The Role of Virgin Olive Oil Components on the Modulation of Endothelial Function

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Abstract

The endothelium is involved in many of the processes related to the development of atherosclerosis, which is considered an inflammatory disease. Actually, traditional risk factors for atherosclerosis predispose to endothelial dysfunction, which is manifested as increase in the expression of specific cytokines and adhesion molecules. There is firm evidence supporting the beneficial effects of olive oil, as the most genuine component of the Mediterranean diet. Although the effects of olive oil and other oleic acid-rich dietary oils on atherosclerosis and plasma lipids are well-known, the roles of minor components have been less investigated. Minor components constitute only 1-2% of virgin olive oil and are comprised of hydrocarbons, polypohenols, tocopherols, sterols, triterpenoids, as well as other components usually found in traces. Despite their low concentration, non-fatty acid constituents may be of importance, since studies comparing monounsaturated dietary oils have reported different effects on cardiovascular disease. Most of these compounds have demonstrated antioxidant, anti-inflammatory and hypolipidemic properties. In the present review we summarize the current knowledge on the effects of these compounds contained in virgin olive oil on vascular dysfunction and the mechanisms by which they modulate endothelial activity. Such mechanisms involve the release of nitric oxide (NO), eicosanoids (protaglandins and leukotrienes) and adhesion molecules, in most cases by activation of nuclear factor κB (NFκB) by reactive oxygen species (ROS).

1. Endothelial dysfunction in cardiovascular disease

1.1. Nature of the atherosclerotic process

Atherosclerosis remains as the leading cause of death in the developed countries and is on track to become the most common cause of disease-related disability and death by the year 2020 [1,2]. Although several theories have been proposed for the pathogenesis of atherosclerosis, the current trend is to consider it as a response of the vascular wall to a variety of agents and mechanisms, which contribute to the development of the atheromatous plaque [3]. The vascular endothelium is an active and dynamic monolayer of cells, which serves as a semipermeable barrier between blood an tissue [4]. Due to this strategic location it is involved in maintaining homeostasis, by sensing changes in hemodynamic forces and signals and responding to them by releasing bioactive substances [5,6].

Traditional risk factors for atherosclerosis, which is considered an inflammatory disease, predispose to endothelial dysfunction and activation of the endothelium [7]. This activation is manifested as an increase in the expression of specific cytokines and adhesion molecules, such as intracellular adhesion molecule (ICAM-1), vascular cell adhesion molecule (VCAM-1) and E-selectin [8,9]. Circulating monocytes are attracted by these molecules and adhered to the endothelium, from which they transmigrate to the subendothelial space. Once within the endothelium, monocytes differentiate into macrophages, which scavenge oxidized LDL and triglyceride-rich lipoproteins (TRL), becoming foam cells and contributing to the formation of the atheroma in the early stages of the atherosclerotic process [7].

1.2. Function of endothelial cells.

The endothelium has a major function in thrombotic and coagulant activities, synthesizing several molecules that are released in response to different stimuli [10]. Heparan sulfate, nitric oxide [NO] and prostacyclin are vasodilators, whereas thromboxane A_2, prostaglandin H_2 and endothelin 1 are vasoconstrictors [11,12]. NO is the primary endothelial-derived relaxing factor playing a pivotal role in vascular reactivity [13]. It inhibits platelet aggregation [13], alters cell adhesion molecule expression and inhibits proliferation of smooth muscle cells [14]. NO is generated in the endothelial cell by NO synthase (eNOS), which converts the aminoacid L-arginine to NO and L-citrulline. NO diffuses from the endothelial cell to the

vascular smooth muscle and increases cyclic guanosine monophosphate, thereby causing relaxation of smooth muscle and dilation of the artery.

Endothelial dysfunction occurs early in the development of atherosclerosis, even before the formation of the plaque [15]. Clinical studies have revealed that risk factors for atherosclerosis, such as smoking, hypercholesterolemia, hypertension, diabetes and hyperhomocysteinemia, predispose to endothelial dysfunction [7]. When dysfunctional, the endothelium increases flow disturbances due to improper vasoreactivity. Most importantly, it initiates inflammatory responses by releasing proinflammatory cytokines and chemokines. Thus, it increases leukocyte activation, promotes monocyte adhesion molecule expression and facilitates entry of monocytes and lipoproteins into the subendothelial space [16].

1.3.Measurement of endothelial function.

Reliable assessment of endothelial function in humans seems to be of major importance and can be achieved by different approaches. The determination of soluble endothelial markers in blood is the most common and will be discussed later in this review. However, direct measurement of endothelial function would be desirable. Vasodilation can be measured after intra-arterial pharmacologic stimulation with substances that enhance NO release but this method is highly invasive. The most promising non-invasive method is the measurement of the flow-mediated dilation (FMD) in the brachial artery by Doppler ultrasonography. This technique is based on the endothelium sensitivity to shear stress, which elicits NO release and dilation of underlying smooth muscle [17]. Shear stress is caused by hyperemia (induced by cuff inflation and then deflation) and, in these conditions, FMD appears to be mediated mostly by NO (70%) and prostacyclin (30%) [17,18]. Thus, FMD may also serve as an index of NO bioavailability [19]. The endothelial signaling cascade responsible to concert mechanic stimuli into the release of vasodilatory molecules is nor fully known. Although some mechanisms have been suggested, it is probable that it involves phosphorylation of a serine residue of eNOS by shear stress, altering its sensitivity to intracellular calcium levels and hence increasing NO formation [20].

There is ample evidence that FMD is diminished in patients with atherosclerosis and coronary risk factors and that it can detect endothelial dysfunction in hyperlipidemia, hypertension and diabetes [21-23]. FMD has been shown to be decreased after a single high-

fat meal and postprandial lipemia [24], however there are very scarce data on the effect of olive oil. Vogel et al. [25] found a decrease in FMD after olive oil-rich meals compared with canola oil and salmon, which was attributed to oxidative stress because the decrease in FMD was reduced by the concomitant administration of vitamins C and E. However, these results should be confirmed with further investigations.

2. Factors that influence endothelial function

2.1. Reactive oxygen species impair the activity of nitric oxide

There is ample evidence indicating that increased vascular free oxygen levels are the prime mechanism for endothelial dysfunction [6]. ROS may be produced during normal metabolism or after oxidative processes and include superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) [26]. The contribution of NO and ROS to the vascular tone is inversely proportional to each other and the appearance of one could likely compensate for the absence of the other [27]. The formation of ROS is balanced by a range of antioxidant defenses, but the excess, as in several cardiovascular risk factors like hypercholesterolemia, hypertriglyceridemia or high postprandial triglyceridemia, can overwhelm these systems, leading to oxidative stress.

2.1.1. Effect of hypercholesterolemia on endothelial dysfunction

Hypercholesterolemia [28,29], diabetes mellitus [30] and hypertension [31,32] are related to increased production of superoxide anion. This anion reacts rapidly with NO to form peroxynitrite ($OONO^-$) thus inactivating NO and leading to endothelial dysfunction. ROS can also react with polyunsaturated fatty acids (PUFA) contained in lipoproteins in the vessel wall initiating lipid peroxidation. The hydroperoxides formed in this process can in turn react with NO to form peroxynitrites, inactivating NO and can directly decrease the endothelial synthesis of NO [33]. In addition, altered LDL are not recognized by LDL receptors, which are saturable, and instead are taken up by macrophages through scavenger receptors, not saturable, eventually becoming foam cells. Antioxidants constitute a diverse group of compounds among whose effects includes inhibition of LDL oxidation by both reductions in the concentration and reactivity of oxidants and improved resistance of the particle to them [34]. The antioxidants that have received the most attention are vitamin C (ascorbic acid), vitamin E (α-tocopherol) and phenolic compounds. In contrast to LDL, HDL provides an

atheroprotective effect by inhibiting cytokine-induced endothelial cell adhesion molecule expression [35] and by enhancing agonist-induced vasodilation in coronary arteries [36].

2.1.2. Effect of hypertriglyceridemia on endothelial dysfunction

There is evidence that hypertriglcyeridemia-induced endothelial dysfunction plays a critical role in this pathology. Traditionally, fasting triglyceride (TG) concentrations have not been considered as an indicator of coronary artery disease (CAD). The metabolic relation between TRL and HDL, the heterogeneity of TRL and the imprecision by which serum TG are determined contribute to the erosion of the relationship between TG and CAD [37,38]. However, the serum TG concentration is often more strongly correlated with future CAD incidence in multivariate analysis than is serum cholesterol [39]. Considering this evidence and that the TG metabolism also determines the fate of some other lipoproteins, such as LDL or HDL [40], TG seem to have a central role in the pathogenesis of atherosclerosis.

Disorders in TG metabolism may promote atherogenesis by increasing expression of vascular cell adhesion molecules. The concentrations of E-selectine, ICAM-1 and VCAM-1 are increased in the serum of patients with hypertriglyceridemia, independently of other risk factors [41].

2.1.3. Effect of postprandial triglcyeridemia on endothelial dysfunction

Postprandial triglyceridemia is also a good predictor of the presence and progression of atherosclerosis [42]. TRL, comprising TG contained in chylomicrons (CM) and VLDL, as well as their remnants, can cross the endothelial barrier and enter the arterial wall [43] and can be taken up by macrophages without need of further oxidation of the particles [44]. It has been suggested that the entry of lipoproteins into the arterial wall is inversely related to the size of the particles, and for that reason, it was thought for many years that TRL were not atherogenic, since they are too large to penetrate the tissue [45,46]. However, the TRL remnants formed after the hydrolytic activity of lipoprotein lipase (LPL) and endothelial lipase (EL) can enter the arterial intima and thus, be more atherogenic than their nascent precursors [47].

In vitro studies of endothelial cells reported that LPL-derived TRL remnants isolated from hypretriglyceridemic subjects have the ability of disrupt endothelial integrity [48,49]. It has been shown that remnant-like lipoprotein particles (RLPs) have a causative role in endothelial vasomotor dysfunction in human coronary arteries and that RLPs directly induced endothelial dysfunction in the isolated rabbit aorta [50,51]. In fact, TRL and their remnants stimulate endothelial cell plasminogen activator inhibitor-1 [PAI-1] production [52], which is a marker of endothelial dysfunction, and cause a profound depression of fibrinolytic activity [53]. TRL remnants also promote an enhanced thrombogenic tendency by increasing circulating factor VII levels [54].

In addition, it has been reported that monocyte adhesion to endothelial surface is enhanced by TRL [55,56]. CM preparations obtained from human plasma 4h after a standard fat-containing meal, were shown to up-regulate the expression of E-selectin and VCAM-1. Interestingly, TRL did not require LPL to promote the expression of adhesion molecules, suggesting that these particles may be pro-inflammatory themselves independently of their ability to release fatty acids [57]. Doi et al. [56] confirmed the up-regulation of protein and mRNA expression of these adhesion molecules when endothelial cells were incubated with TRL obtained from hypertriglyceridemic patients and they extended it to ICAM-1 and tissue factor (TF).

The oxidative mechanism can at least partially explain the adverse effects of TRL on endothelial cell function. TRL accumulation in plasma leads to increased oxidative stress and decreased NO availability as it is associated with increase of vascular superoxide anion production [58]. In their study, Doi et al. [56] observed increased cellular oxidant levels in the endothelial cell incubation medium containing TRL and that co-incubation with α-tocpherol decreased the concentrations of ICAM-1 and VCAM-1.

Hydrolytic enzymes, such as LPL and EL may be directly implicated in endothelial dysfunction, as their synthesis is up-regulated in early atherosclerosis [59]. Apart from smooth muscle cells, LPL is produced by monocytes and macrophages [60-63]. There is evidence that LPL activity can enhance the retention of LDL and VLDL to the arterial wall [64,65], but also it facilitates proteoglycan-mediated monocyte adhesion to the endothelium [66]. Actually, inflammatory cytokines, such as TNF and IL-1β can up-regulate endothelial derived LPL mRNA [67]. In contrast to LPL, EL is synthesized in the endothelium and has a

primary phospholipase activity [68]. However, its mRNA expression is also increased in endothelial cells by TNFα and IL-1β [67]. The upregulation of these enzymes is thought to be mediated via the nuclear factor κB (NF κB) pathway [69]. NFκB can be activated by a number of stimuli, including lipids and ROS. These stimuli cause the phosphorilation of IκB and subsequent proteolytic degradation of this inhibitor subunit, allowing NF κB to translocate into de nucleus, where it binds to recognition sequences in DNA in order to induce gene expression [70].

2.2.Beneficial and adverse effects of dietary fatty acids.

It has been postulated that as energy needs are increased during acute inflammation, lipolytic enzymes are up-regulated as a way of generating free fatty acids from circulating lipoproteins to be used by the tissues, including the endothelium. The increase in free fatty acid concentrations in the endothelium has been shown to decrease endothelial NO bioactivity, due to both superoxide generation and reduction in eNOS activity [71,72]. *In vitro* studies suggest that PUFA are more pro-inflammatory than monounsaturated (MUFA) and saturated (SFA) fatty acids [73]. In fact, linoleic acid (18:2, n-6) has greater capacity to induce oxidative and inflammatory stress than other fatty acids. Incubation of this fatty acid with endothelial cells promotes NFκB activation and transcriptional activity, being this effect is attenuated by vitamin E [74]. Additionally, exposure of endothelial cells to linoleic acid can lead to production of cytokines, such as IL-6 and IL-8 [75], which are involved in the initiation and progression of atherosclerosis [76,77].

Conversely, n-3 PUFA are believed to exert an endothelial protective effect. Particularly, docosahexaenoic acid (22:6, n-3; DHA) decreases expression of VCAM-1 on the vascular endothelium and monocyte adhesion [78-80] and eicosapentaenoic acid (20:4, n-3; EPA) increases NO production. Although these results from *in vitro* studies are promising, *in vivo* studies were more controversial. Abe et al. [41] found no reduction in soluble adhesion molecules after 6 weeks in patients receiving n-3 fatty acids but did find reductions in ICAM-1 and E-selectin after 7 months. Seljeflot et al. [81] supplemented male smokers with n-3 fatty acids for 6 weeks finding reductions in pro-thrombogenic von Wilebrand factor but increase in VCAM-1 and E-selectin. Their results were corroborated by Johansen et al. [82].

Among the key inflammatory mediators released by the endothelium are the eicosanoids derived from the n-6 PUFA arachidonic acid (20:4, n-6; AA). Prostaglandin E2 (PGE_2) can cause pain and vasodilation and leukotriene B4 (LTB_4) is a chemoatractant and activator of neutrophils. They are formed from AA via the cyclooxygenase (COX) and 5-lipooxygenase (LOX) pathways, respectively. However, EPA is also a potential COX substrate and can compete with AA for this enzyme leading to formation of PGE_3 which is synthesized with very low efficiency [83]. EPA is also substrate for LOX forming LTB_5, with less inflammatory activity compared to LTB_4 [84,85]. Thus, increasing the n-3 content in the diet, the balance of the eicosanoids produced can be shifted to a less inflammatory mixture.

3. Evidences of the benefits of olive oil on cardiovascular disease

A number of epidemiological studies developed in different countries constitute a firm and reliable experimental base supporting the beneficial effects of the Mediterranean diet, rich in olive oil, in regard to the reduction of CAD [86].

The Seven Countries Study, initiated by Ancel Keys in 1970 [87], was designed to investigate relationships between diet and CAD comparing different populations. The results of this study showed that the population of the Mediterranean island of Crete had the lowest rates of CAD and cancer, concluding that the cause might be the low saturated fat and high oleic acid intake, in terms of olive oil, of the Mediterranean diet. Subsequently, the Lyon Diet Heart Study [88] developed on patients recovering from a myocardial infarction, was the first clinical evidence in support of the health benefits of a Mediterranean-style diet, similar to that of Crete. The protective effects were ascribed to higher intakes of oleic acid and α-linolenic acid (18:3, n-3) and lower intakes of saturated fatty acids and linoleic acid (18:2, n-6). Trichopoulou et al. [89], demonstrated that the adherence to a Mediterranean diet reduced mortality from cancer and coronary heart disease in a Greek population of more than 22,000 individuals. These results were more recently confirmed in subjects diagnosed of CHD [90].

Cumulative evidence suggests that MUFA may be key components in the protective role of the Mediterranean Diet [91]. In fact, MUFA are believed to be as effective as n-6 PUFA at lowering total and LDL-cholesterol when replacing saturated fatty acids, as supported by two meta-analyses [92,93]. In addition, it has been reported that a controlled olive oil-based diet

can even lower plasma triacylglycerol levels [94], although there is some controversy in this regard [95,98].

It is probable that arterial hypertension resulted to be "quantitatively" the most important risk factor for CHD, due to its repercussion on cardiovascular mortality [99]. Diets enriched in n-3 PUFA are known to reduce blood pressure in humans [100,101] and in Spontaneously Hypertensive Rats (SHR) [102], which has been related to increased synthesis of series n-3 eicosanoids, with a stronger vasodilator effect than their series n-6 homologous [103]. However, there is increasing evidence showing that olive oil reduces systolic and diastolic blood pressure in normotensive [14,105] and hypertensive individuals [106]. Recently, another study including participants that had never received a diagnosis of hypertension confirmed these results. It concluded that the Mediterranean diet is inversely associated with arterial blood pressure and that olive oil intake, per se, is inversely associated with both systolic and diastolic blood pressure [107]. The modification in blood pressure by dietary olive oil has been related to changes in the fatty acid composition of cell membrane, which affects its functionality [106,108].

Oxidative modification of LDL is an important determinant in the development of atherosclerosis, as it accelerates the uptake of LDL by macrophages, which is the beginning of formation of a fatty streak. LDL may be protected against attacks of free radicals by antioxidants in plasma and in the particle itself. Lipoproteins rich in MUFA after long-term consumption of olive oil have been shown to be less susceptible to oxidation compared to particles enriched in PUFA [109-113].

An increasing number of studies point out that the content of oleic acid alone can not fully explain the impact on health of olive oil. This conclusion has been drawn from studies comparing the effects of diets enriched in different monounsaturated oils [114], among which VOO and high oleic sunflower oil (HOSO) have been most widely used.

Unlike VOO, HOSO was not able to reduce the blood pressure, in hypertensive patients [106]. Further studies in humans revealed that this differential effect was related to the composition and functionality of the cell membrane of hypertensive patients [106,115]. In SHR, VOO and HOSO yield to different liver, adipose tissue and myocardium lipid composition [116-118] and vascular reactivity [119]. These differences may be consequence

of a differential incorporation of the components of VOO and HOSO into TRL [120-122]. Actually, postprandial lipoproteins obtained after ingestion of VOO or HOSO are differentially incorporated into vascular cells [123], from which release of eicosanoid substances is also affected [124]. Furthermore, it has been reported that LDL from olive oil-fed rats were more resistant to oxidation *in vitro* than those isolated from plasma of triolein-fed rats [125]. The differential effects of different oleic acid-rich oils on LDL oxidation were confirmed in ten normolipidemic subjects by Nicolaiew et al., [126] after administration of VOO or HOSO. These data support the idea that the protective effects of olive oil against CAD must not be attributed exclusively to oleic acid, but to some other components of the oil.

Among the differential characteristics of VOO, the quantitatively most important is the TG molecular species composition. Compared to HOSO, which contains mainly triolein, VOO contains also important amounts of dioleoy-palmitoyl-glycerol. In much lower concentrations, but with growing evidence of important biological effects, minor components of olive oil can be also used to differentiate this oil from others.

4. Chemical composition of olive oil

In contrast to most dietary oils, which are obtained from the seeds of the plants by means of solvent extraction, and refined before being edible, olive oil is obtained from the whole fruit of *Olea Europaea L*, only by physical pressure. This procedure makes olive oil unique, since some compounds that cannot be found in other dietary oils are transferred from the leaves and skin of the fruit to the oil.
Olive oil can be classified into two fractions from a quantitative point of view. The major fraction constitutes 98-99% of the oil and it is mainly composed of saponifiable glyceridic compounds as triacylglycerols (TG). The abundance of oleic acid is peculiar to olive oil and ranges from 60-84% of total fatty acids in TG, while linoleic acid, the major essential fatty acid and the most abundant polyunsaturated acid in our diet, is present in concentrations between 3-21%.

Minor compounds account for the rest 1-2%, comprising unsaponifiable compounds, phenolics and waxes, and despite of being a little proportion, they confer important biologic activities. The minor compounds of VOO, classified in growing order of polarity after developed by thin-layer chromatography are: hydrocarbons, tocopherols, fatty alcohols, 4-

methylesterols, sterols, triterpenic dialcohols, polar-coloured pigments and phenolic compounds [127].

One of the greatest differences between VOO and the rest of edible oils is the composition in hydrocarbons [128]. Among them, the most important is squalene [129], a polyunsaturated triterpene that appears at high concentration and makes up 60-75% of the unsaponifiable fraction of the olive oil [130], and it is a precursor in the biosynthesis of cholesterol and steroid hormones. β-carotene is also found within this group, a triterpenic polyunsaturated hydrocarbon which plays an important role as precursor of vitamin A and, along with lycopene, conferring the yellowish colour to the oil. The analysis of the sterol fraction is of importance because it helps to the characterisation of the species from which the oil has been extracted [131]. The main sterol found in virgin olive oil is β-sitosterol [95%] but campesterol, Δ-7 stigmastenol, stigmasterol, spinasterol and avenasterol are also present. VOO contains α, β, γ and Δ-tocopherols, but α-tocopherol typically accounts for more than 85% of total tocopherols. Triterpenic dialcohols and acids, from the skin of the fruit and from the leaves are incorporated in second pressing oil and the final concentration is higher than in VOO. The main dialcohol is known as erythrodiol, which in some cases, is accompanied by another triterpenic-tetracyclic diol identified as uvaol. Among acids, oleanolic and maslinic have recently revealed some pharmacological properties, which will be discussed below.

Phenolic compounds are rarely determined in routine analysis because of their solubility in water and diluted bleach, what explains their absence from both unsaponifiable and glyceridic fractions. These substances constitute the 'polar fraction' in VOO and underlay its exceptional thermal stability [132-134], contribute to its characteristic flavour and taste, and prevent its autooxidation, contributing to the resistance of VOO to oxidative rancidity [135]. Phenolic compounds have emerged as potent antioxidants present in VOO. Among these compounds, oleuropein itself and its derivatives, tyrosol and hydroxytyrosol, have been reported to have a protective role against LDL oxidation *in vitro,* equivalent to vitamin E [136]. Although it has been demonstrated that VOO phenolics are dose-dependently absorbed in humans [137], in plasma they are mostly present as conjugates of glucuronic acid. The *in vivo* antioxidant effect of VOO phenolics and the antioxidant capacity of the glucuronidates are scarce and controversial. However, interventional studies administrating increasing doses

of phenolic compounds in VOO have reported reduced oxidative status in healthy [138,139] and dyslipemic subjects [140].

5. Effects of the components of olive oil on the endothelial function

Very few studies have addressed the effects of long-term olive oil consumption on the endothelial function. Mediterranean diet, rich in olive oil, has been shown to improve endothelial function in diabetic [141] and hypercholesterolemic patients [142], as assessed by measuring endothelium-dependent vasoreactivity. Fuentes et al., [142] also observed a reduction in the plasma levels of P-selectin. In a randomized crossover trial, Ros et al. [143] confirmed the improvement on endothelial function in 22 hypercholesterolemic subjects receiving a Mediterranean diet. However, the results were more evident when part of the dietary olive oil was replaced with walnuts. Sondergaard et al., [144] observed a greater improvement in flow-mediated vasodilation (FMD) in patients receiving fluvastatin when they were advised of following a Mediterranean diet. Esposito et al. [145] carried out a randomized trial among 180 subjects with metabolic syndrome, who were instructed to follow a Mediterranean-style diet, including olive oil. After two year follow-up, they observed improved endothelial function as a measure of blood pressure and platelet aggregation response to L-arginine, the natural precursor of nitric oxide. They also reported a significant reduction of markers of systemic vascular inflammation, such as C-reactive protein and interleukins 6 (IL-6), 7 (IL-7) and 18 (IL-18), apart from total and LDL-cholesterol. However, the mechanisms by which dietary olive oil elicits these effects and which are the actual components of the oil responsible for the effects are poorly elucidated. Below, we show the current knowledge on the effects of the components of olive oil on endothelial activation. A graphic summary of the mechanisms is depicted on Figure 1.

5.1. Effects of major components of olive oil on endothelial function

5.1.1. Effects of oleic acid

Oleic acid, the main fatty acid contained in olive oil, accounts for about 29% of the daily caloric intake in some Mediterranean countries. Recent studies testing the role of different unsaturated fatty acids in endothelial cell activation and injury, suggest that oleic acid does

not activate endothelial cells confirming its benefits on early events in atherosclerosis compared with other unsaturated fatty acids [146]. Carluccio et al. [147] reported that plasma concentration of oleate under conditions of high olive oil consumption are likely to be fully in the range of concentrations exerting biological effects in our system, likely between 10 and 100µmol/L.

Tsimikas et al. [148] assessed the proinflammatory potential of LDL isolated from Greek subjects consuming a diet naturally rich in olive oil. Oleic acid content in LDL was significantly higher in the Greek compared Americans and was inversely correlated with the extent of *in vitro* LDL oxidation and the induction of monocyte adhesion by mildly oxidized LDL. To confirm that dietary fatty acids influence the proinflammatory properties of mildly oxidized LDL, native LDL were also isolated from American subjects after consumption of a liquid diet supplemented with either oleic or linoleic acid. The oleic acid-supplemented group had higher oleic and lower linoleic acid content in LDL than the linoleate-supplemented group. When exposed to oxidative stress, the LDL enriched in oleic acid promoted less monocyte chemotaxis and reduced monocyte adhesion, showing a strong negative correlation between oleic acid LDL content and monocyte adhesion. This study demonstrated that the level of dietary enrichment with oleic acid necessary to obtain these benefits is realistic and readily achieved by using diets currently in use in Mediterranean countries. Also, this study suggested that LDL enriched with oleic acid and reduced in PUFA may be less easily converted to a proinflammatory, minimally modified LDL, which has the ability to enhance monocyte chemotaxis and adhesion.

The main challenge after these results is to discover which are the underlying mechanisms implicated in oleic acid beneficial effects. Initially, Tsimikas et al. [148] explained the mechanism by which oleic-acid enriched diets decreased lipoprotein susceptibility to oxidation, presumably, as a result of the decreased linoleic acid content within lipoprotein. However, previous experiments with liposomes progressively enriched in oleic acid but with constant amounts of linoleic acid, showed that particles with higher oleic acid concentration were less susceptible to oxidation, and monocyte chemotaxis and adhesion were nearly completed inhibited when exposed to mild oxidative stress, suggesting that oleic acid may have an additional independent mechanism of action [149].

Studies on endothelial cells *in vitro* have shown that the main dietary PUFA and oleic acid may prevent endothelium activation either by inhibiting the expression of adhesion molecules or by improving the NO production [150]. Supplementation of endothelial cells with oleic acid *in vitro* reduces endothelial cell sensitivity to oxidants, creating a reduced prooxidant environment as a consequence of reduced intracellular ROS [151,152]. In this environment, oleate reduces the activation and mRNA expression of NF-κB and AP-1, thereby interfering with the endothelial expression of adhesion molecules for circulating monocytes and contributing to a direct vascular atheroprotective effect [153-156]. Additionally, cellular treatment with this fatty acid protects endothelial cells against cytokine-induced VCAM-1, ICAM-1, or E-selectin overexpression [147].

This condition might occur by two mechanisms: reduced enzymatic production of ROS or increased scavenging after their production. It has been suggested that the inhibitory potency of unsaturated fatty acids is directly proportional to their number of double bonds [153, 156]. According to this, DHA, with 6 double bonds would be 6 times more potent than oleic acid. However, addition to oleic acid to the culture medium significantly increases the unsaturation index [147], probably through selective displacement of SFA, but not PUFA, in cell membrane phospholipids, with a consequent modulation of gene expression for molecules involved in monocyte recruitment [153]. Actually, the kinetics of VCAM-1 inhibition by oleic acid resembles that of DHA. Similarly to what has been previously shown for DHA, the effect is totally independent of the stimulus used: cytokines acting on totally different receptors, such as IL-1, TNFα, IL-4, or LPS, and inducing different responses [157].

Oxidation of fatty acids might help scavenging of ROS, thus reducing the formation of superoxides that can be dismutated to hydrogen peroxide, which is in turn responsible for NF-κB activation [158,159]. Therefore, an alteration of hydrogen peroxide metabolism in vascular cells may contribute to the ability of fatty acids to modulate cellular oxidant susceptibility [160].

Experiments with porcine artery endothelial cells (PAEC) supplemented with oleic acid and exposed to oxidant conditions by means of hydrogen peroxide treatment showed an attenuated increase in intracellular hydrogen peroxide [161]. The consequence was an increase in resistance to derangements caused by oxidized LDL and a reduction in oxidant mediated

dysfunction [160,162]. Massaro et al. [163] showed that incubating of oleate with cytokine-stimulated endothelial cells, prevents the depletion of glutathione (GSH) and partially prevents stimuli-induced increase of intracellular ROS. This occurred without any change in the activity of GSH-related antioxidant enzymes, superoxide dismutase and catalase. These authors suggested that oleate may exert direct vascular atheroprotective effects by inhibiting endothelial activation through quenching of ROS. In contrast, incubation with linoleic and stearic acids reduces GHS levels and increases NFκB [146,155]. Additionally, coincubation of linoleic acid with TNFα doubled the production of IL-6 compared with TNFα alone. This was concomitant to an increment in the content of arachidonic acid in membrane phosphatidylethanolamine.

In contrast with these results, other authors have suggested negative effects of circulating non-esterified fatty acids like oleic acid in diabetic patients. Experiments with cultured rat aortic smooth cells maintained in media containing oleic acid concentrations similar to those in diabetic patients showed a significant increase in endothelin-1 receptor amount suggesting that it may contribute to the acceleration of atherosclerosis in diabetic patients. [164,165].

5.2 Effects of minor components of olive oil on endothelial function

5.2.1 Effects of olive oil phenolic compounds

The major phenolic compounds in olive oil, oleuropein, hydroxytyrosol and tyrosol; are strong antioxidants and radical scavengers [166], which can help to revert the imbalance between increased oxidative stress and impaired antioxidant defence that affects endothelial function, and therefore contributes to atherosclerotic disease progression.

Associations between oxidative stress and impaired endothelial function have been demonstrated in experimental animal models of atherosclerosis, hypertension, hypercholesterolemia and diabetes [167-169]. Reduced bioavailability of NO in a setting of increased superoxide anion levels seems to be constant biological changes that occur in the vessel wall under these conditions [170]. In fact, it has been described that endothelial vasomotor dysfunction could be reversed in these kind of patients by the administration of agents capable to scavenging ROS [171-174].

A number of papers have reported *in vitro* experiments evaluating and confirming the antioxidant activities and the scavenging potencies of olive oil and its isolated constituents [175-179].

The low unsaturation of olive oil fatty acids, in addition to water soluble antioxidant protection in the form of phenolic compounds favourably influences a reduced susceptibility to oxidation of olive oil-derived lipoproteins [180]. Recently, it has been reported that an olive oil intake of 25 mL in a single dose does not promote exacerbated hypertriglyceridemia or hyperglycemia, which are linked to postprandial oxidative stress. These authors found a postprandial increase of both tyrosol and hydroxytyrosol, two phenolic compounds frequently used as markers of olive oil intake. The increased concentration of these phenols in plasma may be related to the reduced oxidative stress, since their increase was concomitant to a postprandial decrease in plasma oxidized LDL.

Oleuropein and hydroxytyrosol are potent scavengers of ROS and superoxide anion in neutrophils [177,179]. Saija et al. [175] hypothesized that whereas hydroxytyrosol can serve as scavenger of aqueous peroxyl radicals near the membrane surface, oleuropein acts also as scavenger of chain-propagating lipid peroxyl radicals within the membranes. Additionally, oleuropein has been shown to increase NO production from LPS-stimulated mouse macrophages [181] and to possess a tonic effect toward the inducible form of NO synthase (iNOS). Tuck et al. [166], reported that these two phenolic compounds, as well as caffeic acid but not tyrosol, can scavenge free radical NO (NO) and peroxynitrite in a concentration dependant fashion. Altogether, these features of oleuropein and hydroxytyrsol might contribute to increase of NO levels and to prevent formation of the powerful oxidant peroxinitrite [182].

Besides the antioxidant properties of the phenolic compounds from extra VOO, anti-inflammatory effects have been demonstrated in several cell types. De la Puerta et al. [183] studied a range of VOO phenolics in rat peritoneal leukocytes, finding inhibition of LTB_4 production by oleuropein glycosyde, caffeic acid and tyrosol. This had already been described in human platelets and leukocytes [184,185]. Hydroxytyrosol has also demonstrated inhibitory effects on LOX [185] in leukocytes, presumably by penetrating in cell membranes [186]. The inhibition of LTB_4 production from arachidonic acid may lead to reduced platelet aggregation [184,187].

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It has been described that some phenolic compounds may inhibit cytokine and eicosanoid production by inhibiting the IL-1β mRNA and protein expression, and the activity and transcription of COX-2 [183,185,188], which may contribute to the antiatherogenic properties ascribed to extra VOO. Miles et al., [188] showed a very strong effect of oleuropein glycoside but none of the phenolic compounds from olive oil studied was able to affect the production of IL-6 or TNF-α. However, these authors did not use oleuropein aglycone or hydroxytyrosol in their experiments.

Monocyte adhesion to endothelial cells can also been modulated by VOO phenolic compounds. Carluccio et al. [189] incubated oleuropein, hydroxytyrosol and tyrosol with LPS or cytokine-stimulated HUVECs and observed inhibition of the expression of VCAM-1, I-CAM-1 and E-selectin and the adhesion of monocytes. The oleuropein aglycone and hydroxytyrosol were the most potent phenolic compounds, which is consistent with their higher antioxidant activity. In contrast, to the study of Miles et al., [188] the oleuropein glycoside showed a low activity on adhesion molecule expression, which was attributed to its lower lipophilicity, and the consequent lower incorporation into membranes and interaction with lipids. mRNA expression for VCAM-1 was also affected, indicating a pretranscriptional action of the phenolic compounds. These authors observed a repression of the transcription factors NFκB and AP-1, the interaction of which are known to amplify VCAM-1 promoter activation [190]. Very recently, Turner et al. [191] also reported increased VCAM-1 and ICAM-1 production by VOO phenolics (namely oleuropein, hydroxytyrosol, tyrosol and homovanillic alcohol) but no influence on NO production or platelet aggregation. Activation of NF-κB involves complex signal transduction pathways that ultimately result in the activation of a specificκB kinase (IKK) and translocation of NFκB from cytoplasm to the nucleus. In an attempt of elucidating the mechanisms by which phenolic compounds inhibit NFκB, Ma et al. [192] showed that these compounds do not inhibit activation of IKK activity, degradation of κBα, a component of the cytoplasmic NFκB complex, or translocation of activated NFκB to the nucleus, but they block the formation of NF κB/DNA binding complexes. These authors suggested that this blockade involves the antioxidant property of the phenolic compounds.

5.2.2 Effects of the unsaponifiable fraction

The unsaponifiable fraction of VOO is also rich in other minor components with antioxidant and anti-inflammatory properties, such as tocopherols, sterols or terpenic compounds [193-195].

The investigation of the effects of the unsaponifiable fraction of olive oil as a whole is almost an unexplored field. Ochoa et al., [196] investigated the influence of VOO and HOSO, with different unsaponifiable fractions on the fatty acid composition and lipid peroxidation of LDL in rabbits. The main outcome of the study was the lower susceptibility of LDL oxidation and higher antioxidant content in LDL of animals fed the diet enriched in VOO, which was attributed to the higher phenolic content in this oil. Unfortunately, as stated above, the phenolic compounds of olive oil are not included in the unsaponifiable fraction.

We recently incubated endothelial cells with postprandial TRL derived from the intake by healthy subjects of meals containing VOO, HOSO and VOO enriched in its unsaponifiable fraction (EVO) to a final concentration of 2.4% [124]. The unsaponifiable fraction of VOO was richer in squalene, terpenic compounds and waxes (long-chain fatty alcohols), whereas HOSO presented a higher concentration in tocopherols. The total concentration in sterols was similar between VOO and HOSO but in EVO, was almost double. We found a reduction in the production of PGE_2 and TXB_2 after the incubation with EVO-TRL, compared with VOO and HOSO (Figure 2), but no effect on NO production. These results suggest that minor components from VOO that are transported postprandially in TRL may have favourable effects on endothelial function by improving the balance between vasoprotective and prothrombotic factors released by endothelial cells. Despite the beneficial effects attributed to phenolic compounds it is very unlikely that they were responsible for the effects observed in this study: firstly, because its concentration in VOO and EVO was very similar, secondly, because due to their hydrophilic nature they are readily transported into plasma and not to TRL and thirdly because they appear not to affect COX activity [183]. Therefore we suggested that tocopherols, sterols or terpenoids might be responsible for the effects observed.

Vitamin E, comprising tocopherols, tocotrienols and some of their derivatives, has a protective role against the attacks of free radicals, by acting as lipid based radical chain breaking molecules [197]. More recently, non-antioxidant functions of vitamin E have been

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proposed, in particular as gene regulator, which seems to be unrelated to its radical chain breaking potential [198].

Apart from protecting LDL from lipid peroxidation [199-202], α-tocopherol has an inhibitory effect on LDL- and cytokine-induced production and expression of adhesion molecules [203-205] and adhesion of monocytes to endothelial cells, probably by inhibition of ICAM-1 expression [204,206]. In cells stimulated with IL-1β, α-tocopherol reduces the upregulation of ICAM-1, VCAM-1 [207] and E-selectin [206], but more importantly, it can also regulate the production of IL-1β by down-regulating its gene expression [208]. However, the effect on adhesion molecules seems not be mediated by NFκB mobilisation [159,206].

Vitamin E can also modulate eicosanoid metabolism in endothelial cells. Actually, PGI$_2$ synthesis in impaired in vitamin E-deficient mice [209-210] and it can restore reduced PGI$_2$ synthesis in endothelial cells [211,212]. In addition, there are data indicating that α-tocopherol inhibits 5-LOX [213] and COX-2 [214]. In a series of studies carried out by Meidani et al. [215-217] these authors demonstrated that α-tocopherol can eliminate the increase in PGE$_2$, TXA$_2$ and TXB$_2$ by reducing the activity of COX in LPS-stimulated macrophages from aged-mice. This effect was found not to be due to regulation of COX transcription or translation, but to the effect of α-tocopherol scavenging hydroperoxydes and NO , which leads to lower production of peroxynitrites. There is data suggesting that peroxinitrites may modulate COX activation via Ca^{2+}-dependent PLA2 activity and AA release [218].

Finally, pretreatment of endothelial cells with vitamin E, prevents alterations in the plasma membrane by hydrogen peroxide [219] and decreases oxidized LDL-mediated degradation of I-κB and apoptosis [220].

However not all tocopherols have same effects, α-tocopherol, but not β-tocopherol can regulate AP-1 [221] and integrin [222] gene expression. Inhibition of the induction of VCAM-1 and E-selectine by IL-1β was time and dose-dependent for α-tocopheryl succinate, but not for IL-1-β [223]. In fact, α-tocopheryl succinate can inhibit cytokine-induced mobilisation of NFκB [223], by activating caspase-3 and caspase-6, which cleave the p65 subunit of the transcription factor [224,225]. According to Christen et al. [226] γ-tocopherol

can inhibit peroxynitrite-induced peroxidation more effectively than α-tocopherol. Hence, α-tocopherol isomers might inhibit COX activity more effectively than α-tocopherol itself [214]. Nevertheless, there is controversy regarding the *in vivo* and long-term effects of vitamin E and it is not clear what amounts or combinations may be beneficial in preventing chronic diseases [197].

There are studies in humans and animals evidencing that plant sterol supplementation, including the most abundant in VOO, β-sitosterol, can cause a decrease in serum cholesterol concentration [227, 228]. One of the principal mechanisms by which this effect has been explained is through inhibition of the absorption of cholesterol [229]. Despite of this, a recent study carried out by Ho et al. [230] suggests that the mechanisms also involve decreased production of the apoB-containing lipoproteins from the liver and intestine. However, very little is known about their effect on vascular function. De Jongh et al, [231] administrated a mixture of phytosterols (β-sitosterol, campesterol, stigmasterol and others) to 41 children with familial hypercholesterolemia, finding a reduction in LDL-cholesterol but no effect on endothelial dysfunction as measured by FMD. However, de la Puerta et al. [194] had reported that β-sitosterol has antiinflamatory effects as it can reduce the auricular edema induced by TPA in mice. The effect was as high as that of hydroxytyrosol or oleuropein. Moreno [232] incubated phorbol ester (PMA)-stimulated RAW 264.7 macrophages with β-sitosterol, which was responsible for a reduction in ROS production and arachidonic acid release. ROS modulation may regulate the release of arachidonic acid by phospholipase A2, as well as the induction of COX-2 through NF-κB activation. By this mechanism β-sitosterol might reduce PGE$_2$ and LTB$_4$ production by macrophages, as observed in this study. Subsequently, this author reported that β-sitosterol can regulate the GSH redox cycle, enhancing GSH peroxidase and superoxide dismutase activities, hence decreasing superoxide anion levels although no ROS scavenger activity was found.

The potential therapeutic importance of olive oil triterpenoids, encompassed of acids and alcohols, has not been extensively studied. Although their presence in VOO is very low, in olive pomace oil is very significant, as it can be as high as 120 mg/kg. For that reason, the concentration of triterpenic alcohols is used as a parameter of purity for the presence of pomace oil in VOO [233].

Oleanolic acid has been identified in multitude of medicinal plants [234] and has been reported to possess a number of biological pharmacological activities, including some affecting inflammation [235]. Oleanolic acid inhibits LOX and COX-2 activities [236,237], therefore reducing the production of PGE_2 and LTB_4. Additionally, it has been shown that oleanolic acid can inhibit the generation of the superoxide anion by human neutrophils, which might occur through the protein-kinase independent pathway [238]. Although it has been attributed anti-inflammatory activities to the triterpenic alcohol erythrodiol, its mechanism of action is still unknown. Erythrodiol is able to reduce the edema caused by TPA, with a possible action on PLA2 [194].

We recently developed a study to evaluate the properties as vasodilator agents of oleanolic acid and erythrodiol and to determine their mechanism of action [195]. The vasorelaxant effect induced by these triterpenoids was studied in isolated thoracic rat aorta. Results from this work introduced the first *in vitro* evidence that oleanolic acid and erythrodiol evoke an endothelium-dependent vasorelaxation in rat aorta, and suggested that the mechanism of relaxation is mainly mediated by the endothelial production of NO (Figure 3). According with the pharmacological effects obtained, it was concluded that oleanolic acid and erythrodiol may have interesting therapeutic potential as new vasodilator drugs, being able to protect the cardiovascular system.

6. Conclusion

It is becoming clear that the content of oleic acid alone can not fully explain the impact on health of olive oil and that being VOO an unique fruit-derived oil it is rich in a number of minor compounds with relevant physiological and pharmacological functions. Among these compounds, tocopherols and phenolic compounds have demonstrated antioxidant properties, which may improve the endothelial function by reducing levels of ROS in the endothelium and, consequently, the production of eicosanoids and adhesion molecules. However, there are other less studied compounds that have been proved to exert important effects on the endothelial function. Phytosterols and triterpenoids have anti-inflammatory and vasorelaxant effects, respectively but their roles in the endothelium need to be further studied. The increasing investigations on the properties of these minor compounds are not only helping to explain some of the classical beneficial effects of the Mediterranean diet and VOO itself but

also to the emergence of other olive-derived oils, such as pomace olive oil, which being more enriched in these minor components, might be helpful for the prevention of CVD.

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Table 1. Minor Component Composition of Virgin Olive Oil.

Sub-fraction Component		Concentration (mg/kg)
UNSAPONIFIABLE		
Hydrocarbons	Squalene	200-7500
	β-Carotene	0.3-0.7
	Polycyclic aromatic hydrocarbons	Traces
Sterols	β-Sytosterol	1800-2600
	Campesterol	<4.0% of total sterols
	Δ7-Stigmasterol	<0.5% of total sterols
	Brassicasterol	<0.1% of total sterols
Terpenic Dialcohols	Erythrodyol + Uvaol	6-10 + 18
Tocopherols	α-Tocopherol	60-200
	β+γ-Tocopherol	3% of total tocopherols
	Δ-Tocopherol	<2% of total tocopherols
PHENOLIC COMPOUNDS	Tyrosol	
	Hydroxytyrosol	
	Caffeic acid	50-800 (total phenols)
	Oleuropein	
OTHERS	Flavour components	Traces

Figure 1. Proposed model for the action mechanisms of the oleic acid and minor compounds from olive oil based on the literature gathered for the present review. Despite the number of studies contributing to this model several gaps are still present, which should be filled with further investigations. The main mechanism by which the components of olive oil influence endothelial activation involves inhibition and/or scavenging of reactive oxygen species (ROS). Oleic acid and β-sitosterol may reduce intracellular ROS by creating a less oxidant environment through inhibition of intracellular ROS production. β-sitosterol may also enhance superoxide dismutase (SOD) activity, hence decreasing superoxide anion levels O_2^-. This reduction has also been observed for the terpenoid oleanolic acid, although the mechanism is not presently known. Tocopherols and phenolic compounds are potent antioxidants that may help to reduce lipid peroxidation and to scavenge intracellular ROS and free radical nitric oxide (NO), reducing the formation of peroxynitrite (OONO). ROS can activate the nuclear factor κB (NFκB), which is then translocated into the nucleus, where it binds to recognition sequences in DNA in order to induce gene expression. This mobilisation of NFκB is blocked by α-tocopheryl succinate but not by α-tocopherol. In contrast, phenolic compounds have been proposed to act blocking the formation of NFκB/DNA binding complexes. NFκB modulates the expression of cytokines, 5-lipooxygenase (LOX) and cyclooxygenase (COX), thereby affecting the levels of adhesion molecules and eicosanoids. However, some of the minor compounds of olive oil may act directly on these enzymes and cytokines. LOX and COX activities are inhibited at different points by phenolics and triterpenoids and IL-1β expression by phenolics and tocopherols, contributing to protect the endothelium against vasoconstriction, platelet aggregation and monocyte adhesion. Vasodilation is also suggested to be enhanced by oleuropein and oleanolic acid through increase of the production of nitric oxide (NO).

Figure 2. Effect of the unsaponifiable fraction of olive oil in TRLs on eicosanoid production. HUVEC were incubated for (24 h) with TRL obtained 2 h after the ingestion of high-oleic sunflower (HOSO), virgin olive (VOO) or enriched-virgin olive (EVO) oils. Eicosanoid released to the medium were determined by EIAs. Results corresponding to TXB_2, PGE_2 and 6-keto-PGF$_{1\alpha}$ are shown in panels A, B and C respectively. a: p<0.05, vs. Control; b: p<0.05, vs. HOSO; c: p<0.05, vs. VOO.

Figure 3. Relaxant effect of oleanolic acid (A) and erythrodiol (B) in phenylephrine (10^{-6} M) precontracted rat aortic rings. Addition of the NO-synthase inhibitor L-NAME (3x10^{-4} M) produced a significant reduction of the endothelium-dependent relaxation to both triterpenic compounds (circles) compared to control (squares). ***: p<0.001, vs. Control.

Figure 3
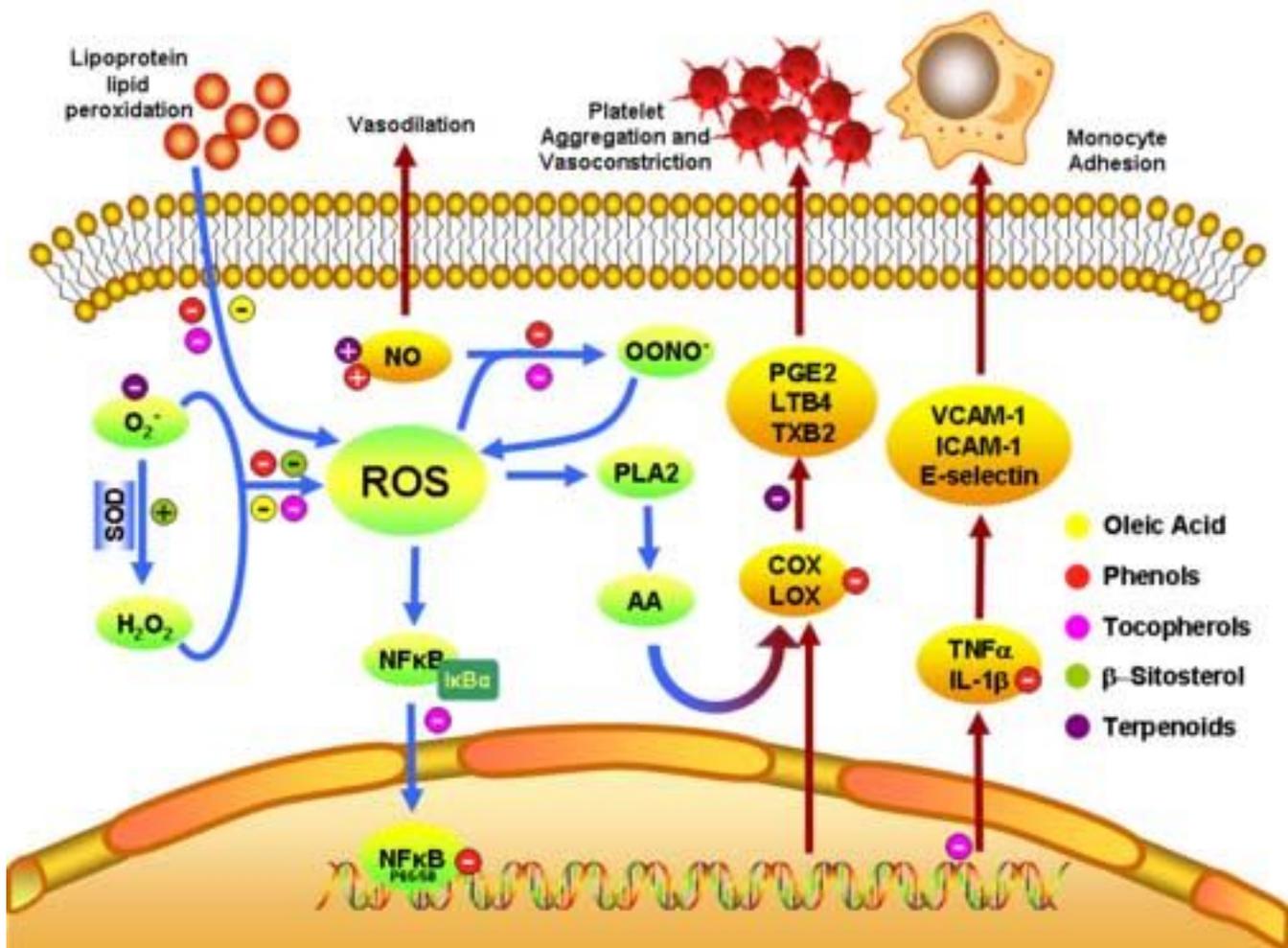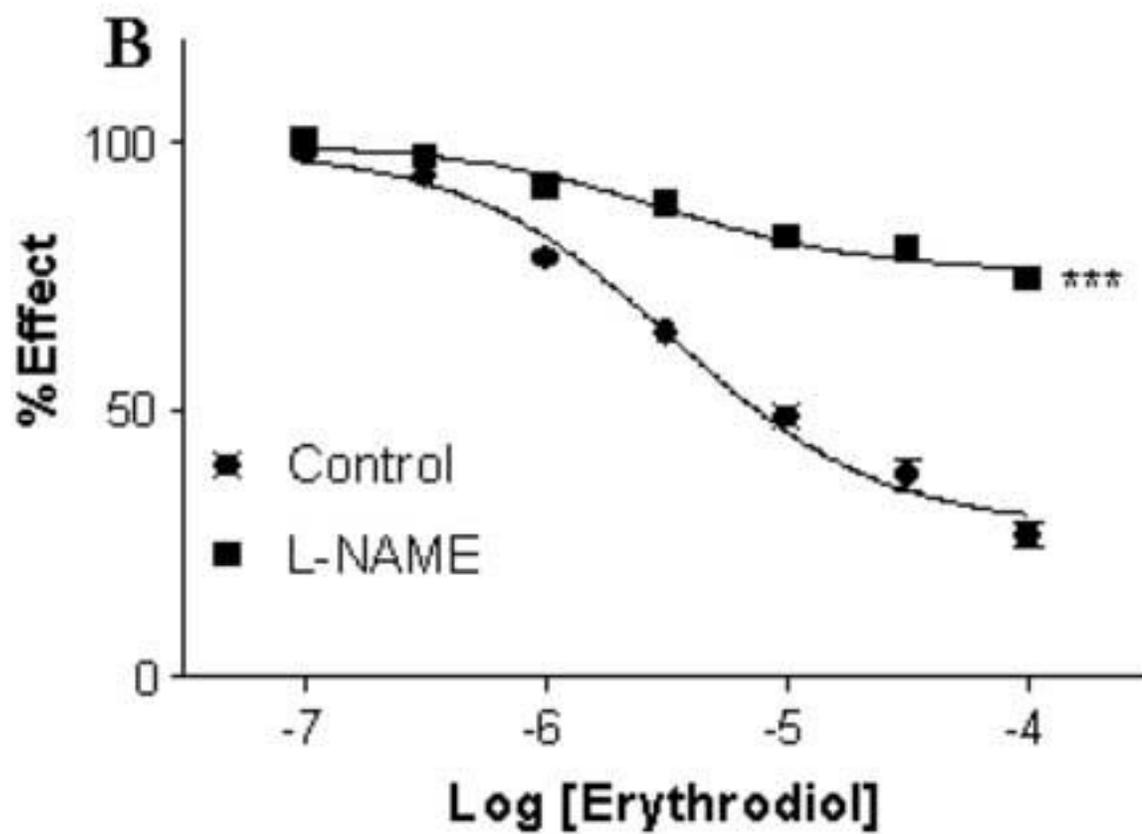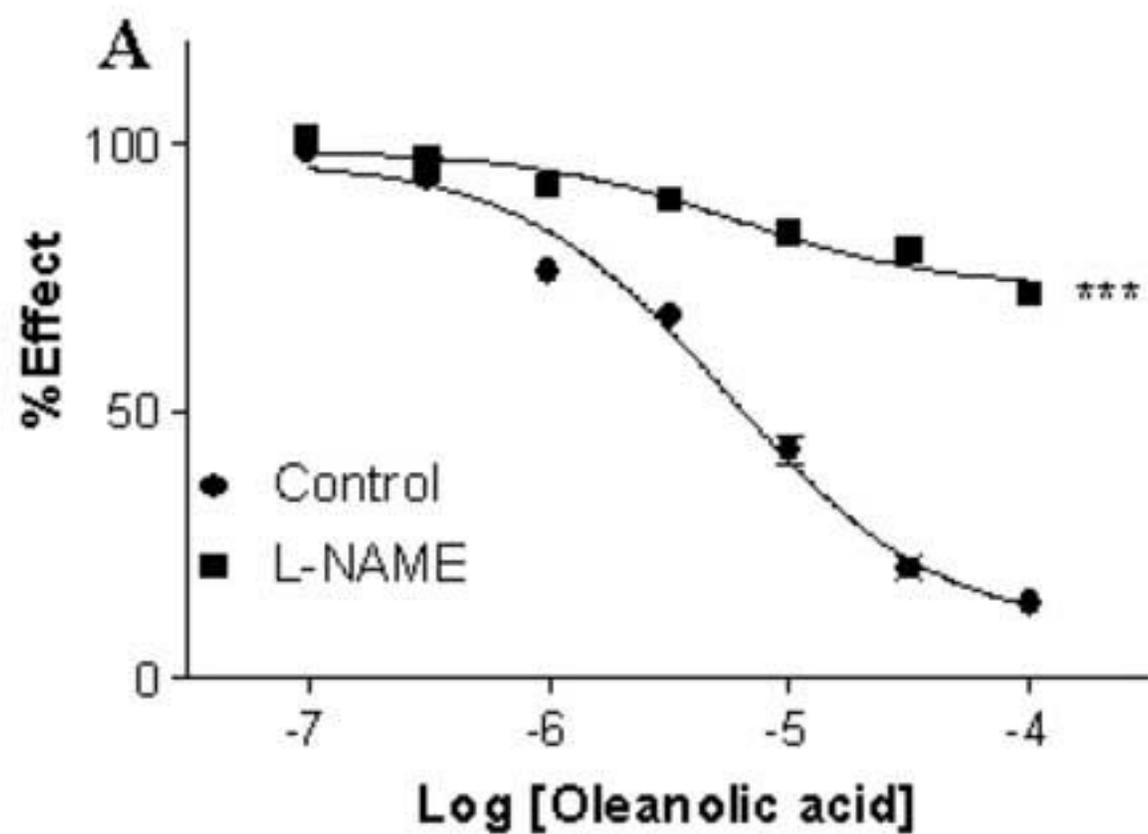[Click here to download high resolution image]