 42, 43 and 44, HG induced a significant decrease of occludin, ZO-1 and JAM-A level in HUVEC, with a percentage of about 25-30% reduction with respect to the CTRL (100%) at 3 h, confirming the role of HG in endothelial barrier alteration. From 6 h to 24 h the disrupting effect of HG on all three TJ was not significant, as we detected levels similar to CTRL.

Fig 45, 46 and 47 show the protective effect against HG-induced decrease in occludin, JAM-A and ZO-1 levels, exerted by the pre-treatment of HUVEC with the phenolic compounds and their sulphated and glucuronidated metabolites before incubation with HG for 3 h. HG led to a decrease of about 25-30% of TJ proteins level compared to CTRL (100%). Pretreatment with Tyr, HT and their sulphated and glucuronidated metabolites were capable to limit the decrease of occludin, JAM-A and ZO-1 levels, caused by HG, in a significant manner, similar for all three TJ proteins.

4.3.4 Modulation of p38 and ERK 1/2 exerted by phenolic compounds in HUVEC cell monolayers treated with HG

It was evaluated the capacity of HG to activate MAPK signalling pathways in HUVEC cell monolayers, through Western blotting, investigating on the modulation of phosphorylation state of p38 and ERK1/2 with time. Cells were treated with HG (30 mM), for 1, 2, 3, 4 and 6 h and the protective effect of the phenolic compounds and their metabolites was evaluated in HUVEC pretreated with HT, Tyr and their glucuronidated and sulphated metabolites (1 μM) and treated with HG (30 mM) for 3 h.

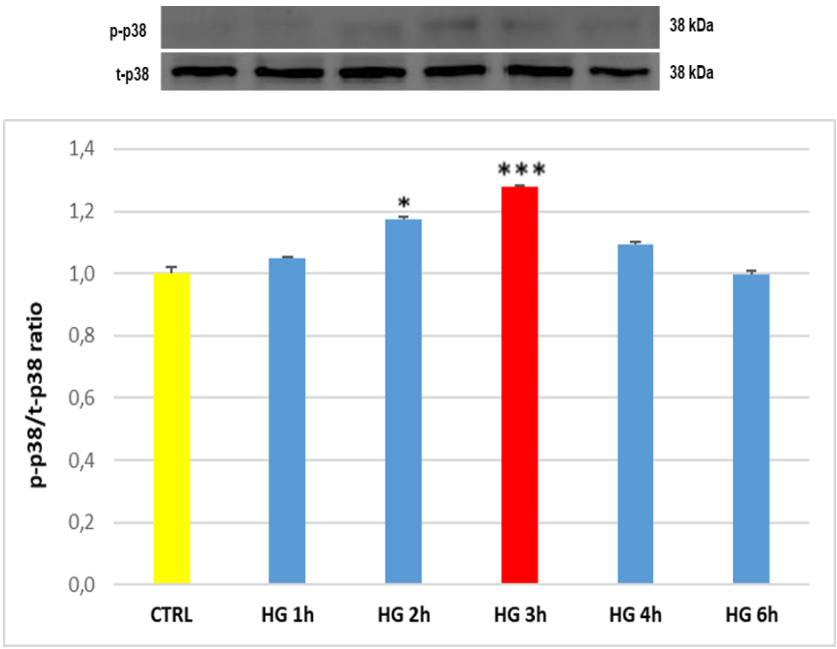


Fig 48. p-p38/t-p38 ratio measured in HUVEC cells not treated (CTRL) or treated with HG 30 mM at different incubation times. CTRL samples come cells treated with low glucose medium (5 mM). * = p< 0,05 HG vs CTRL; *** = p< 0,001 HG vs CTRL (n=3).

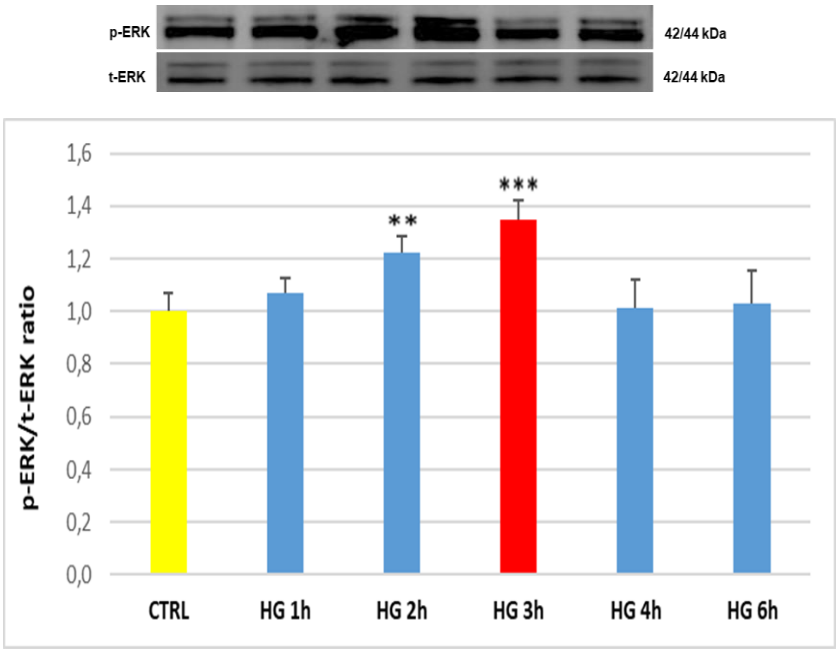


Fig 49. p-ERK1/2/t-ERK1/2 ratio measured in HUVEC cells not treated (CTRL) or treated with HG 30 mM at different incubation times. CTRL samples come from cells treated with low glucose medium (5 mM). ** = p< 0,01 HG vs CTRL; *** = p< 0,001 HG vs CTRL (n=3).

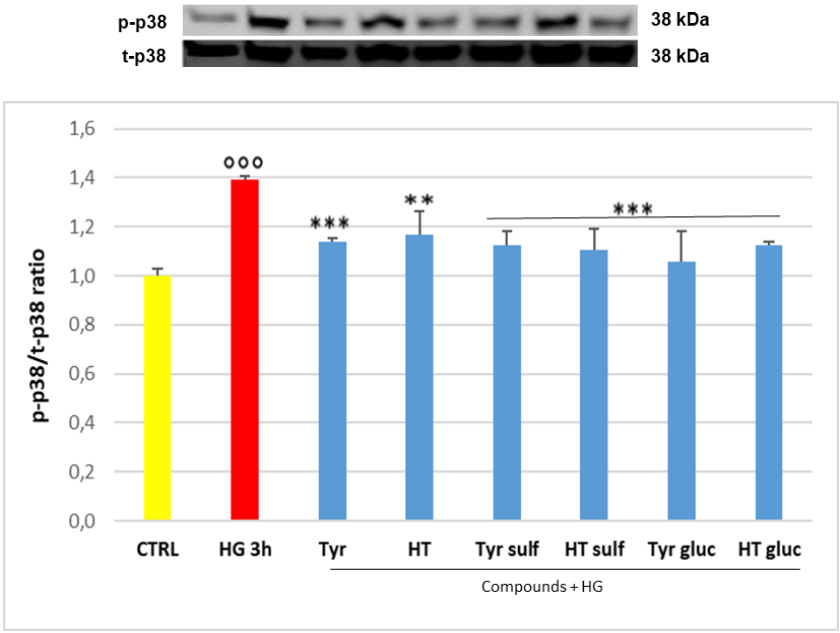


Fig 50. p-p38/t-p38 ratio measured in HUVEC cells not treated (CTRL), pretreated with Tyr, HT, Tyr sulf, HT sulf, Tyr gluc, HT gluc (1 µM) and treated with HG (30 mM) for 3 h. CTRL samples come from cells treated with low glucose medium (5 mM). °°° = p< 0,001 HG vs CTRL; ** = p< 0,01; *** = p< 0,001 Compounds vs HG; p> 0,05 Compound vs Compound (n= 3).

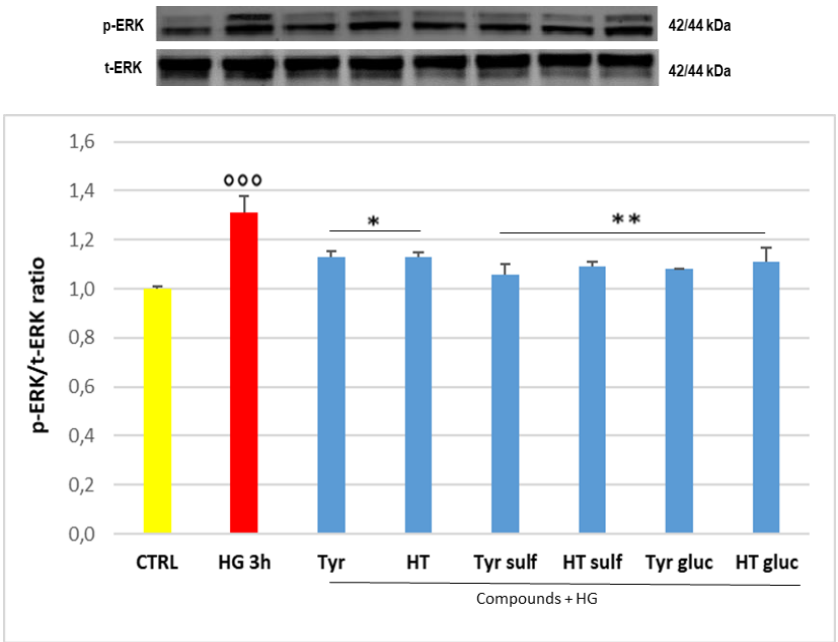


Fig 51. p-ERK1/2/t-ERK1/2 ratio measured in HUVEC cells not treated (CTRL), pretreated with Tyr, HT, Tyr sulf, HT sulf, Tyr gluc, HT gluc (1µM) and treated with HG (30 mM) for 3 h. CTRL samples come from cells treated with low glucose medium (5 mM). °°° = p< 0,001 HG vs CTRL; * = p< 0.05; ** = p< 0,01 Compounds vs HG; p> 0,05 Compound vs Compound (n= 3).

Fig 48 and 49 show the phosphorylation rate of p38 and ERK1/2 detected in HUVEC cells treated with HG, at 1-6 h of incubation. After 2-3 h of incubation HG induced a significant phosphorylation of both proteins respect to the CTRL, where the values reached their maximum at 3h, with about 30% for p-p38 and about 35% for p-ERK1/2. At 6 h the level of both MAPK was similar to the CTRL.

Fig 50 and 51 show the phosphorylation of p-38 and ERK1/2 induced by HG and the effect exerted by phenolic compounds and their metabolites on HUVEC cells. HG was able to significantly enhance the levels of p-p38 and p-ERK1/2 compared to CTRL after 3 h of incubation (as previously observed), when the ratio p-p38/t-p38 was about 30% higher in HG sample and p-ERK1/2/t-ERK1/2 ratio was about 40% higher in HG sample than CTRL (100%). Pretreatment with compounds and metabolites limited in a significant manner the phosphorylation state respect to the cells treated with only HG. The action of compounds and metabolites was similar in the inhibition of the analized MAPK ($p > 0,05$).

4.3.5 Protective effect exerted by Tyr, HT and their sulphated and glucuronidated metabolites against activation of NLRP3 inflammasome in HUVEC cell monolayers treated with HG

The protein levels of NLRP3 inflammasome were investigated on HUVEC cell monolayers treated with HG with time, through Western blotting. Cells were treated with HG (30 mM), for 1, 2, 3, 4, 6, 18 and 24 h. Protective action of Tyr, HT and their metabolites to modulate HG induced increase of NLRP3 protein levels, was evaluated in HUVEC cells pretreated with the phenolic compounds (1 µM) and treated with HG (30 mM) for 3 h.

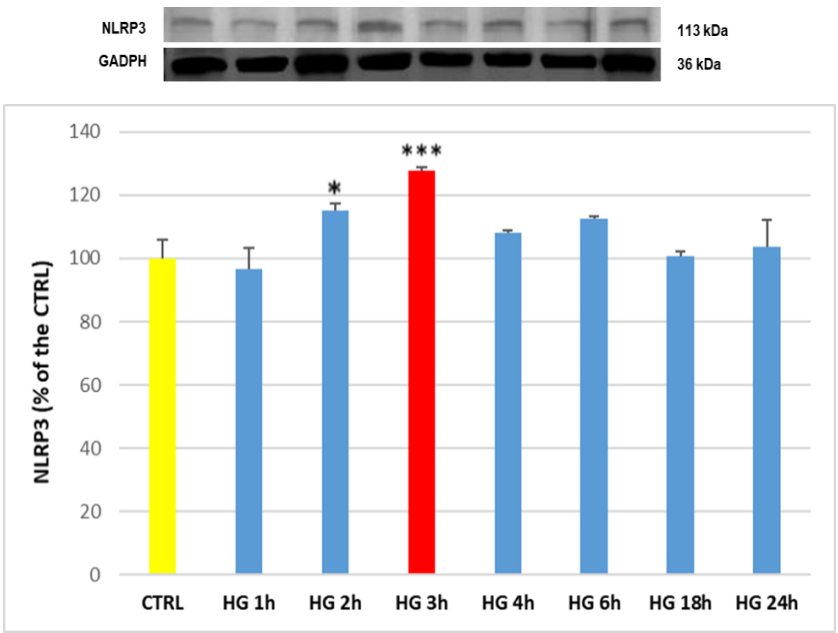


Fig 52. NLRP3/GADPH ratio measured in HUVEC cells not treated (CTRL) or treated with HG 30 mM at different incubation times. Data are reported as percentage compared to CTRL for each time. CTRL samples come from cells treated with low glucose medium (5 mM). * = p< 0,05; *** = p < 0,001 CTRL vs HG (n=3).

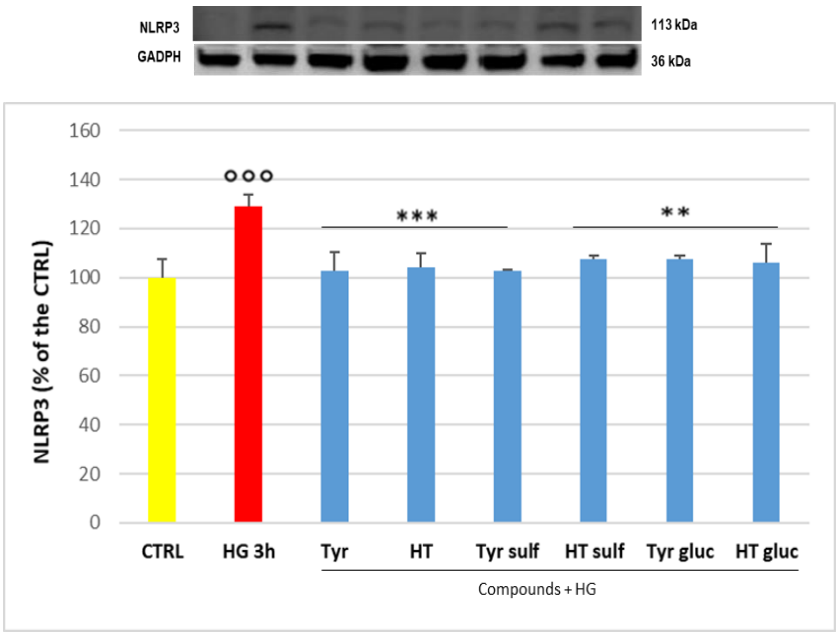


Fig 53. NLRP3/GADPH ratio measured in HUVEC cells not treated (CTRL) and pretreated with Tyr, HT, Tyr sulf, HT sulf, Tyr gluc, HT gluc (1 µM) and treated with HG (30 mM) for 3 h. CTRL samples come from cells treated with low glucose medium (5 mM). °°° = p< 0,001 HG vs CTRL; ** = p< 0,01; *** = p< 0,001 Compounds vs HG; p> 0,05 Compound vs Compound (n= 3).

In Fig 52 is shown the result of the treatment of HUVEC with HG, that determined a significant increase of NLRP3 protein levels of about 25-30% compared to CTRL (100%), and the higher value occurred at 3 h of incubation with HG. After 3 h, the level of NLRP3 decrease and at 24 h showed no significant differences with CTRL values.

Fig 53 reports the percentage of NLRP3 protein (about 30%) with respect to the CTRL, and protective effect of Tyr, HT and their sulphated and glucuronidated metabolites in HUVEC cells treated with HG for 3 h. Pretreatment with all phenolic compounds tested reduced the levels of NLRP3 protein to the extent of 22-30% compared to HG group. This protective effect was observed almost equally for all tested phenolic compounds, with no significant differences (p> 0,05).

4.3.6 Modulation of NF-κB Inhibitor α (NF-IκBα), IL-1β and IL-6 gene expression by Tyr, HT and their sulphated and glucuronidated metabolites in HUVEC cell monolayers treated with HG

In order to further evaluate the role of HG and phenolic compounds and their metabolites on the modulation of inflammatory response, we evaluated the activation of NF-κB, connected to MAPK and NLRP3 inflammasome signaling pathways, measured as degradation of its inhibitor NF-IκBα. Activation of NLRP3, MAPK and NF-κB leads to proinflammatory cytokine release, thus HG treatment in HUVEC cells may enhance the production of such inflammatory mediators. For this reason, we determined gene expression of NF-IκBα and the gene expression of IL-1β and IL-6 (products of NLRP3 and NF-κB respectively) by Real-Time PCR in HUVEC pretreated with HT, Tyr and their glucuronidated and sulphated metabolites (1 µM) and treated with HG (30 mM), at 2 h and 6 h, in order to evaluate their protective action.

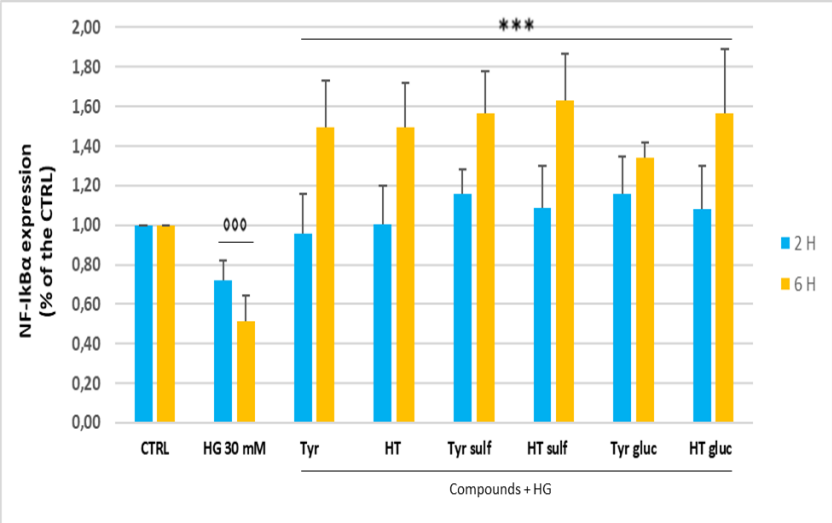


Fig 54. NF-IκBα expression measured in HUVEC cells not treated (CTRL), pretreated with Tyr, HT, Tyr sulf, HT sulf, Tyr gluc, HT gluc (1µM) and treated with HG (30 mM) for 2 h and 6h. Data are reported as percentage compared to CTRL for each sample. CTRL samples come from cells treated with low glucose medium (5 mM). °°° = p< 0,001 HG vs CTRL; *** = p<0,001 Compounds vs HG; p> 0,05 Compound vs Compound (n= 9).

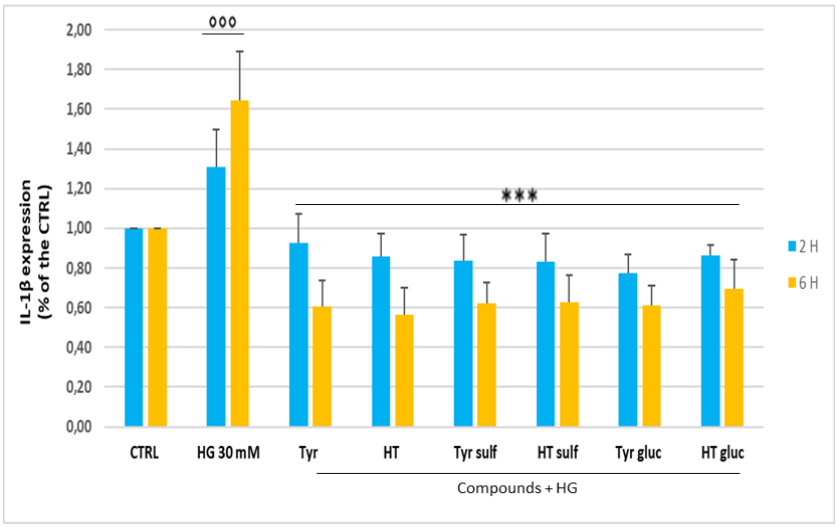


Fig 55. IL-1β expression measured in HUVEC cells not treated (CTRL), pretreated with Tyr, HT, Tyr sulf, HT sulf, Tyr gluc, HT gluc (1 µM) and treated with HG (30 mM) for 2 h and 6h. Data are reported as percentage compared to CTRL for each sample. CTRL samples come from cells treated with low glucose medium (5 mM). °°° = p< 0,001 HG vs CTRL; *** = p<0,001 Compounds vs HG; p> 0,05 Compound vs Compound (n= 9).

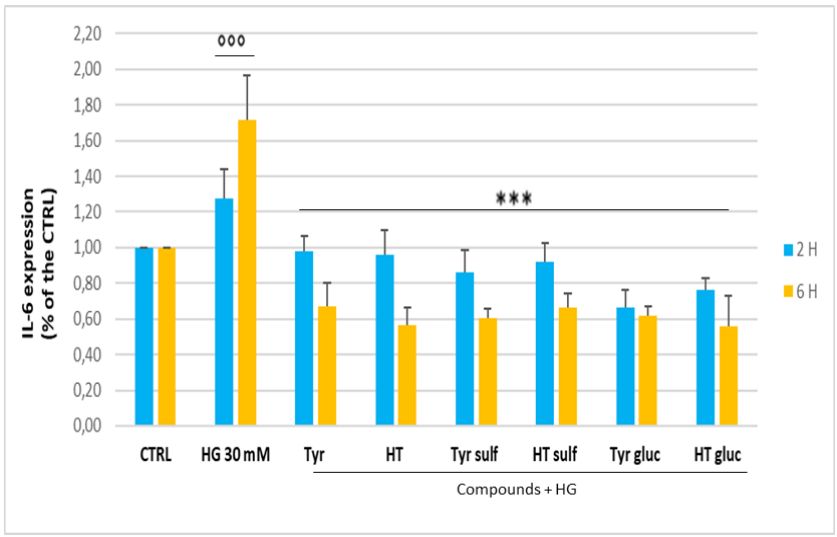


Fig 56. IL-6 expression measured in HUVEC cells not treated (CTRL), pretreated with Tyr, HT, Tyr sulf, HT sulf, Tyr gluc, HT gluc (1 µM) and treated with HG (30 mM) for 2 h and 6 h. Data are reported as percentage compared to CTRL for each sample. CTRL samples come from cells treated with low glucose medium (5 mM). °°° = p< 0,001 HG vs CTRL; *** = p<0,001 Compounds vs HG; p> 0,05 Compound vs Compound (n= 9).

Modulation of NF-IκBα gene expression by Tyr, HT and their sulphated and glucuronidated metabolites in HG-induced inflammation in HUVEC cells, is reported in Fig 54, as percentage of NF-IκBα respect to the CTRL. As shown in the figure, HG treatment resulted in a significant decrease of NF-IκBα expression both at 2 h (about 30%) and 6 h (about 50%) of incubation, in comparison with the CTRL, thus inducing the activation of NF-κB signalling pathway. Pretreatment with phenolic compounds and their metabolites led to a significant increase of NF-IκBα expression, particularly evident at 6 h, suggesting their role in HUVEC protection from HG exposure. All compounds promoted NF-IκBα gene expression, in a similar way.

Fig 55 and 56 show the modulation of IL-1β and IL-6 gene expression by Tyr, HT and their sulphated and glucuronidated metabolites in HG-induced inflammatory reaction in HUVEC cells. Treatment with HG determined a significant increase of both cytokines' expression at 2 h and 6 h (from about 30% to 70% at 6 h of incubation), in comparison with the CTRL values. Pretreatment with the phenolic compounds and their metabolites led to a significant containment of IL-1β and IL-6 production, exerting a protective action. The greatest effect was at 6 h. For IL-6, only glucuronidated metabolites were more active from 2 h than the other compounds. However, all compounds limited the expression of both citokines, in a similar manner, with no significant differences ($p > 0,05$).

5 Discussion

Intestinal epithelial cells and EC are key components of the barriers that contribute to the maintenance of organism homeostasis, playing a critical role in the body's defense functions, against microorganisms, potentially toxic compounds, and pathologic stimuli **(Chang J et al, 2017; Jeon D et al, 2017)**. Epithelial cells and EC are directly adjacent, indicating active inter-barrier communication and are associated with each other via intercellular TJ protein complexes **(Remenyik J et al, 2022)**, including transmembrane proteins, such as occludin and JAM, and cytosolic scaffold proteins, such as ZO-1, which anchor the transmembrane proteins to the perijunctional actomyosin ring**,** that connect the cytoskeleton and signalling molecules **(Cao SG et al, 2018; Komarova YA et al, 2017)**.

It is widely accepted that a defective intestinal epithelial barrier and abnormalities of EC structure and function, are initiating factors of intestinal and vascular disorders, resulting in an increased permeability that plays a critical role in the pathogenesis of intestinal diseases, such as IBD **(Schoultz I et al, 2019)** and of atherosclerosis, and thus CVD **(Rajendran P et al, 2013)**.

Several pro-inflammatory stimuli have been proven to directly affect the intestinal epithelial **(Cui W et al, 2010)** and endothelial barrier **(Remenyik J et al, 2022)** by reducing the localization and expression of TJ proteins. Among these, LPS constitutes a common proinflammatory agent and for this reason it is widely used in experimental studies about intestinal **(He S et al, 2020; Lan H et al, 2021)** and endothelial barriers **(Bian Y et al, 2019; Zhang X et al, 2013; Chen W et al, 2020)**. Stimulation with LPS leads to the activation of signaling pathways involved in the production and/or release of proinflammatory mediators **(Cao SG et al, 2018; Wang X et al, 2019; Ma MM et al, 2015)** at intestinal **(Feng Y et al, 2019)** and vascular level **(Goldberg EL and Dixit VD, 2015)**.

Among the main risk factors involved in the onset and progression of vascular complication there is hyperglycemia **(Rezabakhsh A et al, 2017)**, whose effect is most likely

due to the accelerated formation of ROS, RNS and AGE, which lead to hemostasis disorders and/or cellular biochemical abnormalities **(Popov D, 2010)**.

On the other hand, barrier function may be enhanced with the dietary intake of compounds with antioxidant and anti-inflammatory properties. The MD is one of the richest in bioactive compounds, to which its numerous health beneficial effects against degenerative diseases are due. Bioactive compounds are mostly present in fruits, vegetables and EVOO, the symbol of the MD, which are particularly rich in polyphenols, the most relevant of these bioactive compounds **(Ditano-Vázquez P et al, 2019)**, that have been proven to exert a significant protective action against chronic diseases by modulating numerous patho-physiological processes **(Luca SV et al, 2019)**.

Dietary polyphenols, however, are poorly absorbed and/or extensively metabolized within enterocytes and liver by phase I and phase II enzymatic reactions **(Zeka K et al, 2017)**. In addition, they undergo intensive biotransformation by gut microbiota to a wide variety of new chemical structures than can pass easily into the systemic blood circulation **(Williamson G and Clifford MN, 2017)**. It is considered that less than 5% of the total polyphenolic intake is absorbed and reaches the plasma unchanged **(Cao H et al, 2015)**, therefore the parent compounds reach very low plasmatic levels that are unlikely to supply efficient cellular concentrations to justify the overall efficacy **(Chiou YS et al, 2014)**. Thus, in the last decade, an increasing interest has been paid to the metabolism of dietary polyphenols and bioactivity of their metabolites in respect to the parent compounds **(Costa V et al, 2022)**. HT and Tyr are the main bioactive phenolic compounds present in EVOO and have been shown to possess scavenging properties. They are also able to exert an indirect antioxidant action by modulating intracellular signaling pathways and improving cellular response to oxidative stress and pro-inflammatory stimuli **(Serreli G and Deiana M, 2018)**. After ingestion, both HT and Tyr are biotransformed in the gut and liver to produce maily glucuronidated and sulphated metabolites,

that have been demonstrated to maintain the beneficial effects of their parent compounds **(Serreli G et al, 2019)**.

In this context, the present study aimed at evaluating the protective effect of HT, Tyr and their sulphated and glucuronidated metabolites, on the inflammatory response both at intestinal and endothelial level, induced by pro-inflammatory stimuli, such as LPS and/or a HG conditions. Their modulatory action was investigated in relation to TJ alteration and activation of cellular pathways, which are linked to chronic inflammatory diseases. The compounds of interest were tested in two cell models at the concentration of 1 µM, considered to be biologically relevant after ingestion of foods containing these phenols **(Serreli G et al, 2019; Jenner AM et al, 2005; Manach C et al, 2004; Pastor A et al, 2016)**.

5.1 LPS-induced alteration of Caco-2 cell monolayer permeability and protective effect exerted by Tyr, HT and their sulphated and glucuronidated metabolites

The first part of the research project was focused on the investigation of the protective effect exerted by Tyr, HT and their sulphated and glucuronidated metabolites, on LPS-induced alteration of Caco-2 monolayer permeability. Among commercially available cell lines, Caco-2 cells have been widely used to study absorption, metabolism, and bioavailability of drugs and xenobiotics **(Smith MC et al, 2018; Tran VN et al, 2020; Serreli G et al, 2021 B)**. They possess the ability to spontaneously differentiate into a monolayer (approximately 14-21 days post-seeding with well-defined TJ) expressing many properties typical of absorptive enterocytes with a brush border layer, TJ and efflux and uptake transporters as found in the small intestine **(Iftikhar M et al, 2020**).

In order to induce an alteration of monolayer permeability, Caco-2 cells were treated with LPS, the major cell wall component of Gram-negative bacteria, a pro-inflammatory stimulus abundant in the gut lumen, reported to increase intestinal permeability and cause inflammatory reaction **(Tang X et al, 2018 B; Serreli G et al, 2020)**.

Variation of monolayer permeability was assessed through a well established experimental procedure, the measurement of the TEER **(Srinivasan B et al, 2015)**. Cells, pretreated with Tyr, HT, and their sulphated and glucuronidated metabolites, were exposed to LPS at the concentration of 10 µg/mL, a pathological concentration able to cause intestinal inflammatory response, but not cell death, in agreement with what was reported by **He S et al (2020)**. As previously shown **(Serreli G et al, 2020)**, in our experimental conditions LPS treatment caused a temporary alteration of the monolayer permeability, which returned to the initial integrity after 24 h of incubation. Derangement of differentiated Caco-2 cell monolayers was due to a decrease in the level of TJ proteins primarily involved in the regulation of paracellular permeability, ZO-1, occludin and JAM-A **(Oshima T and Miwa H et al, 2016)**, as shown by Western blot analyses. It has been reported that LPS triggers an inflammatory signaling cascade to reduce TJ proteins expression, leading to increased intestinal permeability and disrupting intestinal epithelial barrier function **(Bian Y et al, 2019; Tunisi L et al, 2019; Lan H et al, 2021)**.

The protection exerted against the functional impairment of the monolayer was due to the compounds ability to preserve TJ level. Sulphates and glucuronides exerted a protection comparable to that of the parent compounds without significant differences among them. Similar efficacy in protecting against monolayer alteration was highlighted for the phenolic fraction derived from a particular Sardinian cultivar of olives, found to restore the loss of the epithelial integrity and repair membrane oxidative damage in enterocyte-like cells **(Serreli G et al, 2017)**.

TJ disruption by LPS has been associated to its interaction with MAPK signaling. In particular, the maintenance of the TJ proteins to the junctional complex has been mainly correlated to the inhibition of MAPK and NF-κB pathways **(Paradis T et al, 2021)**.

Previous studies, conducted by **Serreli G et al (2019)** on Caco-2, confirmed that LPS caused downstream cascade reactions, including the activation of MAPK and NF-κB pathways. In particular, LPS induces an increase in the phosphorylation state of the MAPK p38 and ERK, together with IκB-α phosphorylation and degradation, leading to the activation of NF-κB. The turning on of these pathways leads to the production of proinflammatory mediators, including IL-1β and IL-8, which promote inflammation and increase the expression and activity of myosin light chain kinase, a crucial molecule that regulates the interaction of TJ with the cytoskeleton, thus disrupting intestinal barrier integrity **(Serra G et al, 2018; Cao SG et al, 2018; Wang X et al, 2019; Ma MM et al, 2015)**.

Thus, investigating the mechanism of the tested compounds protective action, we evaluated their ability to modulate p38 and ERK activation in our experimental conditions. HT, Tyr and their metabolites were able to inhibit p38 and ERK phosphorylation, induced by LPS treatment, in agreement with what observed by our group in a previous study where the same compounds modulated LPS-induced MAPK activation **(Serreli G et al, 2019)**. Our results are also in line with what reported by **Muto E et al (2015)**, who showed the ability of an EVOO phenolic extract to inhibit LPS-induced IL-8 secretion, by interfering with NF-κB signaling and p38 pathway in Caco-2 cells and by **Aggarwal S et al (2011)** that proved the involvement of ERK on TJ integrity in Caco-2 cell monolayers. Actually, the TLR4/MAPK/NF-κB pathway, seems to be the major mechanism involved in the LPS-induced secretion of pro-inflammatory cytokines, alteration of intestinal TJ and thus increase of permeability **(Chen M et al, 2019; Nighot M et al, 2017)**.

However, it has been reported in the literature that LPS interaction with TLR4 can also promote an over-activation of NLRP3 inflammasome, involved in the abnormal expressions of TJ proteins in inflammatory gut disorders **(Feng Y et al, 2019; Zhuang Y et al, 2015; Lei-Leston AC et al, 2017)**. A growing body of evidence highlights the relevance of the citosolic

multiprotein complex NLRP3 inflammasome (constitute from NLRP3, ASC and pro-caspase-1) in regulating the integrity of intestinal homeostasis and in shaping innate immune responses during bowel inflammation **(Elinav E et al, 2013)**. Once being activated, NLRP3 recruits and cleaves pro-caspase-1 into its active forms, in turn processing pro-IL-1β and pro-IL-18 into their mature forms**,** contributing to the amplification of the inflammatory response **(Schroder K and Tschopp J, 2010)**. In our experimental conditions LPS treatment induced a significant expression of the complex with time, as determined by Western blot detection of an increased level of NLRP3 protein, that was significantly inhibited by pretreatment with the phenolic compounds. EVOO phenols have been reported to modulate TLR4/NLRP3 pathway, thus exerting an anti-inflammatory effect **(Taticchi A et al, 2019)**. **Miao F (2022)** showed that HT supplementation exerts anti-inflammatory effects in DSS-induced ulcerative colitis by enhancing colonic antioxidant capacities, inhibiting NLRP3 inflammasome activation, and modulating gut microbiota *in vivo*. **Montoya T et al (2018)** investigated on the anti-inflammatory effects of a new derivative of HT, peracetylated hydroxytyrosol (Per-HT), compared with HT, on LPS-stimulated murine macrophages, highlighting the involvement of the NLRP3 signaling pathway.

5.2 LPS and HG-induced alteration of HUVEC cell monolayer permeability and protective effect exerted by Tyr, HT and their sulphated and glucuronidated metabolites.

The second part of the project was focused on the investigation of the protective effect exerted by Tyr, HT and their sulphated and glucuronidated metabolites, on LPS-induced alteration of HUVEC monolayer permeability. HUVEC cultures simulate the vessel endothelium and are a good model to study *in vitro* the endothelium pathophysiology and vascular inflammation **(Jingyan L et al, 2017; Gao P et al, 2018)** but also for studying endothelial barrier function because of their defined TJ and AJ **(Wuest DM et al, 2013)**.

EC are the first responder to LPS because they line all blood vessels **(Dauphinee SM and Karsan A, 2006)**, and LPS is one of the most common factors that trigger inflammatory response in EC, as alteration of endothelial barrier permeability. Thus, LPS-induced injury to HUVEC is commonly used as an *in vitro* model for exploring the mechanism of EC injury **(Zeuke S et al, 2002; Jeon D et al, 2017)**. In our experimental conditions HUVEC were treated with LPS at 10 µg/mL, accordingly with **Lee YM et al (2003)**, who demonstrated that at this concetration LPS determines a pro-inflammatory response but does not reduce HUVEC viability.

To assess alteration of HUVEC monolayers permeability following LPS treatment we measured the leakage of FITC-Dextran through the monolayer, in accordance to**Wang L et al (2017)**. The loss of endothelial monolayer permeability was observed after 2 h of incubation, conferming the role of LPS in slacking the linkage between EC with consequent aggravation of the vascular endothelial tissue permeation **(Lee W et al, 2014)**.

LPS is reported to cause changes of the cytoskeleton structure and endothelial barrier function in HUVEC through the decrease of the expression of ZO-1 and occludin **(Flemming S et al, 2015; Zhang X et al, 2013; Chen X et al, 2019)**, that play active roles in regulating paracellular permeability of the endothelium **(Harhaj NS and Antonetti DA, 2004)**.

The loss of the monolayer integrity was prevented by all the tested compounds, that significantly reduced LPS-induced FITC-Dextran permeation. The protective action of the metabolites was comparable to that of the parent compounds and was due to their ability to inhibit TJ protein loss.

As already reported, LPS binds TLR4 and can activate several intracellular signaling pathways, such as the MAPK and NF-*κ*B, that participate in endothelial inflammation by modulating the expression of adhesion molecules and pro-inflammatory cytokines, leading to endothelial permeability alteration **(Unenkhuu B et al, 2021; Opal SM, 2007)**. Previous

studies in mouse showed that the degradation of IκBα increased NF-κB activity after stimulation with LPS and the activation of NF-κB inhibited the expression of TJ proteins **(Gerondakis S et al, 2006)**. Our data confirm the involvement of the MAPK pathway in LPS-induced endothelial TJ alteration in HUVEC cultures, where an increase of phosphorylation state of p38 and ERK1/2 was observed in LPS treated samples with respect to the controls. In support of our data, **Xia JL et al (2014)** demonstrated that p38 is responsible for LPS-induced F-actin rearrangement and increased endothelial monolayer permeability. All the compounds tested in our study were effective in counteracting p38 and ERK1/2 LPS-induced activation. These findings suggest that the tested phenolic compounds exhibit their protective effect on LPS-induced injury, through their ability to modulate NF-κB and MAPK signaling pathways, in agreement with other studies regarding EVOO phenolics and their capacity of down-regulating MAPK activity in EC **(Catalan U et al, 2018)**.

Emerging evidence indicates that NLRP3 inflammasome initiates inflammatory response also at vascular level **(Liu W et al, 2014; Abderrazak A et al, 2015)**, and it is known that LPS triggers the activation of TLR4/NLRP3 inflammasome pathway in HUVEC, enhacing the expression of its components and promoting the activation of the complex **(You L et al, 2021)**. In our experimental conditions LPS treatment determined a significant increase of NLRP3 complex level in HUVEC, that occurred mostly at 2 h and 3 h. The presence of Tyr, HT and their sulphated and glucuronidated metabolites inhibited NLRP3 activation.

The ability of the phenolic compounds to maintain endothelium integrity was also evaluated under hyperglicemic conditions, that represent one of the most likely cause of endothelial damage that may accelerate the development of vascular diseases **(Liu TS et al, 2014; Lee W et al, 2012**; **Busik JV et al, 2008)**.

HUVEC were treated with a glucose concentration of 30 mM, considered as the ideal concentration to analyze, in *in vitro* models, the effects of HG-induced dysfunction of the

vascular endothelium **(Leng B et al, 2019; Li B et al, 2017; Chang Y et al, 2014)**. In particular, **Chao AC et al (2016)** reported that HG 30 mM mediated the increase of vascular permeability in HUVEC without cause significant cytotoxicity.

Changes of the function of HUVEC monolayers induced by HG were evaluated through the measurement of the FITC-Dextran flux. As **Chao AC et al (2016)** reported, in our experimental system HG 30 mM determined an increase of basal endothelial permeability in HUVEC, which was reflected by the increased passage of FITC-Dextran to basal compartment. When cells were pretreated with the phenolic compounds before LPS treatment, monolayer permeability was significantly reduced. Tyr, HT and their metabolites exerted their protective action without any significant differences among them.

HG-mediated alteration of endothelial barrier is likely to be due to a direct effect of glucose in the alteration of the endothelial TJ. **Chao AC et al (2015)**, demonstrated that hyperglycemia increases the production of amyloid β-peptide, leading to decreased endothelial TJ, with an attenuated expression of ZO-1, claudin-5, occludin, and JAM-C in HUVEC, with consequent increase of the paracellular endothelial permeability. **Li B et al (2017)** reported HG-induced suppression of claudin-5 and claudin-11 protein expressions in human cardiac microvascular EC. Our results showed that HG triggered downregulation of TJ molecules, occludin, JAM-A and ZO-1, during time, thereby leading to increased EC permeability. TJ level was preserved by pretreatment with all the tested phenolic compounds.

The protective effect of Tyr, HT and their sulphated and glucuronidated metabolites was likely due in part to their scavenging abilities, as HG treatment led to a significant and time-dependent increase of ROS production in HUVEC, detected using the fluorescent probe 2',7'-DCFH-DA. Pretreatment with Tyr, HT and their metabolites counteracted HG-induced alteration of cellular redox status, inhibiting ROS production and notably all compounds prevented ROS formation in a similar way. Hyperglycemia has been well documented to

potentiate intracellular ROS generation, which is considered another contributor to the dysfunction of EC **(Lin F et al, 2020; Qian Y et al, 2010)**.

Alteration of cellular redox status activate intracellular signaling patways, as MAPK sensible to oxidative stress **(Plotnikov A et al., 2011)**. In our HUVEC cultures HG-induced alteration of cellular redox status led to the activation of p38 and ERK1/2, confirming the involvement of MAPK pathway in HG-induced endothelial monolayer alteration. The activation of p38 and ERK pathways by HG in HUVEC cells has also been reported by **Kim SW et al (2011)** and **Kim MH et al (2017)** as upstream event in the up-regulation of ICAM-1. MAPK may activate the transcription of pro-inflammatory genes, such as NF-κB, which induces adhesion molecules, such as VCAM-1 and ICAM-1, increasing the migration and adhesion of monocytic cells to EC, a key event in the inflammatory process (**Gloire G et al, 2006)**. Previous studies have shown that HG activates NF-*κ*B **(Lee W et al, 2012)**, thus we investigated its activation in our HG treated HUVEC cultures, measuring the expression of its inhibitor NF-IκBα. HG treatment resulted in a significant decrease of NF-IκBα gene expression.

When HUVEC were pretreated with HT, Tyr and their metabolites HG activation of the MAPK/NF-kB pathway was inhibited, confirming the ability of the phenolic compounds to interfere in the intracellular signaling.

HG has been shown to activate NLRP3 inflammasome in several cell types **(Wan Z et al, 2019)** as well as ROS production **(Gicquel T et al, 2015)**. In our experimental model, HG treatment induced an increase of NLRP3 protein level with time, which was inhibited by exposure to all tested phenolic compounds. HT, Tyr and their metabolites were also effective in inhibiting the expression of the proinflammatory cytokines IL-1β, product of NLRP3 activation **(Pavillard LE et al, 2018)** and IL-6, product of NF-kB activation **(Liang H et al, 2018)**, overexpressed in HG treated HUVEC.

Thus, HG-induced inflammatory responses and EC dysfunction may be ameliorated by the suppression of the NF-κB and NLRP3 inflammasome pathways, as also reported by previous studies **(Bruder-Nascimento T et al, 2016; Wang W et al, 2017; Zhu X et al, 2018)**.

5.3 General Discussion and Conclusions

The maintenance of the intestinal epithelial and endothelial barrier integrity and the modulation of inflammatory responses by exogenous bioactive molecules are efficient strategies for the prevention and treatment of IBD **(Mennigen R et al, 2009; Zhang H et al, 2021)** and cardiovascular complications **(Wettschureck N et al, 2019)**.

Dietary polyphenols are considered the most active substances that may exert a protective effect in the development of degenerative diseases, being able to modulate signaling and regulatory pathways. EVOO polyphenols are characteristic contributors on total phenolic intake in the MD, the dietary pattern associated to diseases prevention and longevity. Among EVOO phenolic compounds, HT and Tyr are the most extensively studied, and their beneficial effects have widely been explored in hundreds of studies, mainly *in vitro*, carried out in the last three decades **(Bordoni L et al, 2019; Serreli G and Deiana M, 2018)**.

Exploring their bioavailability however, researchers are aware by now that the *in vivo* systemic concentrations of free compounds are much lower than those of their metabolites (**Pastor A et al, 2016; Rodriguez-Morato J et al, 2016; Lopez de las Hazas MC et al, 2018)**. In the process of crossing enterocytes, EVOO phenolic compounds are subjected to an important first pass metabolism through a classic phase I/II biotransformation, to the extent that the un-metabolized forms could be almost undetectable in biological matrices **(Vissers MN et al, 2004)**. More than 10 metabolites of HT and Tyr have been found in several animal and human studies, however, sulfate and glucuronide 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 **(Miro-Casas E et al, 2003)**. HT and Tyr metabolites have shown biological activity *in vitro* studies. Research on the inflammatory responses in primary human keratinocytes showed that HT and its acetate ester (HT-Ac), a natural HT derivative found in EVOO, interfere with NF-κB signaling by reducing IκB degradation and activating NF-kB **(Rosignoli P et al, 2013)**. HT and its metabolites, HT sulf, HT-4-gluc and 3-O -gluc, were able to inhibit the endothelial activation and expression of VCAM-1 and ICAM-1 in HUVEC and in the intestinal Caco-2 cells stimulated by LPS or TNF-α or IL-1β **(Dell'Agli M et al, 2006; Bordoni L et al, 2019)**. A previous report from our group evaluating the antioxidant effect of HT and Tyr sulphate metabolites in intestinal cells (Caco-2), found that they displayed an efficiency in protecting cells comparable to that of the parent compounds **(Atzeri A et al, 2016)**. HT and Tyr sulphate showed antioxidant activities in HUVEC preventing the increase of ROS, the depletion of glutathione (GSH), and the down-regulation of glutathione peroxidase-1, glutamatecysteine ligase catalytic subunit, and heme oxygenase1 genes **(Muriana FJG et al, 2014)**. **Catalan U et al (2015)** tested the bioactivity of HT and a mixture of its metabolites at physiological concentrations in human aortic EC (HAEC) incubated with TNF-α and demonstrated that HT its and metabolites significantly reduced the secretion of E-selectin, P-selectin, ICAM-1, and VCAM-1. In HAEC cultures, our group highlighted the ability of HT and Tyr conjugated metabolites to promote NO production and availability, through the activation of Akt and eNOS, thus suggesting a contribution to the effects of parental phenolics in the prevention of CVD **(Serreli G et al, 2021 A)**.

Overall data obtained in our study show that metabolites of EVOO-derived phenolics, as well as their parental free forms, possess the ability to contribute to the maintenance of barrier integrity at intestinal and vascular level, preventing inflammation and oxidative stress, through the modulation of MAPK and NF-κB signaling and the NLRP3 inflammasome, with an

efficiency comparable to that of the parent compounds. Acquired information so far suggests a significant biological role of these metabolites, able to exert beneficial effects other than through antioxidant and scavenging properties, by modulating intracellular signaling; however, the actual contribution of phenolic metabolites to the biological activity of the parent compounds is still quite controversial.

It is not known yet if metabolites are able to exert a biological action themselves or if they work just as "pro-drugs" of their free forms, since it has been observed in many cases that metabolites undergo deconjugation before entering the cells, releasing free forms which may be partially converted into other metabolites inside the cell environment acting as conjugated forms. Polymorphic variations of the enzymes involved in the conjugation process of Tyr and HT and consequently of their ability to form sulphates and glucuronides, could modulate the metabolic disposition of Tyr and HT and therefore the global biological effects observed following EVOO intake that could be dependent at least in part on the genetic background of each individual **(Boronat A et al., 2021)**.

Stability and uptake studies undertaken by our group in Caco-2 cells demonstrated that HT, Tyr and their metabolites enter the cells after 30 minutes of incubation and undergo extensive metabolism, giving rise to a pool of metabolites, mainly sulphate and methyl sulphate metabolites and to a lesser extent glucuronides **(Atzeri A et al, 2016)**.

HUVEC are also reported to possess glucuronidase **(Shimoi K and Nakayama T, 2005)** and sulphotransferase **(Garcia-Vallejo JJ et al, 2006)**, thus similar metabolic outcomes are expected in this cell type. Preliminary data obtained by our research group in collaboration with the research group of Prof. De la Torre at the Hospital del Mar Medical Research Institute (IMIM) in Barcelona, showed an extensive metabolization of parent compounds and metabolites, that originated a mixture of sulphated, metilated and glucuronidated compounds (data not shown).

All these findings therefore suggest that EVOO-derived phenolics parental free forms and their major *in vivo* formed metabolites, which represent the largest part of a continually changing pool of compounds, are responsible, as a whole, for the observed beneficial effect in the prevention and amelioration of the major degenerative diseases. This evidence could open the way to further studies aimed to corroborate the anti-inflammatory EVOO actions by investigating their effect *in vivo* models of inflammation, in order to identify effective dosage and timing of administration that could be translated into clinical practice. This could help to maintain and promote health and provide solid and definitive evidence of their positive effects in a number of human pathologies, as CVD and IBD. Therefore, further clinical intervention studies, as well as studies on the mechanism of their activity at molecular and genetic levels, should be greatly encouraged.

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