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**Role of Bioactive components in inflammation and
oxidative stress in vascular endothelium**

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ABSTRACT

An increasing number of scientific evidence supports the preventive value of dietary patterns that favor the consumption of plant food. Especially fruit, vegetables, grains and legumes; the correlation between the reduction of risk of chronic disease such as adherence to the Mediterranean diet is the most significant example. The central role of the endothelium in maintaining vascular homeostasis and the correlation between endothelial dysfunction and the development of cardiovascular diseases makes this tissue a primary target for dietary strategies aimed at cardiovascular diseases prevention. Research in the field of nutrition is therefore directed to the identification of food bioactive components with beneficial effects on the endothelium.

This study first focused on the evaluation of the potential vascular protective effects of a wheat peptide belonging to the family of non-specific lipid transfer proteins type 2 (nsLTP2). nsLTP2, at physiological concentrations, showed antioxidant and cytoprotective effects in HUVECs undergoing oxidative/inflammatory stimulation and demonstrated modulatory capacity on the expression of adhesion molecules and heme oxygenase-1, both involved in endothelial inflammation. Polyphenols are widely studied antioxidant compounds and research supports the preventive/protective role of a polyphenol-rich diet. Despite experimental evidence of their positive influence on human health, to date there is no clear indication of the compounds responsible for this protective role. In fact, upon ingestion polyphenols are extensively metabolized and the molecule that will act at cellular level will more likely be a metabolite. For this reason the second part of the study focused on the protective effect of polyphenol metabolites belonging to two families: cinnamic acids and anthocyanins. Overall the tested compounds demonstrated antioxidant and cytoprotective activities at endothelial level in oxidative/inflammatory conditions, being also able to affect adhesion molecules expression.

These observations may support and characterize biological activities of bioactive peptides and polyphenols metabolites beneficial to vascular health.

CHAPTER 1: Vascular Endothelium

1.1 *The endothelium and its structure*

The endothelium is a monocellular layer that separates all tissues from the circulating blood. Previously considered only as a selective barrier able to facilitate bidirectional passage of macromolecules and blood gases between blood and tissues (Gibbons 1993), the vascular endothelium is now regarded as a dynamic organ with important roles in regulating vascular homeostasis (Gori et al. 2007). This is mediated thanks to a balanced release of a number of autocrine and paracrine molecules in response to biological, physical and chemical stimuli (Gori et al. 2007). These substances released from endothelium can regulate vascular tone, thrombosis and thrombolysis, cellular adherence, smooth muscle cell proliferation and vessel wall inflammation. Given the functional relevance of the endothelium, it appears clear how endothelial dysfunction plays a key role in vascular diseases onset and development/progression. The disruption of these endothelial-balanced actions, in fact, represents the initial step of many disease including atherosclerosis, hypertension, sepsis and some inflammatory syndromes.

In the blood vessel, the endothelium is anchored to a thin layer of connective tissue with which it forms the inner part of the vessel called *tunica intima*. The lower layer, *tunica media*, divided from the first layer by an internal elastic membrane, consists of smooth muscle fibers, connective tissue, rich in collagen, and elastic fibers; *tunica media* has a variable thickness, depending on the type of vessel and the district of origin in the capillaries). The outer part of the vessels (*tunica adventitia*) is formed only by connective tissue consisting of collagen and elastic fibers, which have a containment function (*Figure 1.1*). Flat ECs (endothelial cells) are typical of arterial vessels, while in veins they could be also round or cuboidal and the thickness is variable from 0,1 μm in capillaries and veins to 1 μm in the aorta (Florey 1966; Aird 2007). Every EC has 3 different surfaces: the cohesive surface (ECs with each other) facilitates transport processes and consists of specialized intracellular junctions which can be tight or adherent; the adhesive surface (between cells and basal lamina) and the luminal surface (between cells and luminal side) (Favero et al. 2014).

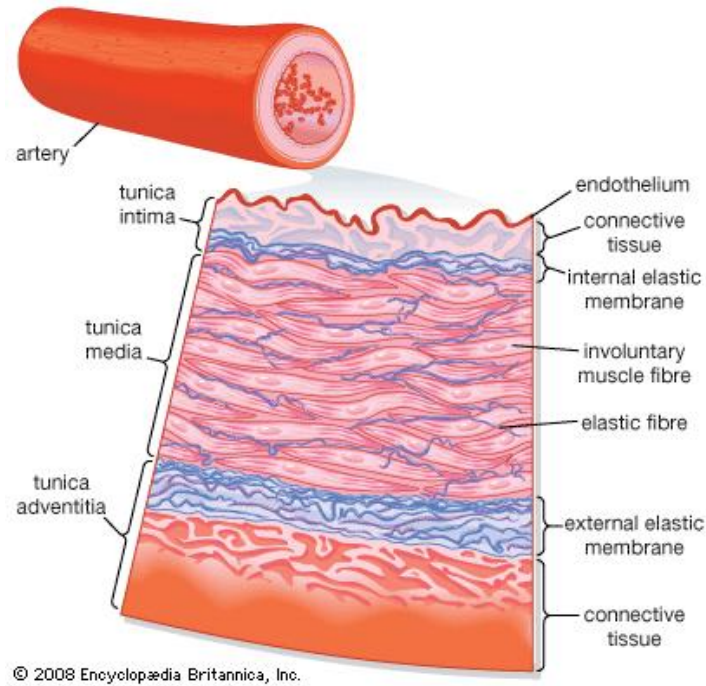


Figure 1.1: Traverse section of an artery (© 2008 Encyclopedia Britannica Inc.)

The endothelium may be continuous and discontinuous. The continuous endothelium may be fenestrated or non-fenestrated, where *fenestrae* are transcellular pores of about 70 nm in diameter possessing a thin non membranous diaphragm across their opening (Aird 2007). Continuous endothelium is characterized by ECs tightly connected and surrounded by a continuous basement membrane; discontinuous endothelium is instead characterized by the presence of fenestrae, frank gaps and pores and a poorly formed underlying basement membrane (Aird 2007). Arteries and veins are both lined with continuous non-fenestrated endothelium and the endothelial junctions in arteries are tighter compared with those in veins; this type of endothelium is also present in capillaries of brain, skin, and lung. Fenestrated continuous endothelium is characteristic of zone of high filtration or transcellular transport such as glands capillaries, intestinal mucosa, glomeruli and renal tubules. Discontinuous endothelium can be found in certain sinusoidal vascular beds, especially in the liver, with larger fenestrations of 100 to 200 (Wisse 1970; Aird 2007; Favero et al. 2014a).

1.2 Functions of Endothelium

The vascular endothelium is involved in many functions. First, it represents a selective barrier that avoid the entrance of injurious substances and it is also involved in many transport functions. It also exerts its functions in maintaining the vascular homeostasis thanks to a balanced release of autocrine and paracrine molecules in response to physical, chemical and biological stimuli (Gibbons 1993). This happens thanks to production of vasoactive factors like vasodilators and vasoconstrictors, inflammatory and anti-inflammatory factors, oxidizing and anti oxidizing factors and many others. A wider vision of all these functions is graphically explained in *figure 1.2*.

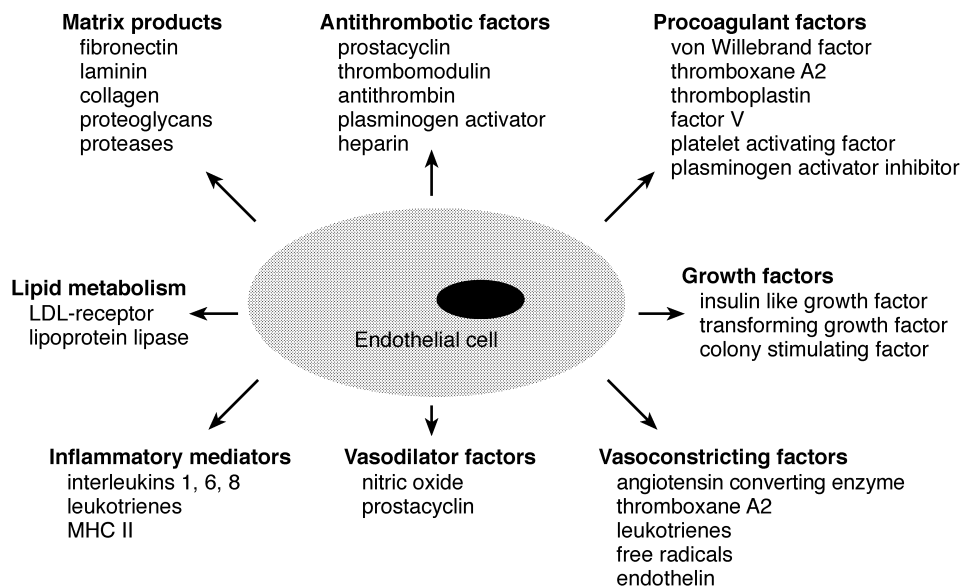


Figure 1.2: Synthetic and metabolic functions of the endothelium. Mediators released are able to influence cellular function throughout the body (Galley & Webster 2004)

1.2.1 Endothelium transport functions

The endothelium represents a semi-permeable barrier, which separates blood from the surrounding tissues and controls solute and macromolecule transfer across the blood vessel wall in response to specific metabolic needs of tissues. There are two different way of transport in the endothelium: the transcellular way use transporters, pores and simple diffusion; the paracellular way use the tight junctions of endothelium, which act as a selective barrier restricting passage of solutes larger than 3 nm in radius (Galley & Webster 2004).

The transcellular transport involves caveolae, membrane microdomains rich in cholesterol and glycosphingolipids, coated, on the cytoplasmic surface, with caveolin-1, which represent their key structural and signalling protein (Rothberg et al. 1992). Numerous signalling molecules, such as G-proteins, kinases, eNOS or ion channels, are associated with caveolin-1 (Lockwich et al. 2000; Absi et al. 2007; García-Cardena et al. 1996). Thus, caveolin-1, by regulating protein-protein interactions, concentrates signalling molecules and optimizes their functions (Minshall & Malik 2006). Albumin is an important molecule transported across the endothelium by this means. Cav-1 is also involved in NO production. In fact Cav-1 has been shown to bind directly eNOS and inhibit eNOS-derived NO release under physiological condition (Bernatchez et al. 2005).

Within the transporters one of the most important is the glucose transporter, GLUT. There are seven different facilitative glucose transporters in this family, but ECs express only GLUT-1 and GLUT-4 (Galley & Webster 2004). Amino acids use a multiple transport system in endothelial cells. The most relevant in this group is the cationic amino acid transporter as L-Arginine (substrate for nitric oxide). Experimental evidence have shown that some cytokines as Tumor Necrosis Factor α (TNF- α) are able to stimulates the L-arginine transport in ECs causing an increase in NO production (Galley & Webster 2004) (Bogle et al. 1995).

The tight junctions are responsible for paracellular transport and permit transport of ions, gas and some cells like leukocytes or cancer cells, working as specific gates and letting selected substance pass through. (Sawada et al. 2003). The paracellular transport became relevant when transporters reach saturation or during immunity and inflammatory processes with a release of leukocytes in the luminal side (Galley & Webster 2004).

1.2.2 Role of the endothelium in vascular tone regulation

The Vascular endothelium is involved in regulation of vasomotor tone producing a number of vasodilators such as NO and prostacyclin which, in non pathogenic conditions are balanced by the action of vasoconstricting molecules like angiotensin II and Endothelin (Gibbons 1993).

Nitric oxide and prostacyclin are powerful vasoactive substances, able to affect the underlying smooth muscle cells, released from the endothelium in response to both mechanical and humoral stimuli (Galley & Webster 2004).

NO is produced by NO-synthase (NOS) which converts the L-Arginine, in presence of Oxygen, into L- Citrulline and NO (*Figure 1.3*).

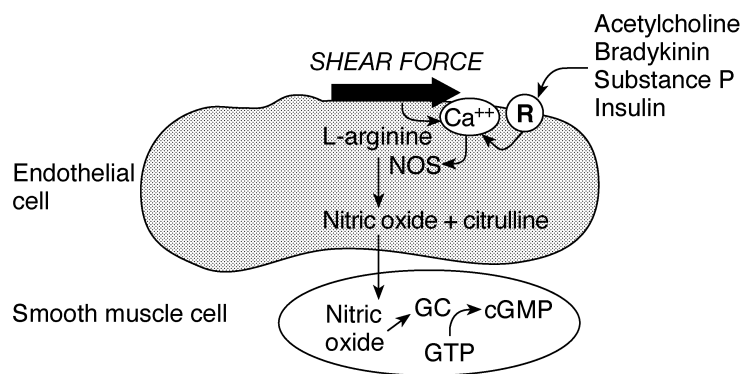


Figure 1.3: Nitric Oxide synthase (NOS) catalyzes the production of NO. The enzyme is activated via changes in intracellular calcium. Released nitric oxide activates soluble Guanylate cyclase (GC) in smooth muscle cells, converting GTP in cGMP responsible of vasodilation (Galley & Webster 2004).

Three different isoforms of NOS have been identified: neuronal NOS (nNOS), endothelial NOS (eNOS) and cytokines-inducible NOS (iNOS). The nNOS and eNOS are constitutive, Ca^{2+} dependent and produce small quantity of NO at basal level. iNOS promotion is supported by an inflammation-mediated stimulation; thus iNOS produces large quantities of NO upon stimulation, such as by pro-inflammatory cytokines (Interleukin-1, Tumor necrosis factor alpha and Interferon gamma) (Green et al. 1994). These three NOS isoforms are characterized by high homology regions (reductase and oxygenase domains), but at the same time each isoforms develops characteristic and distinctive features, which reflect their *in vivo* activity. In basal

conditions there is a continuous release of NO (Tousoulis et al. 2012); this happens because endothelial cells *in vivo* are continuously exposed to stimuli, which affect nitric oxide synthesis. The Ca^{2+} is the major factor for eNOS activation as the enzyme is linked to Calmoduline (CAM), a calcium sensitive protein (Figure 1.4). Increase in blood pressure and inflammation mediators may activate the enzyme.

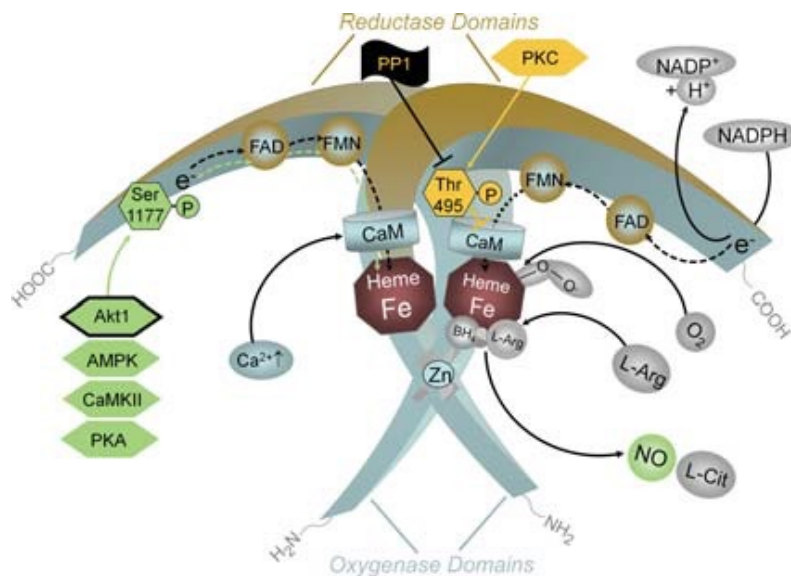


Figure 1.4: regulation of eNOS activity by intracellular Ca^{2+} and phosphorylation (Forstermann & Sessa 2012)

NO is characterized by a short half-life and low solubility, therefore it diffuses through the membranes to the smooth muscle cells and its activity takes place on the neighboring cells. NO has anti-inflammatory properties by inhibiting the synthesis and the expression of cytokines and adhesion molecules which attract inflammatory cells to the endothelial surface. Nuclear factor kappa B (NF- κ B) is the redox-sensitive transcription factor which mainly regulates the gene expression of cytokines, adhesion molecules and enzymes involved in immune and inflammatory response (Galley & Webster 2004). NO regulates basal systemic, coronary and pulmonary vascular tone by increased cGMP in smooth muscles cells (Tousoulis et al. 2012; Bath et al. 1991; De Caterina et al. 1995). NO can also inhibit the activity of Endothelin (ET-1), angiotensin II and NO can also inhibit leucocyte adhesion to vessels interfering with the ability of CD11/CD18 family of adhesion molecules to bind with the endothelial surface or even suppressing the expression of these proteins on leucocytes. NO has also been reported to be a free radical scavenger and its ability to scavenge O_2^- has been reported by Tousoulis et al. both *in vivo* and *in vitro* (Tousoulis et al. 2012). One of the proposed mechanism for this scavenging

action is that the exceeding production of NO leads to an accelerated decomposition of ONOO⁻ to nitrite and nitrate, causing a reduced tissue exposure to ONOO⁻ and OH⁻ (Tousoulis et al. 2012).

Another endothelium-derived vasorelaxant molecule is the prostanoid Prostacyclin (PGI₂). PGI₂ is synthesized from arachidonic acid thanks to a multistep in which cyclooxygenase (COX) is involved, transforming arachidonic acid first in prostaglandin H₂ and then in prostanoids TXA₂ (vasoconstrictor) or PGI₂ (vasodilator). Similarly to NO, PGI₂ diffuses out of the ECs and inhibits both VSMC contraction and platelet aggregation (Mas 2009). These effects are mediated through cAMP production in target cells, resulting in activation of protein kinase A, with a consequent decrease of cytosolic Ca²⁺ and potassium channels opening causing hyperpolarization (Parkington et al. 2004). It is released from endothelial cells in response of “shear stress”, hypoxia and other substances also responsible for NO release. Under normal circumstances, the dominant pathway leads to the generation of the vasodilatory PGI₂, overriding the possible influence of vasoconstrictor prostanoids (Mas 2009). In a dysfunctional endothelium, however, the arachidonic acid metabolism in ECs can be shifted toward the release of TXA₂, normally more relevant in platelet, and other vasoconstrictor prostanoids such as PGH₂ (Mas 2009).

Endothelin (ET), is a strong vasoconstrictor with remarkable effects on vascular tone. There are 3 different isoforms of Endothelin, ET-1, ET-2 and ET-3, each containing 21 amino-acids, but endothelial cells and smooth muscle cells only produce ET-1 although ET receptors are present throughout the body (Galley & Webster 2004). ET-1 synthesis is increased by different stimuli like vasoactive hormones, growth factors, hypoxia, shear stress, lipoproteins and many others. (Agapitov & Haynes 2002).

Two distinct receptors types (ET_A and ET_B) were identified for ET-1, with different pharmacological characteristics. ET_A receptors are found in smooth muscle cells, whereas ET_B receptors are localized on ECs.

The binding to ET_A receptors activates phospholipase C, which leads to long-lasting vasoconstriction. In contrast, the activation of endothelial ET_B receptors stimulates the release of NO and prostacyclin, in endothelial cells (Luscher & Barton 2000).

Between vasoconstrictor agents there is also angiotensin II, which has a pivotal role in the renin-angiotensin system, able to regulate the blood pressure modeling Na⁺ retention and the H₂O reabsorption in kidneys. Angiotensin is produced in a cascade reaction starting from pro-renin at

kidneys level which is converted to renin; renin is responsible for the conversion of angiotensinogen in angiotensin I. The final conversion enzyme of angiotensin II is located on ECs surface and is called Angiotensin converting enzyme (ACE). The angiotensin receptor is in 4 different isoforms (AT₁₋₄R). The better characterized is AT₁R, directly involved in vasoconstriction, which is found in cardiovascular system, liver, kidneys etc.

1.2.3 Function of endothelium in inflammation and host defense

The vascular endothelium both contributes to and is affected by inflammatory processes. Inflammation represents a defence mechanism against pathogens and is mediated by circulating leukocytes that migrate and accumulate in the affected tissues, a process which is actively mediated and precisely controlled by leukocytes, the cytokines they produce, and the vascular endothelium (Zarbock & Ley 2008). However, excessive or uncontrolled inflammatory responses can lead to the pathologic inflammation seen in many rheumatologic and inflammatory disorders. This particular orchestration of immune and inflammatory responses is developed with soluble molecules release such as chemokines, cytokines and growth factors. Part of the cytokines family, the chemokines have a low molecular weight and their main role is related to immune system cells recruitment and stimulate the production of inflammatory mediators. (Galley & Webster 2004). Their spectrum of action is mostly restricted to effects on leucocytes, but some *in vitro* studies have also shown effects on endothelial cells (Colotta et al. 1995)

Leukocyte-endothelial adhesion involves dynamic interactions between leukocytes and endothelial cells, involving multiple steps. These steps must be precisely orchestrated to ensure a rapid response with only minimal damage to healthy tissue (Butcher 1991). Interactions between leukocytes and the endothelium are mediated by several families of adhesion molecules, each of which participates in a different phase of the process. The surface expression and activation of these molecules during an inflammatory response is tightly controlled under normal conditions (Butcher 1991). Three families of adhesion molecules are of particular importance to the process of leukocyte-endothelial adhesion:

Selectins, expressed on both leukocytes and endothelial cells, which primarily mediate cellular

margination and slow rolling; Integrin, responsible for adhesion of leukocytes to the vascular endothelium; members of the immunoglobulin (Ig) superfamily of proteins which include intracellular adhesion molecule-1 (ICAM-1), ICAM-2, and vascular cell adhesion molecule-1 (VCAM-1). These molecules, expressed on ECs, are involved in firm adhesion and transmigration by interacting with integrin on leukocytes (Anon n.d.). ICAM-1 and VCAM-1 in normal conditions have a minimal expression on the endothelial cells membrane but their expression is increased in case of chronic inflammation and also by cytokines and LPS activation (Galley & Webster 2004). These proteins can interact with leucocytes under basal condition via L-Selectin receptor; this interaction is also possible thanks to integrin like LFA-1 (Leucocyte function-associated antigen-1) or VLA-4 (very late antigen-1) which interact both with VCAM and ICAM (Galley & Webster 2004). E-selectin, or CD62E, is expressed on endothelial cells only after their cytokines-derived activation and its expression is restricted to the endothelium, induced by inflammatory cytokines such as TNF- α and IL-1, and mediates the slow leukocyte rolling.

1.2.4 Haemostasis and coagulation

One vital function of the endothelium is to maintain blood fluidity and to provide controlled haemostasis at sites of vascular injury: this is achieved by balancing clotting properties and antiplatelet activity. In this way the endothelium maintains the vascular lumen surface non-adhesive ensuring the fluidity of the blood and avoiding also the formation of clots in districts with a low flow rate as the veins and peripheral arteries and also the valves.

In physiological circumstances endothelial cells carefully prevent thrombosis by different anticoagulant and antiplatelet mechanisms. Endothelial cells are involved in all major homeostatic pathways upon vascular injury and limit clot formation to the areas where homeostasis is needed to restore vascular integrity

Failure of this balanced complex between pro-and anticoagulant systems because of genetic or acquired disturbances, may result in bleeding or thrombosis

Endothelial heterogeneity assures adequate haemostasis in the different organs and parts of the vascular tree. The local environment induces heterogeneous endothelial cell phenotypes determined by local needs. This heterogeneity also explains the diverse pathological responses upon disturbed vascular integrity

The procoagulant properties, as mentioned above, are aimed at repairing a damage of the endothelium. It activates a series of biochemical mechanisms. An emergency basal lamina is formed by fibrin and fibronectin; Then, with the intervention of the FGF and PDGF, is promoted the migration of fibroblasts and endothelial cells, which the healing process. Among the substances synthesized by the endothelium with anticoagulant activity should be mentioned the GAGs (glycosaminoglycans) that include substances such as heparin (Calò L, Semplicini A 1998). The role of GAGs is to bind to antithrombin III and inhibit the coagulation protease (in particular thrombin and factor X). The endothelium also synthesizes thrombomodulin, which reduces the action of thrombin on fibrinogen and platelet activation. (Jaffe 1987). However, the endothelium modulates coagulation also through an important fibrinolytic action: this activity is carried out through the production of substances with the final target of producing plasmin, which in turn degrades fibrin to more soluble products. Between endothelial antithrombotic mechanisms, should also be considered the actions of the endothelium on platelets: inhibition, adhesiveness and aggregation. Prostacyclin produced by the endothelium inhibits platelet activation. Furthermore, the endothelium modulates interactions between platelets and cell wall through the production of NO; on the other hand also the platelets may regulate its own adhesion and aggregation through the production of NO.

CHAPTER 2: Endothelial Dysfunction

2.1 Definition

The term “endothelial dysfunction” first coined in the 1980’s, after the discovery that acetylcholine required ECs to relax the underlying vascular smooth muscle cells (Furchgott & Zawadzki 1980), includes any structural and functional abnormalities of the endothelium under pathological conditions. In response to a wide variety of stimuli, the endothelium acquire a “non adaptive state” which is characterized by the deregulation of all the mechanisms that normally operate in healthy cells (Sitia et al. 2010), leading to impaired endothelium vasodilation, increased vascular reactivity, platelet activation, thrombus formation, increased permeability, leukocyte adhesion and monocyte migration into the vascular wall (Montezano & Touyz 2012). These events underlie endothelial impairment, which by definition is a functional and reversible alteration of ECs function. It is important to underline that ECs may be activated without being dysfunctional, where the term “activation” reflects the capacity of ECs to perform new functions without evidence of cell injury or dysfunction (Favero et al. 2014b). Molecular mechanisms contributing to this include increased expression of adhesion molecules, increased synthesis of pro-inflammatory and pro-atherosclerotic factors, activation of the local renin-angiotensin system and increased ET-1 secretion (Montezano & Touyz 2012). Endothelial dysfunction is observed in the early stage of most cardiovascular diseases (Yang et al. 2010) and is associated with various pathologies including hypertension, diabetes, atherosclerosis pulmonary hypertension, ischaemic heart diseases and chronic kidney disease (Montezano & Touyz 2012). The severity of endothelial dysfunction has been shown to have a prognostic value for cardiovascular pathologic events and its early clinical detection may become a critical point in the prevention of atherosclerosis and cardiovascular disease. Although the mechanisms underlying endothelial dysfunction are complex and multifactorial, a growing body of evidence suggests that increased production of reactive oxygen species (ROS) may have a pivotal role in this phenomenon (Münzel et al. 2010). Since a balance between endothelium-derived vasodilators, especially NO, and ROS modulates endothelial function, an imbalance of these conditions due to increased ROS bioavailability and deregulated redox signalling (oxidative stress) together with decreased NO production because of reduced eNOS activity and increased NO consumption by ROS, may contribute to many of the molecular events underlying endothelial injury (Montezano & Touyz 2012).

2.2 Oxidative stress and endothelial dysfunction

ROS are generated at sites of inflammation and injury, and at low concentrations have a signaling function, participating in regulation of important cell activities such as cell growth and cell adaptation responses. However, at higher concentrations, ROS can cause cellular damage. The vascular endothelium is a primary target of oxidative stress, playing a critical role in the pathophysiology of several vascular diseases. Oxidative stress is responsible for an increased vascular endothelial permeability and for promoting leukocyte adhesion, which is coupled with alterations in endothelial signal transduction and redox-regulated transcription factors (Lum & Roebuck 2001; Hadi et al. 2005).

The susceptibility of vascular cells to oxidative stress is a function of the overall balance between the degree of oxidative stress and the antioxidant defence capability (Higashi et al. 2009). Once defined as an imbalance between pro- and anti-oxidants, oxidative stress is now regarded as an impaired redox signalling and equilibrium ultimately leading to cellular damage (Montezano & Touyz 2012). This condition has been associated to various pathogenetic mechanisms at the basis of different chronic diseases, including CVD and chronic inflammation (Montezano & Touyz 2012). Intracellular produced and biologically relevant oxidants include ROS and reactive nitrogen species (RNS), both of which are very well recognized for playing a dual role being both deleterious and beneficial (Bielli et al. 2015; Brandes et al. 2005). ROS comprise free radicals such as superoxide ($O_2^{\bullet-}$) and hydroxyl (OH^{\bullet}) radicals, characterized by high reactivity and very short half-life. $O_2^{\bullet-}$ is rapidly reduced to hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD) and in turn reduced to water by the activity of glutathione peroxidase, peroxiredoxin and catalase (Brandes et al. 2005; Bielli et al. 2015). Differently from the other radicals, which are characterized by insolubility in lipids and by a low diffusion out of the production site, H_2O_2 may diffuse through the membranes via aquaporin, expanding the tissue damage. H_2O_2 is not a radical but has a strong oxidant power (Valko et al. 2007). The main source of H_2O_2 in vascular tissue is the dismutation of $O_2^{\bullet-}$, which can be either spontaneous or catalysed by SOD (Montezano & Touyz 2012). Other reactive radicals derived from oxygen which can be formed in living systems are peroxy radicals (ROO^{\bullet}) which are involved in fatty acid peroxidation, a chain reaction that may affect particularly cellular membranes (Valko et al. 2007). RNS consist primarily of NO and $ONOO^-$. Nitric oxide is a small molecule and has an unpaired electron in the specific antibonding $2\pi^*$ orbital, so is a radical. It has a half-life of few

seconds in aqueous environment, on the contrary has greater stability in an environment with low oxygen concentration. Due to solubility both in aqueous and lipid media, it easily diffuses through both the cytoplasm and membranes. Overproduction of RNS is called “nitrosative stress” (Valko et al. 2007). The immunity system cells produce for example both superoxide anion and NO during inflammatory processes; under this particular conditions, nitric oxide and superoxide anion may react together producing a relevant amount of ONOO⁻, a potent oxidizing agent that can cause DNA fragmentation and lipid oxidation.

Physiologically, the antioxidant defence systems are capable of adapting to the changing levels of oxidants in order to maintain the oxidant-antioxidant balance (Sies 1997; Sies 2007). This antioxidant defence system includes enzymes such as superoxide dismutase, glutathione peroxidase and catalase, non-enzyme molecules including albumin, bilirubin and glutathione (Wang et al. 2013). However, in pathophysiological conditions, the production of ROS exceeds the natural antioxidant defence of the cells, causing the active ROS to attack and producing cellular alterations (Vaziri 2008; Cai & Harrison 2000) (Sies 1997; Majzunova et al. 2013)

ROS may be generated intracellularly and extracellularly, (Griendling & FitzGerald 2003). They can also be generated by polymorphonuclear lymphocytes via NADPH oxidase, apart from vascular cells (Ebrahimian et al. 2011).

Mitochondria are the first endogenous sources and represent the main producer of ROS in physiological conditions: the enzymes of the respiratory chain are involved in this process, especially complex I (NADH dehydrogenase) and complex III (ubiquinone cytochrome c reductase) (Kovacic & Jacintho 2001). They can also be target for ROS stimulation.

In general, NADPH oxidase, xanthine oxidase, lipoxygenase, myeloperoxidase and, under certain conditions, endothelial nitric oxide synthase (eNOS), are the enzymes primarily involved in ROS production. The vascular production of ROS is mainly driven by the increased activity of the NADPH oxidase isoforms (Ago et al. 2004) The enzyme is responsible for transport of electrons, one by one, thanks to a flavoprotein and a cytochrome C conjugated; the first product of this chain is O₂^{•-} followed by H₂O₂. The Nox4 isoform, which is mainly expressed in the endothelium, is constitutively active (Serrander et al. 2007; Stasi et al. 2010) whereas smooth muscle cells (SMCs) also express Nox1 isoform that requires the binding of additional regulatory subunits for its activation (Murdoch et al. 2011). The Nox2 isoform is expressed in circulating

leukocytes and monocytes, and in endothelial cells to a lesser extent. Both endothelial cells and SMCs are involved in oxidative stress-induced vascular damage (Scioli et al. 2014). An increase in NOX activity has been shown in different pathologies such as hypercholesterolemia, atherosclerosis, diabetes, hypertension and coronary disease in association with endothelial dysfunction and others risk factors (Münzel et al. 2010). ROS can be formed during xanthine metabolism via xanthine oxidoreductase activity. This enzyme is present in two different forms: xanthine dehydrogenase (the physiologic form), which mostly uses NAD⁺ as electron donor. In pathologies like ischemia, this enzyme is converted in oxidase, a form able to react with oxygen and generate ROS as O₂^{•-} or H₂O₂. Another important source of vascular ROS is eNOS. This enzyme, in fact, is a cytochrome P450 reductase-like enzyme that requires some co-factors for its action; among them tetrahydrobiopterin (BH₄), flavin-nucleotides and NAD(P)H for electrons transfer to guanidine nitrogen of L-arginine. BH₄ and L-arginine deficiency are associated with uncoupling of the NO pathway resulting in a decreased NO formation and an increase eNOS mediated generation of O₂^{•-} (Nedeljkovic 2003). Several hypotheses have been proposed for intracellular BH₄ depletion, including an ONOO⁻-mediated oxidation of BH₄: in a positive feedback manner, the increased formation of O₂^{•-} and/or ONOO⁻, oxidizes BH₄ to the BH₃ radical and lead to further ROS and reactive nitrogen species formation (Kuzkaya et al. 2003)

Various physiological stimuli have the ability to contribute to the pathogenesis of vascular diseases and can induce ROS formation. For instance, vasoactive agents such as Endothelin-1 (ET-1), which is a potent vasoconstrictor peptide, has pro-oxidant and pro-inflammatory properties deeply involved in endothelial dysfunction (N et al. 2015). ET-1 expression in ECs is increased by many stimuli like AG II stimulation, aging and diabetes mellitus, etc (LaMarca et al. 2009; Donato et al. 2009). This overexpression activates ROS formation via increasing of NADPH oxidase activity (Barhoumi et al. 2014). ET-1 is also directly involved in vascular cell adhesion molecules and monocyte/macrophages infiltration and in NO bioavailability reduction; this last effect depends to the redistribution of eNOS from plasma membrane to mitochondria causing a phosphorylation of the eNOS responsible for reduction of bioavailable NO (Sun et al. 2014) Moreover the activation of redox signaling pathways leads to inflammatory insults related to pro-inflammatory cytokines release able to up regulate the inducible NOS (iNOS) expression in macrophages and smooth muscle cells. The result is that the excessive amount of NO produced by iNOS reacts with superoxide forming peroxynitrite ONOO⁻.

In recent years, considerable research has been conducted on the critical role of ROS in pathological cellular reactions and in the pathogenesis of vascular diseases, including hypertension, atherosclerosis, diabetes mellitus, and cardiovascular failure (Lee & Griending 2008).

2.3 Role of Inflammation in endothelial dysfunction

Inflammation is another common underlying mechanism of endothelial dysfunction, and it is closely interrelated with oxidative stress (Karbach et al. 2014). Inflammation represents a protective response to injury or infection; a complex process that involves inflammatory cells first identifying the affected tissue, leukocyte recruitment into tissue, elimination of the offending agent, and repair of the site of injury (Dinh et al. 2014). Inflammation requires interactions between cell surfaces, extracellular matrix, and pro-inflammatory mediators (Keane & Strieter 2000). Excessive inflammation state can have negative effects and contribute to the progression of chronic diseases such as atherosclerosis (Lusis 2000), rheumatoid arthritis (Sweeney & Firestein 2004) and systemic lupus erythematosus (Dinh et al. 2014).

A tight relation between inflammation and endothelial dysfunction exists (Huang et al. 2012; Stenvinkel 2001); inflammation, due to many risk factors such as diabetes, hypertension, obesity might turn from a biological defence system in a serious chronic condition, in which a disruption of the normal function of endothelial cells takes place.

Various inflammatory cytokines are involved in this process and, among them, Tumor necrosis factor-alpha (TNF- α), and IL-1 are the most relevant. The cytokines are generally produced by lymphocytes and macrophages after stimulation by injuries, toxins or inflammatory mediators. General properties of cytokines are: short-half life, modulation by immune response, interaction with other cytokines and recognition by specific receptors (Zhang 2008).

TNF- α , mainly secreted by macrophages, is first produced as a type II trans membrane protein which, after some photolytic cleavage performed by TNF- α converting enzyme (TACE) is converted in the active and soluble form (Wajant et al. 2003). This cytokine is involved, together with pro-inflammatory activity, in a wide spectrum of biological process including cell proliferation, differentiation and apoptosis (Wajant et al. 2003).

TNF- α may be considered the major cytokine involved in inflammation and endothelial dysfunction (Huang et al. 2012). On the other hand, the relation between TNF- α mediated

inflammation and oxidative stress and ROS overproduction, is playing a pivotal role in the pathogenesis of endothelial dysfunction (Huang et al. 2012). Generation of ROS comes from multiple sources, including various enzymes such as xanthine oxidase, cytochrome P450, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, together with uncoupled endothelial nitric oxide synthase, all expressed in ECs (Huang et al. 2012). NADPH oxidase, as shown in *Figure 2.1*, is considered one of the primary site of TNF- α mediated ROS production in endothelium. TNF- α , in fact, has been demonstrated to be one of the principal stimuli of NADPH activation via phosphorylation of its subunit p47^{phox}.

TNF- α levels are elevated in various diseases and condition related with endothelial dysfunction, including insulin resistance (Zhang 2008; Fernández-Checa et al. 1997; Fischer & Hilfiker-Kleiner 2007) and in coronary artery disease. Also TNF- α is responsible for inhibition of endothelium-dependent NO-mediated dilation via activation of JNK and production of superoxide anion via xanthine oxidase, responsible for reduction in NO bioavailability (Zhang 2008; Deisher et al. 1993). A property of cytokines is the interaction with other cytokines: TNF- α , in fact, induces the gene expression of several inflammatory cytokines and chemokines and activates the transcriptional factor NF- κ B which initiates some situations such as atherogenesis, thrombosis, impaired NO bioavailability and perpetuate inflammation by increasing expression of adhesion molecules (Zhang et al. 2009).

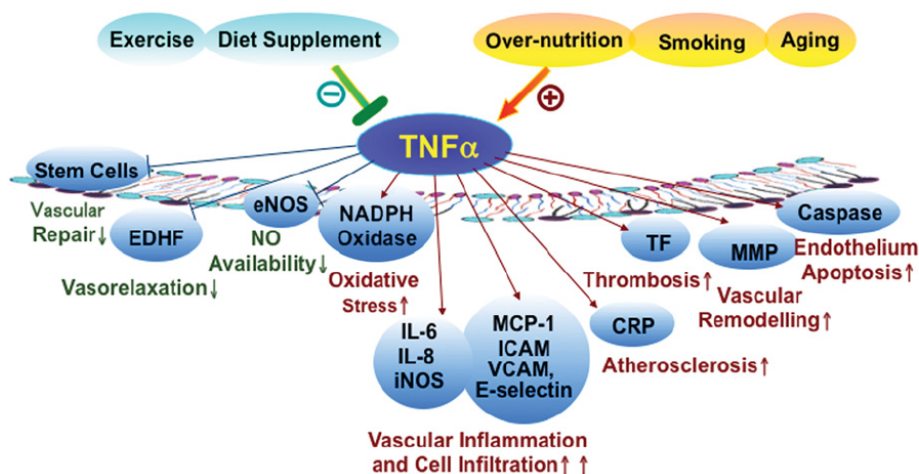


Figure 2.1: Pivotal Role of TNF- α in endothelial dysfunction (Zhang et al. 2009)

A crucial role in inflammation and endothelial dysfunction has to be assigned also to Interleukins. IL-1 is the most involved interleukin in endothelial dysfunction. Particularly IL-1 β is a pro-inflammatory cytokine implicated in inflammation, pain and autoimmune conditions (Ren & Torres 2009). There are two IL-1 receptors: IL-1RI that bind IL-1 α and IL-1 β and IL-1RII, which is considered a decoy receptors as its binding doesn't lead to cell signalling (Ren & Torres 2009). Also a third receptor (IL-1ra) is, present which is an antagonist receptor and prevent the interaction of IL-1 with its receptors, thus acting as a natural inhibitor. In normal conditions IL-1 β has important homeostatic functions and, as reported by Dinarello et al (Dinarello 1996), regulates feeding, sleep and temperature. However, an overproduction of IL-1 β is related with pathophysiological changes which result in different pathologies including endothelial dysfunction and related vascular diseases (Ren & Torres 2009). Production of IL-1 β happens in the inflammasome, an intracellular multi-protein complex really important in regulation of inflammation. Inflammasome is responsible for Caspase-1 cleavage, which then can process the transformation from pro-IL-1 β (inactive form) to its bioactive IL-1 β form (Ren & Torres 2009) .

Numerous scientific evidence showed the role of TNF- α and IL-1 β in endothelial dysfunction. When endothelial cells undergo inflammatory activation IL-1 β and TNF- α , by the acute phase protein CRP, increase the expression of selectins, and adhesion molecules (VCAM-1, ICAM-1) (Zhang 2008).

Adhesion molecules are largely studied as they are considered a biomarker of endothelial activation and inflammation. Adhesion molecules (CAMs) participate not only in pathologies but also in basic vital processes such as cellular growth and differentiation, migration and embryogenesis (Krieglstein & Granger 2001). They mediate the interaction between blood cells (leukocyte, platelet) and the endothelium. This interaction may happen in all sites of vasculature in both physiological (homeostasis) and pathological conditions (inflammation, immune response, cancer etc.) (Krieglstein & Granger 2001; Golias et al.). Looking at the physiological role of CAMs, they are main actor in leukocyte trafficking in microcirculation and their presence is crucial in localization of the inflammatory response. The main classes of CAMs known at present are integrins, caderins, selectins, members of the immunoglobulin gene superfamily (IgSF) and CD44 and each class mediates a different step in migration of leukocyte from the

bloodstream toward inflammatory foci (Golias et al.; González-Amaro et al. 1998; Jaitovich & Jaim Etcheverry 2004).

Selectins mediate leukocyte rolling, while integrin and immunoglobulin supergene families (VCAM1-ICAM1) enable the firm adhesion and migration of leukocytes (Golias et al.). In normal conditions (a dormant state) the interaction between leukocyte and endothelial cells is not possible, in fact, the selectin-binding site is present on leukocyte but not expressed in dormant endothelial cells; CAMs in this moment mediate the interactions between cell matrix and endothelial cells and regulate the vasculature permeability. However, in case of damage or inflammation, there is a pro-inflammatory cytokines release and an increase in ROS production that up-regulate the expression of adhesion molecules and so the adhesion and migration of leukocytes across the vascular wall (Marecková et al. 1999; Golias et al.). *Figure 2.2*

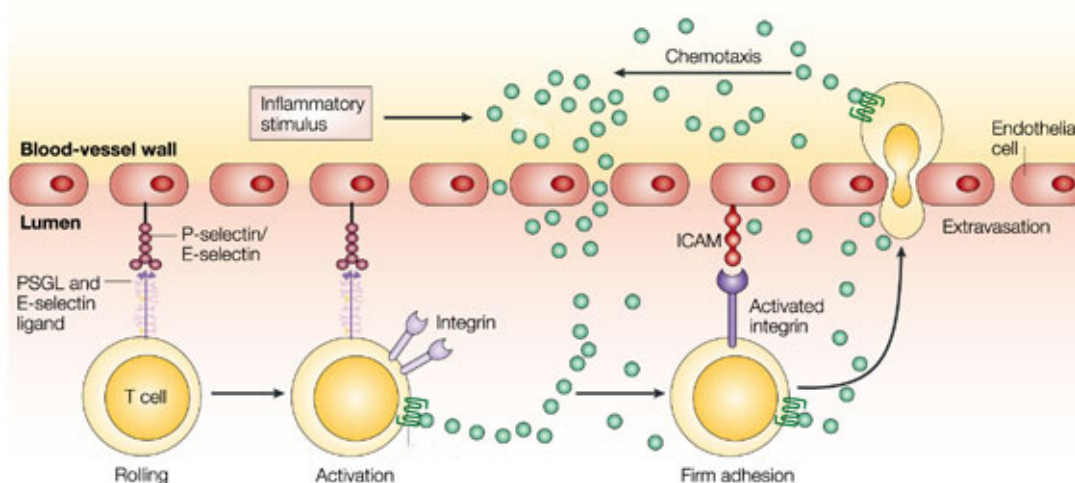


Figure 2.2: Leukocytes are recruited from the circulation to the sites of inflammation (Luster & Tager 2004).

Scientific evidence support the view that leukocytes and adhesion molecules are important participants in endothelial dysfunction and tissue injury and both these situation are linked inflammatory state and cardiovascular diseases (Golias et al.). A deregulation of all the physiological system in which adhesion molecules are involved leads to the activation of transduction pathways that code for cytokines and chemokines expression, which can contribute

to continue the leukocytes recruitment and migration with a consequent unresolved inflammation (Golias et al.)

Another pathologic condition characterized by an inappropriate activation of inflammatory process is systemic septicaemia. Sepsis is, in fact, a clinical syndrome caused by a systemic host response to infections (Schouten et al. 2008). However the clinical and pathological manifestation of sepsis are not directly caused by the pathogens, rather than the hypotension and multisystem dysfunction, mostly associated with inflammation (Schouten et al. 2008). One important characteristic of sepsis is microvasculature dysfunction, in which endothelial activation and dysfunction play a significant role. Particularly, during infections, components of bacterial wall, such as lipopolysaccharide (LPS) activate some recognition receptors on endothelium surface. After that, when the inflammatory response has started, a huge number of inflammatory mediators, including chemokines and cytokines further activate endothelial cells perpetuating the inflammation event (Schouten et al. 2008).

LPS consists of an oligosaccharide core, a series of repeated O-specific polysaccharide chain units and a lipid component responsible for the pro-inflammatory activity. The bond with the membrane of cells promotes its activation resulting in cytokine production and expression of adhesion molecules, also inducing apoptosis in some cases (Dauphinee & Karsan 2005). On immune system cells it promotes the release of other inflammatory mediators.

The first protein to bind LPS is the LBP (LPS-Binding Protein), a molecule of the acute phase and with its substrate forms a ternary complex with CD14 on the membrane of the leucocytes, while in endothelial cells the LPB induces the uptake of LPS (Dauphinee & Karsan 2005).

The receptor activation by LPS increases caspase activity and the activation of transcription factor NF- κ B that leads to the expression of adhesion molecules and other pro-inflammatory proteins. It has been observed that high levels of LPS can lead to the formation of ROS, until induction of apoptosis. In addition, there is an increased permeability of the mitochondrial membrane as a result of the activation of pro-apoptotic proteins (Bannerman & Goldblum 2003).

LPS also activates SOD, in particular the Mn and Cu-Zn SOD, which converts superoxide anion into H_2O_2 , which happens to be in excess, with an increase in the oxidizing potential in the cytoplasm (Bannerman & Goldblum 2003).

2.4 HO-1 and its anti-inflammatory properties

Heme oxygenase (HO) is a microsomal enzyme essential for heme oxidative degradation. Heme catabolism requires molecular oxygen, nicotinamide adenine dinucleotide phosphate (NADPH) and cytochrome P-450 reductase, producing ferrous iron, carbon monoxide (CO) and biliverdin, which is subsequently converted to bilirubin by biliverdin reductase.

Despite its physiological and beneficial effects an excess of intracellular-free heme is responsible for pro-inflammatory and cytotoxic effects caused by increased ROS production and lipid peroxidation (Jeney et al. 2002) (Calay & Mason 2014). The fine balance between heme biosynthesis and catabolism operated by HO is essential for limiting the potential harmful effect of free heme.

Two main isoforms of HO have been identified: HO-1 and HO-2. The first isoform (HO-1) can be induced by different stimuli both physiological and pathological, like oxidative stress, UV, radiation and pro-inflammatory cytokines (Naito et al. 2014). The main organs in which HO-1 is highly expressed are those responsible for the degradation of senescent erythrocytes (spleen, liver, hepatic reticulo-endothelial cells and bone marrow); in other tissues basal expression of HO-1 is very low. In the other tissues not directly responsible for hemoglobin metabolism the basal expression of HO-1 is low but can rapidly be induced by all those stimuli mentioned above (Calay & Mason 2014).

There are numerous scientific evidence in support of the beneficial role of HO-1 at the vascular level. Overexpression of HO-1 in human endothelial cells has a protective effect against the toxicity of tert-butyl hydroperoxide, crotonaldehyde and pro-apoptotic cytokines. The activity of HO-1 reduces oxidative stress induced by H₂O₂ or NADPH oxidase and could attenuate inflammatory responses induced by LPS, TNF- α , ox-LDL or interleukin IL-18; From a mechanistic point of view these effects involve the inhibition of the nuclear transcription factor NF- κ B activation and the consequent up-regulation of cyclo-oxygenase 2 (COX-2), E-selectin, VCAM-1, ICAM-1 and the secretion of chemokines such as MCP-1 (Calay & Mason 2014).

These protective effects of HO-1 depends to its enzymatic products, especially CO and bilirubin. These products, in fact, are responsible for inhibition of apoptosis, oxidative stress, necrosis and inflammation in various cell types, including endothelial cells (Calay & Mason 2014).

In the vasculature CO, at low concentration (1-100 nM). induces vasorelaxation by soluble guanylate cyclase (sGC) activation and NO release from intracellular store, while at higher concentration (1-10 μ M) it promotes vasoconstriction through the inhibition of eNOS activity. CO also exerts anti-apoptotic and anti-inflammatory effects inhibiting the synthesis of pro-inflammatory cytokines TNF- α and IL-1 β by macrophages and inducing the anti-inflammatory cytokine IL-10. The anti-inflammatory effect has been linked to CO ability to inhibit NF- κ B resulting in an attenuation of TNF- α actions and the induction of adhesion molecules. Another positive effect of CO in the vasculature is the reduction of vasoconstrictive protein ET-1 (Calay & Mason 2014).

The biliverdin/bilirubin system also showed some vasculoprotective effects. Epidemiological studies show that raised level of plasma bilirubin are related with a lower risk of cardiovascular diseases (Ollinger et al. 2005; Ollinger et al. 2007; Calay & Mason 2014). *In vitro* studies on endothelial cells have suggested that their protection against the generation of NADPH-dependent superoxide and against the pro-oxidant effects of hyperglycemia and angiotensin II may contribute to the protective effects observed. In HUVECs, bilirubin and biliverdin reduce the oxidative stress induced by various stimuli (Hayashi et al. 1999). However, the beneficial effect of bilirubin / biliverdin system at vascular level are not limited to antioxidant activity: in rats treated with LPS, the administration of biliverdin caused a decrease of IL-6 synthesis (pro-inflammatory cytokine) and a parallel increase in IL-10. (Calay & Mason 2014). Bilirubin has also shown the ability to reduce platelet aggregation and adhesion of leukocytes to the vascular wall (Haines et al. 2012). In human endothelial cells it was also observed that the increase in the activity of HO-1, bilirubin and / or chelation of Fe²⁺ significantly reduce the expression of E-Selectin and VCAM-1, but not ICAM-1, induced by TNF- α (Calay & Mason 2014).

The link between the activity of HO-1 and the wide variety of protective effects observed (antioxidant, anti-inflammatory and anti-apoptotic) make this enzyme a good therapeutic target. Several drugs have been reported to affect HO-1 activity in the vasculature and one of the current challenge is trying to translate it to clinical practice. As an example, drugs currently used in therapy of hypercholesterolemia, such as statins (atorvastatin, simvastatin, rosuvastatin), display vasculo-protective, anti-inflammatory and immunomodulatory activities that are independent from the HMG-CoA reductase inhibitory effect. They have been shown to increase

HO-1 promoter activity and mRNA levels, to induce HO-1 activity and increase its antioxidant activity (Calay & Mason 2014) (Figure 2.3).

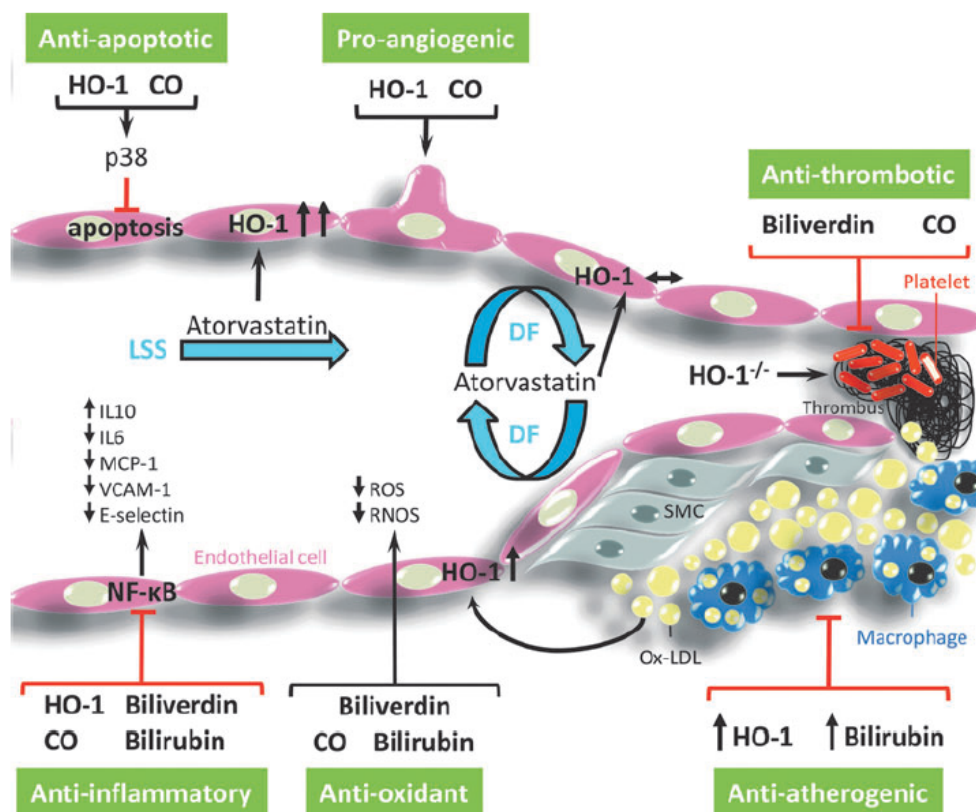


Figure 2.3: Cytoprotective action of HO-1 and its products in the vasculature (Calay & Mason 2014)

2.5 Endothelial dysfunction from risk factors to cardiovascular diseases.

It has already been explained how complex is the dysfunctional condition of endothelium and how wide is the number of factors involved in it. Several risk factors such as hypertension, smoking, high-fat diet and many others are involved in endothelial dysfunction and associated with overexpression of ROS, increase oxidative stress and perpetuated inflammation. Oxidative stress in the vascular wall, for example, may contribute to decrease NO bioavailability and thus interfere with endothelium-mediated vasodilatation. (Grover-Páez & Zavalza-Gómez 2009). In hypertension the two main aspects are hemodynamic alteration and increase of peripheral resistance. A reduction in endothelium-mediated vasodilatation has been highlighted in

numerous models of arterial hypertension (systemic hypertension or salt-sensitive hypertension) (Lüscher 1990)

Also high level of serum cholesterol creates an alteration of the endothelium-dependent vasodilatation, which undergoes morphological changes of endothelial cells. In hypercholesterolemia NO production increases but its bioactivity is decreased. This means an excess of superoxide generation, which is not able to inactivate NO but may increase the LDL oxidation process and take part in the vicious circle that promote endothelium damage. Cholesterol is probably the most established risk factor for coronary artery disease. Moreover, cholesterol levels, even if in a normal physiological range, are inversely correlated with endothelium-dependent vasodilatation; this means that within the normal range, cholesterol may improve the production and release of endothelium-dependent NO (Grover-Páez & Zavalza-Gómez 2009; Masumoto et al. 2001). Diabetes also play a significant role in endothelial dysfunction: Cosentino and others experimentally demonstrated a reduced capacity of NOS to generate NO when endothelial cells are exposed either *in vitro and in vivo* to a diabetic environment (Cosentino & Lüscher 1998; Lambert et al. 1996). Also endothelial dysfunction is closely associated with microangiopathy and atherosclerosis in both types of diabetes (Cosentino & Lüscher 1998; Grover-Páez & Zavalza-Gómez 2009). Diabetes is characterized by an increase tendency for oxidative stress, high levels of ox-LDL and, in human, associated also with pro-thrombotic tendency, increased platelet aggregation and impaired endothelium-dependent vasodilatation in both types I and types II diabetes.

Finally, atherosclerosis is a dynamic and progressive disease directly related with endothelial dysfunction and inflammation. In case of prolonged inflammation there is a decreased eNOS expression and an increase in ET-1 production (Grover-Páez & Zavalza-Gómez 2009). Also, it has been demonstrated that inflammation occurs together with artery wall lipid accumulation in atherosclerosis. Moreover, under inflammation stimuli ECs begin to express on their surface selective adhesion molecules (VCAM-1, ICAM-1) capable to bind leukocytes. Recruitment of leukocytes doesn't happen in normal condition but perpetuated inflammation stimuli create a change in endothelium cells' surface, which starts supporting white blood cells binding (Grover-Páez & Zavalza-Gómez 2009). Once adherent to endothelium, leukocytes penetrates the intima and, thanks to MCP-1 (monocyte chemoattractant molecules) migrate to the site of lesion formation. If the inflammatory process perpetuates the activated leukocytes and ECs can release fibrogenic mediators (peptide, growth factors) that can promote replication of SMCs and

contribute to elaboration of a dens extracellular matrix typical of atherosclerotic lesions (Grover-Páez & Zavalza-Gómez 2009).

CHAPTER 3: Bioactive Peptides

3.1 Bioactive peptides: general description

Bioactive substances can be defined as “food components – essential or not essential- that, beyond their nutritional value, affect physiological processes with a positive impact on body function or condition and ultimately health” (Marques et al. 2012). This definition partly overlay that of “nutraceuticals”. In recent years, increasing epidemiological evidence is linking the prevalence of diseases, such as cardiovascular diseases (CVD), obesity, hypertension, diabetes and some type of cancers, collectively indicated as. non communicable - chronic diseases, to dietary factors (Hernández-Ledesma et al 2011). These pathologies are characterized by multifactorial etiology, in which life style and dietary habits represent modifiable risk factors. Moreover non-communicable diseases in spite of diverse etiologies share many underlying pathological mechanisms, including abnormalities in inflammatory responses and oxidative stress. Thus targeting the common pathological pathways has gained increasing attention in recent years for both prevention and treatment of chronic diseases (Chakrabarti et al 2014). Increasing interest has been directed to food that contain besides essential nutrients, bioactive ingredients endowed with health enhancing activity (Malaguti et al. 2014). Recently, it has been recognized that dietary proteins, usually regarded as a source of energy and essential amino acids, necessary for growth and maintenance of physiological functions, may represent a source of biologically active peptides. Bioactive peptides, usually molecules smaller than 10kDa (Malaguti et al. 2014) may exist naturally in food or be encrypted within protein primary sequence. In order to gain functional activity they have to be released from parent protein mainly through proteolysis (during gastrointestinal (GI) digestion *in vivo*, bacterial fermentation and ripening in food processing or *in vitro* enzymatic hydrolysis) (Kitts & Weiler 2003; Chakrabarti et al. 2014). Depending on the aminoacidic composition of the polypeptide chain these peptides can exhibit a wide range of biological activities, including antimicrobial properties, blood pressure-lowering capacity, ACE – inhibitory effects, cholesterol-lowering ability, antithrombotic and antioxidant activities, mineral binding and opioid-like activities (Malaguti et al. 2014). Amino acid composition and size also influence the mechanism by which these compounds are absorbed: large water-soluble peptides diffuse through tight junctions (paracellular route) or endocytosis, while hydrophobic peptides can be absorbed by passive transcellular diffusion. Some bioactive peptides can exert their activity at gastrointestinal level by directly binding to digestive membrane receptors (e.g. opioid receptors) (Bouglé & Bouhallab 2015). Biologically active peptides derive from both plant and animal sources. Plant sources

include cereals (barley, wheat, corn, rice), pseudo cereals (backwheat and amaranth), legumes (soybean, bean, pea), brassica species and other sources, like sunflower. Protein from animal sources are found in milk, currently the most important source of bio peptides, although these bioactive compounds can also be obtained from meat, egg and fish (Malaguti et al. 2014). Thanks to their health-enhancing potential and safety profile, bioactive peptides may be used as components in functional foods or nutraceuticals and their consumption may play a relevant role in promoting health in various chronic and degenerative disorders such as CVD (Erdmann et al. 2008)

A constantly increasing number of scientific evidence have found an inverse correlation between the reduced risk and occurrence of chronic diseases and the adherence to dietary patterns rich in cereals, legumes and derived products, supporting their preventive role. Particularly in the Mediterranean diet, where cereals, legumes and grains are largely consumed, bioactive peptides can contribute to the observed positive effect in controlling/preventing chronic degenerative diseases.

3.2 Animal Bioactive Peptides

MILK PEPTIDES

Milk comprises two main classes of proteins: caseins (80%) and whey proteins (20%). Caseins are colloidal aggregation of κ -, α s-1, α s2 and β caseins. The class of whey proteins comprises fractions of β and α lacto globulin, lactoferrin, immunoglobulins and serum albumin. Milk proteins can be found in diary products and also as food ingredients in concentrated, dried or liquid forms. After processing they are subjected to enzymatic hydrolysis in gastrointestinal tract and are splintered in smaller peptides, some of them with bioactive roles (Cam & de Mejia 2012). Many studies have been conducted on dairy products and their potential beneficial impact on risk factors for chronic disease like CVD. (Panagiotakos et al 2010), conducted a human study examining the dietary habits of men and women in the Attica region in Greece: volunteers with no clinical evidence of cardiovascular diseases were chosen and administrated with diary products such as milk, cheese and yogurt. People who assumed 11-14 servings of dairy products per week had up to 12% lower level of C-reactive protein (CRP), IL-6 and TNF- α

compared to those that consume less than 8 servings. Furthermore, volunteers who consumed more than 14 servings per week had 29.9%, 18.8% and 20% lower levels of CRP, IL-6 and TNF- α , respectively (Panagiotakos et al. 2010). These results corroborated the association of decrease levels of inflammation biomarkers linked with chronic and inflammatory disease with dairy food consumption (Cam & de Mejia 2012).

Also lactoferrin (LF), a milk glycoprotein from whey, showed anti-inflammatory and anti-microbial properties *in vitro*, inhibiting IL-1, LPS-induced TNF- α and IL-6 expression in THP-12 monocytic cells through an inhibition of nuclear factor Nf- κ B (Yalçin 2006; Zimecki & Kruzel 2007; Håversen et al. 2002; Cam & de Mejia 2012). Puddu et al., demonstrated that LF treatment inhibited LPS-Induced expression of adhesion molecules and pro-inflammatory cytokines in bovine aortic endothelial cells (Puddu et al. 2011). Furthermore in an *in vivo* study in mice a supplementation with LF (Kobayashi et al. 2011) showed a reduction of TNF- α and an increase in IL-10. Milk protein hydrolysates and peptides also captured attention for their antihypertensive activities. Especially two lactotriptides derived from casein, Val-Pro-Pro (VPP) and Ile-Pro-Pro (IPP) showed a reduction of blood pressure via inhibition of Angiotensin Converting Enzyme (ACE) (Sano et al. 2005; Mizuno et al. 2005; Cam & de Mejia 2012). As demonstrated by all this scientific evidence, milk and milk derived peptides have reported a beneficial impact on markers of inflammation linked with CVD; however, some studies did not match with these results and, although significant findings suggest the efficacy of dairy products on the improvement of inflammation and CVD risk factors, the differences in results show the essential need to investigate further in this field.

MEAT PEPTIDES

Bioactive peptides derived from meat and fish showed antioxidant and anti-hypertensive properties. These peptides have been isolated from hydrolysates of meats like beef, chicken, pork and also fish (Arihara 2006; Cam and de Mejia 2012; Liu et al. 2010;). The main activity proposed and investigated for meat peptides was the ACE inhibitory effect, but some recent studies revealed other potential health benefits. For example Shimizu et al., in a mice study, isolated by papain hydrolysis a fraction from defatted pork meat and after oral administration at 70 and 210 mg/kg body weight assessed the antithrombotic activity of the peptide by calculating the total thrombosis size after a laser irradiation of the carotid artery; the results obtained showed

a significant inhibition of thrombus formation comparable with 50mg/kg body weight administration of aspirin (Shimizu et al. 2009; Cam & de Mejia 2012). Two different studies assessed the potential protective effects of chicken collagen hydrolysates (CHH) on atherosclerosis. In the first study, apolipoprotein E-deficient mice were fed a diet supplemented with 10% CCH or a normal diet for 12 weeks; the results from plasma analysis showed a significant reduction in total plasma and total hepatic cholesterol (14, 24 and 48% reduction, respectively) and hepatic triglycerides in the CCH mice compared with the non-supplemented diet group (Zhang, Kouguchi, K. Shimizu, et al. 2010), showing a concrete evidence that CCH supplementation might help in atherosclerosis prevention. In a second study the pre-treatment with CCH in a rat model of cardiovascular damage exhibited a significant reduction in blood pressure and expression of ICAM-1 together with an increase of NO (Zhang, Kouguchi, M. Shimizu, et al. 2010).

3.3 Plant Bioactive Peptides

Soybean was considered in the last decades an interesting source of bioactive peptides. Soy food have been used for centuries in Asian countries diet but only recently they have reached popularity in western countries due to their potential nutraceutical properties (Cam & de Mejia 2012) (Messina 2010). First studies conducted with food containing whole soy indicated that its consumption may reduce cardiovascular risk factors such as LDL, total cholesterol and blood triglycerides and increase HDL. However, whole soy also contains isoflavones, vitamins, minerals and other potential bioactive components, making difficult to attribute the observed effect of soy proteins (Cam & de Mejia 2012). Recently the attention has been focused on Lunasin, a 43-amino acid bioactive component derived from soybean, characterized by a unique arginine-glycine-aspartic acid cell adhesion motif and a polyaspartic acid tail composed of nine aspartic residues responsible for its bioactive properties. (Cam & de Mejia 2012). Peptides containing arginine-glycine-aspartic acid (RGD) cell adhesion motif have also a significant potential in CVD prevention binding to integrin with high specificity and leading to anti-angiogenic and anti-inflammatory effects (Cam and de Mejia 2012).

Whole-grain food consumption has been subjected to a wide variety of epidemiological studies demonstrating high correlation with reduced risk of coronary artery disease, CVD, hypertension

and diabetes, improvement of antioxidant status etc. The initial outcomes in bioactive peptides focused mainly on soybean peptides and their benefic effects in chronic disease, including CVD, while specific bioactivity of peptides in oat, wheat and barley were not widely studied so far and few evidence are now available. However, recent studies concentrated more and more on legumes and cereals peptides and their potential activities on vascular health (Cam & de Mejia 2012). Cheung et al., found that oat protein hydrolysates display ACE inhibitory activity with IC_{50} 35-85 $\mu\text{g}/\text{mL}$, after simulated gastrointestinal digestion (Harris and Kris-Etherton). Yang et al., isolated from wheat germs, five different peptides with ACE-inhibitory activity with IC_{50} value as low as 5,86 μM (Yang et al. 2011).

3.4 Biological activities attributed to bioactive peptides related to cardiovascular health

3.4.1 Cholesterol-lowering activity

Hypercholesterolemia is a well known CVD risk factor and proteins hydrolysates and peptides with hypocholesterolemic bioactivity may represent potential nutraceuticals ingredients with promising applications in development of functional foods in diet aimed at reducing cholesterol levels in population at high risk of cardiovascular disease (Ruiz Ruiz et al. 2014).

The most investigated proteins with lipid-lowering activity are soy protein hydrolysates and peptides. Food protein sources with hypocholesterolemic and hypolipidemic effects include soy, milk, egg white and fish proteins (Sugano et al. 1990; Ruiz Ruiz et al. 2014): however, the plant-derived peptides seem to produce a more notable effect than animal proteins. (Ruiz Ruiz et al. 2014). Lovati et al. (Lovati et al. 1992) demonstrated that a soy protein peptic hydrolysate (SPH) had stronger serum cholesterol lowering activity than intact soy protein. The mechanism responsible for the observed effect was later clarified by an *in vitro* study to be related to cholesterol absorption; in fact, micellar cholesterol solubility was found to be significantly lower in the presence of SPH, and cholesterol uptake in Caco-2 cells was lower from SPH-containing micelles (Ruiz Ruiz et al. 2014; Nagaoka et al. 1999). Moreover, it was observed in rat fed a high-cholesterol diet that administration of soybean 7S globulin hydrolysates resulted in a

decrease in plasma cholesterol and tryglicerides, decreased the low density /high density lipoprotein ratio and up-regulated β -VLDL (Duranti et al. 2004; Ferreira et al.). Peptides from other peptide such as *Lupinus mutabilis* have been investigated and demonstrated a remarkable reduction in both triglyceride and plasma cholesterol levels, confirming the hypolipidemic potential of legumes (Spielmann et al. 2007; Ruiz Ruiz et al. 2014). Sirtori et al demonstrated that lupin protein isolate (50mg/die) significantly reduced both VLDL and LDL and it was also shown in HepG2 hepatoma cell line that conglutin γ isolated from lupin total protein increased LDL uptake by inducing LDL receptor activity.(Sirtori et al. 2004). Rigamonti et al. 2010 investigate a possible impact of protein from *Pisum sativum*, on the expression of gene involved in cholesterol metabolism by measuring the relative mRNA level of sterol regulatory element binding protein (SREBP)-2 and of its target genes and found that pea proteins exert a hypotrygliceridemic effect mainly trough down regulation of fatty acids synthesis. Lunasin has been shown to be responsible for the cholesterol-lowering effect associated with soybean consumption (Ruiz Ruiz et al. 2014). The lunasin mechanism of action involve a reduction in the HMGCoA reductase gene expression (similar to statin action) (Ruiz Ruiz et al. 2014). In addition lunasin enhance the plasma LDL cholesterol clearance by increasing transcription of LDL receptor gene.

3.4.2: Antihypertensive Properties (ACE –Inhibition)

High levels of blood pressure are one of the major independent risk factors for cardiovascular diseases and its control has a large impact on the health state of human population (Harris et al.; Kannel & Higgins 1990; Erdmann et al. 2008). Angiotensin I - Converting enzyme (ACE) regulates blood pressure through its action on the renin-angiotensin system (RAS) as it is responsible for the conversion of Angiotensin I to a potent vasoconstrictive agent, Angiotensin II, which induces the release of aldosterone causing an increase in sodium concentration and blood pressure; ACE also take part of the kinin-kallicrein system as it inactivates the vasodilator bradykinin (*Figure 3.1*) (Erdmann et al. 2008).

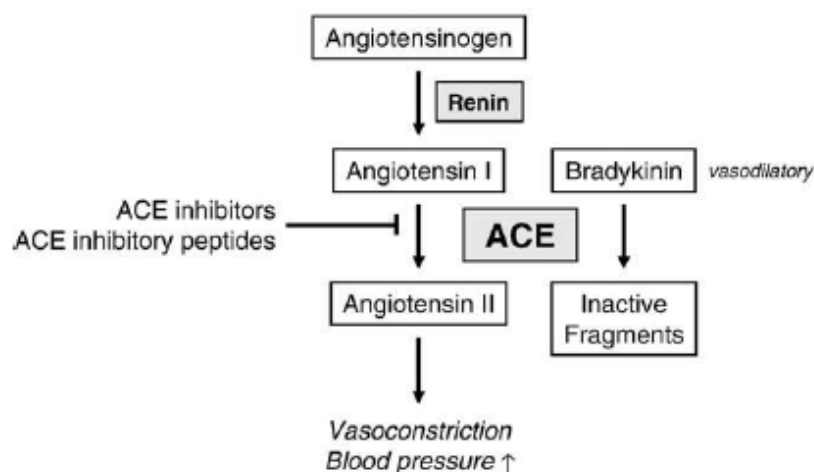


Figure 3.1: Renin-angiotensin system (Erdmann et al. 2008)

Based exclusively on the ability to inhibit ACE, a number of peptides with a capacity to lower blood pressure have been isolated from a variety of foods. The selection of potential antihypertensive peptides from food proteins is usually performed by *in vitro* ACE inhibition assay which allow the determination of IC₅₀ (concentration needed to inhibit 50% of the enzyme activity) that expresses peptide potency. Structure-activity data have indicated that C-terminal tripeptide residues play a predominant role in in competitive binding to ACE active site. It has been reported that inhibitors containing hydrophobic (aromatic or branched side chains) amino acid residues at each of the three C-terminal positions are better substrates for the enzyme. The most effective ACE inhibitory peptides identified contain Tyr, Phe, Trp and/or Pro at the C-terminal. It has also been observed that Leu and other branched chain aliphatic amino acids such as Ile and Val, the positive charge of Lys and Arg at C-terminal may contribute to increase ACE inhibitory potential (Hernández-Ledesma et al. 2011)

The antihypertensive effects can be assessed by *in vivo* experiments using spontaneously hypertensive rats (SHR), an accepted animal model to study human essential hypertension (Hernández-Ledesma et al. 2011). Due to the incomplete and unknown bioavailability of ACE inhibitory peptides after oral administration, it is difficult to predict the *in vivo* antihypertensive effects starting from *in vitro* analysis. Regarding this aspect, the structural properties are really important and it has been showed that di or tri-peptide (especially ones with proline or hydroxyproline residue at C-terminus) are usually more resistant to degradation by digestive enzyme (Erdmann, Cheung, and Schröder 2008; Matsufuji et al. 1994; Vermeirssen, Van Camp,

and Verstraete 2004). The comparison of results obtained from both tests, have evidenced that, in some cases, the correlation between the IC₅₀ value and the antihypertensive action of peptides *in vivo* is lacking. These discrepancies might arise from technical issues, such as the lack of standardization of the different methodologies employed or, more importantly, from physiological transformations that may occur *in vivo* which can influence the pharmacokinetic and pharmacodynamic properties of the peptides (Hernández-Ledesma et al. 2011; Marques et al. 2012).

To date, milk proteins are the main source of ACE-inhibitory peptides, although these bioactive compounds can also be obtained from muscle, ovalbumin, blood, and fish proteins. Plant sources include pea, garlic, rice, soybean, wheat and amaranth proteins (Hernández-Ledesma et al. 2011). Numerous studies have been performed in order to determine the antihypertensive effects of food-derived ACE inhibitors in spontaneously hypertensive rats (SHR) and hypertensive human volunteers after intravenous or oral administration (Erdmann et al. 2008). Results from these trials have revealed that these peptides had little or no effect on blood pressure of normotensive subjects, suggesting that the peptides exert no acute hypotensive effects. Thus, ACE inhibitory peptides might be applied in the initial treatment in mild hypertensive subjects or as a supplemental treatment (Erdmann et al. 2008) eliminating all the associated side effects reported for the synthetic ACE inhibitors (FitzGerald et al. 2004).

The most studied antihypertensive peptides are derived from milk proteins of which the most significant are Ile-Pro-Pro (IPP) and Val-Pro-Pro (VPP) that demonstrated, in animal studies, the ability to attenuate hypertension development after long-term consumption. Their antihypertensive effect was ascribed not only to ACE inhibition but also to the effect on vascular and endothelial function as evidenced by the increased expression of cyclooxygenase (COX) and endothelial nitric oxide synthase (eNOS) gene and the decrease in the expression of NF- κ B subunit. In line with these observations it was found that vasorelaxation of the mesenteric artery of SHR was abolished when COX and eNOS were blocked; moreover, gene expression analyses of the aorta of treated animals, revealed a down regulation of several genes linked to inflammatory processes (chemokine receptor, cell adhesion molecules and interleukin receptor) indicating another possible mechanism of action for antihypertensive effect of lactotriptides, since chronic inflammation is extremely important in the pathogenesis of hypertension and vascular diseases (Marques C c et al 2012).

Commercial fermented milk products and hydrolysates containing peptides IPP and VPP have the most substantiated antihypertensive activity in humans subjects with high-normal blood pressure and mild hypertension (Hernández-Ledesma et al. 2011). Some clinical trials have been performed with consumption of products containing lactotriptides associated with reduction of blood pressure. This evidence showed that IPP and VPP might be used as an additional tool for prevention and treatment of hypertension. Despite some controversial results comparing with placebo group, these evidence might be considered for investigations involving pre-hypertensive patients, where a diet modification could be the first therapeutic approach (Marques et al. 2012)

Other milk peptides RYLGY and AYFYPEL (obtained from enzymatic hydrolysis of bovine casein) have demonstrated the ability to reduce systolic blood pressure in SHR and left ventricular hypertrophy often caused by hypertension, and to cause greater relaxation to acetylcholine in aortic and mesenteric arteries from treated animals. These results suggest an improvement of endothelial function that can be explained by the increased expression of eNOS in the aorta of treated animals, suggesting other mechanism for the antihypertensive effect. In fact, despite the low IC₅₀ observed *in vitro* (0.71 and 6.58 μ M for RYLGY and AYFYPEL, respectively), an increased plasma ACE activity was observed in treated group. Moreover, the high radical scavenging capacity of both peptides could represent an alternative mechanism that has to be considered since oxidative stress is strictly linked to cardiovascular pathologies. Besides, it has been observed that an antioxidant-rich diet also cause significant reductions in SBP of SHR (Marques C. 2011).

Furthermore, grains products, important sources of micronutrients and fibers, also demonstrated to be a source of bioactive peptides exerting an antihypertensive effect (Aoyagi 2006; Rizzello et al. 2008; Malaguti et al. 2014). Matsui et al., (Matsui et al. 1999) discovered a tripeptide IVP with ACE-inhibitory activity from wheat germ hydrolyzed with alkaline protease, while Motoi et al., identified the tripeptide IAP in gliadin hydrolysed with acid protease with similar effect (Motoi & Kodama 2003). Some other small peptide were also identified from corn α -Zein, amaranth and legumes (Silva-Sánchez et al. 2008; Li & Aluko 2010; Malaguti et al. 2014). In addition, some peptides from legumes protein showed an hypotensive effect. Glu-Phe, Lys-Phe and Ile-Arg are dipeptides from pea protein digestion, which exerted an ACE-inhibitory activity (Li & Aluko 2010). In another study Wu et al (Wu et al. 2006) not only demonstrated the potent ACE-inhibitory activity of these two peptides, but also predicted a sequence of some dipeptides with theoretical ACE-inhibitory activity (Malaguti et al. 2014).

3.4.3 Anti-inflammatory properties

Inflammation is characterized by increased endothelial permeability, leakage of protein-rich exudates and infiltration of leukocytes in extravascular tissues; inflammation is essential for resistance to microbial infections and wound healing, but an excessive and uncontrolled inflammatory process may lead to chronic disease such as CVD. The main therapy for inflammation consists in non steroidal anti-inflammatory drugs (NSAIDs), which are widely used to prevent and manage cardiovascular diseases. However, the presence of side effects like gastric bleeding preclude a long-term use of NSAIDs (Chakrabarti et al. 2014). For this reason the research in nutrition is engaged in the characterization of the biological activities of peptides and other nutraceuticals to consolidate their beneficial role in cardiovascular diseases prevention.

Vascular endothelium plays a pivotal role in inflammation, as it represents the main gate for extravasation of leukocytes. The generation of mediator of inflammation produces an increase expression of adhesion molecules-with the consequent recruiting, rolling, activation and finally transmigration of leucocytes across the endothelial barrier. Moreover, mediators like TNF- α , IL-1 β , chemokines and ROS are involved in the generation and propagation of inflammatory response through the activation of signaling pathways such as NF- κ B and MAPK (mitogen activated protein kinase) (Chakrabarti et al. 2014).

Among the bioactive peptides, the tripeptides VPP and IPP, derived from bacterial fermentation of casein demonstrated a potential vasodilatory action in HUVECs via stimulation of NO and bradikinin pathways in a dose dependent manner (Hirota et al. 2011). Egg is another dietary source for many bioactive peptides and a study conducted by Mine et al (Mine 2007) is been demonstrated that generation of a tripeptide (IRW) from ovotransferrin is responsible for a downregulation of cytokines-induced inflammatory responses, mainly via modulation of NF- κ B (Mine 2007; Majumder et al. 2013). Concerning plant peptides, evidence confirm that they can modulate inflammatory processes. Dia et al., (Dia et al. 2009) using a monoclonal lunasin antibody, purified three peptides from soybean flour and investigated the anti-inflammatory activity of lunasin . The pre-treatment of a macrophages cell lines with 100 μ M lunasin before the induction of stress with LPS resulted in the inhibition of pro-inflammatory biomarkers such as IL-6, IL-1 β , NF- κ B activation and COX-2 and iNOS expression; the supposed mechanism of

action is the inhibition of the p65 and p50 Nf- κ B subunits translocation. In the same experimental model Hernandez-ledesma et al. (Hernández-Ledesma & de Lumen 2008) observed that lunasin was able to inhibit IL-6 and TNF- α release. The encouraging findings in cell-based studies brought the research in bioactive peptides and protein hydrolysates in animal model of human diseases. A summary of all the potential anti-inflammatory mechanism in which peptides might be involved is reported in *Figure 3.2*.

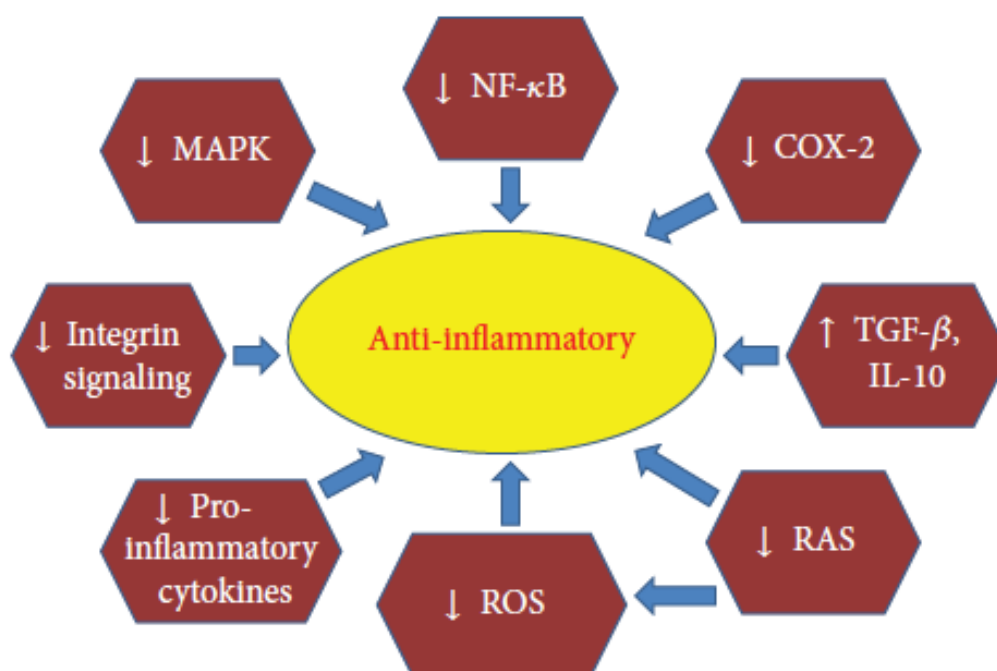


Figure 3.2: Potential mechanism of actions of anti-inflammatory bioactive peptides and peptide-rich protein hydrolysates (Chakrabarti et al. 2014)

3.4.4: Antioxidant Properties

Free radical are generated through normal reactions during respiration in aerobic organism (Sarmadi & Ismail 2010). The ROS Physiologically produced have various functions; however , their increase and/or any excessive amount due to over-production or caused from an impaired cell antioxidant capacity, can be harmful to cell causing damage to main cellular components (nucleic acids, proteins and lipids). In experimental and human hypertension studies, it has been

demonstrated an increased production of superoxide anion and hydrogen peroxide and a decrease NO synthesis. In fact, an excess of ROS is responsible for a reduction of NO bioavailability, due to the reaction of NO and O_2^- to form peroxynitrite ($ONOO^-$), with a consequent impaired vasodilation (Chakrabarti et al. 2014). Moreover, the oxidative modification of LDL can increase their atherogenicity. Therefore, the interest in potential antioxidant activity of numerous food proteins and bioactive peptides in the treatment of several vascular diseases has increased. Although the exact mechanism by which they display antioxidant activity is still not fully understood various activity were proposed including inhibition of lipid peroxidation (Moure et al. 2006; Qian et al. 2008), ROS scavenging (Rajapakse et al. 2005) and metal ions chelation. Erdmann et al., also found that dipeptide Met-Tyr from sardine muscle induced HO-1 and ferritin expression in endothelial cells (Erdmann et al. 2006). Moreover, it has been observed that leaf protein can enhance antioxidant enzyme activity (SOD and GSH-Px) reducing lipid peroxidation by-products levels in vivo (Fu et al 2003)

Antioxidant activity of peptide seems to be related to composition, structure and hydrophobicity (Chen et al. 1998; Sarmadi & Ismail 2010). For example Tyr, Trp, Met, Lys, Cys, amino acids with aromatic residues improves the radical scavenging-properties. In addition. Moreover, the correct positioning in peptide sequence plays a very important role. Val and Leu, instead, exert strong antioxidant properties when situated at the N-terminus and, on the contrary Tyr and Trp when situated at the C-terminus (Medina-Godoy et al. 2012). In a study designed by Chen et al. (Chen et al. 1996) the analysis of 28 different peptides based on the structure of the antioxidative peptide conglycinin (Leu-Leu-Pro-His-His), revealed that the Pro-His-His sequence had the greatest antioxidant activity among all the tested peptides. Other studies as well (Saito et al. 2003) confirmed that tripeptides arrangement results in a good antioxidant activity. Legumes and grain peptides have also confirmed the antioxidant activity. Jong et al. demonstrated the lunasin antioxidant properties via chelation of Fe^{2+} ions (J. B. Jeong et al. 2010), García-Nebot et al, instead, investigated its scavenging activity both against peroxy and superoxide radical (García-Nebot et al. 2014). moreover Lunasin, at physiological concentration (0.5-25 μ M), decreased intracellular ROS levels in Caco-2 cells treated with hydrogen peroxide (Yin et al. 2014).

3.5 Conclusions

Bioactive peptides have been demonstrated to possess different properties, which may positively affect cardiovascular health, such as the ability to reduce blood pressure (ACE-inhibitory activity) , lipid levels, and free radical formations as well as the anti-inflammatory activity. A full screen of all these peptides activity is shown in *Figure 3.3*.

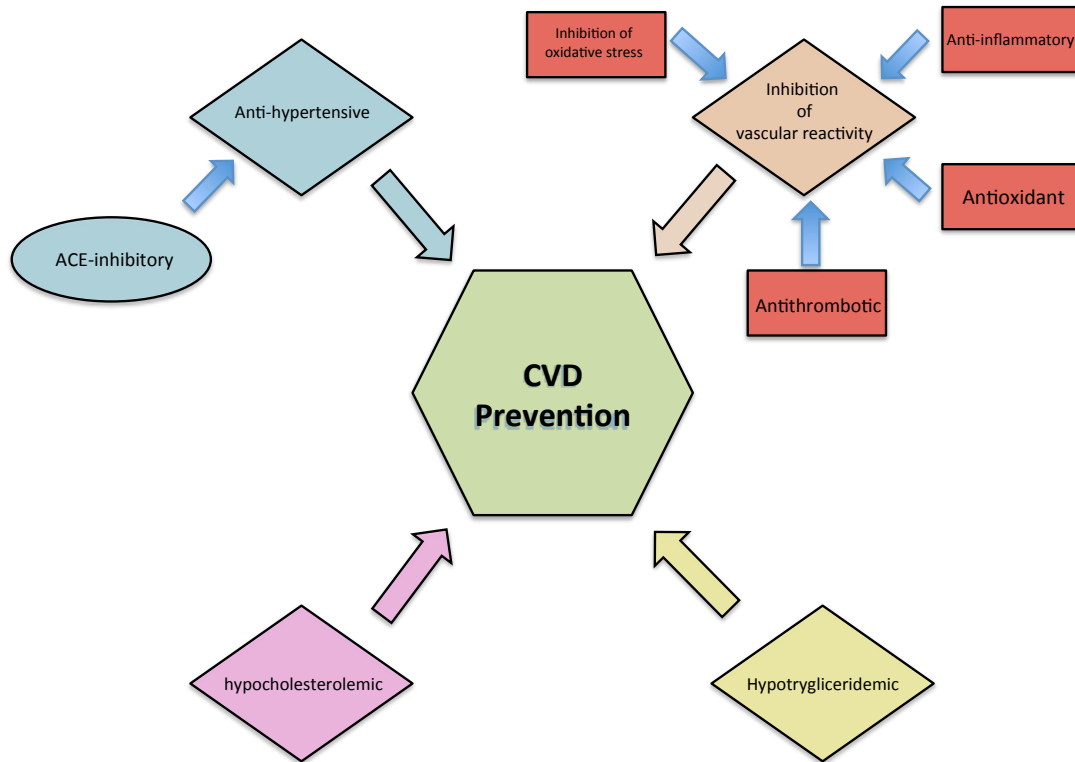


Figure 3.3: Regulatory pathways of CVD potentially modulated by bioactive peptides

Since cardiovascular diseases represent public health issue worldwide, especially in western countries, bioactive peptides may have a crucial role in maintaining and potentially increasing the worldwide health. Many peptides and small proteins showed important biological actions also in prevention of chronic/degenerative diseases (Malaguti et al. 2014). The number of products available in our markets containing bioactive peptides is constantly increasing, however, nowadays the most relevant effects of peptides were only observed *in vitro* or in animal studies. The number of clinical human studies supporting nutraceutical properties of bioactive peptides is still very limited and it is really difficult to quantify the optimal plasma levels of bioactive peptides. Moreover, processes like digestion can modify peptides and convert

them from a bioactive form into an inactive one and *vice versa*. Therefore, further researches on human clinical trials are needed in the future to clarify protective, preventive and potential therapeutic role of peptides in human.

CHAPTER 4: Polyphenols

Polyphenols are a complex group of molecules naturally present in plants. Their family consists of over 5000 organic molecules whose structural characteristic is to have one or more aromatic with more than one hydroxyl group in their structure (*Figure 4.1*).

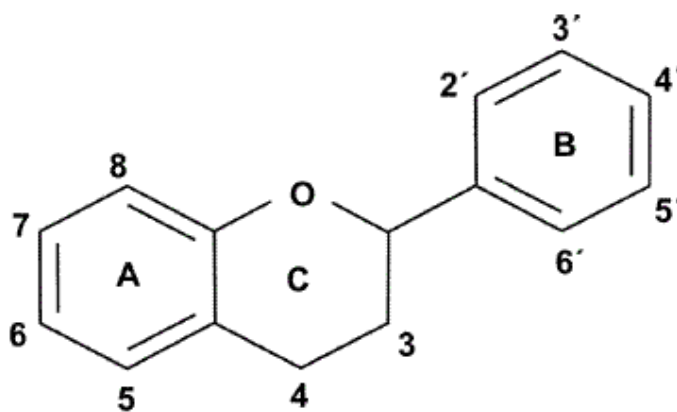


Figure 4.1: General structure of Polyphenols

They represent plant secondary metabolites and their functions is to enhance plants defense to biotic (e.g. pathogens growth and survival inhibition protection against herbivores) and environmental stress (e.g. protection against UV light and oxidative stress) (Scalbert & Williamson 2000; Parr & Bolwell 2000). The interest in researching the link between dietary polyphenols and human health followed the results of epidemiological studies which highlighted an inverse association between the intake of polyphenols rich-food and the incidence of various chronic degenerative diseases such as cardiovascular disease, diabetes and cancer (Hertog et al. 1993). The first property ascribed to polyphenols was the antioxidant activity: this capacity is closely related to their chemical structure; for example, the phenols with two hydroxyl groups in position "ortho" on the aromatic residue are better "scavengers" than those with a single hydroxyl group. In general, all polyphenols are reducing agents and, in addition to scavenge free radicals, participate in the regeneration of other antioxidants such as vitamin E (Jovanovic & Simic 2000; Rice-Evans et al. 1996).

Epidemiological studies have shown that polyphenols are able to slow or even inhibit the cellular damage, including endothelial dysfunction. Biological activities are often tested on cell cultures

or isolated tissues using polyphenols in the form in which they are found in food, without taking into account the fact that the polyphenols are widely metabolized from both tissues and microbiota. So it is important to identify and determine the biological properties of both parental compounds and metabolites in both in vivo and in vitro conditions. (Kuiper et al. 1998; D'Archivio et al. 2007; Divisi et al. 2006).

4.1 Polyphenols Classification

Phenolic compounds can be divided into several sub-groups according their structural characteristics. However, those commonly found in plant food can be categorized into two main sub-groups: flavonoids and non-flavonoids. Flavonoids group may be further divided in flavons, isoflvaons, flavonols, flavanols and anthocianins; the non-flavonoids group includes phenolic acids, stilbenes a lignans. About 30% of the free or bound forms of dietary phenolic in plants are phenolic acids, which can be further divided in benzoic acids and cinnamic acids, based on the C1-C6 or C3-C6 backbone (Zhang & Tsao 2016).

Flavonoids have a common carbon skeleton of diphenyl propane and two benzene rings joined by a linear three-carbon chain. The central three-carbon chain may form a closed pyran ring with one of the benzene rings. Flavonoids are furthermore divided into 6 subclasses depending on the oxidation state of the central pyran ring (D'Archivio et al. 2007b).

In *figure 4.2* a general overview of polyphenols is shown together with their presence in food.

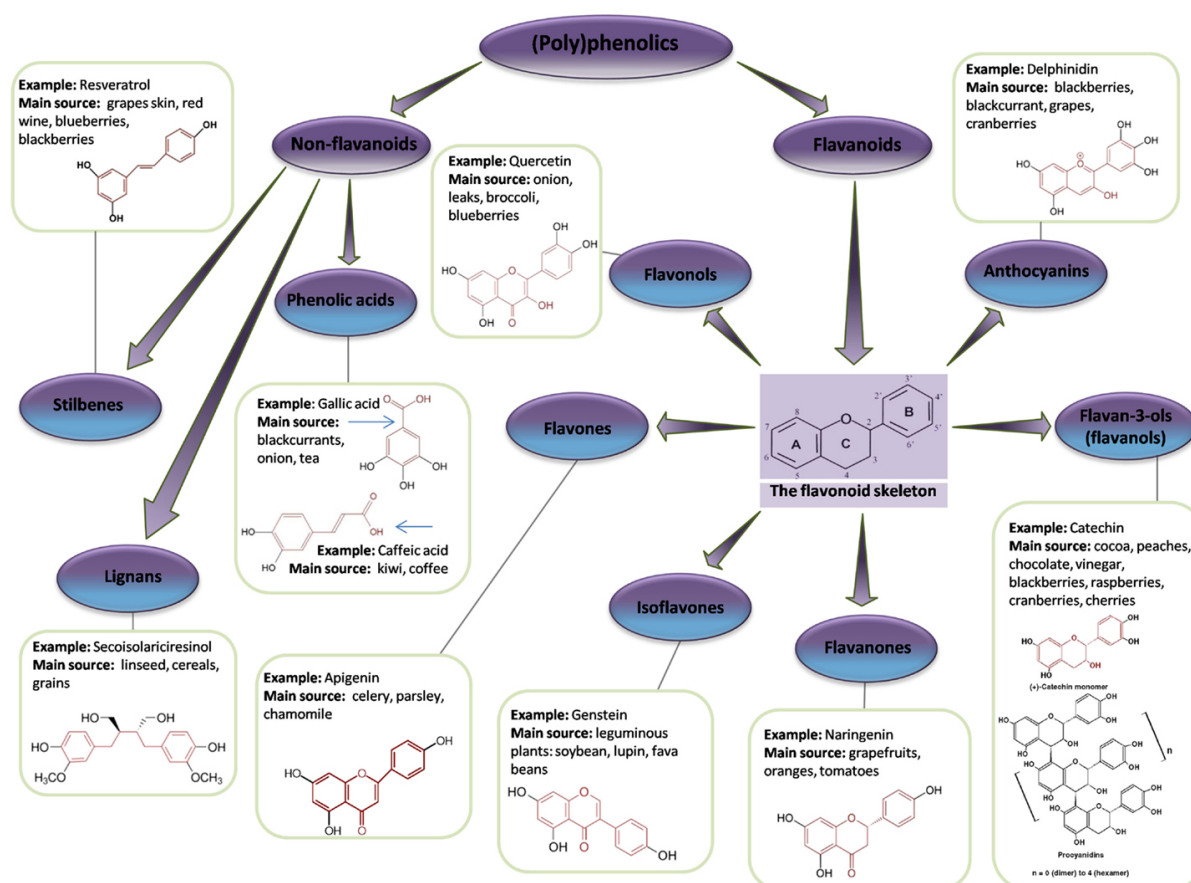


Figure 4.2: Polyphenols classification overview: structure and presence in food. (Goszcz et al. 2015)

4.2 Dietary intake of Polyphenols

4.2.1 Food Content of Polyphenols

The most important sources of polyphenols are generally vegetables and fruits together with beverage such as red wine, green and black tea, coffee, chocolate and extra virgin olive oil. They represent polyphenols rich-food, which are consumed in large quantities. Spices, herbs and nuts represent other rich sources, but their relevance depend on culinary habits (Visioli et al. 2011). Some polyphenols like quercetin are present almost in all plant products (fruits, vegetables, cereals and legumes, tea, infusion etc), but some others are very specific for particular foods (ex.

flavanones in citrus fruits). Moreover, food contain mostly a mixture of polyphenols which may be present both as aglycones or glycosylated forms (Manach et al. 2004). In addition, for a large quantity of products, the polyphenols characterization is definitely less known and some foods such as exotic fruits and some cereals have not been studied yet. Furthermore, numerous factors may affect plant polyphenol content; these include environmental factors, plant variety, ripeness at harvesting, food processing, cooking and storage, (Manach et al. 2004).

Environmental factors have probably the major influence on polyphenol content and may be pedoclimatic or agronomic. Sunlight exposure has the most relevant effect on flavonoids and also the degree of ripeness has a remarkable effect on polyphenols concentration (Anon n.d.; Manach et al. 2004). Polyphenols, especially phenolic acids have a role in plant response to stress and it has been observed that sustainable culture may increase their content with respect to vegetables grown without stress (Manach et al. 2004). Storage has also a remarkable effect on polyphenols content; in fact, polyphenols may be rapidly oxidized, resulting in formation of polymerized substances, which bring to a change of food color and organoleptic properties (Manach et al. 2004).

Culinary preparation and industrial processing can affect polyphenol content: the simple peeling of fruit and vegetable removes a relevant portion of polyphenols. In fruit juices production, the clarification step is aimed at removing some polyphenols responsible for discoloration. Crozier et al., (Crozier et al. 1997) demonstrated that onions and tomatoes lose around 80% of the initial quercetin content after 15 min boiling, 65% after cooking in microwave and 30% after frying. On the contrary maceration helps the diffusion of polyphenols in juice as happens in vinification of red wine resulting in a final concentration of polyphenols 10 times higher in red wine than white wine and even higher than the grape juice (Vinson & Hontz 1995; Abu-Amsha et al. 1996; Manach et al. 2004).

To date, two databases on polyphenol contents have been developed. The first one from the USDA and the more recent Phenol- Explorer, which is more comprehensive and include more than 35000 values related to 500 different polyphenols from all classes and their content in 400 different food sources.

4.2.2: Polyphenols in Diet

The estimation polyphenol intake with the diet is complicated by different factors: the complexity of polyphenols into food sources, their presence in a wide variety of food, the numerous factors that may affect their content in foods, together with the methods used for dietary assessment (food frequency questionnaires, 24-h dietary recall, food diaries), the limited understanding of the metabolic fate of individual polyphenols and the inter-subject variability which may affect the way polyphenol are metabolized. Moreover, eating habits in different countries must be considered. Consumption of polyphenols has been estimated around 20-25 mg/die in USA while in Holland and Denmark is in the order of 67-69 mg/die. In Italy this range might be higher (from 5 to 125 mg/die with a mean value of 35 mg/die) (Manach et al. 2004). The major dietary sources flavonones are represented by citrus fruits which are mainly consumed in the areas of production such as southern Europe. Accordingly, in Scandinavia and Northern Europe, consumption of berries is relevant and the average anthocyanins intake is 82 mg/die with maximum intake up to 200 mg/die. Soy is, instead, the main source of polyphenols in Asian countries with 10-35 g/die. This means an intake of isoflavones of 25-40 mg/die with a maximum intake of 100 mg/die (Adlercreutz et al. 1991; Kimira et al. 1998). Obviously, Europeans and Americans eat very low amount of soy and assume very few mg of isoflavones per day (Manach et al. 2004). Consumption of hydroxycinnamic acids is related with coffee consumption; people who drinks numerous cups per day may ingest up to 500-800 mg of hydroxycinnamic acids, while people who do not drink coffee and eat low quantity of fruit and vegetables don't reach 25 mg/die (Clifford 2000). Total polyphenol intake per day has been estimated to be about 1g/die in people who eat several servings of fruit and or vegetables (Manach et al. 2004).

4.3. Bioavailability, metabolism, uptake and excretion of polyphenols

4.3.1: Absorption and Bioavailability

The chemical structure, together with pH, solubility, hydrophilic/lipophilic balance and electrons configuration of polyphenols, regulate the efficiency of absorption and the nature of plasma metabolites (D'Archivio et al. 2007a). In general the aglycones are absorbed from the small intestine, but the majority of polyphenols are present in foods in form of esters, glycosides (linked to one or more sugars) or polymers forms. For this reason these compounds must be hydrolyzed by intestinal enzymes or by gut microbiota during intestinal absorption. and then in the liver polyphenols are conjugated by methylation, sulfation and glucuronidation (Manach et al. 2004). Conjugation is so effective that aglycones are almost absent in blood or, at least, present in very low quantity. Anthocyanins are the only glycosides that pass in an intact form through the intestinal barrier and can be found in plasma, this due to the stability of the glycosylated forms, or for the presence of specific uptake mechanisms for these compounds (D'Archivio et al. 2007b). Hydroxinnamic acids, instead, cross the intestinal barrier via transporters localized on the membrane of the epithelial cells, which are the carriers of monocarboxylic acids (MCT) (Scalbert & Williamson 2000). Furthermore, fractions of polyphenols can be absorbed via passive diffusion, like acetylated compounds such as flavanols: epicatechin and gallic acid, which cross biological membranes without undergoing hydrolysis processes or deconjugation (Scalbert & Williamson 2000).

A direct evidence of polyphenols absorption and bioavailability is obtained by measuring their plasma and urine concentration after ingestion and the quantification, identification of metabolites represent probably the most important field of research concerning polyphenols.

Bioavailability of a given compound is more important than its total content in a specific food or dietary supplements (D'Archivio et al. 2007a). This is particularly valid for polyphenols and it is really important to realize that the most common polyphenols in the diet are not necessarily the most bioactive. This is due to multiple reasons such as low intrinsic activity, poor absorption, high metabolism or rapid elimination (Manach et al. 2004). It has been also observed a strong inter-individual variability in the amount of active circulating metabolites, probably due to polymorphisms of the phase II enzymes, but also to the different composition of the gut microbiota. The time to reach the maximum blood peak is different from species to species and

depends on the state of glycosylation. Isoflavones are the ones that demonstrate the highest bioavailability among all classes of flavonoids; in this case the bioavailability does not change between aglycones and glycosides although the absorption of both forms is very slow since it occurs at the colon level. Generally, plasma concentration of the intact flavonoids rarely exceeds the μM range and the maintenance of high plasma concentration requires repeated administrations (Adlercreutz et al. 1991).

4.3.2: Metabolism

Polyphenols metabolism begins to be unraveled and follows the same rule of drugs metabolism. Metabolism initiates at small intestine level; however, the major part of polyphenols are in esters, glycosides or polymers forms and must be hydrolyzed by intestinal enzymes or colon microbiota (D'Archivio et al. 2007b). Essentially there are two kind of enzymes: Phase I enzymes add some functional groups to the molecule (C=O, -OH etc) or catalyze redox reactions. Phase II enzymes catalyze conjugation reactions adding whole molecules or parts to the polyphenol structure. This conjugation process mainly occur in the liver and includes methylation, sulfation and glucuronidation reactions and represent the common metabolic detoxification process for many xenobiotics which facilitates biliary and urinary elimination (D'Archivio et al. 2007) basically increasing their hydrophilicity.

Among these enzymes catechol-O-methyl transferase (COMT) catalyzes the transfer of a methyl group from S-adenosyl-l-methionine, generally in position C3 or C4-positions (Wu et al. 2002). This enzyme is highly active in liver and kidney but it is also present in other tissues (D'Archivio et al. 2007b).

UDP-Glucuronyl transferases is a membrane-bound enzyme and is normally located in endoplasmic reticulum in various tissues. This enzyme catalyze the transfer of glucuronic acid from UDP-glucuronic acid to polyphenols, steroids and many other dietary constituents. The glucuronidation normally occur in intestine and liver at C3 position (D'Archivio et al. 2007).

Sulfotransferases catalyze, instead, the transfer of a sulfate group from 3'-phosphoadenosine-5'-phosphosulfate to a hydroxyl group. The sulfation occur mainly in the liver even if the position of sulfation for polyphenols has not been identified yet (D'Archivio et al. 2007)

In general most of the ingested dose of polyphenols undergo conjugation processes, in fact, that very low amount of free aglycones circulates in plasma after ingestion. It is actually really important to identify the circulating metabolites as well as their conjugation sites and the nature and number of conjugating groups because it could also affect their biological properties. Moreover, circulating metabolites may bind to proteins in blood stream and the primary proteins for this binding is albumin. This binding is important because it may have consequences for the clearance or tissue delivery of these metabolites; this situation is still partially unclear but it seems that only the free form of a polyphenols may be used by the cells (D'Archivio et al. 2007; Dufour et al. 2007).

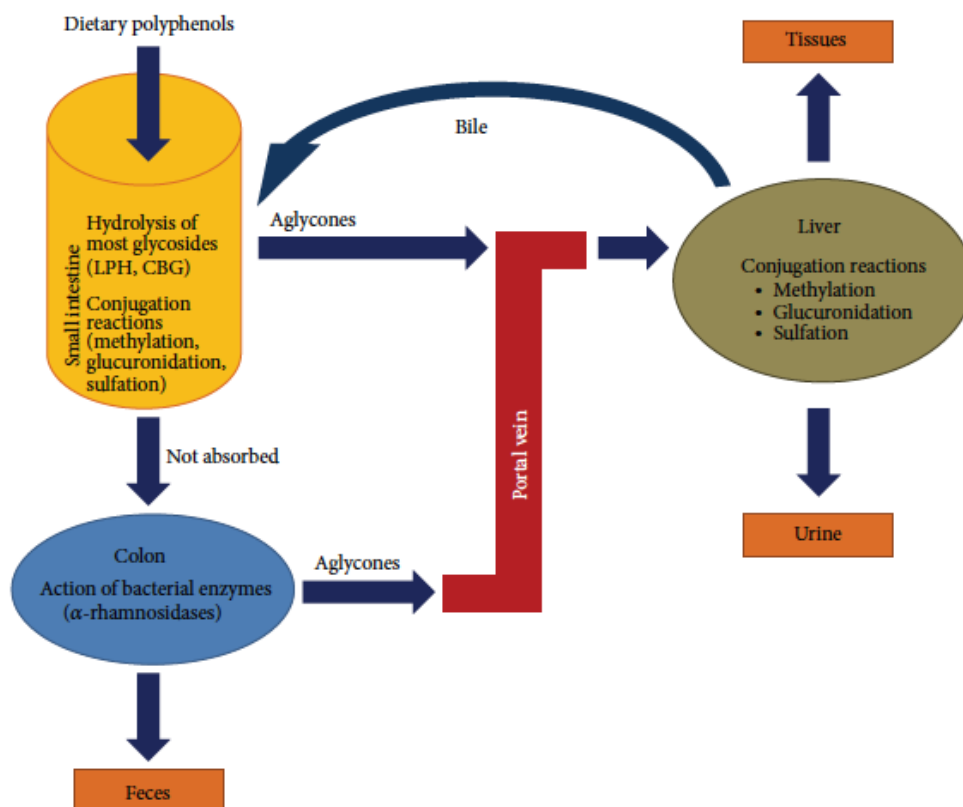


Figure 4.3: Absorption and metabolism for dietary polyphenols and their derivatives in human. (Marín et al. 2015).

4.3.3: Plasma concentrations

The plasma concentrations reached after ingestion of polyphenols-rich foods vary in relation with the nature of polyphenol and food source (D'Archivio et al. 2007b). After taking 80-100 mg of quercetin, contained in apples or onions, plasma levels are in the order of 0.3-0.75 $\mu\text{mol} / \text{L}$; ingested in the form of green tea (0.1 to 0.7 $\mu\text{mol}/\text{L}$ for an intake of 90-150 mg), cocoa (from 0.25 to 0.7 $\mu\text{mol}/\text{L}$ to an intake of 70-165 mg) and red wine (0,09 mmol/L to an intake of 35 mg). In contrast, the plasma concentrations of anthocyanins are very low: the peak concentration which is between 30 min and 2 h after consumption, is in the order of few nanomoles per liter for an intake of about 110 -120 mg of anthocyanins (Manach et al. 2004). However the metabolism is so rapid that maintaining high concentrations of polyphenols requires repeated administration over time. The maximum concentration, in fact, are reached within 2 h after ingestion for the majority of polyphenols (Manach et al. 2004)

4.3.4: Tissue Uptake

Determination of bioavailability of polyphenols metabolites in tissues is much more important than its knowledge of plasma concentrations (Manach et al. 2004). Polyphenols, in fact, can penetrate tissues, particularly liver and intestine in which they are metabolized. However is not fully understood yet why polyphenols accumulate in specific target tissues (Manach et al. 2004). Some studies indicated the presence of specific mechanism to incorporate polyphenols. For example, the endothelium is probably the main site of action for flavonoids and Schramm et al (Schramm et al. 1999)demonstrated the presence of a rapid energy-dependent transport system in HAEC for morin's uptake; this system may be also responsible for phenolic compounds transport ; Manach et al. 2004). In a study conducted in rats or mice administered with single doses of radiolabeled polyphenols (quercetin, epigallocatechin, quercetin-4-glycoside and resveratrol) and sacrificed 1-6 h later, the radioactivity was mainly recovered in stomach, intestine and liver (Manach et al. 2004; Vitrac et al. 2003;), but polyphenols have been detected by HPLC method in a large list of tissues in mice and rat, including brain, ECs, heart, kidney, etc (Abd El Mohsen et al. 2002; Manach et al. 2004; Datla et al. 2001). By now very few studies reported data on polyphenols concentration both in human and animals tissues. In two different studies phytoestrogens and tea polyphenols were measured in prostate tissue. In the first study prostatic

concentrations of genistein in men with benign prostatic hyperplasia were lower than in those with a normal prostate, whereas plasma concentrations were higher in men with benign prostate hyperplasia (Hong et al. 2002; D'Archivio et al. 2007a). In the second study it has been shown that tea polyphenols are available in human prostate after a daily consumption of 1.42 L of green or black tea for 5 days. In prostate tissue epigallocatechin, epicatechin and epigallocatechin gallate reached concentration from 21 to 107 pmol/g tissue (Henning et al. 2006; D'Archivio et al. 2007a). These few study displayed a non-direct correlation between plasma concentrations and target tissues and that distribution between blood and tissue changes between various polyphenols.

4.3.5: Excretion

Metabolites of polyphenols follow two pathways of elimination: biliary route and urinary route. In general, large conjugated metabolites are likely eliminated by bile while small conjugates are excreted by urine (Manach et al. 2004). Biliary excretions is the main pathways of elimination of genistein, epigallocatechin gallate and eriodictyol (Sfakianos et al. 1997; Kohri et al. 2001; Manach et al. 2004). Moreover, intestinal bacteria have also the possibility to release free aglycones from conjugated metabolites secreted in bile thanks to β -glucuronidases: in this way aglycones are reabsorbed and constitute an enterohepatic cycling. Studies about pharmacokinetics in rats have shown a second plasma peak concentration around 7 h after genistein intake, which is explicable with enterohepatic circulation (Coldham & Sauer 2000; Manach et al. 2004). A second plasma peak was also observed in human studies with isoflavones 10-12 h after ingestion of soy. Urinary excretion has been deeply investigated in human studies and the amount of metabolites excreted is approximately correlated with plasma concentration. Some polyphenols like flavonones from citrus fruit demonstrated high urine elimination (4-30% of intake). For flavanols urinary excretion account 0,3-1.4% of intake and for catechins 0.5-6% of ingested those; however, the urinary excretion percentage may be very low for other polyphenols such as anthocyanins (0.005-0.1% of intake). Low values of urinary eliminations might be translated with a pronounced biliary excretion or extensive metabolism. In addition, even if rarely calculated, the half-lives of polyphenols is in the order of 2 h with some specific exceptions. For this reason the maintenance of high plasma concentration of polyphenols requires regular and frequent consumption of fruit and plant products, otherwise plasma concentration fluctuates and there is no final accumulation (Manach et al. 2004)

4.4 Biological effects of Polyphenols

4.4.1: Beneficial effects of polyphenols on CVD

Phenolic compounds in the last decades have shown to be able to exert beneficial effects and to reduce the incidence of vascular diseases (Quiñones et al. 2013; Schroeter et al. 2006; Perez-Vizcaino et al.). Since the link between oxidative stress and cardiovascular disease has been deeply investigated, the first hypothesis of beneficial effects of polyphenols implied their direct antioxidant activity due to their capacity lower ROS levels by different mechanism including the direct interaction with ROS/RNS, the chelation of metal ions, such as iron and copper, which suppress radical generation through Fenton reaction and the inhibition of ROS generating enzymes and NADPH oxidase such as xanthine oxidase (XO) antioxidant enzymes (Quiñones et al. 2013). These effects are dependent on polyphenol structural features such as the number and position of hydroxyl and catechol groups (Quideau et al. 2011). Nevertheless, low polyphenols bioavailability (nM range) pose a debate regarding their direct antioxidant activity in the *in vivo* situation.

On the basis of recent studies the arising concept is that polyphenols, by influencing transcriptional activity of nuclear transcription factor erythroid-2 related factor-2 (Nrf2), are able to induce several enzymes part of the endogenous antioxidant defense system, therefore exerting indirect antioxidant activity (Barbagallo et al. 2012; Zhu et al. 2005). The expression of cytoprotective proteins, coordinated by indirect antioxidants, provide the potential for more efficient and longer lasting up-regulation of antioxidant properties and cell protection

The ability to neutralize free radicals has also a positive effect on the formation of oxidized LDL, which plays a primary role in the onset and progression of atherosclerosis. Some studies have shown that polyphenols are able to bind to the lipids in the serum, and then reduce their oxidation (Adlercreutz et al. 1991). Different beneficial effects to cardiovascular health have been observed for polyphenols including anti-inflammatory, antiapoptotic, antithrombotic and vasodilator activity. These effects could potentially results from polyphenols antioxidant action and from their actions on different targets and mechanisms (Quiñones et al. 2013) (*Figure 4.4*).

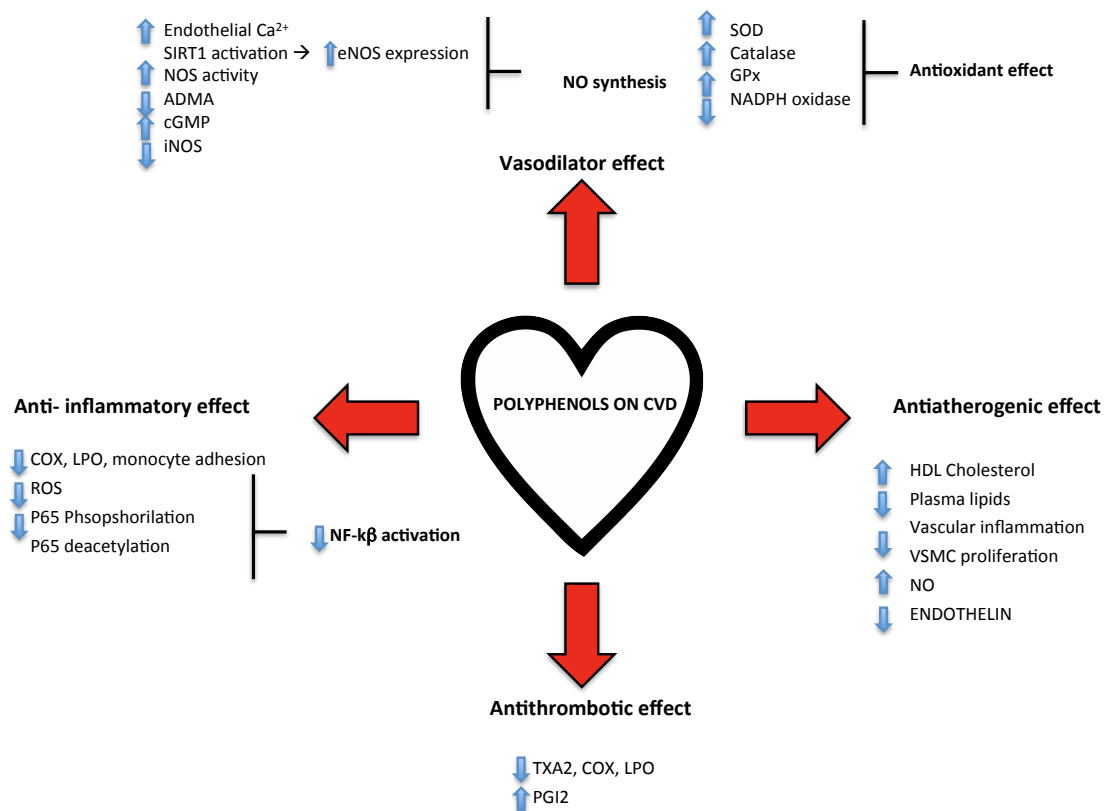


Figure 4.4: beneficial effects of Polyphenols on CVD

VASODILATION

The antioxidant effect exerted by polyphenols through O_2^- radical neutralization can improve also NO bioavailability and consequently have a positive effect on vasodilation; O_2^- is, in fact, responsible for the destruction of NO (Quiñones et al. 2013). Most of the observed effects of polyphenols on vasodilation imply an increased NO production and an increase of cyclic guanosine monophosphate (cGMP) in vascular smooth muscle cells and different mechanisms have been involved. It has been shown that resveratrol and the anthocyanin delphinidin can modulate NO production in endothelial cells by means of increased intracellular calcium concentrations (Martin & Andriantsitohaina 2002; Quiñones et al. 2013) and similar effect was reported for quercetin and it has been observed that a quercetin-rich diet can increase endothelial NO synthase (eNOS) activity. Other authors have observed that some polyphenols modulate

eNOS expression at transcriptional level, while at the same time inhibiting the inducible (iNOS) gene expression (Kim et al. 2008; Ndiaye et al. 2005) and It has also been recently reported that polyphenolic compounds, especially those found in red wine, can inhibit phosphodiesterases (PDEs), which are responsible for cGMP degradation (Behr et al. 2009). In addition, resveratrol can increase eNOS activity by decreasing intracellular levels of asymmetric dimethylarginine (ADMA), an endogenous eNOS inhibitor, and by stimulating eNOS phosphorylation, an effect mediated by the estrogen receptor ER α and a signaling pathway involving the α subunit of G-protein, caveolin-1 and different kinases (Dell'Agli et al. 2005; Quiñones et al. 2013; Park et al. 2012)

ANTI-INFLAMMATORY EFFECT

Inflammation-generated oxidative stress increases the expression of cyclooxygenase (COX) and Lipooxygenase (LPO), which are involved in interleukins and chemokines release (Quiñones et al. 2013). Many polyphenols, particularly quercetin, has been shown to inhibit COX and LPO (Ridker et al. 2004; Quiñones et al. 2013). Also, a moderate wine consumption is related to reduction of monocyte adhesion on endothelial cells and thus with regulation of adhesion molecules on monocytes' surface (Mellor et al. 2010; Quiñones et al. 2013). In fact, the anti-inflammatory effects exerted by dietary polyphenols can be mediated through the influence on transcriptional factors or signaling pathways that affect inflammatory gene expression, promote anti-inflammatory mediators, and NO production thus hindering the vascular-immune cellular interplay and limiting endothelial dysfunction. Dietary polyphenols down-regulate the expression of adhesion molecules, such as E-selectin, (ICAM-1) and (VCAM-1), by the endothelium and limit the recruitment of circulating inflammatory cells and their migration into the sub-endothelial space. Polyphenols also has been shown to reduce pro-inflammatory cytokine secretion (IL-6 and TNF- α). Central in controlling chronic inflammatory diseases are the activation of nuclear factor kappa beta (NF- κ B), a transcription factor that modulate pro-inflammatory genes expression thus representing the major molecular target for anti-inflammatory strategies. NF- κ B family proteins include several members (p50, p52, p65, RelB and cRel) and are found in the cytoplasm in an inactive state bound to inhibitory molecules of κ B (IkB) (including IkB-a, IkB-b, IkB-1, IkB-z, p100, p105 and Bcl-3). Under pro-inflammatory stimulation IkB is phosphorylated by activated kinases and degraded allowing NF- κ B nuclear

translocation.

Polyphenols can inhibit NF- κ B activation through several mechanisms: decreasing ROS-mediated NF- κ B activation blocking subunit p65 phosphorylation necessary for NF- κ B transcriptional activation (Quiñones et al. 2013; Vinson et al. 2001; Ou et al. 2006) and decreasing phosphorylation of I κ B, responsible for its degradation.

In vitro studies demonstrate that resveratrol moderates the overexpression of adhesion molecules (VCAM-1 and ICAM-1) through an inhibition of the NF- κ B pathway in endothelial cells (Pal et al. 2003). This PP also has been shown to reduce pro-inflammatory cytokine secretion (IL-6 and TNF- α) through down-regulation of the NF- κ B (Actis-Goretta et al. 2006).

ANTI-ATHEROGENETIC and ANTI-THROMBOTIC EFFECTS

Improving lipid profile prevents lipid accumulation in vessels wall responsible for the onset and development atherosclerosis (Quiñones et al. 2013). Oxidation of LDL may be increased by ROS production, resulting in endothelial dysfunction and macrophage foam cell-formation. Another negative event is the migration of smooth muscle cells in the neo-intima area (Quiñones et al. 2013). All this happening creates an inflammatory response. Moreover, extracellular matrix deposit increases and permits the formation of atheroma plaques. Beneficial effects of polyphenols are in terms of limiting LDL oxidation. Especially red wine and grapes juice demonstrated to reduce plasma lipid concentration and neointima growth and lipid deposits together with reduced cholesterol levels, triglycerides and apoprotein B in the blood. Chronic administration of cocoa procyanidins in rabbit fed with hypercholesterolemic diet showed a decrease in plasma lipid hydroperoxide levels and an increase in antioxidant capacity (Quiñones et al. 2013)

Platelet aggregation plays a pivotal role in the development of atherosclerosis and the beneficial effect of polyphenols on cardiovascular health can be linked to their antithrombotic activity due to their capacity to inhibit enzymes implicated in the synthesis of eicosanoids (TXA₂, COX and LPO), and other arachidonic acids metabolites which participate in vascular homeostatic control. Anthocyanins demonstrated a positive effect inhibiting platelet function. Cocoa procyanidins inhibit platelet activity through the formation of prostacyclin (PGI₂), a platelet aggregation

inhibitor, and limit vasoconstriction and inflammation through leukotrienes biosynthesis inhibition, which are vasoconstrictor agents and inflammation stimulants.

4.5 Specific actions of Hydroxycinnamic acids, anthocyanins and their metabolites on endothelial dysfunction

Within the huge list of dietary polyphenols the most common are certainly hydroxycinnamic acids and hydroxibenzoic acids. In the study related with this thesis the attention was focused primary on the role of caffeic and ferulic acids and their metabolites in maintaining and improving vascular health. Hydroxycinnamic acids are very common in diet and present on many food sources both fruit and vegetables such as berries, apples, citrus fruits potatoes, cabbages etc.

FERULIC ACID (FA) commonly found in fruit and vegetable such as berries (strawberries) and tomatoes, together with broccoli and eggplant is the most abundant phenolic acid in grains, representing almost the 90% of total polyphenols (0.8-2 g/kg dry wt) (*Figure 4.5*).

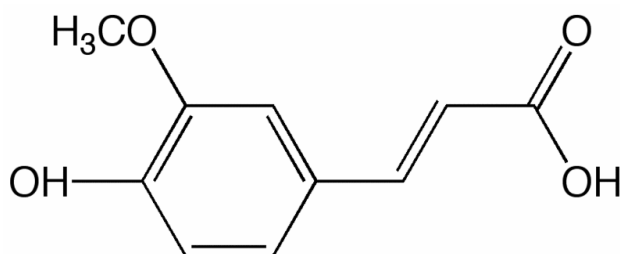


Figure 4.5: Ferulic acid structure

The daily intake of ferulic acid is around 150-250 mg considering the typical Mediterranean diet made of 3-5 serving/day of vegetables, fruit, bread and grains. However, this compounds is normally in conjugated forms (ester-linkage with mono-,di- and polysaccharides glycoproteins) which may influence its bioavailability and biological activity. After ingestion FA is not degraded by the stomach but pass trough stomach and undergoes intestinal transit. (Zhao et al. 2004). Arrived in the colon some esterase brake FA bounds and 90% of FA is absorbed by passive diffusion (Kroon et al. 1997). FA is quickly absorbed after oral ingestion, reaching C_{max} within 30 min (Mancuso & Santangelo 2014), while the complexation with mono and poly-

saccharides reduces the C_{max} and increases the T_{max} . FA undergoes a systemic metabolism through UDP-glucuronosyltransferase in the liver and the most common metabolites are glucuronides and sulfoglucuronides; only a small percentage of unmodified FA is available (9-20%) (Mancuso & Santangelo 2014; Lu et al. 2009). Some of these metabolites were object of studies in this thesis. Plasma concentration of FA metabolites is in the order of nM and the C_{max} is after 1-4 h from ingestion. FA demonstrated radical scavenging activity. Moreover, Trombino et al. showed that FA is able to block the iNOS induction, thereby decreasing peroxynitrite formation and inhibit lipid peroxidation caused by peroxy radical (Trombino et al. 2009; Mancuso & Santangelo 2014). The cytoprotective effect is not limited to these radical since FA showed protecting effects also against H_2O_2 toxicity. Also FA derivatives were shown to be able to up-regulate some anti-oxidant enzymes such as HO-1, SOD and CAT (Mancuso & Santangelo 2014). What is really interesting concerning this thesis and deeply linked with anti-oxidative properties of FA, is its protection at vascular level. FA orally administered at the dose of 1-100 mg/kg, showed a blood pressure decrease in both spontaneously hypertensive rats (SHR) and stroke-prone animals. Spontaneously hypertensive rats (SHRSP) with a maximum reduction reached after 1-2 h from oral administration (Ardiansyah et al. 2008; Mancuso & Santangelo 2014). Moreover, FA at the dose of 50 mg/kg demonstrated an antihypertensive effect comparable to the result obtained with 10 mg/kg of captopril (ACE inhibitor). In conclusion the vasculo-protective effect of FA on endothelium have to be considered multifactorial, as it involve different mechanism: reduction of angiotensin II as a consequence of ACE inhibition, increase of NO synthesis via activation of eNOS and inhibition of iNOS and reduction of NADPH-dependent production of O_2^- (Suzuki et al. 2007; Mancuso & Santangelo 2014). FA has also shown positive effects on triglycerides and cholesterol reduction and on, glycaemia regulation by increasing glucose uptake in the liver and musculature, activating the glycogen synthesis, which may be beneficial in diabetes control (Mancuso & Santangelo 2014)

CAFFEIC ACID (CA) derives from 4-hydroxycinnamic acid (*Figure 4.6*). As previously mentioned, CA forms, together with quinic acid, chlorogenic acid, coffee, the most abundant compound in coffee and it is also found in but in potato's peel (40 mg / 100 g), red fruits (150-300 mg / 100 g), in aromatic and medicinal plants like rosemary angelica, arnica, burdock, flue, lemon balm and in elevated percentage in propolis (Bonita et al. 2007). A single cup of coffee contains from 20 to 675 mg of chlorogenic acid and it was estimated that the daily intake of caffeic acid is about 206 mg / day.

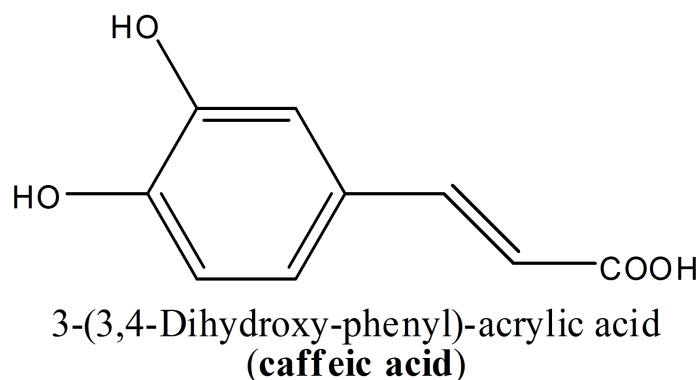


Figure 4.6: Caffeic Acid

Following coffee ingestion, chlorogenic acid is poorly absorbed but undergoes a relevant metabolism in small intestine, where its hydrolysis release CA .

The free CA is transformed into caffeic acid 3-O-sulfate and enters the blood stream; after ingestion of 200 mL of coffee blood peak concentration of CA conjugates is reached within 1 hour approximately and C_{max} is $7.7 \pm 2.5 \mu\text{M}$ (Stalmach et al. 2009). The absorption of CA, in its rare free form can take place in small amount already in the stomach, through a mechanism of passive diffusion due to very low pH. However, more significant absorption occurs in the gut, using an active Na^+ -dependent transport. 5 h after the intake of 200 mL of coffee, different metabolites such as dihydrocaffeic 3-O-sulfate appear in blood, , in a concentration of 325 ± 99 nM (Stalmach et al. 2009). CA demonstrated at concentration 10-20 $\mu\text{g/mL}$ a strong antioxidant activity *in vitro*; it inhibits lipid peroxidation and scavenge O_2^- produced by NADPH- oxidase. Also, treatments with CA dihydroderivatives demonstrated up-regulation of eNOS increasing NO production and developing a protective action in vascular endothelium. In HUVECs models, cells treated with chlorogenic acid have been shown to inhibit monocytes adhesion and CA metabolites directly inhibited the IL-1 β -mediated expression of adhesion molecules such as ICAM-1, VCAM-1 and e-Selectin (Stalmach et al. 2009)

ANTHOCYANINS

Anthocyanins are flavonoids with a characteristic C3-C6-C3 carbon structure. In vegetables they have a protective role against environmental stress such as cold temperature and UV light (*Figure 4.7*) (Wallace 2011)

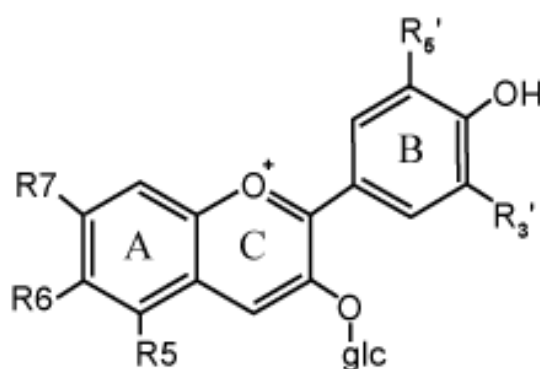


Figure 4.7: structure of Anthocyanins

Daily intake of polyphenols has been estimated to be around 1g/die which is relevant if compared with daily intake of other micronutrients like vitamin E and C (12-90 mg/die). Within polyphenols the dietary intake of anthocyanins is high (180- 215 mg/die) compared with other flavonoids such as genistein and quercetin (20-25 mg/die). Anthocyanins, differently from other flavonoids, can be detected in plasma in their intact active form (glycosides) and, despite the initial studies referring a low bioavailability (<1%) for anthocyanins, recent studies reveal that this percentage might be underestimated, as many metabolites have not been identified yet (Manach et al. 2005; Wallace 2011).

Anthocyanins are absorbed not only in the intestine, but also in the stomach. The small amount that reaches the bloodstream consists of intact anthocyanins, methylated derivatives, glucuronides and/or sulfate. The peak plasma concentration is reached within 1-3 hours after ingestion, depending on the individual consumption, then metabolites in urine persist up to 24 hours (Wallace 2011). Immediately after oral administration, anthocyanins are rapidly degraded, mostly in phenolic compounds that are further metabolized in the gut. It was found that after oral intake of 250 mg/g of elderberry extract the most abundant compound was protocatechuic acid (68.3%); In the same study, researchers found that this compound reaches a peak concentration in plasma (358 nM) 3 h after ingestion (de Ferrars et al. 2014). Excretion of anthocyanins

derivatives occur primarily at kidney level and these compounds seem to come from degradation operated by gut microbiota.

Several epidemiological studies investigated food sources rich in anthocyanins (red wine and several species of berries) and the correlation with reduction of cardiovascular risk. In fact, a decreasing trend for CVD was observed in studies in which volunteers consumed high amounts of strawberry and moderate consumption of red wine (Wallace 2011). Mechanistic studies support the beneficial effect of flavonoids, including anthocyanins, on specific biomarkers of cardiovascular risk, including NO, inflammation and endothelial dysfunction. The ability of anthocyanins to improve endothelial function and protect the vascular endothelium from the onset of endothelial dysfunction may be linked to increased levels of NO, thus preventing the loss of NO-mediated vasodilation. The up-regulation of eNOS in the endothelium in animal models is evident after an exposure of 6 hours to a derivative of cyanidin (cyanidin 3-glucoside) at a concentration of 0.1 mol/L. Furthermore, pelargonidin showed ability to inhibit the expression of iNOS in a dose dependent manner in macrophages exposed to an inflammatory stimulus mimicked by treatment with LPS. In intervention studies in humans there was a direct correlation between the plasma antioxidant capacity and plasma concentration of anthocyanins after ingestion of 1.2 g of anthocyanins in adults: a decrease in the activity of NADPH oxidase can lead to an increase in plasma antioxidant capacity; This suggests, among all the possible mechanisms, that anthocyanins and their derivatives can reduce the production of superoxide by acting directly on the NADPH oxidase.

Anthocyanins also seem to protect endothelial cells from the action of chemokines, molecules that play a key role in controlling the migration of leukocytes into the inflamed site and responsible for the propagation of inflammation. Particularly, it has been observed a protective effect from the MCP-1 (Monocyte chemotactic protein 1) induced by TNF- α . Cyanidin metabolites also reduced oxidative stress induced by interleukins and TNF- α (Wallace 2011).

The anthocyanins exert an action on specific molecules involved in the modulation of inflammation including the endothelium-derived growth factor (VEGF) and adhesion molecules, such as ICAM-1; delphinidin and cyanidin inhibit the expression of VEGF-induced growth factor derived from platelets, preventing the activation of redox-sensitive kinase, such as p38 MAPK and JNK. Anthocyanins have demonstrated a protective action against the expression of adhesion molecules induced by activated platelets. In many studies, the suppression of pro-inflammatory

chemokines, growth factors and adhesion molecules has been associated with inhibition of NF- κ B transcription factor sensitive to oxidative stress, which controls the expression of genes involved in the inflammatory response. Following administration of anthocyanins, the levels of chemokines, cytokines and mediators of inflammation NF- κ B-dependent significantly decreases (Wallace 2011).

Much knowledge about specific action of anthocyanin metabolites came from *in vivo* clinical studies. A large number of studies indicated that, cranberry juices and grape juices improve FMD and vascular functions (Rodriguez-Mateos et al. 2013; Stein et al. 1999; Dohadwala et al. 2011). However these studies were mostly based on the amount of berries consumed rather than delivery of polyphenols amount, causing a difficult interpretation (Rodriguez-Mateos et al. 2013). (Rodriguez-Mateos et al. 2013) designed a clinical study around the total blueberry polyphenols investigating the endothelial function changes correlated with variation FMD, Identifying a total of 32 metabolites in plasma after blueberry consumption, 6 of them related with a significant increase in plasma concentration 2 h after consumption and increase in FMD providing interesting information about those metabolites which, more than others, are responsible for acute improvement in endothelial function.

CHAPTER 5: Aim of the Thesis

Vascular homeostasis is tightly regulated by the endothelium through the release of several biochemical factors that act locally in the vessel wall and the lumen. In fact, in physiological conditions, the synergistic action of endothelial factors like NO, PGI₂, ET-1 and others allows the maintenance of normal vascular tone, vascular permeability, blood pressure and blood flow. In pathological conditions functional and reversible alteration of endothelial cell function occur, resulting in impaired vasodilation, increased vascular reactivity, platelet activation, thrombus formation, increased permeability, leukocyte adhesion and migration into the vascular wall (Montezano & Touyz 2012).

Endothelial dysfunction has been observed in the early stage of most cardiovascular diseases (Yang et al. 2010) and is associated with various pathologies including hypertension, diabetes, atherosclerosis, pulmonary hypertension, ischaemic heart diseases and chronic kidney disease (Montezano & Touyz 2012). The severity of endothelial dysfunction has been shown to have a prognostic value for cardiovascular pathologic events.

Although the mechanisms underlying endothelial dysfunction are complex and multifactorial, a growing body of evidence suggests that increased production of ROS may have a pivotal role in this phenomenon (Münzel et al. 2010).

Low levels of ROS are produced at physiological levels with the function of signaling molecule (Reczek & Chandel 2015). In particular conditions, the release of some factors induces an increase in ROS generation, in parallel with the inability by antioxidant defenses to neutralize them. Normally, the endothelium is able to adapt itself to a stress condition by transiently changing its morphology and function, but if the stimulus is not limited to the endothelium it passes to a state of permanent oxidative stress, which results in "endothelial dysfunction". This condition is characterized by a dysregulation of the homeostatic mechanisms. A prolonged production of ROS causes damage at the level of proteins, lipids, DNA and membranes. In this context the endothelium-mediated vasodilation is impaired, platelets are activated, pro-thrombotic factors are released, vascular reactivity, permeability, and leukocyte adhesion and transmigration are enhanced (Favero et al. 2014a). Moreover, the endothelial dysfunction has been recognized as the main link between risk factors and development of cardiovascular pathologies such as atherosclerosis, diabetes and hypertension.

Numerous studies have shown that oxidative stress and inflammation are closely interrelated and play a key role in the pathogenesis of endothelial dysfunction. A condition of "chronic" oxidative stress mediates a progressive endothelial damage through the reduction of the bioavailability of NO, the proliferation and migration of vascular smooth muscle cells, the alteration of the extracellular matrix, the apoptosis of endothelial cells and the production by the endothelial cells of inflammatory mediators including cytokines, such as interleukin-1 β (IL-1 β), tumor necrosis factor- α (TNF- α), IL-6, IL-8, chemokines (monocyte chemo attractant protein-1 (MCP-1) and adhesion molecules (ICAM-1, VCAM-1, E-Selectin). The expressions of genes whose products are involved in the inflammatory response are under the control of the transcription factor NF- κ B. On the contrary, it was demonstrated that an increased expression of the enzyme heme oxygenase 1 (HO-1) plays an important role in the resolution of inflammation and may therefore represent a protective factor part of the antioxidant endogenous system (Ryter et al. 2006).

The etiology of cardiovascular disease (CVD) is multifactorial and is a process with a strong correlation with lifestyle. Some already well known cardiovascular risk factors, such as hypertension, diabetes, smoking and high cholesterol levels, are associated with endothelial dysfunction (Versari et al. 2009). A perpetuated inflammatory state plays a pivotal role in development and continuation of endothelial dysfunction. The constant stimulation of the endothelium by pro-inflammatory factors promotes the recruitment of other immune system cells, the expression of cytokines, integrins, selectins (both on endothelial cells and immune system cells), and the increased production of ROS, resulting in depletion of endogenous antioxidants and thus the propagation of the inflammation itself.

A growing number of scientific evidence supports the beneficial effects of plant-derived foods for human health maintenance. In fact, numerous studies have highlighted an inverse correlation between the risk of developing chronic diseases and the adherence to dietary pattern that favor the consumption of food of vegetable origin, with the Mediterranean diet as the most significant and popular example.

The central role of the endothelium in maintaining vascular homeostasis and the correlation between endothelial dysfunction and the development of cardiovascular diseases make this tissue a primary target for the dietary strategies aimed at the containment of cardiovascular diseases. The research in nutrition is now addressed to the identification of bioactive components responsible for this beneficial effects and characterization of their biological activities in order to

define and sustain their nutraceutical value. Among the bioactive compounds of natural origin and with nutraceutical properties, polyphenols and bioactive peptides are main characters.

Cereals and legumes are key components of a healthy, balanced diet, as highlighted by their location at the base of different national nutritional pyramids. Although cereals represent the primary source of carbohydrates in our daily diet, they also contain significant amounts of protein, thus constituting a dietary source of bioactive peptides. In order to identify an effective dietary approach for the prevention/control of chronic degenerative diseases, nutrition research has recently directed itself to the characterization of the healthy properties of bioactive peptides which, through various mechanisms, including the ACE inhibitory action, antioxidant, antithrombotic and cholesterol lowering (Erdmann et al. 2008) may contribute to maintaining the functionality of the cardiovascular system.

Polyphenols are a class of phytochemicals in the diet and are well known especially for their antioxidant action. The results of research in nutrition support their preventive role against chronic degenerative diseases, including cardiovascular disease (Hooper et al. 2008). However, it was not yet possible to clearly identify a specific component responsible for the observed beneficial effects; this is mainly due to the intense Phase II metabolism, to which polyphenol and I are subjected. In this context is relevant to consider all the aspect related to polyphenols intake: absorption, bioavailability, and metabolism. Although experimental *in vitro* studies seems to confirm the potential influence of polyphenols in CVD, and epidemiological evidence of beneficial effects of polyphenols-rich food consumption are strong, intervention studies using specific phenolic compounds, have produced less convincing evidence. Also the characterization of metabolites involved in these benefic effects is crucial.

Therefore, the overall aim of the study presented in this thesis was to investigate the potential protective effect of bioactive compounds (bioactive peptides and polyphenols metabolites) against oxidative and inflammatory damage using endothelial cells (HUVEC) as a model system.

Among bioactive peptides present in cereals we have focused our attention on non-specific lipid-transfer protein (nsLTP) type 2, which was identified in wheat.

Since, to date, no biological activities in mammalian cells are present in the literature, the research activities were first focused to the analysis of potential biological activities of this

newly isolated peptide, that may be related to vascular health protection and to the characterization of cellular and molecular mechanisms underlying these effects. With this aim, experiments were targeted to the evaluation of nsLTP2 ability to influence intracellular ROS levels and to prevent cell damage under oxidative (H₂O₂) and inflammatory agents (TNF- α , IL-1 β , LPS) stimulation. Immunblotting analyses were subsequently conducted in order to analyze nsLTP2 capacity to modulate specific marker of inflammatory processes, such as adhesion molecules (ICAM-1, VCAM-1, E-Selectin) and cellular regulators of endothelial inflammation, such as heme oxygenase-1 (HO-1), whose role in the pathogenetic process of different cardiovascular diseases has been established.

As previously mentioned, polyphenols present in foods are largely metabolized within human body, following dietary intake. For this reason the molecules that will act at cellular and tissues level, will be most likely a metabolite, rather than its parental compound. Particularly, in this study the effect of metabolites of polyphenols belonging to two different classes, anthocyanins and caffeic acid derivatives, was evaluated. Experiments aimed at examining whether these compounds are able to reduce the levels of ROS and develop cytoprotective effects in endothelium during oxidative stress and inflammatory conditions. It was subsequently deepened the role of polyphenol metabolites by assessing their effect on the expression of adhesion molecules such as VCAM-1 and ICAM-1 and cytoprotective enzymes such as HO-1.

The experimental work of this thesis is part of a larger research project “Beneficial effects of dietary bioactive peptides and polyphenols on cardiovascular health in humans - BACCHUS”, funded by the European Union (BACCHUS FP7 European commission grant agreement 312090).

CHAPTER 6: Materials and methods

6.1 Chemicals and Reagents

Medium M200 phenol red free #M-200-500, LSGS supplement kit #S-003-K, Recombinant Human TNF- α #PHC3015, recombinant human IL-1 β #PHC0814 and HUVECs (Human umbilical endothelial vein cells) #C-015-10C were purchased from GIBCO (Thermo-Fischer Scientific, Waltham, MA USA). Fetal bovine serum (FBS) #F7524, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) #M5655, dichlorodihydrofluorescein diacetate (H₂DCFDA) #D6883, hydrogen peroxide solution #H1009 (H₂O₂), L-glutamine #G1753, 0,25% Trypsin-EDTA Solution #T4049, lipopolysaccharides (LPS) #L2630, Hanks's balanced modified salts solution (HBSS) #H4891, bovine serum albumin (BSA) #A4503, Glycine for electrophoresis #G8898, Trizma® base #T1503, gelatin from bovine skin #G9391, Dimethyl sulfoxide (DMSO) #D8418, Phosphate buffered saline (PBS) #P3813, mammalian protease inhibitor mixture #P8340, Sodium Fluoride (NaF) #450022, Phenylmethanesulfonyl fluoride (PMSF) #P7626, Sodium pyrophosphate dibasic #P8135, Sodium orthovanadate #S6508, sodium pyruvate #P8574 and β -Nicotinamide adenine dinucleotide reduce disodium salt hydrate (NADH) #N8129 were purchased from Sigma-Aldrich St. Louis, MO, USA). Nitrocellulose membrane # 10600002 was from GE-Healthcare. Mini protean® TGX Gels, Clarity™ Western ECL Blotting Substrate #102030652, DC protein assay kit #210012250 and Precision Plus Protein™ All Blue Prestained Protein Standards #1610373 were purchased from BIORAD (Hercules, CA, USA). Phenolic metabolite Dihydrocaffeic acid sulfate (M1) and Dihydroferulic acid sulfate (M2) were kindly provided by University of Leeds – UK; Isoferulic acid 3-glucuronide (M3), Protocatechuic acid 4-glucuronide (M4), Protocatechuic acid 3-*O*-sulfate (M5), Protocatechuic acid 4-*O*-sulfate (M6), Protocatechuic acid 3-glucuronide (M7) and purified peptide nsLTP2 were provided by Institute of Food Research – Norwich – UK within the European FP7 programme Bacchus.

6.2 Human Umbilical Vein Endothelial Cells (HUVECs) culture and treatments

Human Umbilical Vein Endothelial Cells (HUVECs) are probably the most used model of human endothelial cells by researchers around the world. They represent the most simple and available human EC type, suitable for the preparation of large quantities of cells. They are widely used in studies of angiogenesis, inflammation, immunity and atherosclerosis, representing the best model for endothelial dysfunction analysis. HUVECs can be obtained as a primary culture from human umbilical cord vein. To isolate the cells, the umbilical vein is washed with PBS in order to remove all blood cells, subsequently is incubated with a 0.2% collagenase solution and cells obtained by enzymatic digestion are collected into a tube. After centrifugation, the obtained cell pellet is separated from the supernatant and dispersed in the culture medium. At this point the cells can be counted and seeded at a desired concentration (Baudin et al. 2007). HUVECs are also commercially available and are supplied as cryopreserved, already isolated "pool" of donors and must be stored in liquid nitrogen until use.

HUVECs show up as a monolayer of polygonal colonies closely leaning against each other, with a well-defined nucleus and cell edges hardly distinguishable. Individual colonies may merge to form large areas of confluent cells with tiled morphology (*Figure 6.1*). HUVECs were grown in standard conditions (humidified atmosphere at 37°C and 5% CO₂) in Phenol red free M200 medium (Thermo-Fischer Scientific) supplemented with 10% decompemented fetal bovine serum (FBS) (Sigma-Aldrich), L-glutamine, growth factors and antibiotics (LSGS Kit - Thermo-Fischer Scientific), which was changed every 48h. The cells were cultured until they reached 80% confluence and successively seeded into gelatine-coated multiwell plates for treatments. Experiments were conducted with cells between passages 3-7.

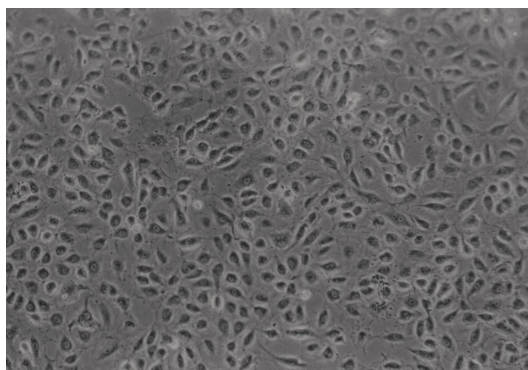


Figure 6.1: HUVECs appearance by inverted microscopy.

HUVECs were treated with nsLTP2 at final concentration of 0.01 μM for 24h. For experiments with polyphenols, HUVECs were treated with caffeic acid (CA) metabolites M1, M2 and M3 (Dihydrocaffeic acid sulphate, Dihydroferulic acid sulfate and Isoferulic acid 3-glucuronide, respectively) and cyanidin (CY) metabolites M4, M5, M6, M7 (protocatechuic acid 4-glucuronide, protocatechuic acid 3-sulfate, protocatechuic acid 4-sulfate and protocatechuic acid 3-glucuronide, respectively). Stock solutions were prepared at 10 mM concentration and stored at -80°C . Working solution were prepared in complete medium at a concentration of 0.1 μM . The polyphenols were all solubilized in water with the exception of M3 and M7, which were solubilized in DMSO.

After 24 h treatment with nsLTP2 and polyphenols metabolites, oxidative and inflammatory stress were induced using H_2O_2 (100 μM final concentration), inflammatory cytokines as TNF- α (20ng/mL) and IL-1 β (10ng/mL) and Lipopolysaccharides (LPS) (0.1-10 $\mu\text{g}/\text{mL}$).

6.3 Cell viability assay

Cell viability was evaluated by the MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, which measure metabolically active cells.

The MTT assay, developed by Mosmann (Mosmann 1983), is a quantitative colorimetric assay, which detects living cells. This method is based on the reduction of the reagent 3- [4,5-dimethylthiazole-2-yl] -2,5- diphenyltetrazolium bromide (MTT) into a formazan salt, insoluble, purple in color, by the intracellular reducing power (*Figure 6.2*). The quantity of formazan is directly proportional to the number of viable cells.

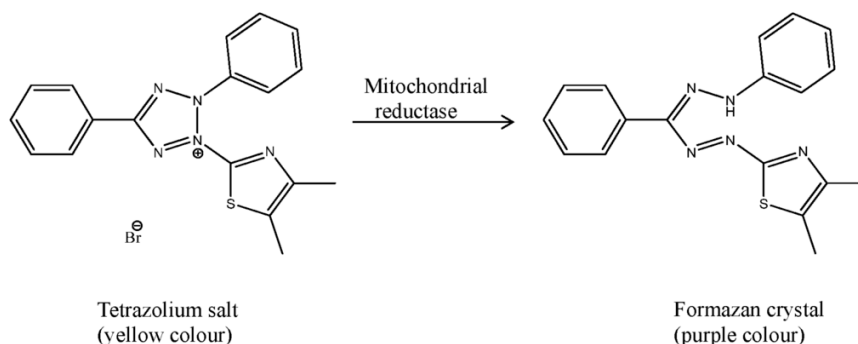


Figure 6.2: The formation of formazan crystals from the tetrazolium salt (Barahuie et al. 2014).

Briefly, HUVECs (7×10^3 /well) were seeded in a 96 well plate and incubated for 24 h. After that, cells were pre-treated with nsLTP2 or polyphenols metabolites for 24 h and subsequently treated with oxidising (H_2O_2) and inflammatory agents ($TNF-\alpha$, $IL-1\beta$, LPS) for further 24 h. The MTT reagent was added to the culture medium to a final concentration of 0.5 mg/mL. After 4 hours of incubation, the MTT containing medium was aspirated and the formazan crystals were solubilized by the addition of dimethylsulfoxide (DMSO). The absorbance of this solution was determined with spectrophotometric reading at $\lambda=595$ nm, using a multiwell plate reader (Wallac Victor², PerkinElmer).

6.4 Lactic dehydrogenase (LDH) release assay

The assay was performed on cell medium aliquots according to Hrelia S *et al.*, 2002. LDH is a cytosolic enzyme, which catalyzes the NADH dependent reversible reaction of oxidation of lactate to pyruvate (Figure 6.3).

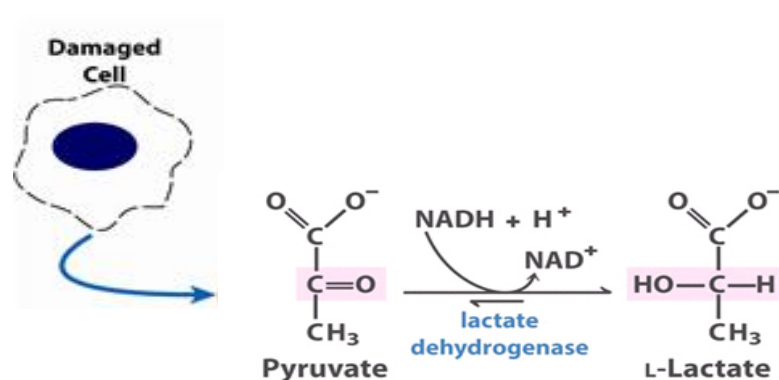


Figure 6.3: transformation of Pyruvate to Lactate

Following cell exposure to different stimuli, the activity of LDH is increased in culture medium as a result of cell damage that, by altering the permeability of the cell membrane, causes the leak of LDH outside the cell. The assay thus represents a method for the evaluation of non-specific cell damage correlated to cell viability. After 24 h pre-treatment with 10 nM nsLTP2 or 0,1 μ M polyphenol metabolites. HUVECs were treated with 100 μ M H₂O₂, 20 ng/mL TNF- α , 10 ng/mL IL-1 β or 10 μ g/mL LPS. Enzyme activity was analysed spectrophotometrically ($\lambda=340$ nm) by measuring the decrease in absorbance of NADH in an assay mixture containing pyruvate 10 mM, NADH 1.5 mM, phosphate buffer pH 7.4 and cell culture medium as a source of LDH.

6.5 Measurement of intracellular reactive oxygen species (ROS) levels

Intracellular ROS levels were evaluated by means of a spectrofluorimetric method implying the probe 2',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA). At the end of 24 h pre-treatment with 10 nM nsLTP2 or 0.1 μ M polyphenol metabolites, cells were washed twice with PBS and incubated with 5 μ M H₂DCFDA for 30 min at 37°C. Subsequently, cells were incubated with different stressors (100 μ M H₂O₂, 20 ng/mL TNF- α , 10 ng/mL IL-1 β or 0.1 μ g/mL LPS) for 30 min. H₂DCFDA is a small non-polar, non-fluorescent molecule that diffuses into the cells, where it is enzymatically deacetylated by intracellular esterases to a polar non-fluorescent compound, that is oxidised to the fluorescent 2',7'-dichlorofluorescein (DCF) by intracellularly generated ROS (*Figure 6.4*). The fluorescence of the oxidized probe was measured at $\lambda_{\text{ex/em}}=485/535$ nm using a multiwell plate reader (Wallac Victor², PerkinElmer).

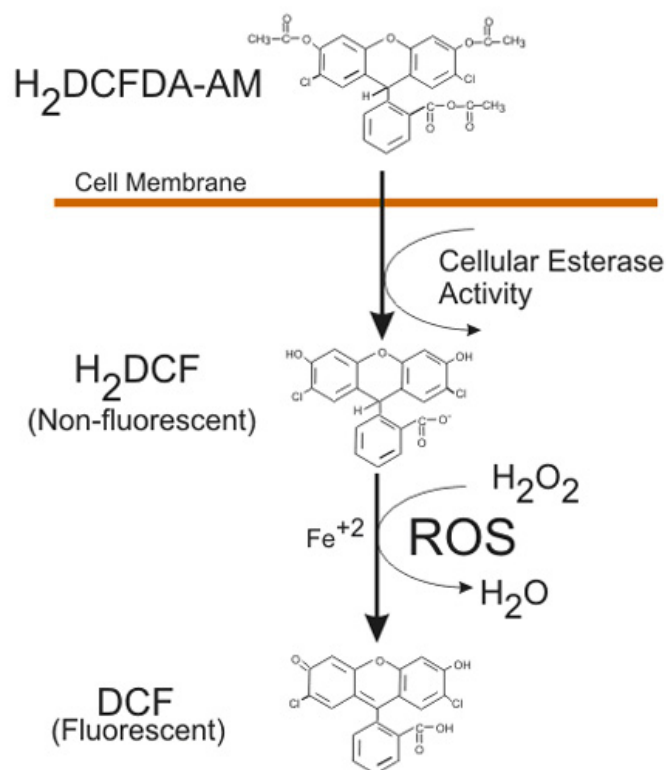


Figure 6.4: Formation of fluorescent compound DCF by ROS.

6.6 Immunoblotting Analysis

6.6.1 Preparation of whole cell lysates

HUVECs (2×10^5 /well), seeded in 6-well plates were pre-treated for 24 h with nsLTP2 or polyphenols metabolites and then treated for 24 h with oxidative and pro-inflammatory agents. (12 h for E-selectine detection). At the end of the treatment cells were washed twice with ice-cold PBS and lysed in Radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-hydroxymethyl-aminomethane, 150 mM NaCl, 0.1% sodium-dodecyl-sulfate (SDS), 1% NP-40 and 0.5% sodium deoxycholate) supplemented with protease inhibitors mixture (Sigma) and phosphatase

inhibitors. Cells were detached from the plastic support using cell-scraper, left on ice to solubilize for 15 min with periodic vortexing and subsequently centrifuged at 12000 rpm for 15 minutes to separate the supernatants.

6.6.2 Protein concentration measurement

Supernatant protein content was determined by DC protein assay kit, (Bio-Rad) using Bovine Serum Albumin (BSA) solubilized in RIPA buffer as a standard. The assay, similar to the colorimetric Lowry assay and modified in order to increase the stability of the products that are formed upon reaction of the dye with proteins, consists on a 2 steps reaction of proteins with an alkaline Cu^{2+} tartrate solution and Folin reagent:

- 1) the reaction between proteins and Cu^{2+} in an alkaline environment;
- 2) the reduction of the Folin reagent by the action of Cu^{2+} -protein complex.

The colour formation is mainly due to the reaction of the dye with amino acids such as tyrosine and tryptophan and secondarily with cysteine and histidine. The complex that is formed has maximum absorbance at $\lambda=750$ nm. 5 μL of standards and samples in triplicate, were loaded into a 96-well plate to which 25 μL of solution A (Alkaline solution of Cu^{2+} tartrate) and with 200 μL of solution B (Folin reagent) were added. After 15 min of incubation, the absorbance was read at 750 nm using a multiwell plate reader.

6.6.3 SDS-PAGE and Western Blot Analysis

After protein concentration determination, samples were boiled with loading buffer for 5 min and 10 μg of each sample were loaded on a 10% SDS-PAGE Mini-Protean[®] TGX[™] (Bio-Rad Laboratories) and electrophoresed at 200 V for 30-40 minutes. Samples were loaded alongside 5 μL of Precision Plus Protein[™] All Blue Prestained Protein Standards (Bio-Rad) to enable identification of proteins of interest.

The separated proteins were then blotted on a nitrocellulose membrane (Hybond-C; GE Healthcare, Buckinghamshire, UK) by applying to the electrophoretic cell, an electric field of 110 V for 90 minutes. Non-specific binding to membrane was blocked with Tris-buffered saline/Tween (TTBS), pH 8.0, containing 5% non-fat dried milk or BSA for 1 hour at room

temperature. Blots were probed with primary antibodies overnight at 4 °C with gentle shaking, washed with TTBS (3x 5 min) and then incubated for 1 h at room temperature with secondary horseradish peroxidase conjugates antibodies. For this research work different primary antibody were used as reported in *Table 6.1*.

Antigen	Species	Dilution	Solvent	kDa	supplier
HO-1	Rabbit	1:1000	BSA 5% p/v TBS-T	28 kDa	Cell Signaling
ICAM-1	Rabbit	1:1000	Milk 2,5% p/v TBS-T	89 kDa	Abcam
VCAM-1	Rabbit	1:1000	BSA 5% p/v TBS-T	95-120 kDa	Cell Signaling
E-selectin CD62E	Rabbit	1:500	Milk 2,5% p/v TBS-T	67 kDa	Abcam
β -actin	Mouse	1:5000	Milk 5% p/v TBS-T	42 kDa	Sigma- Aldrich

Table 6.1: table of primary antibodies

Anti-rabbit IgG, HRP-linked Antibody	1:10000	Cell Signaling
Anti-mouse IgG, HRP-linked Antibody	1:10000	Cell Signaling

Table 6.2: table of secondary antibodies

Protein bands were detected by chemiluminescence, using Clarity™ Western ECL Blotting Substrate (Bio-Rad) according to manufacturer's protocol. Densitometric analysis of specific immunolabeled bands was performed using the Chemidoc MP (Bio-Rad, Hercules, CA) software. Band intensity was normalized to the intensity of the corresponding β -actin band.

6.7 Fluorescence Immunostaining

HUVECs were seeded on glass cover-slips and treated with 10nM nsLTP2 for 24h. At the end of the treatment cells were washed with PBS, fixed with 3% paraformaldehyde for 15 min, washed with 0.1 M glycine in PBS and permeabilized in 70% ice cold ethanol. After fixing, the cells were incubated overnight at 4 °C with anti HO-1 primary antibody. Subsequently the samples were washed with 1% BSA in PBS and incubated with DyLight 488 labeled secondary antibody (KPL, Gaithersburg, MD) for 1 h at room temperature. Nuclei were labelled with DAPI (0.01µg/ml final concentration) for 10 min at room temperature. Preparations were mounted on microscope slides with Vectashild (Vector Laboratories, Burlingame, CA) and images were acquired with Axio Scope.A1 microscope (Zeiss) at 100x magnification.

6.8 Statistical Analysis

Data represent mean values with \pm respective standard deviation (SD) corresponding to three or more independent experiments. Differences between means were analysed by unpaired Student's t-test with $P < 0.05$ considered to be significant.

CHAPTER 7: Results

7.1 Antioxidant activity of nsLTP2

Experiments were conducted in order to evaluate nsLTP2 antioxidant activity in the presence of different stressors using HUVECs, as a model system. The evaluation of the antioxidant activity was performed by measuring the ability of nsLTP2 to decrease intracellular ROS levels, using a spectrofluorimetric assay. Oxidative stress was mimicked by treating cells with 100 μM H_2O_2 , a small molecule, highly diffusible through cell membranes, which represents the main reactive oxygen species involved in cell signaling. Inflammation was induced by treating cells with a panel of cytokine (20 ng/ml TNF- α , 10 ng/ml IL-1 β) and bacterial endotoxin lipopolysaccharide (0.1 $\mu\text{g}/\text{ml}$ LPS), as molecules involved in inflammatory processes. Cells were pre-treated with 10 nM nsLTP2 for 24h. The concentration used for treatments was chosen according to data presented in the literature, which reports a similar plasma concentration for lunasin (previously considered the reference peptide) in men after soy intake (Dia *et al.*, 2009).

Results presented in *Figure 7.1a* show that treatment of HUVECs with 100 μM H_2O_2 for 30 min caused a significant increase in intracellular ROS levels, which was significantly reduced by 24h pre-treatment with 10 nM nsLTP2. No significant reduction of basal ROS intracellular levels was caused by cell pre-treatment with nsLTP2 alone.

In HUVECs treated with 20 ng/ml TNF- α , 10 ng/ml IL-1 β and 0.1 $\mu\text{g}/\text{ml}$ LPS a significant increase in intracellular ROS was observed. Cells pre-treatment with 10 nM nsLTP2 for 24h before the induction of inflammatory stress significantly reduced intracellular ROS levels, with respect to stressed cells (*Figures 7.1 b, c, d*). In cells receiving the peptide alone, with no subsequent induction of inflammatory stress, no significant differences in intracellular ROS levels with respect to control were observed.

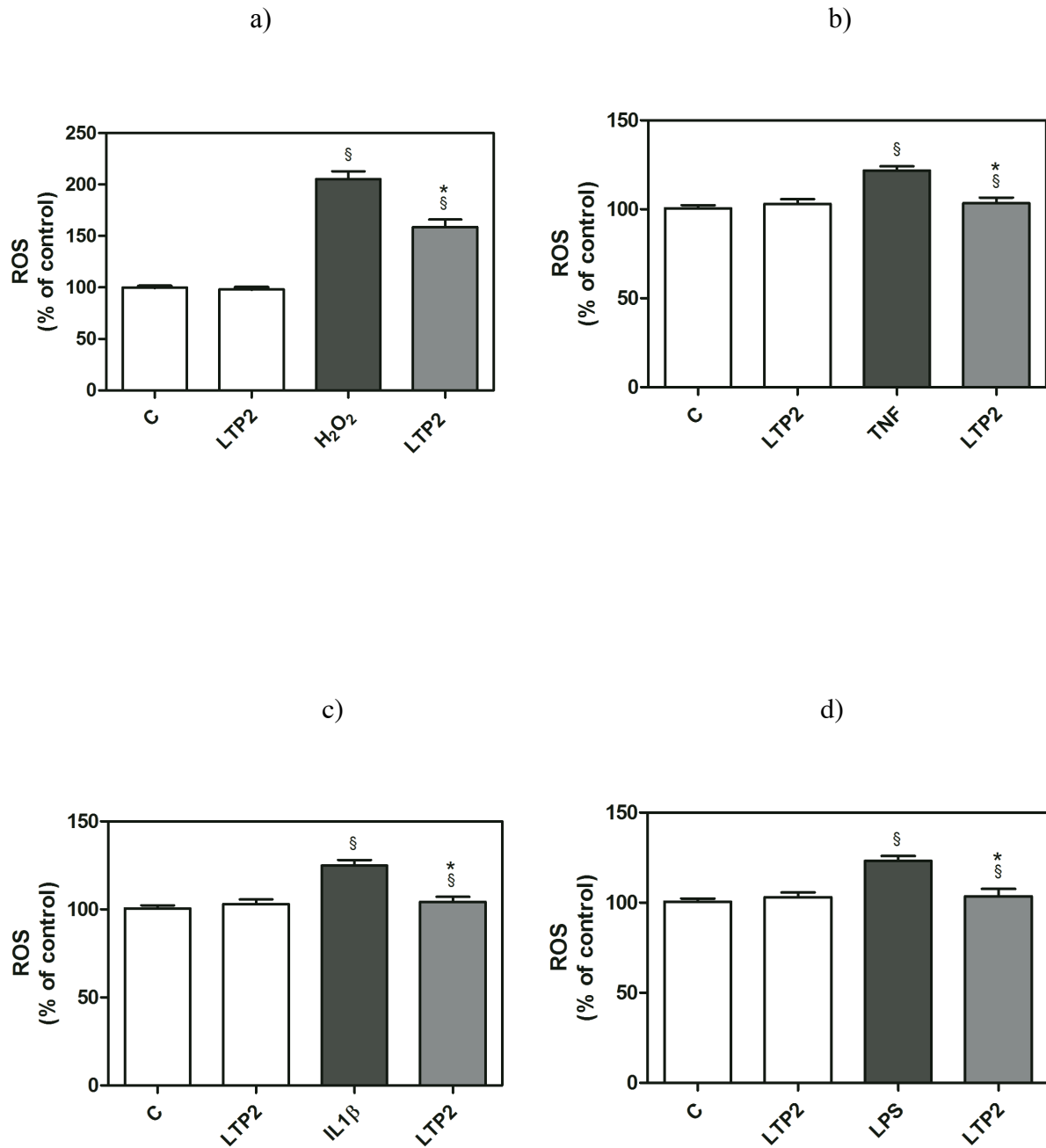


Figure 7.1: Evaluation of nsLTP2 effect on intracellular ROS levels in HUVECs. HUVECs were pre-treated with 10nM nsLTP2 for 24 h and subsequently treated with 100 μ M H₂O₂ (a), 20 ng/mL TNF- α (b), 10 ng/mL IL-1 β (c) and 0.1 μ g/ml LPS (d) as reported in Materials and Methods. Data are expressed as % of control cells. Values represent means \pm SD of at least 3 independent experiments. Statistical analysis was carried out by the Student's t test. §p<0.05 significantly different from control cells; *p<0.05 significantly different from stressed cells.

7.2 Cytoprotective activity of nsLTP2

Cytoprotective activity of nsLTP2 was evaluated measuring lactate dehydrogenase (LDH) activity in cell culture medium as a marker of non-specific cell damage caused by oxidant/pro-inflammatory agents. As depicted in Figure 7.2, treatment of HUVECs with 100 μM H_2O_2 caused a significant increase in LDH activity in tissue culture supernatants with respect to control, untreated cells. In HUVECs pre-treated for 24 h with 10 nM nsLTP2 a decrease in LDH activity was observed, which was significantly different from H_2O_2 treated cells (Figure 7.2 a). HUVECs treatment with different inflammatory stressors (20 ng/mL TNF- α , 10 ng/mL IL-1 β , 10 $\mu\text{g}/\text{mL}$ LPS) caused a significant increase in LDH activity. 10 nM nsLTP2 was able to significantly reduce the increase in LDH activity caused by 20 ng/mL TNF- α or 10 ng/mL IL-1 β (Figures 7.2 b, c), but not cell damage induced by 10 $\mu\text{g}/\text{mL}$ LPS (Figures 7.2 d).

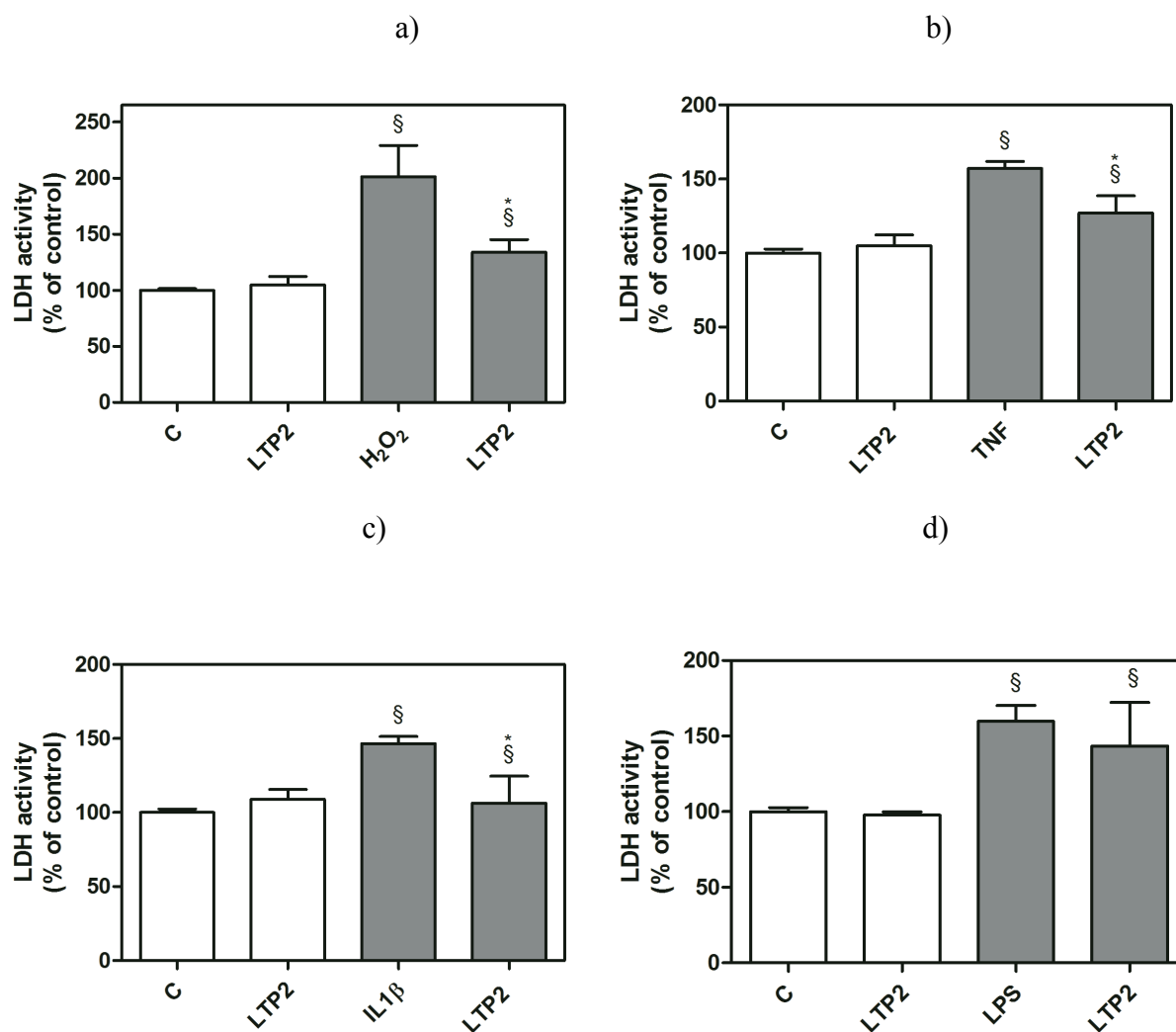


Figure 7.2: Evaluation of nsLTP2 effect on LDH release in HUVECs exposed to different stressors. HUVECs were pre-treated with 10nM nsLTP2 for 24 h and subsequently treated with 100 μ M H₂O₂ (a), 20 ng/mL TNF α (b), 10 ng/mL IL1 β (c) and 10 μ g/ml LPS (d) as reported in Materials and Methods. Data are expressed as % of control cells. Values represent means \pm SD of at least 3 independent experiments. Statistical analysis was carried out by the Student's t test. §p<0.05 significantly different from control cells; *p<0.05 significantly different from stressed cells

7.3 Anti-inflammatory activities of wheat nsLTP2: effects on adhesion molecules expression

Scientific evidence support the view that leukocytes and adhesion molecules are important participants in endothelial dysfunction and tissue injury and both these situations are linked to an inflammatory state and ultimately to different cardiovascular disease onset and progression (Golias et al. 2007). The endothelial expression of cell-surface adhesion molecules, such as VCAM-1, ICAM-1 and E-Selectin represents a marker of endothelial activation, which is typically induced by pro-inflammatory cytokines. With the aim of assessing a potential anti-inflammatory activity of wheat nsLTP2, experiments were conducted in order to verify its potential modulatory effect on adhesion molecule expression.

To this purpose, HUVECs were pre-treated with 10 nM nsLTP2 for 24 h before the induction of inflammatory stress by TNF- α (Figure 7.3a), IL-1 β (Figure 7.3b) and LPS (Figure 7.3c).

Immunoblotting analysis revealed that 24 h treatment with 20ng/mL TNF- α caused a significant increase in the expression of VCAM-1, ICAM-1 and E-Selectin in HUVEC (Figure 7.3a). A 24 h pre-treatment with 10nM nsLTP2, before the inflammatory stimulus, caused a significant decrease in the expression of all adhesion molecules analyzed, which resulted to be significantly different with respect to stressed cells (Figure 7.3a).

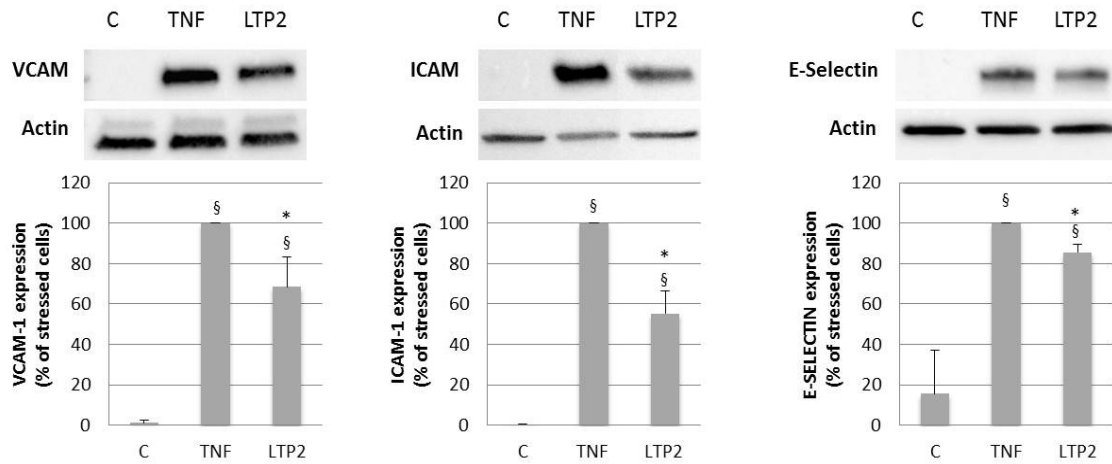


Figure 7.3a: Evaluation of the modulation of cell adhesion molecule expression exerted by nsLTP2 in HUVECs treated with TNF- α . After 10 nM nsLTP pre-treatment for 24 h, HUVECs were stressed by 20ng/mL TNF- α . Cell lysates were subjected to SDS-PAGE and Western blotting analysis, using antibodies directed to VCAM-1, ICAM-1 and E-Selectin. Images show a representative experiment of three independent analyses. Densitometry analysis of bands corresponding to different cell adhesion molecule expression, normalized to actin, was expressed as % of stressed cells and was carried out with the ChemiDoc MP System (BIORAD). Data represent means \pm SD of three independent experiments. §p < 0.05, compared with control HUVECs; *p < 0.05, compared with HUVECs stressed with TNF- α .

As reported in *Figure 7.3b*, results of immunoblotting analysis indicate that the significant increase in the expression of VCAM-1, ICAM-1 and E-Selectin, which was observable in HUVECs after treatment with 10 ng/mL IL-1 β , was reduced by 24 h cell pre-treatment with 10 nM nsLTP2 and adhesion molecules expression was significantly different with respect to stressed cells (*Figure 7.3b*).

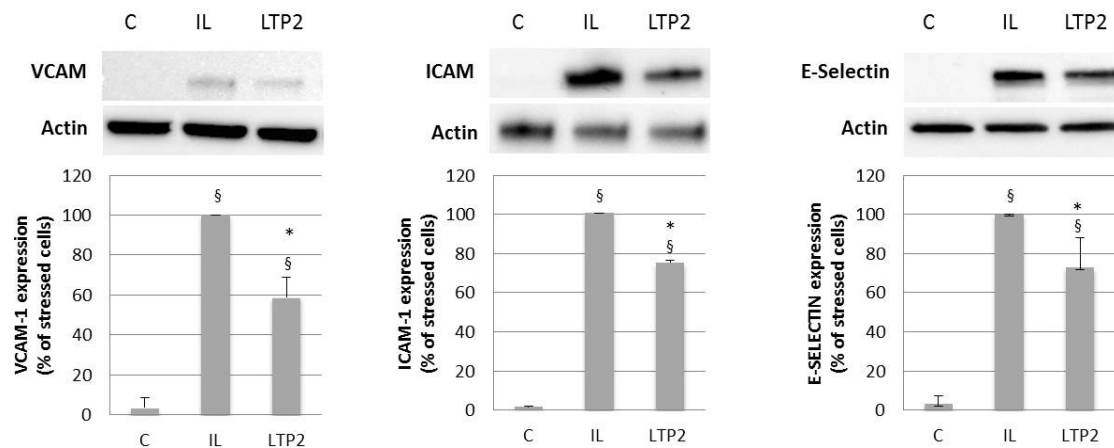


Figure 7.3b: Evaluation of the modulation of cell adhesion molecule expression exerted by nsLTP2 in HUVECs treated with IL1- β . After 10 nM nsLTP pre-treatment for 24 h, HUVECs were stressed by 10 ng/mL IL1- β . Cell lysates were subjected to SDS-PAGE and Western blotting analysis using antibodies directed to VCAM-1, ICAM-1 and E-Selectin. The images show a representative experiment out of three independent analyses. Densitometric analysis of bands corresponding to different cell adhesion molecule expression, normalized to actin, is expressed as % of stressed cells and was carried out with the ChemiDoc MP System (BIORAD). Data represent means \pm SD of three independent experiments. § $p < 0.05$, compared with control HUVECs; * $p < 0.05$, compared with HUVECs stressed with IL1- β .

Immunoblotting analysis of the expression of VCAM-1, ICAM-1 and E-Selectin in HUVECs showed a significant increase, with respect to control, after treatment with 10 μ g/mL LPS. In HUVECs pre-treated with 10 nM nsLTP2 for 24 h, before the induction of inflammatory stress, the expression of all adhesion molecules decrease significantly with respect to stressed cells (Figure 7.3c).

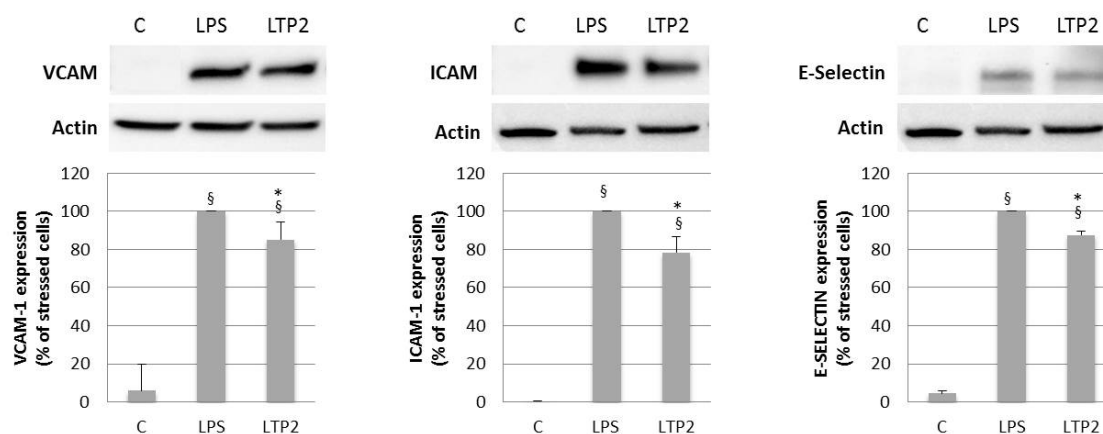


Figure 7.3c: Evaluation of the modulation of cell adhesion molecule expression exerted by nsLTP2 in HUVECs treated with LPS

After 10 nM nsLTP treatment for 24 h, HUVECs were stressed with 10 µg/mL LPS. Cell lysates were subjected to SDS-PAGE and Western blotting analysis using antibody directed to VCAM-1, ICAM-1 and E-Selectin. The images show a representative experiment out of three independent analyses. Densitometric analysis of bands corresponding to different cell adhesion molecule expression, normalized to actin, is expressed as % of stressed cells and was carried out with the ChemiDoc MP System (BIORAD). Data represent means ± SD of three independent experiments. §p < 0.05, compared with control HUVECs; *p < 0.05, compared with HUVECs stressed by LPS.

7.4 Anti-inflammatory effects of nsLTP2: role of heme oxygenase (HO-1) in vascular endothelium.

In order to verify if the observed anti-inflammatory effect of wheat peptide nsLTP2 could be mediated through the modulation of HO-1, HUVECs were treated with 10 nM nsLTP2 for 24 h and cell lysates were subjected to immunoblotting analysis. As reported in *Figure 7.4*, HUVECs treatment with 10 nM nsLTP2 caused a significant increase in HO-1 protein expression, compared to control cells. This, at cellular level, might be translated in an indirect anti-inflammatory and anti-oxidant activity exerted by nsLTP2 peptide, due to the activation of one of the enzyme part of the endogenous antioxidant system.

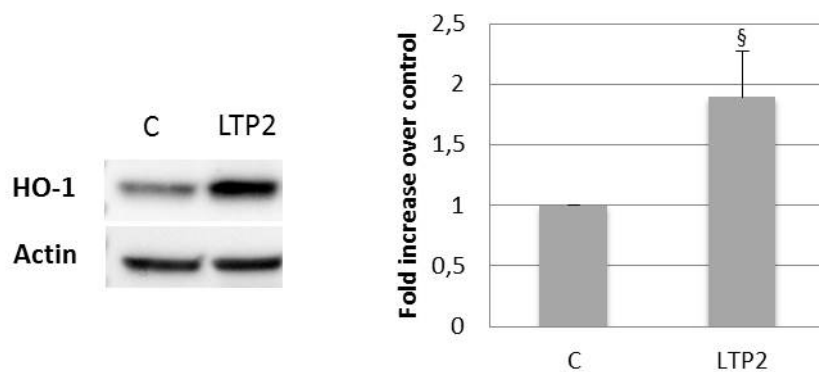


Figure 7.4: effects of nsLTP2 on HO-1 protein expression level

After treatment with 10 nM nsLTP2 for 24 h, HUVECs lysates were subjected to SDS-PAGE and Western blotting analysis of HO-1 protein expression levels. The image shows a representative experiment of three independent analyses. Densitometric analysis of HO-1 expression, normalized to actin, is expressed as fold increase over control and was carried out with the ChemiDoc MP System (BIORAD). Data represent means ± SD of three independent experiments. §p < 0.05, compared with control HUVECs.

In order to corroborate these results, a fluorescent immunostaining analysis was performed in the same experimental conditions. *Figure 7.5* shows that in HUVECs treated with 10 nM nsLTP2 for 24 h a strong increase in cell fluorescence was detectable.

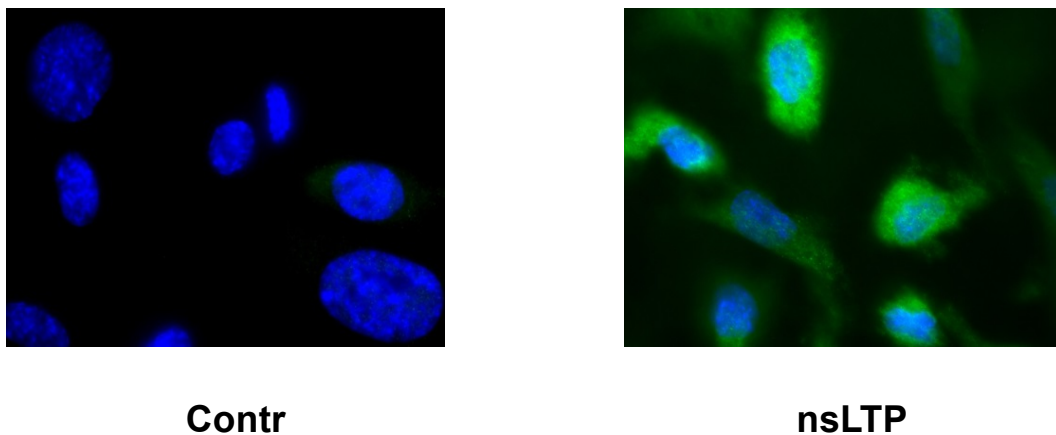


Figure 7.5: Immunofluorescence staining of HO-1 in HUVECs after 10 nM nsLTP2 treatment for 24 h HUVECS, incubated with 10nM nsLTP2, were fixed in 3% (w/v) paraformaldehyde for 15 min. Cells were then immunolabeled with anti-HO-1 antibody, treated with fluorescent FITCH-conjugated secondary antibody and visualized using immunofluorescence microscopy (Zeiss Axio Scope.A1, magnification 100x). Nuclei were stained with DAPI.

To confirm the obtained results regarding nsLTP2 effect on HO-1 expression, a comparison between nsLTP2 and statins effect was performed. HUVECs were treated with simvastatin, atorvastatin or nsLTP2 for 24 h and then analysed for their effect on HO-1 protein expression by immunoblotting. As represented in *Figure 7.6*, HO-1 expression was significantly up regulated by treatment with both statins and nsLTP2 used at the same concentration (10 nM).

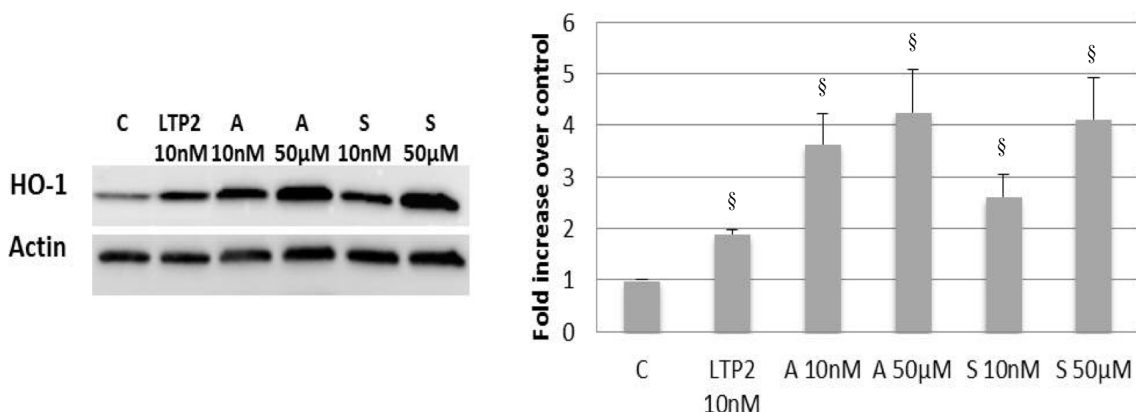


Figure 7.6: Effect of nsLTP2, Atorvastatin and Simvastatin on HO-1 protein expression level.

HUVECs were treated with 10 nM nsLTP2 (LTP2), 10 nM - 50 μ M Atorvastatin (A) or 10 nM - 50 μ M Simvastatin (S) for 24 h. Cell lysates were subjected to SDS-PAGE and Western blotting analysis of HO-1 protein expression level. Densitometric analysis of HO-1 expression, normalized to actin, is expressed as fold increase over control and was carried out with the ChemiDoc MP System (BIORAD). Data represent means \pm SD of three independent experiments. $^{\S}p < 0.05$, compared with control HUVECs.

In order to clarify the role of HO-1 in inflammation and to evaluate its link with the modulatory effect exerted by nsLTP2 on adhesion molecules, HUVECs were treated with two HO-1 inhibitors: Zinc protoporphyrin IX (ZnPPiX) and Tin Protoporphyrin IX (SnPPiX).

Results presented in *Figure 7.7* show that pre-treatment of HUVECs with 10 nM nsLTP2 for 24h resulted in a decrease in the expression of VCAM-1 induced by TNF- α . This effect is abrogated when HUVECs were treated with both ZnPPiX and SnPPiX suggesting that the induced expression of HO-1 may be involved in the modulatory effect of nsLTP2 on adhesion molecules.

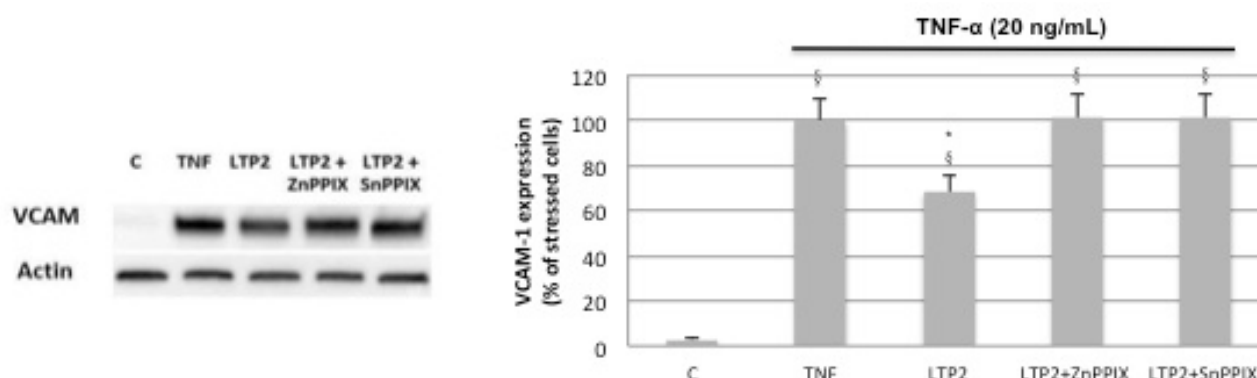


Figure 7.7: Evaluation of the effect of nsLTP2 on VCAM-1 expression in HUVECs treated with TNF- α in the presence or absence of HO-1 inhibitors

HUVECs were treated with 10 nM nsLTP2 in the presence or absence of HO-1 inhibitors (10nM ZnPP IX and SnPP IX) and subsequently treated with 20ng/mL TNF- α . Cell lysates were subjected to SDS-PAGE and Western blotting analysis of VCAM protein expression level. Densitometric analysis of VCAM expression normalized to actin is expressed as % of stressed cells and was carried out with the ChemiDoc MP System (BIORAD). Data represent means \pm SD of three independent experiments. $^{\S}p < 0.05$, compared with control HUVECs, $^*p < 0.05$, compared with HUVECs stressed by TNF- α .

7.5 Antioxidant activity of polyphenol metabolites

As previously described for nsLTP2, the antioxidant activity of polyphenol metabolites was evaluated with a spectrofluorimetric assay implying the DCFH-DA probe. To this purpose, HUVECs were pre-treated with polyphenol metabolites at 0.1 μ M concentration for 24h followed by treatment with 100 μ M H₂O₂, and 20 ng/mL TNF- α .

As reported in *Figure 7.8a*, HUVECs treatment for 24 h with CA metabolite M1 (dihydrocaffeic acid sulfate) at 0.1 μ M concentration causes a significant reduction of intracellular ROS levels compared to cells stressed with 100 μ M H₂O₂. On the contrary, 24 h treatment with other CA metabolites M2 and M3 (Dihydroferulic acid sulfate and Isoferulic acid 3-glucoronide) and with CY metabolites M4-M7 (protocatechuic acid 4-glucoronide, protocatechuic acid 3-*O*-sulfate, protocatechuic acid 4-*O*-sulfate and protocatechuic acid 3-glucoronide) did not show a significant intracellular ROS level decrease.

HUVECs pre-treatment with metabolites M1-M3 and M4-M7 was able to significantly reduce intracellular ROS levels produced after treatment with 20 ng/mL TNF- α (*Figure 7.8b*).

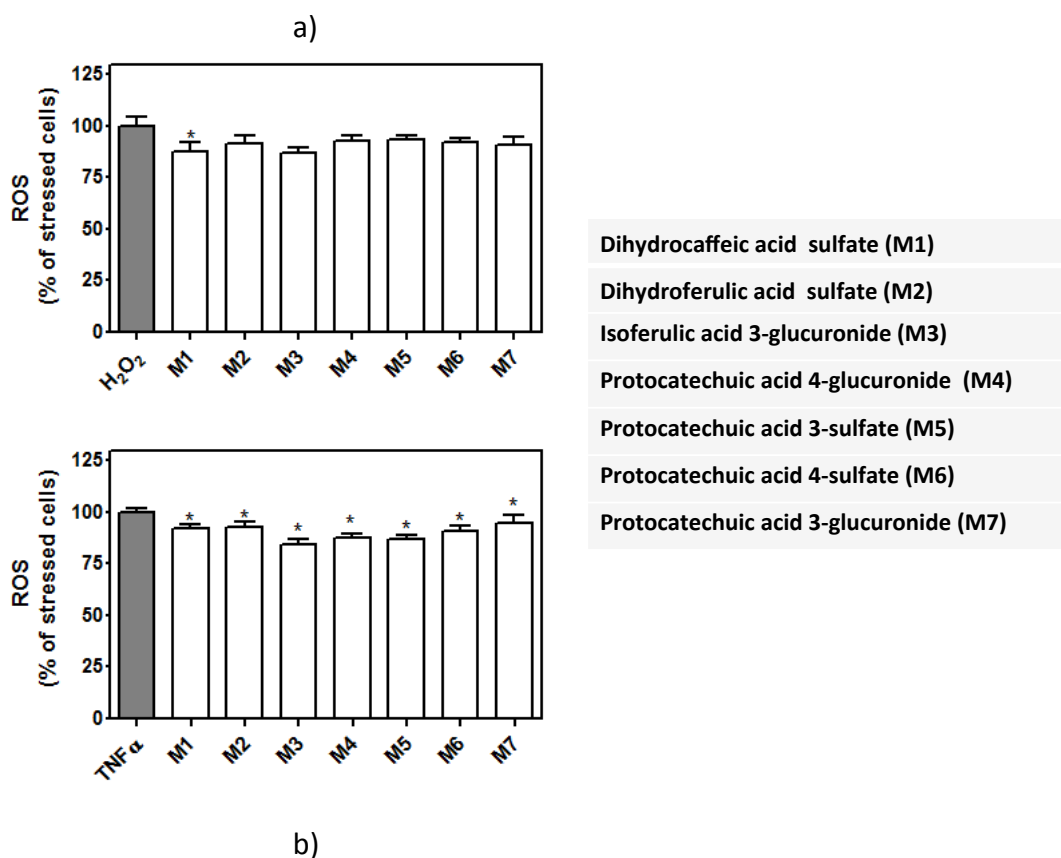


Figure 7.8: Evaluation of intracellular ROS production in HUVECs treated with 100 μM H_2O_2 and 20ng/mL $\text{TNF-}\alpha$ in the presence / absence of polyphenol metabolites. ROS levels were evaluated spectrofluorimetrically using DCFH-DA dye as reported in Material and Methods. HUVECs were pre-treated with tested compounds for 24h at 0.1 μM concentration and then stressed with 100 μM H_2O_2 (a) and 20 ng/mL $\text{TNF-}\alpha$ (b) for 30 min. Data are expressed as % of stressed cells. Values represent means \pm SD of at least 3 independent experiments. Statistical analysis was carried out by the Student's t test. * $p < 0.05$ significantly different from stressed cells.

7.6 Cytoprotective activity of polyphenol metabolites

In order to investigate the potential cytoprotective effect exerted by polyphenol metabolites in HUVECs subjected to oxidative stress and inflammatory stimulation, cell viability was assessed

by means of MTT assay. This spectrophotometric assay is based on reduction of a tetrazolium salt to formazan by the intracellular reducing power. MTT, a yellow tetrazole, is reduced to purple formazan in living cells. This method is widely regarded as an accurate indicator of cell redox activity, correlated to cell viability.

Treatment with 100 mM H₂O₂ for 24 h causes a significant decrease in cell viability compared to control. Treatment with CA metabolites M1 and M2 was able to increase cell viability, which resulted to be significantly different from that of H₂O₂-treated cells. On the contrary, pre-treatment with M3 did not modify cell viability whose values did not differ from that of H₂O₂-stressed cells. All CY metabolites, with exception of M6, were able to significantly increase cell viability (*Figure 7.9a*). In cells treated with 20 ng/ml TNF- α a significant decrease in cell viability was measured. In cells pre-treated with polyphenol metabolites, a significant increase in cell viability values with respect to TNF- α treated cells was observed, with the exception of metabolite M3 (*Figure 7.9b*).

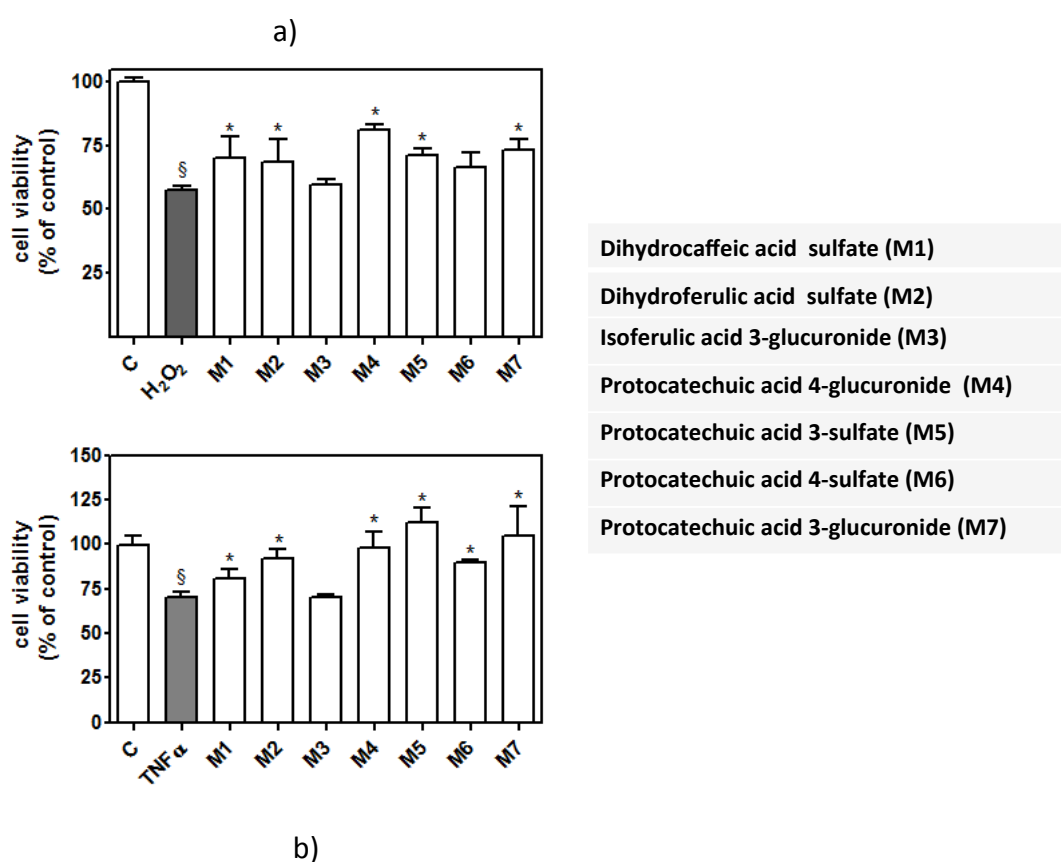


Figure 7.9: Analysis of cell viability in HUVECs treated with 100 μ M H_2O_2 and 20ng/mL $TNF-\alpha$ in the presence / absence of polyphenol metabolites. Cell viability was assayed spectrophotometrically by means of MTT test as described in Materials and Methods. HUVECs were pre-treated for 24 h with the test compounds at 0.1 μ M concentration and subsequently treated with 100 μ M H_2O_2 (a) and 20 ng / mL $TNF-\alpha$ (b) for 24 h. Data are mean \pm SD of 3 independent experiments. Statistical analysis was performed by Student t-test, * $p < 0.05$ vs. H_2O_2 or $TNF-\alpha$ treated cells; § $p < 0.05$ vs. control.

Further evaluation of the potential protective effects of polyphenols against cell damage generated by H_2O_2 or by $TNF-\alpha$ was performed by measuring the activity of the enzyme lactic dehydrogenase (LDH) in cell culture media, as a specific index of cell damage. *Figure 7.10* shows the effect of treatment with polyphenol metabolites in HUVECs subjected to oxidative stress (H_2O_2) (*Figure 7.10a*) or treated with pro-inflammatory cytokine $TNF-\alpha$ (*Figure 7.10b*).

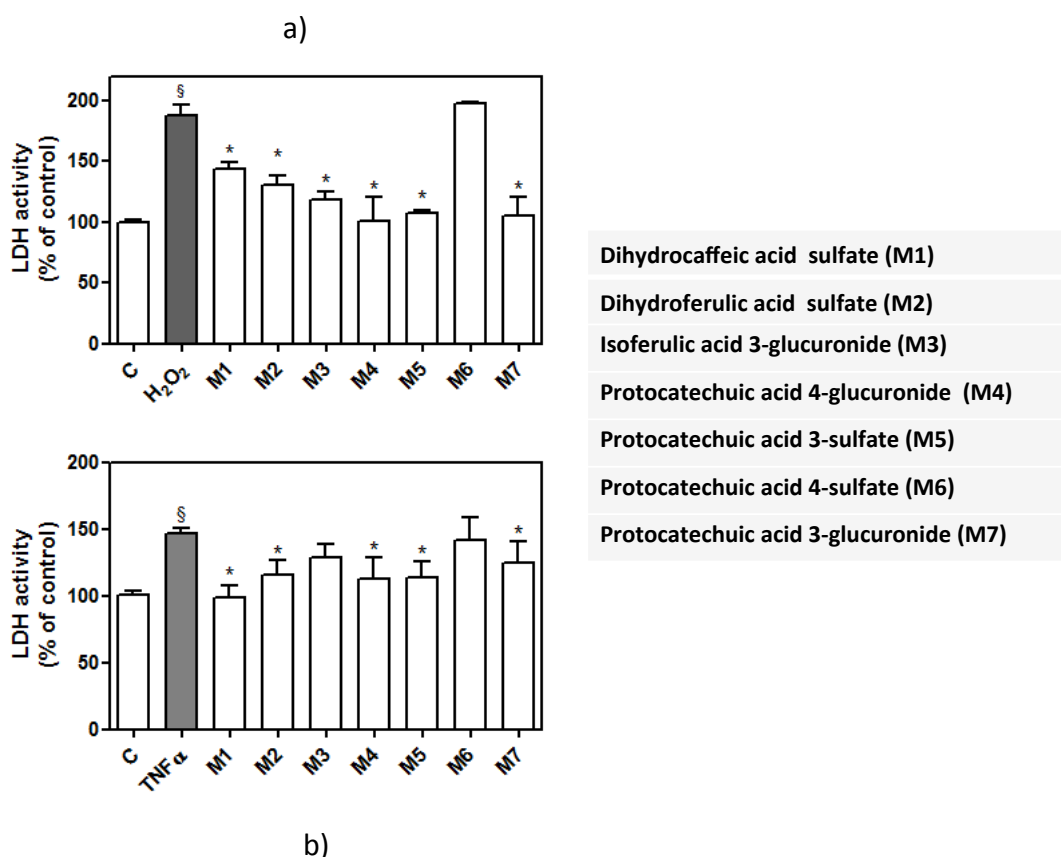


Figure 7.10: Evaluation of LDH release HUVECs treated with 100 μ M H_2O_2 and 20ng/mL $TNF-\alpha$ in the presence / absence of polyphenol metabolites. The LDH activity was assayed spectrophotometrically as described in Materials and Methods. HUVECs were pre-treated for 24 h with the tested compounds at

0.1 μM concentration and subsequently treated with 100 μM H_2O_2 (a) and 20 ng/mL TNF- α for 24 h (b). Data are mean \pm SD of 3 independent experiments. Statistical analysis was performed by Student t-test * $p < 0.05$ vs. H_2O_2 or TNF- α treated cells; § $p < 0.05$ vs control.

Cell treatment with 100 μM H_2O_2 caused cell damage, as evidenced by a significant increase in the activity of LDH in the culture medium. CA metabolites M1, M2, M3 at 0.1 μM concentration significantly reduced LDH release (*Figure 7.10 a*). Among CY metabolites, pre-treatment with metabolites M4, M5, M7 significantly decrease LDH activity with respect to H_2O_2 treated cells, while no significant differences were observed in cells pre-treated with metabolite M6 (*Figure 7.10 a*).

In cells treated with TNF- α , metabolites were able to significantly reduce the increase of LDH activity, with the exception of compounds M3 and M6, among metabolites of CA and CY, respectively (*Figure 7.10b*).

7.7 Anti-inflammatory effects of polyphenol metabolites in vascular endothelium.

To better characterize the effect of polyphenols metabolites at vascular levels, the effect exerted on the expression of adhesion molecule VCAM-1 and ICAM-1 was analyzed in HUVECs treated with TNF- α .

As represented in *Figure 7.11a*, HUVECs pre-treatment with metabolites M1 and M2 was able to significantly decrease the expression of VCAM-1 in cell treated with 20 ng/mL TNF- α (*Figure 7.11 a*). This down-regulation in the expression of VCAM-1 does not occur when cells were pre-treated with metabolite M3. CY metabolites M4-M7 were able to reduce the expression of VCAM-1 induced by 20 ng/mL TNF- α as shown in *Figure 7.11 b*. Moreover, CA metabolites M1 and M2 were able to significantly decrease the expression of ICAM-1 in cells treated with 20 ng/ml TNF- α (*Figure 7.11c*). Cyanidin metabolites M4-M7 were also able to reduce ICAM-1 expression induced by 20 ng/ml TNF- α (*Figure 7.11d*).

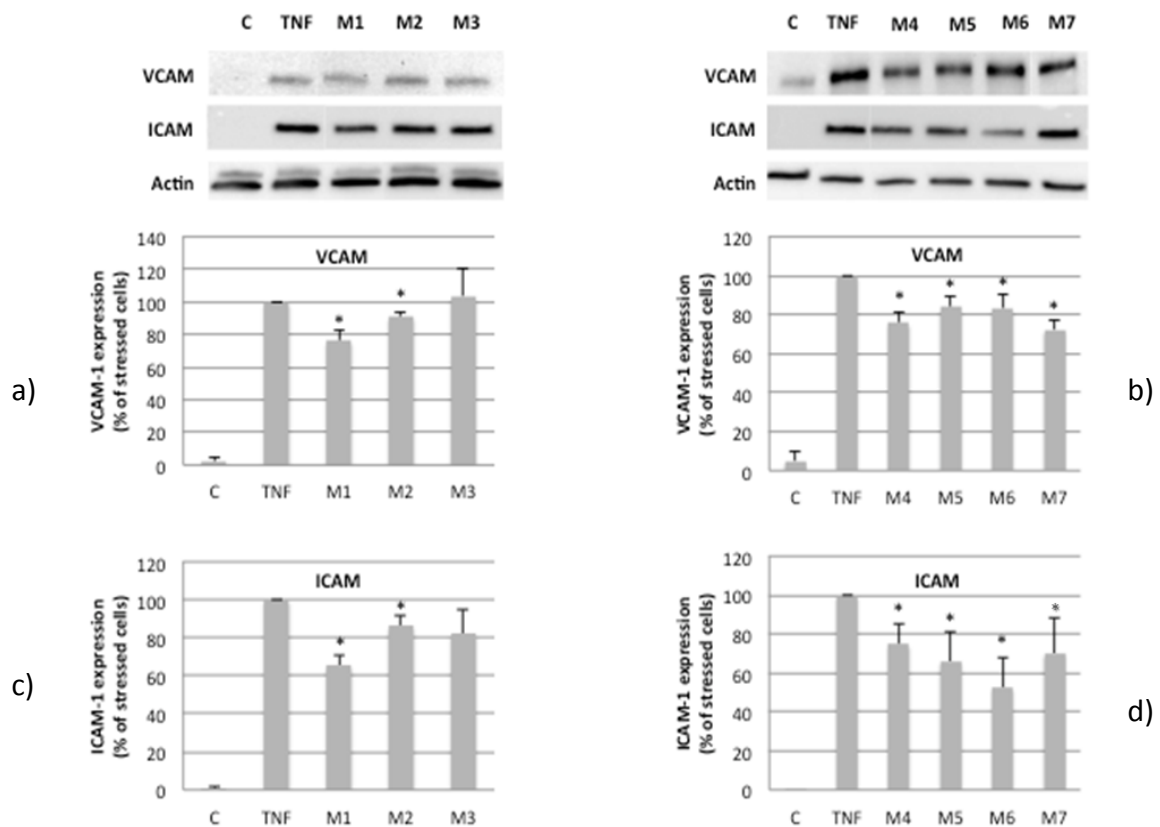


Figure 7.11: Immunoblotting analysis of expression of VCAM -1 and ICAM-1 in HUVEC cells exposed to TNF- α in the presence/absence of metabolites of polyphenols. HUVECs were treated for 24 h with polyphenol metabolites at a concentration 0.1 μ M and subsequently treated with 20 ng / mL TNF- α for 24 h. Cell lysates were subjected to SDS-PAGE and Western blotting analysis of VCAM-1 and ICAM-1 protein expression level. Densitometric analysis of VCAM-1 (a, b) and ICAM-1 (c, d) expression, normalized to actin, is expressed with respect to stressed cells and was carried out with the ChemiDoc MP System (BIORAD). Data are mean \pm SD of 3 independent experiments. Statistical analysis was performed by Student t-test, * p < 0.05 vs. TNF- α treated cells;

7.8 Effects of polyphenol metabolites on HO-1 expression

In order to analyse the effect of polyphenol metabolites on the expression of HO-1, HUVECs were treated with 0.1 μ M polyphenol metabolites for 24 h.

As represented in *Figure 7.12*, in the experimental conditions used in this study, no significant variations in the expression of HO-1 were observed following treatment of HUVECs with metabolites.

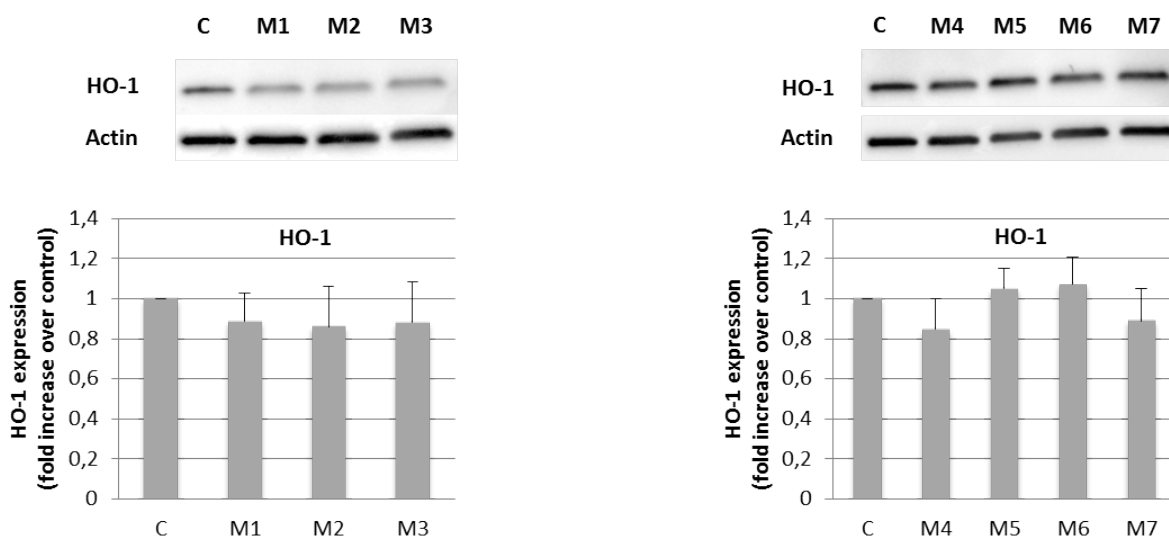


Figure 7.12: Immunoblotting analysis of the expression of HO-1 in HUVECs treated with polyphenol metabolites. HUVECs were treated for 24 h with polyphenol metabolites at a concentration 0.1 μ M for 24 h. Cell lysates were subjected to SDS-PAGE and Western blotting analysis. Densitometric analysis of HO-1 expression, normalized to actin, is expressed with respect to control cells and was carried out with the ChemiDoc MP System (BIORAD). Data are mean \pm SD of 3 independent experiments. Statistical analysis was performed by Student t-test.

CHAPTER 8: Discussion

Numerous epidemiological studies have highlighted the direct correlation between the intake of whole grains, including wheat and derived products (pasta, bread, etc) and the reduction in risk of developing chronic degenerative diseases. Initially attributed to the abundance of fibers and micronutrients, this beneficial effect is today more centred on the nutraceutical value of these foods and related to the specific (poly)phenolic profile which characterize the different varieties (Leoncini et al. 2012).

Recently, nutritional research has been directed towards the characterization of the health benefits of physiologically active peptides that, through different mechanisms, including the ACE inhibitory action, the antioxidant, antithrombotic and cholesterol lowering properties, may contribute to the maintenance of the functionality of the cardiovascular system (Erdmann et al. 2008). An interesting aspect and one of the aims of this research work is the valorisation of commonly consumed foods, such as wheat and wheat-derived products, on the basis of their content in bioactive ingredients endowed with nutraceutical properties. The *in vitro* study of the effects of food bioactive components is essential for the validation of dietary strategies aimed at diseases prevention, and represents an essential step in the definition of food "functionality" (Diplock et al 1999).

This study is part of a wider project "Beneficial effects of dietary bioactive peptides and polyphenols on cardiovascular health in human" BACCHUS, financed within the 7th European Framework Programme. The main objective of BACCHUS is to develop tools and resources that will facilitate the generation of robust and exploitable scientific evidence that can be used to support claims of a cause and effect relationship between consumption of bioactive components, particularly peptides and polyphenols, and beneficial physiological effects related to cardiovascular health maintenance in humans.

Bioactive peptides present in food from both animal and plant sources, have drawn researchers' attention as they demonstrated nutraceutical properties. Among peptides of vegetable origin, one of the most currently studied is lunasin. Lunasin was originally isolated in soy, but its presence also has been reported in other plant sources, including cereals (Jeong et al. 2007). Numerous studies have investigated its anticancer, anti-inflammatory and antioxidant properties (J. B. Jeong et al. 2010) (Hernández-Ledesma & de Lumen 2008). Moreover, *in vivo* studies both in animal and humans have shown that this peptide is partially resistant to enzymatic digestion and is distributed to different tissues, including heart, where it retain biological activity thus

demonstrating its bioavailability (Hsieh et al. 2010). Based on these evidence a cardio-protective role for lunasin peptide was hypothesized.

Preliminary results indicated that lunasin, in the concentration range 0.1-100 μ M is not cytotoxic to HUVECs and positively affected cell viability and the expression of HO-1 and decreased pro-caspase-3 in cells undergoing oxidative/inflammatory stimulation (data not shown).

Galvez e de Lumen (Galvez & de Lumen 1999) reported that cDNAs encoding lunasin corresponds to the small sub-unity of the 2S albumins of soybean, a family of storage proteins present in dicotyledonous plants, but not yet reported in cereal seeds. Following the observation of the biological activity displayed by lunasin, research has been conducted in order to identify lunasin and related peptides in different plant species. In particular, lunasin presence was reported in *Solanum amaranthus* (Silva-Sánchez et al. 2008) and cereal seeds including wheat (Jeong et al. 2007) rye (H. J. Jeong et al. 2010), barley and triticale (Nakurte et al. 2012). In order to determine whether lunasin identified in cereals was also derived from a 2S albumin-like precursor, Mitchell et al., (Mitchell et al. 2013) recently conducted an extensive database search of known sequences of major cereals. They found that no sequence encoding for lunasin was present in these databases, nor were similar or related sequences. Based on these results, Authors concluded that lunasin is not a protein present in cereals and the observed presence could be attributed or derive from a contamination of the raw material analysed by other organisms, such as *fungi*.

Thus, with the aim of searching for peptides, which may characterize different wheat varieties for their biological activity and nutraceutical properties, a study was conducted in the attempt to clarify lunasin presence or absence in wheat. The study was conducted in collaboration with Professor G. Dinelli group of the Department of Agricultural Sciences (UNIBO) and with the company Bioaesis, both partner of the EU founded project BACCHUS, and thanks to the collaboration with Professor R. Gotti and Dr. J. Fiori of the Department of Pharmacy and Biotechnology (UNIBO).

In this study a double approach was used: a Liquid Chromatography/Mass Spectrometry system with a electrospray interface (LC-ESI-MS) was used for the identification of lunasin, while PCR techniques were applied for the detection of the specific gene sequence.

Analyses were conducted on 12 different wheat varieties, belonging to the collection of the Department of Agricultural Sciences (6 varieties of durum wheat, 5 varieties of soft wheat, 1

ecotype of khorasan wheat) covering a broad spectrum, although not fully representative, of the wheat genome. A protein-enriched extract was obtained from each variety included in the study following a method reported in the literature (Dinelli et al. 2014). These varieties were compared with the standard lunasin peptide, commercially available.

Chromatograms of wheat extracts didn't show any peaks with a retention time corresponding to that of lunasin standard. Accordingly, mass spectrum of different extracts corresponding to peaks in the area close to the retention time of the standard peptide evidenced the absence of compounds with a molecular mass similar to that of lunasin.

These differences could not be ascribed to a lack of sensitivity of the method utilized for the analyses since the limit of detection of the technique was 10 times lower than the concentration of lunasin reported in the literature in different cereal matrices.

In order to verify that the apparent absence of lunasin in wheat extracts could be due to the lack of expression of the corresponding gene in the varieties studied, a molecular analysis was conducted using PCR techniques. Primers used for lunasin gene amplification were designed within the sequence encoding 2S sub-unity of soy albumin, according to the sequence reported by Galvez et al (Galvez & de Lumen 1999), introducing two degeneration for each primer in order to allow the possible amplification of sequences, present in wheat genome, not perfectly matching that of soy.

PCR analysis showed the presence, in soy DNA, of electrophoretic bands corresponding to the amplicons of the expected molecular weight. The same amplicons were not observed in the DNA extracted from the different wheat varieties, which were analysed as single accession and as pool of varieties. Sequencing and following BLAST search of the specific 67bp amplicon, present in soy DNA confirmed a 100% match with the lunasin gene sequence. The same analysis conducted on a 77bp amplicon observed only in DNA extracted from wheat revealed a 95.7% match with sequences referred to the genus *Triticum* and a 4.3% match with sequences referred to a botanical species representing an ancestor of modern wheat, thus excluding any possible overlapping with lunasin gene. The obtained results (absence of 4 sequences related to lunasin in 12 different wheat genotypes analysed) indicated the absence of lunasin gene in the extracted DNA (Dinelli et al. 2014).

According to these results, the research work then focused on the identification of the wheat peptide responsible for the observed biological effects that other Authors have previously ascribed to lunasin.

After the purification of the main peptide in the mass range 7-9 kDa by gel filtration and ion exchange chromatography, the isolated peptide was identified by LC-MS/MS analysis on the tryptic digest. Mascot processing data, obtained searching SwissProt and NCBI databases, allow to ascribe the signal of about 7 kDa to a non-specific lipid-transfer protein (nsLTP) type 2 from *Triticum aestivum* (92% protein sequence coverage, according to NCBI). Peptide identity was further confirmed observing the inhibition of *Staphylococcus aureus* growth by a specific microbiological test. In fact, as reported in the literature, peptides belonging to nsLTP2 family proteins are characterized by antimicrobial activity (Boutrot, Chantret, & Gautier, 2008).

To date, two families of LTPs have been isolated and characterized, and are referred to as nsLTP1 and nsLTP2, with molecular masses of ~9 kDa and 7 kDa, respectively (Blein et al. 2002). A number of biological roles (antimicrobial defense, signaling, assembly of cutin) have been proposed for nsLTPs, but conclusive evidence is generally lacking (Yeats & Rose, 2008). However, currently no data regarding nsLTP type 2 biological activity in mammalian cells are present in the literature, therefore considering that the specific aim of the study was related to cardiovascular health maintenance, the research work focused on the characterization of the biological activity related to protective effects on vascular endothelium of the newly isolated peptide and on the analysis of potential mechanism by which nsLTP2 can contribute to cardiovascular health.

Since oxidative stress has been recognized as a key factor in the onset and development of several cardiovascular diseases, experiments were conducted in order to evaluate the potential antioxidant activity of wheat nsLTP2 in the presence of different stressors, both oxidants (H₂O₂) and pro-inflammatory (cytokines and LPS). Results demonstrated that nsLTP2 was able to reduce intracellular ROS production, displaying antioxidant activity.

The observed effect could be related to the specific amino acids composition of the polypeptide chain. The antioxidant activity has been linked to the presence of Tyr, Trp, Met, Lys, Cys, and His due to the chemical properties of the -R group (Sarmadi & Ismail 2010). In particular, Cys sulfhydryl group can directly interact with radicals; aromatic residues contribute to radical-scavenging activity thanks to their ability to donate protons to electron deficient radicals and imidazole group of His display metal ion-chelating, hydrogen donating and lipid peroxy radical trapping ability (Liu et al. 2010; Wang et al. 2010). Considering nsLTP2 sequence

(ACQASQLAVC ASAILSGAKP SGECCGNLRA QQGCFQYAK
DPTYGQYIRSPHARDTLTSC GLAVPHC), the observed antioxidant effect could be ascribed, at least in part, to the presence of several cysteine residues.

Cytoprotective activity of nsLTP2 was evaluated by measuring lactate dehydrogenase (LDH) activity in cell culture medium as a marker of non-specific cell damage caused by oxidant/pro-inflammatory agents. Results show that pre-treating HUVECs for 24 h with 10 nM nsLTP2 cause a significant decrease in LDH activity induced by 100 μ M H₂O₂, 20 ng/mL TNF- α or 10 ng/mL IL-1 β . In the same experimental setting 10nM nsLTP was not able to reduce cell damage induced by 10 μ g/mL LPS. The reason for discrepancy is not known but it could possibly be ascribed to different mechanisms regulating cell survival/death in the presence of LPS.

The relevance of these results is strengthened by the fact that the observed antioxidant/cytoprotective activity of nsLTP2 was observed at physiologically achievable concentrations that were chosen according to data presented in the literature, which reports a similar plasma concentration for lunasin (previously considered the study reference peptide) in men after soy intake (Dia *et al.*, 2009).

Scientific evidence support the view that leukocytes and adhesion molecules are important participants in endothelial dysfunction and tissue injury and both these situation are linked to inflammatory state and cardiovascular diseases (Golias et al 2007.). The endothelial expression of cell-surface adhesion molecules, such as VCAM-1, ICAM-1 and E-selectin, represents a marker of endothelial activation, which is typically induced by pro-inflammatory cytokines. A deregulation of all the physiological system in which adhesion molecules are involved leads to the activation of transduction pathways that code for cytokines and chemokines expression, which can contribute to continue the recruitment of leukocytes, their attachment to the vessel wall and subsequent migration with a consequent unresolved inflammation (Golias et al. 2007). Since the inflammatory response of endothelial cells is of critical importance in different cardiovascular diseases, the control of inflammation at the endothelial level may efficiently retard or reverse the pathogenic process.

With the aim of assessing a potential anti-inflammatory activity of nsLTP2, experiments were conducted in order to verify a possible modulatory effect on adhesion molecule expression.

Immunblotting analyses suggest that nsLTP2 at physiologically achievable concentration (10 nM) is able to significantly reduce the increased expression of cell adhesion molecules observed

following HUVECs treatment with TNF- α (20 ng/ml), IL-1 β (10 ng/ml) and LPS (10 μ g/ml) in HUVECs.

To further analyze the potential anti-inflammatory effect of nsLTP2, its effect on HO-1 expression was evaluated. HO-1 is a well known microsomal enzyme essential in heme catabolism where it cleaves heme to form biliverdin, subsequently converted to bilirubin by biliverdin reductase, and carbon monoxide within a process in which are involved cytochrome P-450, NADPH and molecular oxygen (Durante 2010). Moreover, this enzyme has been recognized as being part of endogenous antioxidant defense system (Talalay 2000). Several scientific evidence reported the important physiological and beneficial role of HO-1 in the vasculature and, many of them, focused on vascular endothelium (Calay & Mason 2014). Overexpression of HO-1 in human endothelial cells has been shown to protect against tert-butyl hydroperoxide cytotoxicity and pro-apoptotic cytokines. In addition, HO-1 decreases oxidative stress induced by H₂O₂ or NADPH oxidase and may attenuate inflammatory response induced by LPS, TNF- α and other inflammatory cytokines. Mechanistically, this involved the inhibition of NF- κ B activation thus decreasing the subsequent up-regulation of cyclo-oxygenase-2 (COX-2), E-selectin, VCAM-1, ICAM-1, and monocyte chemoattractant protein-1 (MCP-1) secretion (Calay & Mason 2014). HO-1 exerts pleiotropic effects on the vascular endothelium, including antiapoptotic, anti-inflammatory and antioxidant actions (Calay & Mason 2014).

Therefore, to verify if the observed anti-inflammatory effect of nsLTP2 could be mediated through the induction of HO-1 expression, HUVECs were treated with 10 nM nsLTP2 for 24 h and cell lysates were subjected to immunoblotting analysis. HUVECs treatment with nsLTP2 caused a significant increase in HO-1 protein expression, compared to control cells. This data suggest an anti-inflammatory activity exerted by the selected peptide due to the activation of one of the endogenous antioxidant system.

The cytoprotective properties of increased HO-1 activity, directed the interest in its modulation. Some common dietary phytochemicals are known HO-1 inducers (Haines et al. 2012) as well as drugs currently used in therapy (Calay & Mason 2014) for example, statins (HMG-CoA reductase antagonists), which exhibit LDL cholesterol-independent immunomodulatory, vasculoprotective and anti-inflammatory activities (Greenwood & Mason 2007). The main effect of statins is to block the conversion of HMG-CoA to mevalonate during the hepatic cholesterol biosynthesis; however, statins have also demonstrated the ability to increase HO-1 expression. Particularly, simvastatin, rosuvastatin and atorvastatin have been shown to increase HO-1 promoter activity and increase the antioxidant capacity in endothelial cells (Mrad et al. 2012).

In order to confirm the observed effect on HO-1 expression exerted by nsLTP and compare it to statins effect, HUVECs were treated with simvastatin, atorvastatin or nsLTP2 for 24 h and then analysed for their effect on HO-1 protein expression by immunoblotting. Results reveal a HO-1 up-regulation with nsLTP2 and statins at the same concentration (10 nM), this up regulation increases at higher statin concentrations (50 μ M), commonly used to evaluate the effect of these drugs on biological activities, as reported in literature (Yang et al. 2012).

In recent studies, HO-1 has been shown to ameliorate inflammation, in part through its ability to inhibit endothelial adhesion molecules expression (Olszanecki et al. 2007).

Considering the promising results obtained, it is of interest to examine whether up-regulation of HO-1 induced by nsLTP2 is correlated to the down-regulation of adhesion molecule expression (i.e. VCAM) induced by TNF- α . Therefore, experiments were conducted using two HO-1 inhibitors: Zinc Protoporphyrin IX (ZnPPIX) and Tin Protoporphyrin IX (SnPPIX). Results indicate that pre-treatment of HUVECs with nsLTP2 resulted in a decreased expression of VCAM-1 induced by TNF- α and this effect is counteracted by HO-1 inhibitors, suggesting that the induced expression of HO-1 may contribute to the underlying mechanism of the anti-inflammatory activity exerted nsLTP2. The inhibition of CAMs expression, mediated through HO-1 expression in HUVECs, has been described for other natural compounds (Li et al. 2014).

Since the endothelium tightly regulates vascular hemostasis and the loss of this balance results in endothelial dysfunction, which has been directly correlated to cardiovascular diseases development, it is evident that this tissue has to be considered a major target for dietary strategies aimed at cardiovascular diseases prevention. Among physiologically active compounds of natural origin, polyphenols represent the main class of antioxidant compounds in diet due to their wide distribution in plants. Several epidemiological studies have recently pointed out that regular consumption of foods and beverages rich in polyphenols is associated with a reduction of cardiovascular diseases, many of which are associated with endothelial dysfunction (Tangney & Rasmussen 2013). Despite scientific evidence, derived from both *in vitro* and epidemiological studies, supporting the preventive role of polyphenols-rich food against cardiovascular diseases, to date, it has not yet been possible to clearly identify the specific compounds responsible for this protective role. This discrepancy likely depends on several factors related to the experimental procedures. For example, many *in vitro* experiments used polyphenol concentrations that are much higher (sometimes orders of magnitude greater) than those that are likely to be found *in*

vivo, after oral ingestion. Another important aspect is represented by the impact of digestive system and cell metabolism on the native form of polyphenol compounds. In fact, it is well known that polyphenols present in foods are subjected, following ingestion, to an extensive metabolism and the molecules that will act at the cellular and tissue level will be, most likely, a metabolite rather than a polyphenolic compound in its native form. For these reasons, part of this research work was dedicated to the analysis of the effect of polyphenols metabolites, derived from caffeic acid and cyanidin and their potential protective effects against oxidative and inflammatory stress (Speciale et al. 2014).

In particular, dihydro caffeic acid 3-*O*-sulfate (M1) and dihydroferulic acid 4-*O*-sulfate (M2) were analyzed as they represent compounds found in post-prandial plasma samples of individuals drinking coffee, which represent the main source of phenolic acids in the diet and isoferulic acid 3-*O*-glucuronide (M3) a metabolite present in both plasma and urine. Antocyanins metabolites, protocatechuic acid 4-glucuronide (M4), protocatechuic acid 3-sulfate (M5), protocatechuic acid 4-sulfate (M6) and protocatechuic acid 3-glucuronide (M7), were chosen as they were found in plasma samples following anthocyanins rich extract intake.

All the metabolites have been tested at 0.1 μM concentration corresponding to the peak plasma concentration range of polyphenol metabolites, according to the literature (Stalmach et al. 2009). The antioxidant capacity represents a widely studied parameter in the analysis of bioactivity of polyphenols and its relevance in the study of a potential protective role in vascular health lie in the observation that oxidative stress is considered a major determinant of endothelial dysfunction (Siti et al. 2015).

Results obtained from the evaluation of the capacity of these molecules to reduce the levels of intracellular ROS show that only dihydrocaffeic acid sulfate (M1), a caffeic acid metabolite, is able to exert an antioxidant effect when the cells are subjected to oxidative stress (mimicked by treatment with H_2O_2) while, in a situation of inflammatory stress, triggered by $\text{TNF-}\alpha$, all the tested polyphenol metabolites display antioxidant capacity. The apparent discrepancy between results of the two assays is due to the fact that the concentration of H_2O_2 used to mimic oxidative stress is much higher than the concentration usually generated at physiological level by growth factors and cytokines, including $\text{TNF-}\alpha$; therefore, the effectiveness of polyphenols to act as antioxidant is more evident against $\text{TNF-}\alpha$ generated ROS, when polyphenols are used at a low concentration (0.1 μM), comparable to that present in the blood following dietary intake. The capacity to counteract H_2O_2 displayed by metabolite M1, is likely due to the presence in its

structure of the phenolic –OH, unlike other caffeic acid metabolites M2 and M3. Factors responsible for the *in vitro* radical scavenging activity are various: structural aspects, thermodynamic and kinetic must be taken into account (Amorati et al. 2006; Rice-Evans et al. 1996). The presence of a catechol ring in the molecule is one of the most studied characteristics, as it is directly implicated in the antioxidant capacity: electron donor groups (-OH) linked to the phenolic ring increase the antioxidant capacity more strongly than -OCH₃ group. The lower efficacy of protocatechuic acids in scavenging H₂O₂ is instead due to the presence of electron withdrawing groups (-COOH) on the aromatic ring.

Moreover, the antioxidant capacity is linked to pKa of hydroxyl groups present on the antioxidant molecule; therefore it is influenced by pH of the buffer. It is also essential to contemplate the different hydrophilicity of the molecules, which determines a different allocation between the aqueous phase and the lipophilic phase, which in turn directly affects the solubility of the molecules and the ability to penetrate inside cells (Bors et al. 2001).

Another key factor to consider when assessing dietary molecules capacity to exert a vascular protective action on the endothelium is not simply the functionality of the endothelium, but its capacity to react to different injuries. Tissue damage at the vascular wall, infections, or the aging process itself leads to stress in the endothelium, which has to respond with sufficient cell replacement (Delgado-Lista et al. 2014). Considering the correlation between oxidative stress and inflammation and their link to the endothelial dysfunction, the cytoprotective effects of polyphenols metabolites in endothelial cells undergoing oxidative and inflammatory stress was investigated. Phenolic acids and antocyanidins metabolites were able to decrease the cytotoxic effect generated by H₂O₂ and TNF- α treatment, evaluated by means of both MTT assay, which correlates cell viability to intracellular reducing power, and LDH release. Only metabolites M3 and M6 did not exert cytoprotective effect, but at the moment it is not possible to define to which of the previously indicated variable this lack of efficacy could be attributed.

To deepen the study of the protective action of polyphenol metabolites, the modulatory activity on the adhesion molecules VCAM-1 and ICAM-1 was evaluated by immunoblotting. It was demonstrated that pro-inflammatory cytokines, such as TNF- α , activate endothelial cells in the site of inflammation and lead to an up-regulation of endothelial adhesion molecules, including VCAM-1 and ICAM-1. Moreover it is well known that the overexpression of adhesion molecules is responsible for leukocytes recruitment, adhesion and their subsequent passage in the sub-endothelial space, which is a critical step in the development of atherosclerosis (Nizamutdinova et al. 2012). Other research works suggest that the induction of adhesion

molecules by TNF- α appears to be regulated at the gene level by the activation of transcription factors such as AP-1 and NF- κ B; some of these are redox sensitive factors and may be activated in conditions of oxidative stress. VCAM-1 and ICAM-1, in fact, might be triggered by high levels of intracellular ROS induced for example by TNF- α (Huang et al. 2012). It is therefore interesting to investigate the action of polyphenols and their metabolites on the expression of these adhesion molecules.

The results obtained in this study pretreating HUVEC cells with caffeic acid metabolites reveal a negative modulation of the expression of VCAM-1 and ICAM-1 when induced by TNF- α . These results are in accordance with Moon et al., in HUVEC cells, using different treatment times and concentrations of caffeic acids (Moon et al. 2009), and Lee et al., in HUVEC treated with a different stressor and polyphenol concentrations (Lee et al. 2012). This down-regulation in expression of adhesion molecules also occurs when cells are pretreated with cyanidin metabolites as reported by Amin (Amin et al. 2015) and Huang (Huang et al. 2014), although the concentrations tested were higher than that used in our study.

Finally, attention has been paid to the effect of the polyphenol metabolites on the expression of (HO-1), enzyme involved in heme degradation and currently recognized to belong to the class of phase II detoxification enzymes (Talalay 2000). The classic phase II enzymes such as glutathione-S-transferase and UDP-glucuronosyl transferase have the role to conjugate xenobiotics with endogenous ligands such as glutathione and glucuronic acid. This conjugation reaction gives rise to molecules of easier excretion. Currently, phase II enzymes include enzymes whose expression is subject to redox regulation and which exert a cytoprotective effect against oxidative stress.

However, in the experimental conditions used in this study, no significant variations in the expression of this enzyme following treatment of HUVECs with metabolites was observed. These results are in line with observations of Wang et al (Wang et al. 2010). In endothelial cells, a treatment with caffeic acid at a 20 μ M concentration, 200 times higher than the concentration used in our experimental setting, did not caused an induction of HO-1 expression in any of the analyzed time treatment. Even in RAW264.7 macrophages treatment with caffeic acid at the concentration 10 μ M for 6-24 h does not induce the transcription of HO-1 (Suzuki et al. 2006).

Despite the absence of induction of HO-1, the antioxidant and cytoprotective actions of caffeic acid metabolites may be justified by the fact that induction of HO-1 cannot be decisive for the

anti-inflammatory action. It has been observed that the anti-inflammatory effect of caffeic acid phenethyl ester (CAPE) persisted even after the silencing of HO-1 (Mapesa et al. 2011). This could also be the reason for the absence of HO-1 induction when cells were treated with 0.1 μ M cyanidin metabolites even if data present in the literature suggest that the parental compound cyanidin is able to exert an inducing capacity although at higher concentrations (Sorrenti et al. 2007; Speciale et al. 2014)

In conclusion, although the polyphenol metabolites have demonstrated overall cytoprotective activity, further studies are needed to deepen the exact mechanism of action.

An ever-increasing number of scientific evidence supports the preventive value of dietary patterns that favor the consumption of plant foods especially fruits, vegetables, grains and legumes; the correlation between the reduction of risk of chronic diseases and adherence to the Mediterranean diet is the most significant example. The central role of the endothelium in maintaining vascular homeostasis and the correlation between endothelial dysfunction and the development of cardiovascular diseases makes this tissue a primary target for the dietary strategies aimed at the prevention of cardiovascular diseases.

Research in the field of nutrition is therefore directed to the identification of food bioactive components with beneficial effects on the endothelium and to the characterization of their biological activities in order to define and support their nutraceutical value.

The activities conducted in this research were particularly focused on the evaluation of the potential beneficial effects in vascular endothelium of peptide belonging to the family of non-specific lipid transfer proteins type 2 (nsLTP2), identified in wheat and of polyphenols metabolites of two families: cinnamic acids and anthocyanins.

The obtained results demonstrated that nsLTP2 is able to perform a protective action on vascular endothelium in conditions of oxidative stress and inflammation, thus supporting the role of bioactive peptides in cardiovascular health.

About polyphenols, their beneficial effects on vascular endothelium in terms of antioxidant activity are unquestionable, as reported by numerous scientific evidence. However, polyphenols undergo an intensive metabolism, which need to be taken into account when investigating their mechanisms of action.

Therefore, in this study, the attention has been focused on the characterization of vascular protective effects of polyphenols metabolites. Caffeic acid and cyanidin metabolites demonstrated antioxidant and cytoprotective activities in endothelium in both oxidative stress and inflammation conditions. These observations are of particular interest in order to characterize the mechanisms of

action and specific biological activities of metabolites at physiological concentrations.

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