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PROTECTIVE ROLE OF BIOACTIVE QUINONES IN STRESS-INDUCED SENESCENCE PHENOTYPE OF ENDOTHELIAL CELLS USING TOBACCO SMOKE

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ABSTRACT

Atherosclerosis is the major cause of CVD and death in the world. The initial stage of atherosclerotic lesions development is represented by endothelial dysfunction which occurs physiologically during aging process, however it could be accelerated by external factors such as incorrect diet, sedentary behavioral and tobacco smoke. Cigarette smoking is known to promote oxidative stress and cell damages. Therefore the development of an *in vitro* model of vascular cells exposed to chemicals contained in cigarette smoke could be useful to elucidate the mechanism of action of protective molecules with anti-inflammatory and antioxidant effects. Ubiquinol (QH) is known to have a potent antioxidant capacity, while vitamin K could act as antioxidant and anti-inflammatory. Both these molecules play a fundamental role in vascular health in physiological conditions, therefore in the present study we aimed at verifying whether they counteract the damages caused by Cigarette Smoke Extract (CSE). Moreover, to investigate if the protective effects of vitamin K were mediated by vitamin K-dependent proteins, the administration of Warfarin, a vitamin K inhibitor, was also tested.

In the present study, the Cigarette Smoke Extract (CSE) toxicity and pro-senescent effects were evaluated in a model of endothelial cells (HUVECs) comparing CSE exposed young cells with untreated replicative senescent cells. Subsequently, the protective effects of ubiquinol, K vitamers and Warfarin pretreatment before exposure to CSE were investigated.

Treatment for 24h with the CSE decreased cellular viability in dose-dependent manner, however this result was not associated with a parallel increase in the percentage of dead cells, but rather with a significant increase in the percentage of apoptotic-like cell. Moreover, CSE increased the imbalance between radicals production (cytosolic ROS and mitochondrial $O_2^{-\bullet}$) and antioxidant defenses resulting in an increased oxidative stress that promotes mitochondrial dysfunction (mPTP opening) and inflammation (caspase-1 activation). These changes, together with specific senescence markers (SA-β-galactosidase and p16 expression) were observed in both young CSE exposed and in senescent HUVECs; suggesting that CSE exposure accelerated the aging process of endothelial cells.

The supplementation with 10 μM of QH and MK7 and 100 μM of Warfarin was found to have a strong protective effect counteracting the oxidative stress and inflammation increase, resulting in a significant improvement in viability inhibiting the apoptotic shift and in the prevention of SA-β-galactosidase increase. However, neither quinones were able to counteract CSE-induced mPTP opening and p16 expression.

The other K vitamers tested, MK4 and K1, showed only a slight improvement in viability and cytosolic ROS content. In addition, MK4 amplified the CSE-induced increase of $O_2^{-\bullet}$ and consequently increase the mPTP opening, causing the amplification in mitochondrial dysfunction.

The minor protective effect of MK4 and K1 could be the consequence of their lower bioavailability compared to MK7, although they were dissolved in a micellar suspension with cremophor and glycerol, the best carrier compared to EtOH and THF. The *in vivo* test confirmed the relevant role of solvent showing the best MK7 bioavailability when supplemented in milk as oil emulsion with arabic gum than as powder.

In conclusion, CSE exposure, at the tested condition, was able to promote the Stress Induced Senescent Phenotype (SISP) in young endothelial cells. These molecular changes likely underlie endothelial dysfunction *in vivo* and the onset and progression of atherosclerotic lesions. Our data show that these effects could be counteract by ubiquinol and menaquinone-7 pretreatment. In particular the latter was the most bioactive K vitamers in counteracting of CSE damage. However, intriguingly, a protective effect was also observed by antagonizing vitamin K by Warfarin. This effect could be due to compensatory mechanisms involved with alternative pathways of the vitamin K cycle that deserve further investigations.

KEY WORDS: Endothelial dysfunction, cigarette smoke, aging, vitamin K, menaquinone, ubiquinol, Warfarin

CONTENTS

ABBREVIATIONS

CoQ_{10}	Coenzyme Q_{10} - Ubiquinone
CVD	Cardiovascular Disease
CSE	Cigarette Smoke Extract
eNOS	endothelial Nitric Oxide Synthase
GRP	Gla-rich protein
HDF	Human Dermal Fibroblast
HUVEC	Human Umbilical Vein Endothelial Cell
K1	Phylloquinone
MGP	matrix Gla protein
MK4	Menaquinone-4
MK7	Menaquinone-7
mPTP	mitochondrial Permeability Transition Pore
NQO1	NAD(P)H Quinone Oxidoreductase-1
QH	Ubiquinol (reduced CoQ_{10})
ROS	Reactive Oxygen Species
SA-β-gal	Senescence-associated β-galactosidase
SISP	Stress Induce Senescence Phenotype
THF	Tetrahydrofuran
VKA	Vitamin K Antagonist
VKDP	Vitamin K Dependent Protein
VKORK	Vitamin K Epoxide Reductase

INTRODUCTION

CARDIOVASCULAR DISEASE

Cardiovascular disease (CVD) are referred to as a group of disorders interesting the heart, blood vessels and are strictly associated to dysfunction of the vascular system in the brain (neurovascular disease). More specifically CVD include: coronary artery disease, cerebrovascular disease, peripheral arterial disease, rheumatic heart disease, congenital heart disease, deep vein thrombosis and pulmonary embolism.

Heart attacks and strokes are usually acute events and are mainly caused by a blockage that prevents blood from flowing to the heart or to the brain. The most common reason is a build-up of fatty deposits on the inner walls of the blood vessels (atherosclerotic lesion). Atherosclerosis is therefore considered a degenerative condition associated with the progressive occlusion of the vascular lumen leading to a limited afflux of blood and oxygen to brain and heart. Ischemia is particularly dangerous to these tissues that mainly rely on oxidative metabolism. Moreover, strokes can be also caused by bleeding from a blood vessel in the brain or by blood clots.

The underlying mechanisms leading to these severe acute events may vary considerably depending on the disease, but in most cases these events represent complications of atherosclerotic disease. This pathology can be considered an age-related disease since it begins early in life and progresses gradually, remaining usually asymptomatic for a long period of time. Major risk factors for atherosclerosis are represented by endothelial dysfunction and dyslipidemia in particular in oxidative imbalance condition, frequent in aging.

EPIDEMIOLOGY

Over the past half century, advances in public health with a focus on life style (healthy diet, exercise training, and smoking cessation), clinical cardiology (chest pain units, coronary stenting, and cardiac defibrillation), and advancement of pharmacological research (lipid-lowering drugs, angiotensin-converting enzyme inhibitors) have contributed to a steady decline in deaths from CVDs in highly developed countries. However, despite these improvements, atherosclerosis-associated vascular diseases, such as IHD (ischemic heart disease) and stroke, remains the leading cause of morbidity and mortality worldwide; some countries even reported an increase in the number of new CVD cases [*Roth 2017; Ezzati 2015*].

According to the European Cardiovascular Disease Statistics 2017 edition, CVDs accounting for 45% of all death in European region (49% among women and 40% among men), equivalent to 3,9 million

deaths each year. Interestingly, the share of all deaths attributable to CVDs in the European Union is slightly lower than that in the continent as a whole, with CVDs responsible for 37% of all deaths (40% among women and 34% among men). IHD is the most common single cause of death in Europe, resulting in 19% of all deaths in men and 20% of all deaths in women, much greater than breast cancer in women (2%) and lung cancer in men (6%) (Fig. 1) [*Wilkins 2017*].

In 2015 there were an estimated 422,7 million prevalent cases of CVD in the world and 85 million in Europe, 11,3 million of which are new cases [*Roth 2017; Wilkins 2017*].

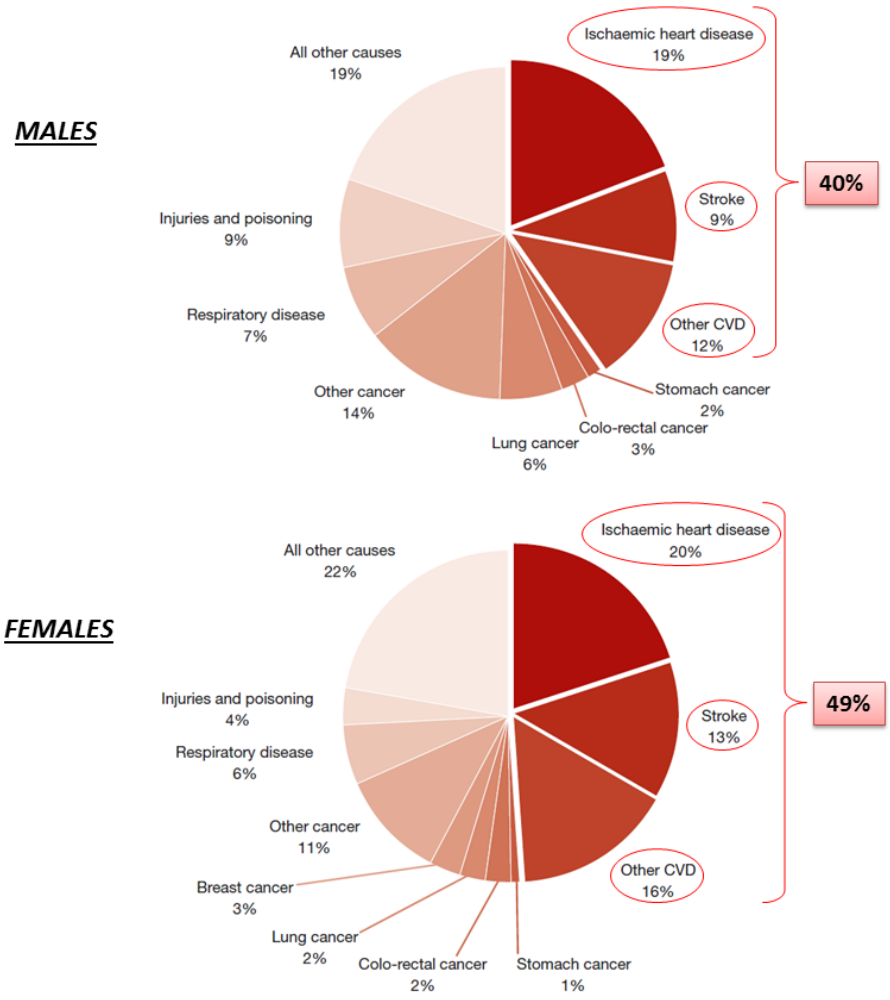

Figure 1: Deaths by cause in male and female in Europe.

RISK FACTORS

The risk factors for CVD can be either non-modifiable such as age, gender, ethnicity and genetic predisposition or modifiable. The latter category includes eight major CVD risk factors fall into two groups: behavioral or medical risk factors.

Behavioral Risk Factors:

- **Diet**: high consumption of dietary fat, especially trans and saturated fats, increases the risk of atherosclerosis, while diets high in sodium increase the risk of hypertension [*Mente 2009*].
- **Sedentary lifestyle** increases the risk of CVD by increasing the risk of hypertension, high triglycerides, low HDL cholesterol, diabetes and obesity; while participation in regular physical activity and/or aerobic exercise training is associated with a reduction in cardiovascular disease prevalence and mortality.
- **Tobacco smoking** raises the risk of IHD by elevating blood pressure and the tendency of blood to clot, promotes atherosclerosis through inflammation of the arteries, reduces plasma levels of HDL, and decreases exercise tolerance. Smoking cessation can substantially reduce the risk of cardiovascular death over time.
- **High alcohol consumption**, particularly binge drinking, increases the risk of CVD by raising blood pressure and blood levels of triglycerides. In addition, high levels of alcohol consumption increase the risk of liver cirrhosis, injuries, and some forms of cancer. While modest levels of alcohol intake (one or two drinks a day) may have a protective effect on the risk of some cardiovascular diseases, on balance, the positive effects of alcohol on the health of populations, beyond very low levels of consumption, are generally outweighed by its negative effects [*Nichols 2012*].

Clinical conditions associated with CVD:

- **Hypertension**: unhealthy diet, physical inactivity and obesity all increase the risk of raised blood pressure, while high blood pressure can be effectively lowered in response to lifestyle changes such as weight loss, increased physical activity and reduced salt and alcohol intake, as well as drug treatment.
- **High blood cholesterol**, in particular LDL are easily oxidized in the presence of ROS. The oxidase LDL (oxLDL) are a major trigger of atherosclerosis since they are actively phagocitated by macrophages that, once engulfed with lipids, form foam cells responsible for plaque formation. Several evidences support a causative role of LDL for atherosclerosis: genetic mutations that impair receptor-mediated removal of LDL cholesterol from plasma produce severe atherosclerosis at an early age; animal models with low LDL cholesterol plasma levels have no atherosclerosis, whereas increasing these levels experimentally leads to disease; similarly, human populations with low LDL-cholesterol levels show residual levels of atherosclerosis, and the process increases in direct proportion with the level of LDL cholesterol in the blood [*Nabel and Braunwald 2012*].
- **Overweight and obesity:** as well as increasing the risk of CVD directly, obesity is a risk factor for high blood pressure, raised blood cholesterol, diabetes and impaired glucose tolerance.
- **Diabetes mellitus:** high blood sugar causes damage to blood vessels, thus increasing the risk of CVD directly, as well as exacerbating the effects of other CVD risk factors such as raised blood pressure, raised cholesterol levels, smoking and obesity.

VASCULAR STRUCTURE

Blood vasculature consists of three principal types of blood vessels that circulate blood throughout the body, namely arteries, veins and their interconnecting capillaries. It forms a closed system that transports gases, nutrients, metabolites, cells and various signaling molecules to surrounding tissues [*Potente and Mäkinen 2017*].

Mammalian vessel walls comprise of three histological layers, namely *tunica intima*, *media* and *adventitia* (Fig. 2). The innermost layer, the *intima*, faces the blood flow, and consists of the endothelial monolayer and the underlying connective tissue on top of the internal elastic lamina. Endothelial cells are connected to each other through tight junctions and gap junctions, which are involved in intercellular cohesion and electrochemical coupling, respectively. The thin sub-endothelial layer of connective tissue, the extracellular connective tissue matrix, serves as a transducer of physical and chemical microenvironmental signals, a reservoir of growth factors and an adhesive scaffold required for anchorage-dependent survival of endothelial cells. In normal arteries the sub-endothelial space is thin, whereas in atherosclerosis intimal thickening is one of the earliest signs of the disease. The intermediate layer below *intima*, *tunica media*, is separated from the *intima* by the internal elastic lamina and from the adventitia by the external elastic lamina. The main constituents of media are smooth muscle cells and extracellular matrix components, such as collagen, elastin and proteoglycans. The outer layer of the vessel is *tunica adventitia*. The main ingredients of adventitia are fibroblasts and loose connective tissue, which contains a network of small blood vessels, the *vasa vasorum*, that supply the walls of the large vessels with oxygen and nutrients. In healthy vessels, the *intima* and the inner part of *media* are nourished by diffusion from the vascular lumen, but in an atherosclerotic plaque, the *vasa vasorum* penetrates the media and extends to feed the pathological, thickened *intima* [*Camaré 2017*].

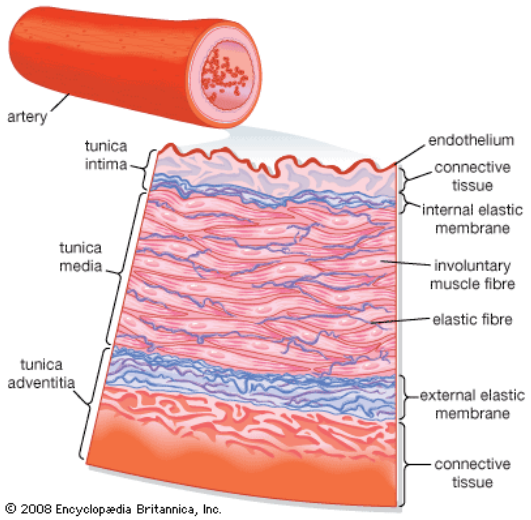

Figure 2: Structure of the vascular wall.

ENDOTHELIAL FUNCTIONALITY

Endothelium is not just a selective barrier between the vessel lumen and the surrounding tissue allowing the exchange of nutrients and gases between the blood and tissues, but it actively secretes signals that modify its own and neighbouring cell and tissue function. For example, endothelium controls vascular tone through secretion of vasoactive substances, which influence smooth muscle cell function, and participates in chemokine and growth factor signaling to regulate normal vascular growth and angiogenesis. Endothelium is also an active modulator of thrombotic events through secretion of anti- and pro-coagulant agents and fibrinolytic substances. Moreover, it protects the surrounding tissues from exogenous pathogens by evoking inflammatory responses and expressing adhesion molecules, which attract immune cells to the site of injury or infection. The most important of the endothelium derived molecules is nitric oxide (NO). NO is particularly relevant for vascular homeostasis by regulating VSMC relaxation, leading to vasodilatation, and inhibiting vascular cellular proliferation and fibrosis and preventing immune cell infiltration of the vascular wall [*Maron and Michel 2012*]. In particular, NO decreases endothelial expression of adhesion molecules, which are implicated in cellular migration, proliferation, atherogenesis, and thrombosis, such as ICAM-1, VCAM-1, P-selectin, β-1 integrin and monocyte chemoattractant protein 1 (MCP-1) [*Gimbrone and García-Cardeña 2016*].

In endothelial cells, the synthesis of NO is catalyzed from L-arginine by endothelial nitric oxide synthase (eNOS). The eNOS gene is stimulated by various stimuli, e.g. acetylcholine, endothelin-1 (ET-1), vascular endothelial growth factor (VEGF), bradykinin and particularly by fluid mechanical forces (share stress), such that endothelial cells exposed to undisturbed laminar flow exhibit enhanced NO-forming capacity [*Chiu and Chien 2011*]. Once NO is produced, it can rapidly diffuse across cell membranes to act as a potent paracrine mediator.

In the presence of low superoxide levels ($O_2^{-\bullet}$), NO reacts with molecular oxygen to form nitrogen dioxide (NO_2). NO_2 upon reaction with another NO molecule, forms the nitrosating species dinitrogen trioxide (N_2O_3), which is subsequently hydrolyzed to nitrite. This is not only a mechanism for scavenging NO but also serves to transport NO and is the molecular basis for biological effects in its own right. By contrast, in the presence of high superoxide levels, NO reacts with $O_2^{-\bullet}$ to form peroxynitrite ($ONOO^-$). Peroxynitrite is a very reactive oxidant and can influence gene expression, interfere with cell signaling pathways, react with proteins, cause lipid peroxidation and promote endothelial dysfunction [*Uppu 2007*].

The observations in animal models and in humans suggest that a deficiency in endothelial NO production or its bioavailability, in both humans and animals, might actually precede the formation of clinically significant atherosclerotic lesions [*Gimbrone and García-Cardeña 2016*].

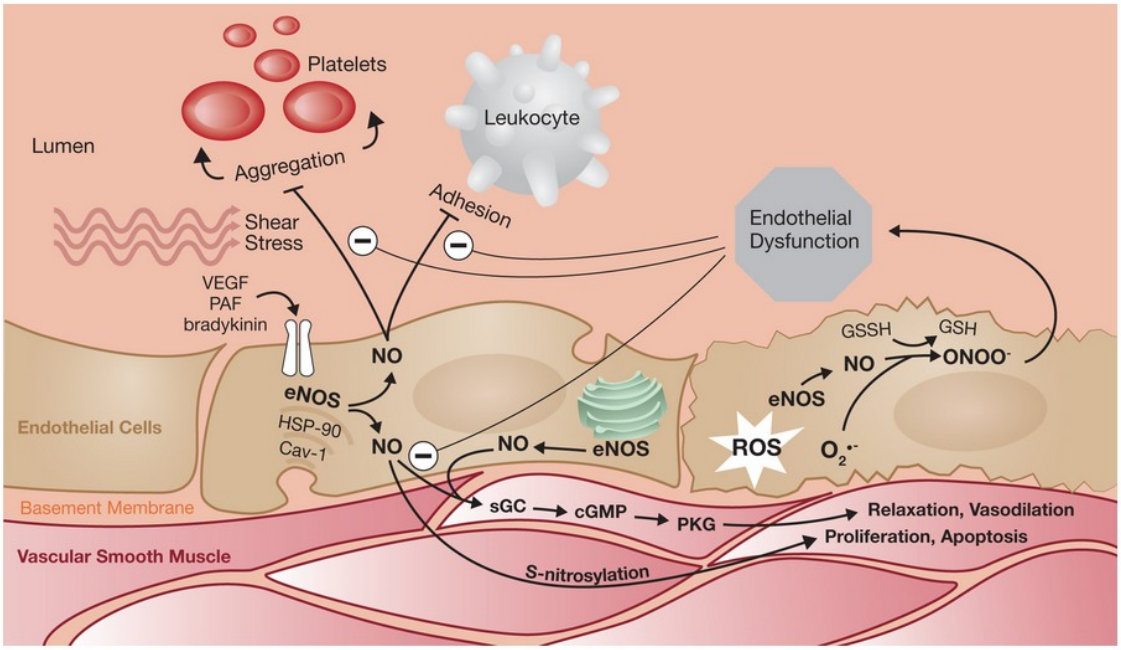

Figure 3: Schematic representation of endothelial Nitric Oxide (NO) signaling.

ATHEROSCLEROSIS

Atherosclerosis is a chronic inflammation of arteries and is a silent process starting early in life and continuing over several decades during which plaques form along the inner walls of the arteries narrowing the lumen and reducing blood flow to target tissues and organs. Early atherosclerotic lesions develop during adolescence in everyone, and with aging and other contributing risk factors, can progress to advanced stages and become symptomatic [*Libby 2011*].

Atherogenesis develops in three stages: initiation, progression and advanced stage.

INITIATION: ENDOTHELIAL DYSFUNCTION

Atherogenesis begins as a qualitative change to intact endothelial cells; when subjected to oxidative, hemodynamic, or biochemical stimuli (from smoking, hypertension, or dyslipidemia) and inflammatory factors, they change their permeability to promote the entry and retention of blood-borne monocytes and cholesterol-containing LDL particles.

Atherosclerotic lesions develop site-specifically primarily at the branching points of large and medium arteries where the turbulent flow results in low shear stress. While in the unbranched parts, blood flows in streamlined, known as the laminar flow, which causes high shear stress on the inner vessel wall. Endothelial cells are specialized and have various roles depending on their location and microenvironment. Arterial endothelial cells have adapted to pulsatile, high pressure and high shear

stress conditions, which inhibit proliferation and induce cell survival and quiescence. However, despite their plasticity, endothelial cells are sensitive to disturbed flow, and the probability of endothelial activation is higher. The consequent increase in endothelial turnover and senescence disrupts the normal barrier function of the endothelium increasing its permeability, oxidative stress, inducing inflammatory signals and leading to endothelial dysfunction [*Gimbrone and García-Cardeña 2016*].

All atherosclerosis risk factors amplify the endothelial activation enhancing oxidative stress and inducing eNOS uncoupling in the vascular wall [*Li and Forstermann 2013*]. Uncoupling of eNOS leads to not only reduced endothelial NO production but also a potentiation of oxidative stress. Oxidative stress is the imbalance in favor of increased generation of reactive oxygen species (ROS) and/or reduced body's innate anti-oxidant defense systems [*Sies 1985*].

ROS excess and decreased NO bioavailability cause the NF-kB nuclear traslocation resulting in the expression of various effector proteins with important pathophysiologic implications. These effectors include adhesion molecules, such as ELAM-1 (E-selectin) and VCAM-1; pro-coagulant molecules and secreted chemokines, such as Interleukin-8 (IL-8) and Monocyte Chemotractant Protein-1 (MCP-1) via MAPK (Mitogen Activated Protein Kinase). In particular VCAM-1 was found to have selective adhesivity for mononuclear leukocytes and lymphocytes, enabling a rapid recruitment into early-stage lesions [*Gimbrone and García-Cardeña 2016*].

ROS may also oxidase LDL, oxLDL are responsible for the progression of atherosclerosis and cause direct damage to endothelial cells promoting cellular apoptosis via LOX-1 down-regulating anti-apoptotic Bcl-2 proteins and upregulating caspase-3 and caspase-9 which cleave the anti-apoptotic proteins [*Li and Mehta 2009*]. In addition, Bcl-2 inhibition increase the mPTP opening and the cytochrome c release, which activates caspase-1.

Also the mitochondria can participate to the development of atherosclerosis. In fact due to their role in cellular respiration these organelles represent an important site of ROS production, mainly as superoxide anion via NADPH oxidase system, but also a sensitive site for oxidative damage. Accumulation of oxidative damage in mitochondria triggers the so called mitochondrial vicious-cycle, leading to membrane depolarization and the uncoupling of oxidative phosphorylation, mtDNA mutation, and a subsequently altered cellular respiration with higher levels of ROS production [*Hsich 2000; Di Lisa and Bernardi 2005*].

Active vascular cells thus generate a complex paracrine milieu of cytokines, growth factors and reactive oxygen species (ROS) within the vessel wall, that once challenged perpetuates a chronic pro-inflammatory state and promotes atherosclerotic lesion progression [*Gimbrone and García-Cardeña 2016*]. This process is inevitable during the aging process however intrinsic and extrinsic factors may play a role on the onset and the rate of development.

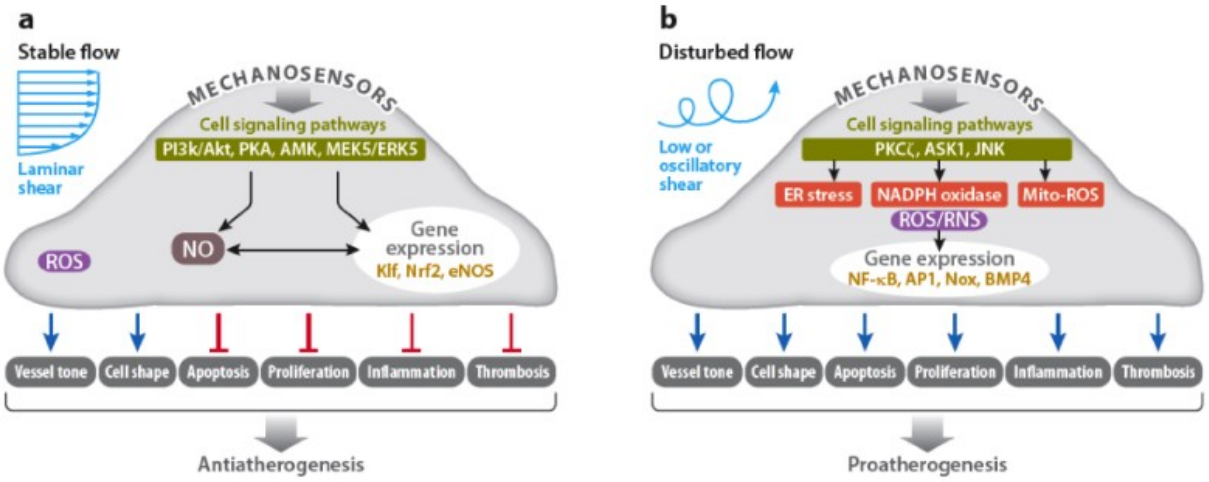

Figure 4: Mechanotransduction signaling pathways in endothelial cells in response to either laminar (high shear stress) or disturbed flow (low shear stress).

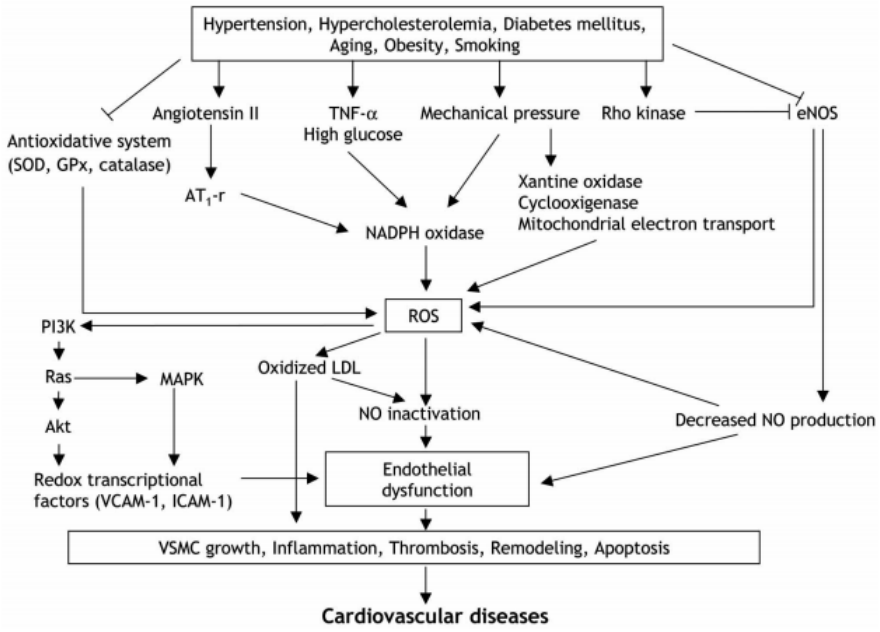

Figure 5: Mechanisms by which CVD risk factors induce endothelial dysfunction increasing ROS production.

PROGRESSION

Endothelial dysfunction, which is not a pathological condition, represents the earliest detectable manifestation of atherosclerosis. Lowered NO bioavailability is in act the first step leading to focal permeation, trapping and modification of circulating LDL particles in the sub-endothelial space, the intima [*Gimbrone and García-Cardeña 2016*]. Furthermore, activated endothelial cells express adhesion molecules on the cell surface, which attracts monocytes to the site and causes their infiltration into the intima. In the intima, monocytes differentiate into macrophages and internalize the retained lipoproteins. Lipoprotein uptake promote the intracellular accumulation of various lipids causing the macrophages conversion to foam cells. The intimal accumulation of oxLDL products further increases oxidative stress and inflammatory signaling thus stimulating endothelial activation and sustaining chronic inflammation in the vessel wall [*Gimbrone and García-Cardeña 2016*].

ADVANCED STAGE

As the lesions progress, foam cells form fatty streaks. Macrophages and endothelial cells produce more chemokines and growth factors, which induce vascular smooth muscle cell proliferation and their migration from the medial layer into the intima. In addition, intimal production of extracellular matrix components increases. This progressive structural remodeling results in the formation of a fibrous cap overlaying a lipid-rich necrotic core of oxidized lipoproteins, cholesterol crystals, cellular debris, and varying degrees of remodeled matrix and calcification. The lateral edges of these complicated plaques contain a rich population of inflammatory cells (activated macrophages and T-cells, natural killer T-cells, dendritic cells), which further modulate the endothelial pro-inflammatory phenotype, and contribute to structural instability of the plaque through the proteolytic modification of its extracellular matrix components [*Gimbrone and García-Cardeña 2016*].

Furthermore, induction of angiogenic factors promotes angiogenesis and neovascularization to increase the local flow of oxygen and nutrients to enable plaque growth [*Camaré 2017*].

In unstable or vulnerable plaques, this may result in a catastrophic transition in the life history of an atherosclerotic lesion-frank plaque rupture, with luminal release of the highly thrombogenic contents of the necrotic core, triggering an atherothrombotic occlusion [*Gimbrone and García-Cardeña 2016*].

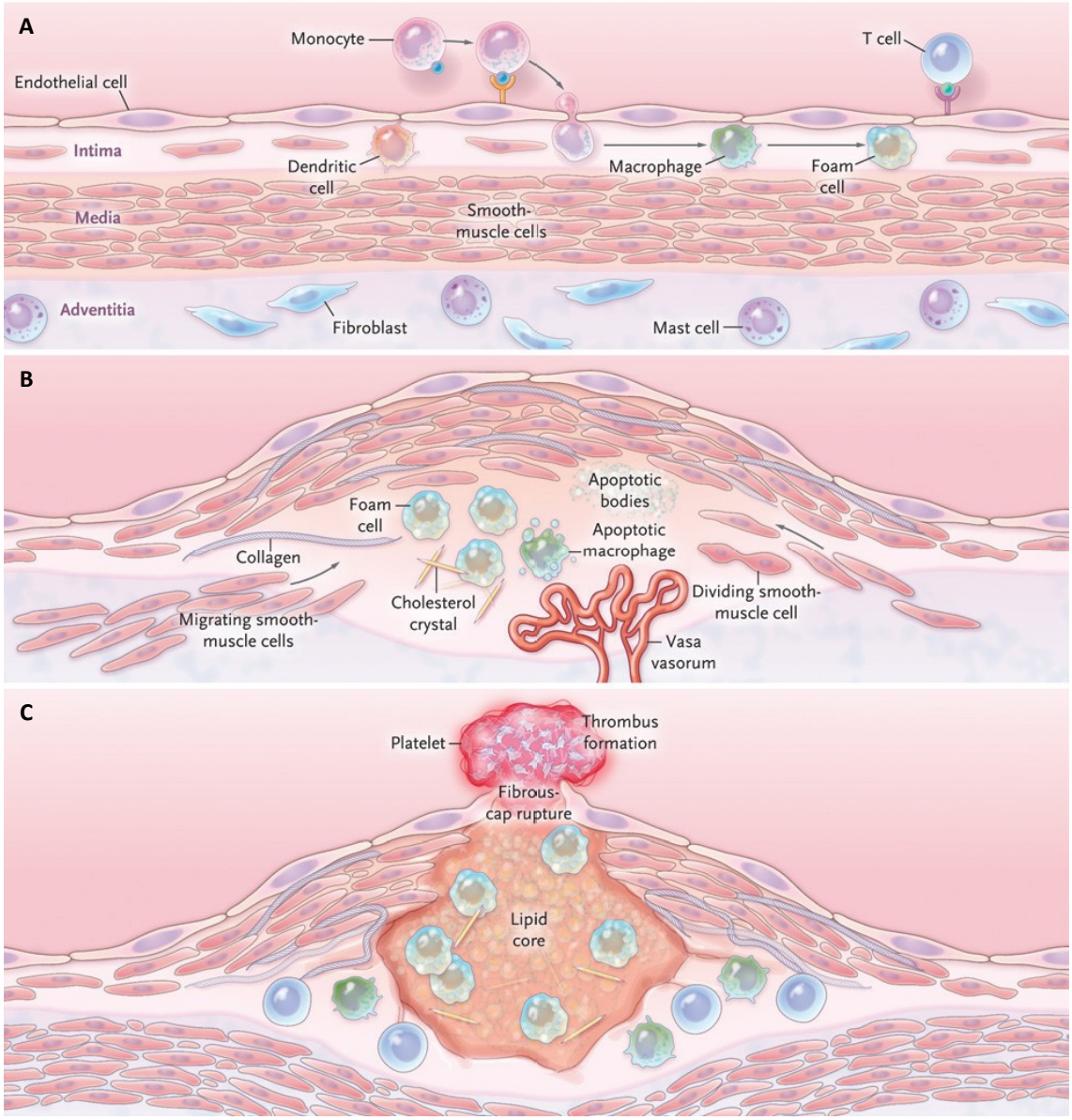

Figure 6: Stages in the development of atherosclerotic lesions: initiation (A), progression (B) and advanced stage (C).

AGING

Advanced age is a known risk factor for atherosclerosis and its thrombotic complications, such as acute myocardial infarction and stroke. Independent of other systemic atherosclerotic risk factors, aging leads to significant biochemical, histological and structural alterations in the vasculature, which promote an atherogenic environment for the classical risk factors to act upon, thereby favoring the progression and destabilization of stable atherosclerotic plaques [*Tesauro 2017*].

In vivo studies have demonstrated that in human atherosclerotic lesions, endothelial cells present senescent phenotype and are biologically older than the same cells in "normal" vascular wall. Furthermore, endothelial cell senescence seems to be induced by the shortening of telomeres and may contribute to atherogenesis [*Erusalimsky and Kurz 2005; Minamino and Komuro 2007*].

Senescence is a natural biological process that occurs when the cells reach their replicational limit, also known as the Hayflick's limit, for this reasons is called "replicative senescence" [*Hayflick and Moorhead 1961*].

Several lines of evidence suggest that accumulating oxidative stress is a major contributor to the aging-associated endothelial dysfunction and the changes lead to endothelial senescence [*Davalli 2016*]. The oxidative stress can be increase in response to different type of external stressors, such as turbulent blood flow, oxidants, smoke, radiation and oncogenes activation, thus induces sub-lethal cell damage and prevent cellular replication, causing Stress Induce Senescence Phenotype (SISP) [*Guo 2016; Farhat 2008*]. Therefore, differently from replicative senescence, SISP is not programmed and its onset is independent of telomeric mechanisms.

However, when compared replicative senescence and SIPS, despite the fact that they involve different mechanisms of action, they share many common properties. In fact, both senescence cell types display senescence-associated morphological changes e.g. flattened and enlarged morphology, vacuolization and increased SA-β-galactosidase activity. The initiation of senescence involves the recognition of DNA damage, which leads to the activation of the cell-cycle checkpoints and induction of irreversible cell cycle arrest through the p53 and $p16^{INK4A}$ pathways [*Wright 1996; Kuilman 2010*]. Moreover, the Senescence Associated Secretory Phenotype (SASP), characterized by transcriptional changes resulting in the secretion of many factors such as pro-inflammatory cytokines, chemokines and extracellular matrix proteases, is also common in both senescent cell types [*Kuilman 2010*].

Thus, therapeutic target to slow down a senescence process is an attractive approach for improving vascular function and reducing the risk of developing aging-related vascular dysfunction.

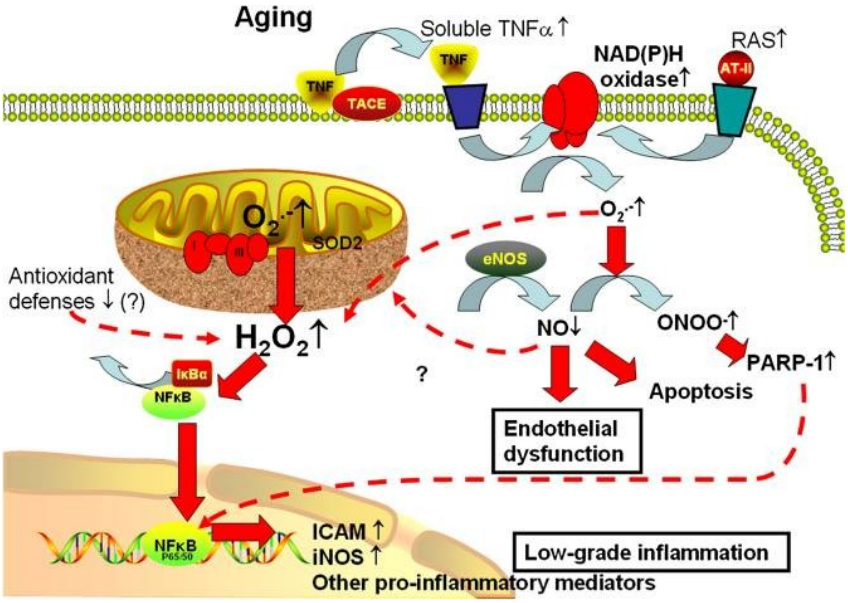

Figure 7: Pathways contributing to cellular oxidative stress and NF-κB activation in aged endothelial cells. Increased levels of $O_2^{-\bullet}$ contribute to NF-kB activation resulting in a pro-inflammatory gene expression. In addition $O_2^{-\bullet}$decrease the bioavailability of NO leading to vasodilator dysfunction and endothelial apoptosis.

CARDIOVASCULAR TOBACCO SMOKE TOXICITY

Tobacco smoke is a major modifiable risk factor for a number of diseases and the most common cause of premature death in Europe. There are big differences in the prevalence of smoking across European region, overall, on average around 38% of men and 17% of women smokes. Over the past 35 years, the implementation of tobacco control policies has led to a decrease in the prevalence of smoking in almost all European countries, however smoking remain an important cause of CVD [*Wilkins 2017*].

Cigarette smoke is a highly complex aerosol of >5000 compounds, including reactive oxygen species (ROS), reactive nitrogen species, carbon monoxide, nitric oxides, nicotine, polycyclic hydrocarbons, cadmium, and other metals and oxidants [*Rodgman and Perfetti 2013*]. Importantly, it is likely that it is not just a single compound but a highly complex and changing mixture of smoke compounds that is responsible for disease initiation, progression, and cardiovascular outcome. In particular the oxidant fraction of cigarette smoke is thought to be the main stimulus for the formation of atherosclerotic plaques and pathological thrombi [*Farhat 2008*].

Craig et al. [*Craig 1989*] showed a statistically significant correlation between smoking and increased total serum cholesterol, very-low-density lipoprotein (VLDL), low-density lipoprotein (LDL), and triglyceride serum concentrations. Apart from modulating lipid quantities, smoking was also shown to change lipids qualitatively. Free radicals and oxidants present in cigarette smoke, as well as endogenously produced (resulting from the smoke chemical-induced alteration in the cellular redox

system), cause a pro-oxidative environment [*Garbin 2009*]. This general shift is likely to contribute to lipid oxidation and to a general increase in oxidative modification (and inactivation) of biomolecules.

A number of clinical and *in vitro* studies indicate that cigarette-smoke-induced endothelial dysfunction is mediated by a lack of nitric oxide bioavailability, the cardinal feature of all endothelial dysfunction [*Rahman and Laher 2007*]. The superoxide anion present in the cigarette smoke, directly reacts with nitric oxide to form peroxynitrite, which causes protein nitration. In addition, smoking compounds is associated with increased acetylation and expression, and decreased activity and uncoupling of endothelial nitric oxide synthase (eNOS), which results in endogenous production of ROS [*Barua 2003; Arunachalam 2010*].

The ROS production increases also from the NADPH oxidases activation caused by the smoke exposure [*Jaimes 2004*]. Under normal physiological conditions, NADPH oxidases are important sources of ROS for signaling pathways regulating vascular tone, and endothelial cell proliferation, migration, and differentiation [*Frey 2009*]. On exposure to cigarette smoke, however, NADPH oxidases produce high levels of ROS, which contribute to endothelial dysfunction and vascular disease [*Jaimes 2004*]. The NADPH-oxidase-activating ability of stable aldehydes present in cigarette smoke suggests a far-reaching pro-oxidative effect of exposure to cigarette smoke extract on the systemic vasculature, which might underlie the dysfunctional endothelial cell phenotype typically encountered in smokers.

The shift to a vascular pro-oxidative state and NADPH activation may also significantly contribute to inflammation. In fact cigarette-smoke-derived ROS trigger the activation of NF-κB and, therefore, promotes the expression of pro-inflammatory cytokines, such as interleukin-6 andinterleukin-8, and adhesion molecules, such as intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1), on the surface of endothelial cells [*Orosz 2007*]. Notably, a positive-feedback loop exists, in which platelet-derived bioactive molecules stimulate increased expression of endothelial adhesion molecules in an NF-κB-dependent manner, which results in further enhanced adhesion of platelets and leukocytes on the arterial wall, therefore in the amplification of inflammatory state. Inflammation is known to constitute an essential element in atherogenesis [*Ross 1999*].

Furthermore, *in vitro* studies have shown that the cigarette smoke is able to induce premature senescence in different cell types, such as skin fibroblasts [*Yang 2013*], airway epithelial cells [*Wu 2016; Liu 2016*], and finally in endothelial progenitor cells exposed to cigarette smoke it has been observed an increased expression of $p16^{INK4a}$, and a consequent block in the cell cycle [*He 2017*].

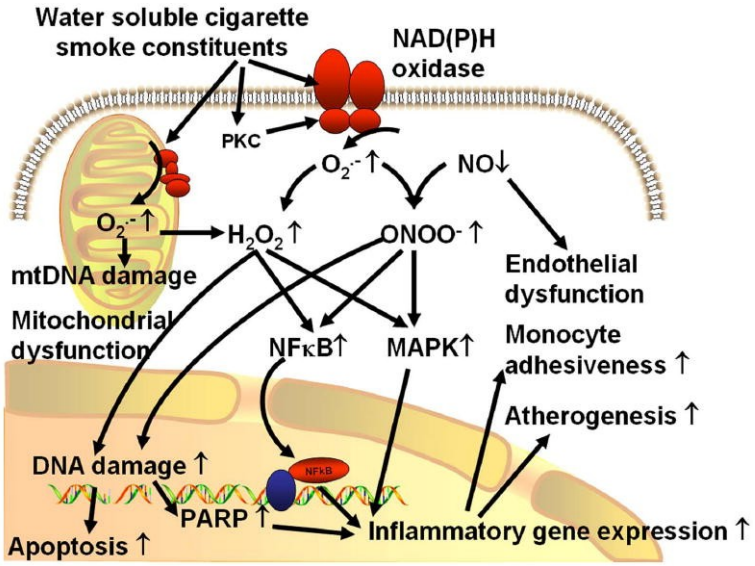

Figure 8: Mechanisms by which water soluble components of cigarette smoke promote pro-inflammatory phenotypic alterations in the blood vessels.

QUINONES

COENZYME Q_{10}

Coenzyme Q_{10} (CoQ_{10}) is an endogenous lypophilic cofactor present in humans. It is composed of a benzoquinone nucleus and a tail of 10 isoprenoid units that are responsible for the redox function and hydrophobicity of CoQ_{10}, respectively. There are two main chemical states of CoQ_{10}: fully reduced (ubiquinol, QH) and fully oxidized (ubiquinone, Q) (Fig. 9). The reduced form (QH) show better bioavailability than the oxidized form, thanks to its more hydrophilic proprieties [*Failla 2014*]. It is ubiquitous, it is present in all organs, in particular is most abundant in heart. Moreover, within cells, CoQ_{10} is present in plasma and subcellular membranes.

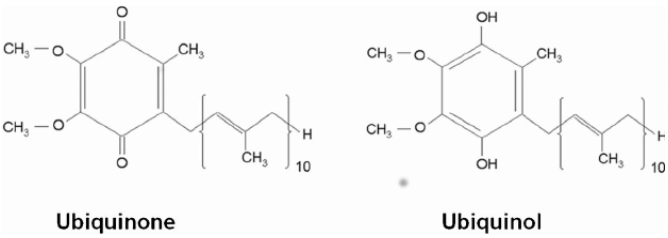

Figure 9: Ubiquinone (Q) and Ubiquinol (QH) chemical structure.

CoQ_{10} has two main biological functions: it supports mitochondrial bioenergetics and in its reduced form, ubiquinol, it is a phenolic antioxidant that acts as an electron donor effective in inhibiting lipid peroxidation of biological membranes [*Bentinger 2007*]. However CoQ_{10} can explain other functions such as modulation of eNOS activity and gene expression. In particular, the non-mitochondrial CoQ_{10}-forming enzyme in Golgi membranes has specific cardiovascular protective functions that modulate eNOS activity through redox reactions involving CoQ_{10} [*Mugoni 2013*]. In addition, expression profiling revealed that CoQ_{10} affects the expression of several hundred genes and may exert many of its effects via the induction of gene transcription [*Schmelzer 2007*], and it exerts anti-inflammatory properties via NF-κB-dependent gene expression; therefore it acts as a potent gene regulator [*Schmelzer 2008*].

Regarding its bioenergetic role, CoQ_{10} is essential for mitochondrial ATP generation transferring electrons from complex I/II to complex III. Moreover, CoQ_{10} analogs affect the mitochondrial permeability transition pore (mPTP) possibly via a common binding site [*Devun 2010*]. Moreover, supplementing mammalian cells with CoQ_{10} prevents the opening of the mPTP and increases survival in response to apoptotic stimuli [*Papucci 2003*]. These findings suggest that CoQ_{10} may modulate the mitochondrial permeability transition pore, potentially via structural effects.

In addition, ubiquinol is a strong hydrogen atom donor, that can regenerates antioxidant state of α-tocopherol [*Stocker 1991*] and can prevents the propagation of lipid peroxidation, as well as protein

and DNA oxidation mediated by lipid hydroperoxides [*Stocker and Keaney 2004*]. CoQ_{10} may also reduce mitochondrial superoxide production by increasing the efficiency of electron transfer from complexes I and II down the mitochondrial electron transport chain [*McCarty 1999*]. These activities, together with its abundance in cellular membranes and the presence of enzymatic systems that maintain CoQ_{10} in the reduced form, make of QH a highly effective cellular antioxidant.

Given the importance of the QH, cells are endowed with efficient CoQ-reducing activities, including cytosolic NADPH-dependent CoQ reductase [*Takahashi 2008*], lipoamide dehydrogenase, thioredoxin reductase, and glutathione reductase [*Nordman 2003*]. Of these, NADPH-dependent CoQ reductase has been identified as the main enzyme responsible for reduction of non-mitochondrial CoQ *in vivo* [*Takahashi 2008*].

Decrease of CoQ_{10} content is known to occur in conditions associated with oxidative stress and mitochondrial dysfunction such as degenerative pathologies and in physiological conditions during the aging process [*Niklowitz 2016; Schottlaender 2016*]. Consequently, in these conditions coenzyme Q_{10} supplementation, in particular in the reduced form, could represent a beneficial integration strategy.

COENZYME Q_{10} IN CARDIOVASCULAR HEALTH AND AGING

CoQ_{10} deficiency has been observed in patients with congestive heart failure, angina pectoris, coronary artery disease, cardiomyopathy, and hypertension; major aging-associated diseases. Furthermore, low plasma CoQ_{10} concentrations are an independent predictor of mortality in patients with heart failure [*Ayer 2015*].

Despite the mechanism by which CoQ_{10} depletion and supplementation affects cellular function and disease pathogenesis is not completely understood, many reviews and meta-analysis have highlighted health effects of CoQ_{10} supplementation in different cardiovascular diseases by reducing morbidity and mortality, in particular improving endothelial elasticity [*Ayer 2015*; *Lei and Liu 2017*]. However Mortensen et al. report no improvement after 16 weeks of CoQ_{10} supplementation (300 mg/day) in chronic heart failure patients, but it became significant after 2 years of treatment [*Mortensen 2014*]. The studies in literature are very varied in CoQ_{10} quantity used (100-300 mg) and in treatment duration (4 weeks to 2 years), but all show a trend toward improvement following CoQ_{10} supplementation [*Ayer 2015*]. The CoQ_{10} also has anti-atherogenic effect reducing the lipid hydroperoxides concentration in atherosclerotic lesions and the size of the same lesions in the aorta, in ApoE -/- mice fed with a high fat diet [*Witting 2000*].

Despite the evidence of theCoQ_{10} supplementation efficacy on CVD treatment, there was a lack of clinical trials to support the use of CoQ_{10} in primary prevention of cardiovascular disease [*Flowers 2014*].

Endothelial dysfunction, the main cause of cardiovascular diseases, is characterized by enhanced oxidative stress, inflammatory state and reduced NO availability. The antioxidant properties of coenzyme Q_{10} can possibly explain the improvement in endothelial function of subjects at risk, as documented in a recent meta-analysis of five trials [*Gao 2012*]. In particular CoQ_{10} may reduce the rate of inactivation of NO and may also affect vascular function indirectly via inhibition of oxidative damage to LDL. An *in vitro* study have demonstrated that in human umbilical vein endothelial cell cultures treated with oxidized LDL, coenzyme Q_{10} protects endothelial cells from oxidative stress-induced injury by up-regulation of eNOS and down-regulation of inducible nitric oxide synthase [*Tsai 2012*].

Other studies have demonstrated that CoQ_{10} possesses a considerable anti-inflammatory effect, showing a negative correlation between CoQ_{10} and inflammatory markers (TNF-α and IL-6) [*Lee 2013*]. Schmelzer et al. proved that coenzyme Q_{10} could exert anti-inflammation effects via the reduction of nuclear factor-κB (NF-κB) dependent gene expression [*Schmelzer 2009*].

Endothelial dysfunction is also characterized by a premature senescence of endothelial cells and the inhibition of this process by CoQ_{10} supplementation was shown through *in vivo*, with senescence-accelerated mice [*Tian 2014*], and *in vitro* studies. In particular, Huo et al. demonstrated that the ubiquinol (QH) supplementation reduced the number of senescence-associated β-galactosidase positive cells and suppressed ROS production and the expression of senescence-associated secretory phenotype-associated genes in H_2O_2-treated HUVECs. Furthermore, QH suppressed the generation of intracellular reactive oxygen species (ROS) but promoted NO production that was accompanied by increased eNOS expression [*Huo 2018*]. Olivieri et al. have shown, not only the anti-aging and anti-inflammatory property of ubiquinol, but also the QH protective role in senescent endothelial cells after LPS treatment, resulting in a lower inflammatory response (miR-146a e IRAK-1) [*Olivieri 2013*].

Therefore, CoQ_{10} can support endothelial functionality improving arterial elasticity and preventing the development and/or progression of atherosclerotic plaque.

VITAMIN K

Vitamin K is a family of fat-soluble vitamin comprising structurally similar molecules (2-methyl-1,4-naphthoquinones) including two main natural forms: phylloquinone (vitamin K1) and menaquinones (collectively known as vitamin K2), which differ from phylloquinone in length and saturation of the side chain (Fig. 10).

The IUPAC-IUB Commission on Biochemical Nomenclature abbreviates VK2 as "MK-n", where "n" signifies the number of unsaturated isoprene units that compose the isoprenyl chain. The most common menaquinones are MK-4 and MK-7. The first is find in animal tissues; while the other one is synthesized by bacteria during fermentation, therefore it's find in cheese and japanese fermented soy dish (natto). MKs are thought to contribute less than phylloquinone to overall vitamin K intakes in Western diets. On the contrary, phylloquinone, found predominantly in dark green leafy vegetables and vegetable oils, is the primary dietary form of vitamin K in Western diets [*Schurgers and Vermeer 2000*].

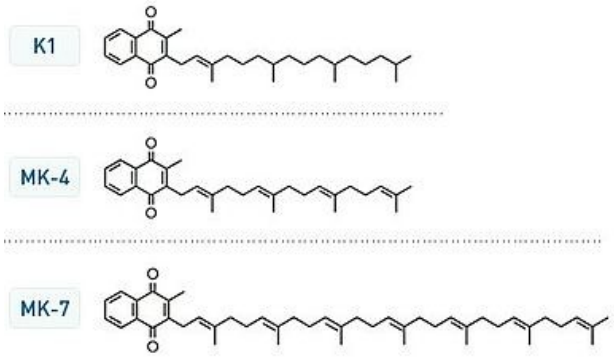

Figure 10: Chemical structure of K vitamers: Phylloquinone (K1), Menaquinone-4 (MK4) and Menaquinone-7 (MK7).

The principal known function of vitamin K is as an enzymatic cofactor for the post-translational carboxylation of certain proteins (called vitamin K-dependent proteins, VKDP). In this contest vitamin K is involved in a recycle reactions namely vitamin K cycle.

VITAMIN K CYCLE

Vitamin K was shown to act as a cofactor for a single microsomal enzyme, namely γ-carboxyglutamyl carboxylase (GGCX), located at the luminal surface of the endoplasmic reticulum. GGCX is needed to catalyze a reaction in which specific peptide-bound glutamate residues (Glu), found in vitamin K dependent proteins, are converted to γ-carboxyglutamate (Gla). This reaction requires the reduced form of vitamin K, hydroquinone (KH2), which is oxidized to vitamin K 2,3-epoxide (KO). KO is

recycled to its hydroquinone form through a two-step enzymatic reduction (first to vitamin K, then to KH2) (Fig. 11) [*Tie and Stafford 2016*]. The second vitamin K reductase activity is necessary because only KH2 can serve as a cofactor for the GGCX.

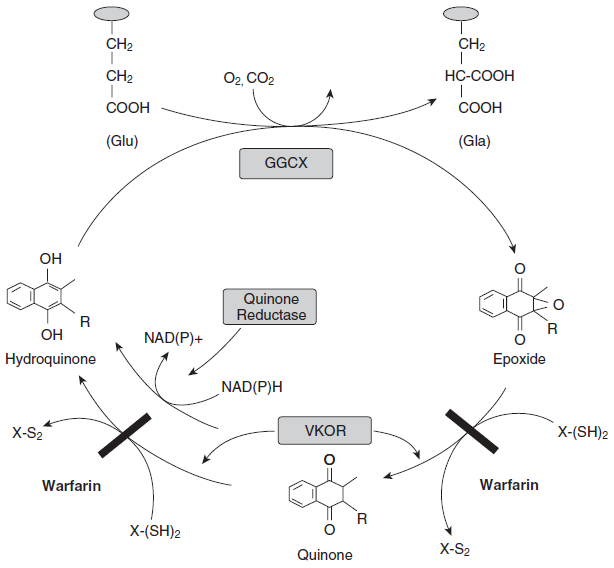

Figure 11: Vitamin K cycle and vitamin K antagonists action.

To investigate the vitamin K reduction mechanisms, researchers used vitamin K antagonists (VKA) such as warfarin and dicoumarol.

Vitamin K epoxide reductase (VKOR) was the first enzyme identified and which is responsible both for the conversion of KO to vitamin K and vitamin K to KH2 [*Chu 2006*]. Studies on VKOR knockout mice [*Spohn 2009*], indicates that VKOR is the main enzyme that can reduce KO to K *in vivo*, at least for the hepatic synthesis of VKD coagulation proteins at physiologically relevant tissue concentrations of vitamin K. However, the question of which enzymes are responsible for the physiological two-electron reduction of K to KH2 is the subject of ongoing research and debate. Furthermore, when VKOR is inactivated by warfarin or knocked out by gene targeting in HEK293 cells, those cells can still efficiently reduce vitamin K to support vitamin K-dependent carboxylation [*Tie 2011*]. Additionally, VKOR deficient mice died due to extensive intracerebral haemorrhage; however, this lethal phenotype could be rescued by the oral administration of vitamin K [*Spohn 2009*]. Therefore, VKOR play an important role, but is not the only reductase enzyme involved in the vitamin K cycle.

VKOR-like 1 (VKORL1) is a paralogous protein of VKOR. An *in vitro* study showed that VKORL1 was capable of reducing epoxides of both K1 and MK-4 to their respective hydroquinones. However, the affinity of VKORL1 for the epoxide forms was lower than their affinities for VKOR [*Westhofen 2011*]. Therefore it can support vitamin K reduction, but probably doesn't play a key role.

NAD(P)H quinone oxidoreductase-1 (NQO1), also known as DT-diaphorase, is a flavoprotein that catalyzes the two-electron reduction of several quinones including vitamin K and has long been known as the warfarin resistant antidotal enzyme for vitamin K reduction [*Wallin 1978*]. Rat liver microsomes depleted of NQO1 displayed reduced vitamin K-dependent carboxylation activity, and this activity could be restored by adding purified NQO1 [*Wallin 1986*]. However, dicoumarol, a strong inhibitor for NQO1, was unable to inhibit vitamin K reduction in HEK293 cells [*Tie 2011*], and NQO1-deficient mice survived at the same frequency as wild-type mice when poisoned with warfarin [*Ingram 2013*]. These results suggest that NQO1 is not the antidotal enzyme for reducing vitamin K to KH2, in other words it is inhibited by vitamin K antagonists.

To date, we know very little about the enzymes that reduce vitamin K, but there are several lines of evidence that demonstrate VKR's (vitamin K reductases) importance. Most notably, patients that are overdosed with warfarin can be rescued by large doses of vitamin K through the action of a warfarin-resistant enzyme which reduces vitamin K, designated as the "antidotal enzyme". The antidotal effect of vitamin K was first discovered in 1966 [*O'Reilly and Aggeler 1966*] and was recently demonstrated in the VKOR-knockout mouse [*Spohn 2009*] and in a cell-based vitamin K cycle enzyme study [*Tie 2011*]. However, despite decades of effort, the identity of VKR is still unknown.

Although several reductase enzymes are known, further studies are needed to clarify the mechanisms of vitamin K reduction, that will also help to understand its functions and regulation.

VITAMIN K DEPENDENT PROTEINS

The vitamin-dependent proteins (VKDP), or Gla proteins, resultant from the vitamin K cycle, are diverse in both structure and function and are found in many cell and tissue types. It is this very diversity of Gla proteins that makes vitamin K a truly multi-functional vitamin. The functions of many Gla proteins remain uncertain, but are suspected to play roles in processes as diverse as bone and cardiovascular mineralization, vascular hemostasis, energy metabolism, immune response, brain metabolism, and in cellular growth, survival, and signaling [*Shearer and Newman 2014*].

Currently, 17 different VKDP are known, but only for a few proteins the functions are well known.

Vitamin K-dependent carboxylation was originally observed in coagulation that involve seven VKDP: prothrombin (factor II), factors VII, IX and X, protein C, protein S and protein Z. In all clotting factors, ten to twelve Gla residues are located in a homologous amino-terminal region referred to as the Gla domain. The multiple Gla residues in this domain adopt a calcium-dependent conformation that promotes clotting factors binding to a membrane surface; such as damaged vascular endothelial cells or activated platelets, which allows the localization of clotting factors near the site of vascular injury to either promote or regulate clotting [*Tie and Stafford 2016*].

Others VKDP well known are: Osteocalcin (OC) and matrix Gla protein (MGP), which play important roles in both bone and vascular system regulating calcium homeostasis. In particular MGP functions as a strong inhibitor of vascular calcification and connective tissue mineralization [*Schurgers 2008*]. In addition, MGP has been described as a critical regulator of endothelial cell function, regulating both physiological and tumor-related angionenesis [*Sharma and Albig 2013*].

Also the growth arrest-specific protein 6 (Gas6) seems to be involved in cardiovascular disease regulating both apoptosis and migration of smooth muscle cells into areas of injury, however the mechanism is not clear [*Son 2006*].

Other vitamin K-dependent proteins include Gla-rich protein (GRP), trans membrane Gla proteins (TMGs), periostin and transthyretin. The metabolic significance of most of these non-coagulation Gla proteins is still poorly understood [*Tie and Stafford 2016*].

VITAMIN K IN CARDIOVASCULAR HEALTH AND AGING

Vitamin K and vitamin K-dependent proteins have been linked to several age-related diseases in observational and intervention studies.

In the Rotterdam Study, the intake of vitamin K2, but not of vitamin K1, of 4807 seniors was inversely associated with aortic calcification and mortality from all causes [*Geleijnse 2004*]. Similar results have also been observed in other studies, such as EPIC study [*Gast 2009*]. However the consumption of vitamins K was analyzed according to validated food frequency questionnaires, which carry inherent limitations.

A systematic review of vitamin K and CVD published in 2015 sought to include only vitamin K supplementation trials of at least 3 months in duration and conducted in healthy adults or adults at high risk for CVD. Ultimately, only one trial that tested the effect of menaquinone-7 supplementation on blood pressure and lipid levels over 12 weeks 60 men and women 45-60 years old was included in the review. Overall, the authors concluded there is insufficient evidence to conclude vitamin K affects CVD and further intervention trials are needed [*Hartley 2015*].

The dephosphorylated-uncarboxylated Matrix Gla-Protein (dp-ucMGP), a VKDP, is considered a marker of cardiovascular disease, such as aortic valve disease, aortic stenosis and heart failure, and mortality in patients with cardiovascular risk. Vitamin K2, but not K1, supplementation has been also associated with the reduction of dp-ucMGP in healthy individuals [*Villa 2017*].

Carboxylated-MGP (cMGP) is an inhibitor of soft tissue calcification, because it is able to strongly bind and inhibit the growth of calcium crystals. In addition it can inhibit VSMCs trans differentiation to osteoblasts in the vessel wall, slowing down the progression of vascular calcification and atherosclerosis [*Wallin 2008*].

The action of vitamin K2 in vascular calcification is also associated with activation of other two important VKDPs: growth arrest specific gene 6 (Gas-6) and Gla-Rich Protein (GRP). Gas-6, when carboxylated, stimulate the anti-apoptotic activity of Bcl-2 and inhibit the pro-apoptotic protein caspase-3, protecting the VSMCs from apoptosis, induced by the starvation of fibroblasts, and calcification [*Son 2006*]. On the other end, GRP has the ability to bind to hydroxyapatite and to be highly accumulated at sites of calcifications in human samples derived from patients diagnosed with skin and vascular calcification pathologies [*Viegas 2009*].

Cardiovascular disease and aging are characterized by a chronic low-grade pro-inflammatory state, which promotes the disease progression. Vitamin K appears to suppress the pro-inflammatory cytokines production through non-carboxylative pathway, rather decreasing gene expression of pro-inflammatory markers [*Ohsaki 2010*].

In a cross-sectional analysis of the Framingham Offspring Study higher vitamin K intake was associated with lower inflammation overall and with lower concentrations of several individual pro-inflammatory biomarkers. Circulating phylloquinone, a global indicator of vitamin K status, has been evaluated in relation to inflammation. In the Framingham Offspring Study, higher plasma phylloquinone was also associated with lower inflammatory-burden cross-sectionally, consistent with the findings of vitamin K intake. However only the plasma phylloquinone, but not ucOC, was associated with a inflammatory burden, suggests that vitamin K role in inflammation is independent of its role as an enzyme cofactor [*Shea 2008*].

In addition, a recent Multi-Ethnic Study of Atherosclerosis (MESA) with 662 participants demonstrated that high serum phylloquinone reduce circulating IL-6, CRP and ICAM-1. In contrast, dietary phylloquinone intake was not associated with any inflammatory biomarker evaluated [*Shea 2014*].

Although phylloquinone supplements (0,5 mg/day) were not effective in healthy elderly [*Shea 2009*], three-month MK-4 supplementation (45 mg/day) significantly reduced serum C-reactive protein (CRP) and matrix metalloproteinase-3 (MMP-3) levels in rheumatoid arthritis patients [*Ebina 2013*].

In sight of these evidence, vitamin K could protect from inflammation, and consequently from aging and cardiovascular disease, via non-carboxylative pathways. In addition, a lot of evidence suggest the cardiovascular protective effect of vitamin K trough VKDP. However additional trials are necessary to confirm the vitamin K role, to understand the mechanism and identify the gold standard vitamin K related and which predict cardiovascular disease.

VITAMIN K ANTAGONIST: WARFARIN

Warfarin (3-[α-acetonyl-benzyl]-4-hydroxycoumarin), one of the vitamin K antagonist, has been used successfully as a clinical agent since 1941. By inhibiting vitamin K, warfarin influences VKDP activity, thus causing significant changes to health: from impaired bone health to artery calcification; however, conflicting data are reported in literature [*Siltari and Vapaatalo 2018*].

Finally, there has been limited investigation of the impact of warfarin treatment on vascular calcification. Available cross-sectional studies have reported warfarin treatment to be associated with aortic valve [*Koos 2009*] and extra-coronary arterial calcification [*Rennenberg 2010*]. In contrast, warfarin exposure has not been associated with extent of coronary calcification in patients without apparent coronary heart disease participating in the Warfarin and Coronary Calcification Study [*Villines 2009*].

In certain patients for unknown reasons, there seems to be a risk of vascular calcification associated with chronic warfarin therapy. No biomarkers are available to recognize the patients at risk of suffering this rare side effect [*Siltari and Vapaatalo 2018*].

Furthermore, the biochemical mechanisms linked to warfarin are not known, for example how the cells behave in the presence of warfarin and how this affects the response to other stimuli.

AIM OF THE THESIS

The endothelium plays a central role in the pathophysiology of atherosclerosis, which underlies most cardiovascular diseases (CVD). A decisive role in the development and progression of this pathology is the cellular senescence that can be physiological but also induced. Tobacco Smoke represents a major risk factor for CVD and is able to induce an early cellular senescence through the increase of oxidative stress and cellular damage.

It is known that molecules such as ubiquinol and vitamin K play a fundamental role in vascular health by protecting the endothelium from oxidative damage, inflammation and ensuring the functionality of important vitamin K dependent proteins involved in vascular health.

However, in the literature there are incomplete and discordant data on their effect and role under stress conditions, particularly on vitamin K and its antagonists, such as warfarin.

Therefore, the aim of this thesis was to elucidate the effect of ubiquinol, K vitamers (K1, MK4, MK7) and warfarin in a model of endothelial cells exposed to tobacco smoke extracts with a particular focus on the prevention of oxidative damage and premature senescence.

The specific experimental aims of this project were to:

- ✓ Establish the effects of tobacco smoke treatment in endothelial cells;
- ✓ Identify the best method to supplement the ubiquinol and vitamers K and their bioavailability;
- ✓ Investigate the roles of the supplementation of: ubiquinol, vitamin K and warfarin on the prevention of tobacco smoke changes in endothelial cells.

PREPARATION OF CIGARETTE SMOKE EXTRACT

Cigarette smoke extract (CSE) was prepared by a modification of the method of Carp and Janoff [*Carp and Janoff 1978*] using a commercial available filter tipped cigarette. Their characteristics are describe in Table1.

	Commercial filter tipped cigarette (mg/cigarette)
Tar	10
Carbon monoxide	10
Nicotine	0,8

Table 1: Commercial filter tipped cigarette composition.

Ten commercial cigarettes with filters were combusted with a modified syringe-driven apparatus represented in Figure 12.

The CSE sampling was performed during a controlled time in which a cigarette was aspirated with a 20 mL syringe which was filled in two seconds. The smoke was bubbled through 50 mL of MEM in 10 seconds. Each cigarette was consumed in 15 puff. The smoke from 10 cigarettes was bubbled in 50 mL of culture medium. The resulting suspension was adjusted to pH 7,4 and filtered through a 0,20 µm pore filter to remove potential bacterial contamination and large particles. The resulting suspension was considered as 100% CSE. At the experimental time, 100% stock solution was diluted in MEM or EGM-2 to HDFs or HUVECs treatment respectively. The reproducibility and the stability of 100% CSE was established treating HDFs for 24h with five CSE batches and measuring the viability and the ROS production.

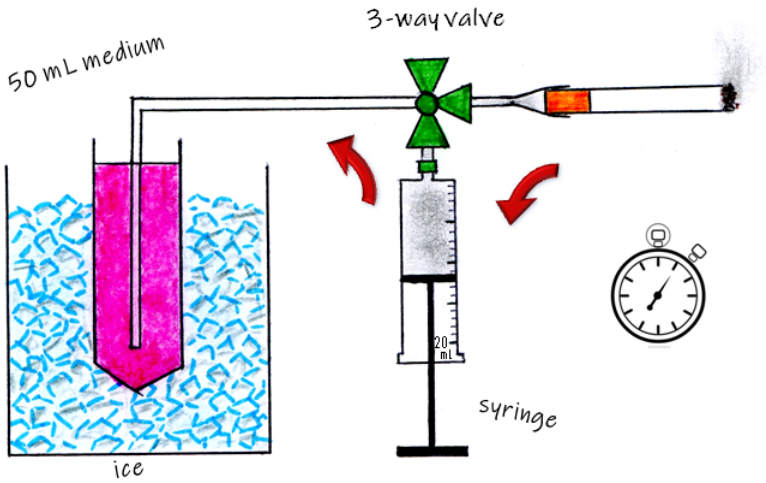

Figure 12: Schematic representation of syringe-driven apparatus used for the cigarette smoke extraction.

QUINONES PREPARATION

Ubiquinol and K vitamers are insoluble in water as lipophilic molecules. Moreover they have different molecular weight and different affinity to organic solvents. Therefore, in the efficacy experiments of these molecules, the bioavailability is a critical element and the use of different carriers can lead to different results. The choice of the optimal carrier for each molecule is therefore critical for the experimental setup.

From previous analysis it has been found that ubiquinol (QH) is more efficiently absorbed by cells when solubilized with a micellar emulsion technique. Therefore QH, kindly donated by Kaneka Corp (Japan), was solubilized in water using a mixture of glycerol and the emulsifying agent PEG 60-hydrogenated castor oil (Cremophor; BASF SE Chemical Co., Germany) (QH:glycerol:HCO60 = 0,4:0,6:1) keeping the mixture at 60°C and diluting with deionized water. A 10 mM stock solution was kept at −80°C until use. Oxidation under these conditions was minimal for several months.

While the K1 (Sigma), MK4 and MK7 (kindly donated by Gnosis s.p.a.; Milano), were solubilized in three different organic solvents: ethanol (Et-OH) and tetrahydrofuran (THF) at room temperature, and in Cremophor with the same method used to solubilized QH. A 10 mM stock solution was kept at −80°C until use.

CELL MODELS

Primary Human Umbilical Vein Endothelial Cells (HUVECs) were purchased from Clonetics (Lonza, Basel, Switzerland). The cells were grown in EGM-2 (Lonza) and maintained at 37°C in a humidified atmosphere of 5% CO_2. Young cells, from passages 5-9, were used for the treatments, while cells from passages 18-20 were used as senescent control. In previous studies it has been observed that endothelial primary cells are able to acquire the senescent phenotype when serially passed in culture.

Primary Human Dermal Fibroblasts (HDFs) were purchased from Experimental Zooprophylactic Institute, Brescia and were cultured in MEM containing 10% Fetal Bovine Serum (South America Origin), 1% L-glutamine, penicillin (100 U/mL) and streptomycin (100 µg). Cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2. Cells from passages 12-15 were used to test the reproducibility and the stability of 100% CSE, and to establish the best method to supplement the ubiquinol and the vitamin K.

For the experiments, cells were seeded in 24 well plates or in 6 well plates an optimal density of $6x10^3$ cells/cm^2 for HUVECs and $11x10^3$cells/cm^2 for HDFs.

DESIGN OF THE STUDY

This study was divided in two parts: 1) Evaluation of the effects of CSE on cell cultures, 2) Evaluation of the effects of ubiquinol, vitamin K and warfarin pretreatment before exposure to CSE.

In the first part was established the reproducibility and the stability of five 100% CSE batches immediately after preparation and after conservation for 6 months at 4°C in the dark. Confluent HDFs were treated with 12,5% CSE for 24h and the viability and the ROS cell content were evaluated. Then HUVECs were cultured with varied concentration of CSE (0; 3,125; 6,25; 12,5; 25; 50%) for 24h and was observed dose-dependent effects in relation to viability decrease and ROS production. Subsequently the CSE at sub-lethal concentration (12,5% CSE) was used to investigate its effect on young HUVECs in terms of oxidative stress, inflammation and senescence markers. Moreover, the results were compared to senescent control cells.

The second part of the study was focused on the role of ubiquinol, vitamin K and warfarin supplement to prevent the CSE damage.

First of all bioavailability of ubiquinol and K vitamers was verified. Therefore confluent HDFs were incubated for 24h with 10 µM K vitamers prepared with different carriers. Increased bioavailability using an emulsion was also investigated *in vivo* in humans administering milk supplemented with MK7 added as powder or as emulsion and quantifying the vitamin K plasma concentration. Once the best carrier was identified, it was verified whether the CSE influences bioavailability in the cellular *in vitro* system. In particular, bioavailability was evaluated in young HUVECs co-incubated with 10 µM of ubiquinol or vitamin K (solubilized with Cremophor in emulsion) in presence or in absence of 12,5% CSE for 24h.

Subsequently the effects of ubiquinol, vitamin K and warfarin pre-treatment on the CSE-treated cells were investigated. Young HUVECs were supplemented for 24h with ubiquinol/vitamin K, or for 48h with warfarin. A range of concentrations (1; 2,5; 10 µM ubiquinol or K vitamers; 10; 50; 100; 200 µM warfarin) was used only for viability, ROS and $O_2^{-\bullet}$ assessment to identify the most effective concentration. Then the medium was replaced with 12,5% CSE medium containing the same molecule for 24h (viability, ROS, $O_2^{-\bullet}$, mPTP and caspase-1 analysis) or 48h (SA-β-galactosidase and p16 analysis).

In vivo MK7 BIOAVAILABILITY

A small *in vivo* study was conducted to verify the importance of the vitamin K administration method. Seven caucasian subjects were recruited: 3 males and 4 females, with an average age of 31,4 ± 6,9 years, non-smokers and without pathologies of the digestive system.

In a fasting condition, they have taken 100 mL of milk with 1% fat, in which vitamin K is not detectable, supplemented with 10 µg/mL of menaquinone 7 in two formulations: as a powder or in emulsion. The emulsion was composed of arabic gum, olive oil and water in proportions: 1:4:2, to which MK7 was added in powder form and mixed vigorously.

To assess the bioavailability the plasma vitamin K was quantified and 4 blood samples were taken: fasting and 2, 4 and 8 hours after taking the supplemented milk. All subjects participated in the same day using one of the two formulations and the tests were conducted after an 8-day wash-out period using the second formulation.

In vitro BIOAVAILABILITY

Bioavailability of K vitamers was verified as a function of the carrier used (Et-OH, THF and micellar suspension with Cremophor). Therefore confluent HDFs cultured in 6 well plates were incubated with 10 µM of K vitamers prepared as indicated in "QUINONES PREPARATION" and diluted in MEM. Untreated cells were used as control. After 24 hours the cells were washed with PBS, detached and counted. After centrifugation, they were resuspended in 50 µL of PBS and stored at -80°C until quantitative analysis with HPLC.

Subsequently, it was verified whether the CSE can influence the bioavailability of these quinones. Therefore sub-confluent young HUVECs cultured in 6 well plates were incubated with 10 µM of ubiquinol and K vitamers prepared in Cremophor and diluted in EGM-2 or in EGM-2 containing 12,5% CSE. After 24 hours the cells were washed with PBS, detached and counted. After centrifugation, they were resuspended in 50 µL of PBS and stored at -80°C until quantitative analysis with HPLC. Incorporated quinones levels were compared with an untreated negative control.

HPLC ANALYSIS

Coenzyme Q_{10}

Coenzyme Q_{10} (CoQ_{10}) levels and its oxidative status were assayed in HUVECs grown on 6 well plates using a dedicated high-performance liquid chromatography (HPLC) system with electrochemical detector capable of detecting both reduced and oxidized forms (ECD oxidation potential, 650 mV; Shiseido, Tokyo, Japan) thanks to a post-separation reducing column (Shiseido) capable of fully

reducing eluted ubiquinone, as reported in [*Olivieri 2013*]. CoQ_{10} concentration was verified by using a single dilution step. Briefly, after cell harvesting the cell pellet was resuspended in 50 µL PBS and extracted with 250 µL propanol. After vigorous vortexing, the extraction mixture was centrifuged for 1 min at 13000g, 4°C and 40 µL of supernatant was injected into the HPLC system with concentrating column (C18 MGII 5 µm, 2,0x35 mm) and analytical column (C18, type AQ, 3 µm, 2,0x150 mm; Shiseido). Two mobile phases were used: mobile phase 1 for loading and concentrating the sample (50 mM sodium perchlorate in methanol/water 95/5 v/v) and mobile phase 2 for the analytical procedure (50 mM sodium perchlorate in methanol/isopropanol 90/10 v/v).

Intracellular total CoQ_{10} levels were expressed as nmol/10^6 cells, while the oxidative status of CoQ_{10} was reported as the percentage of oxidized CoQ_{10}/total CoQ_{10}.

Vitamin K

K vitamers levels were assayed in plasma and in HUVECs suspensions using an HPLC system (YL Instrument 9300) associated with fluorescence detector (Nanospace SI-2; Shiseido) and equipped with a post-chromatographic reducing column (CQ-R 2,0x20 mm; Shiseido). In fact, vitamin K is fluorimetrically detected in its reduced state.

K vitamers were extracted from plasma and cells diluting them 1:6 in Ethanol, and, after vortexing and centrifugation at 20900 g for 2 min, 50 µL of supernatant fraction were injected into the analytical column (2,6 µm C18 100A, 100x4,6 mm; Phenomenex Kinetex). The mobile phase used was ethanol:water (97:3, v/v) and the flow rate was adjusted to 0,7 mL/min. The optimized detection wavelengths were 335 nm (excitation) and 430 nm (emission). In these conditions the K vitamers peaks were at: 7,2 min (MK4), 8,5 min (K1) and 10,8 min (MK7). External standards were used to quantify K vitamers concentrations. K vitamers levels were expressed as ng/mL for the plasma samples, while as nmol/10^6 cells for the HUVECs samples.

FLOW CYTOMETRY ANALYSIS

Intracellular ROS levels, mitochondrial functionality, apoptosis, and caspase activity were assessed by flow cytometry using special probes and a Guava EasyCyte flow cytometer with GuavaSoft 2.7 and excitation source at 488 nm (Merck Millipore, Darmstadt, Germany). Emission fluorescence intensities were recorded in different channels on an average of 5000 cells from each sample. Each condition was analyzed in triplicate and each sample was repeated twice.

VIABILITY AND ROS CELLULAR CONTENT

Intracellular ROS levels were evaluated by carboxy-2,7-dichlorofluorescein diacetate (carboxy-H_2DCFDA) (Invitrogen). Cells were incubated with the dye (10 µM in PBS) in the dark for 30 min at 37°C, washed and detached. An aliquot of each sample was then added to a solution of Guava ViaCount (Merck Millipore), a fluorescent stain formulation that discriminates live, dead and apoptotic cells, on the base of differential permeabilities of two DNA-binding dyes. The nuclear dye stains only nucleated cells, while the viability dye brightly stains dying cells. Therefore, the analysis of ROS and viability were conducted simultaneously using the flow cytometer. Counterstaining with ViaCount was necessary in order to evaluate intracellular levels of ROS only in viable cells. In fact, exclusion of cells with compromised cell membrane integrity is essential in order to avoid false negatives due to loss of carboxy-H_2DCFDA from permeable cells. For data analysis, "high ROS" region was arbitrarily defined in the green channel, while live, apoptotic and dead cells gates are defined in the dot plot produced from the analysis of yellow and red fluorescence channels, using the fluorescence distribution of CSE treated HUVECs as a reference. The same gates were used for the analysis in all subsequent experiments.

MITOCHINDRIAL SUPEROXIDE ANION ASSAY

Mitochondrial superoxide anion production was evaluated flow-cytometrically by means of the reduced nerstian probe MitoSOX™ Red mitochondrial superoxide indicator (Invitrogen), a hydroethidine derivative that is up-taken by actively respiring mitochondria and generates 2-hydroxiethium after reacting with superoxide. Briefly, cells treated with molecules in presence of CSE or CSE alone as well as untreated control cells were incubated for 15 min in a solution 5 µM MitoSOX in PBS at 37°C. After trypsinization and washing cells were analyzed using Guava Easycite flow cytometer (Millipore). For quantitative analysis of distribution, gate defining region with "high" superoxide anion levels were arbitrarily set using untreated and CSE treated cells as a reference. The same values were used for the analysis in all subsequent experiments.

PERMEABILITY TRANSITION PORE OPENING ASSAY

Mitochondrial Permeability Transition Pore (mPTP) opening was evaluated using MitoProbe™ Transition Pore Assay Kit (Invitrogen) based on the manufacturer's protocol. The assay is based on the calcein quenching method as described by Petronilli et al. [*Petronilli 1999*]. Acetoxymethyl ester of calcein (CalceinAM) is a non-fluorescent dye that diffuses to all cellular compartments where intracellular esterases release the polar fluorescent dye calcein. Addition of cobalt chloride quenches cytosolic calcein, while mitochondrial calcein, which is not accessible to the quencher unless the mitochondrial membrane is permeabilized, remains fluorescent. Fluorescence intensity can therefore be used as an indicator of mPTP opening.

Adherent endothelial cells were washed in PBS without Calcium and incubated with 250 nM calcein AM and 400 µM cobalt chloride ($CoCl_2$) in PBS with 1,3 mM calcium for 15 min at 37°C in the dark. They were then detached, washed twice, and the green fluorescence was quantified by flow cytometry. For data analysis regions of fluorescence were set using as a reference control cells stained either with calcein alone (total cellular fluorescence); calcein and $CoCl_2$ (mitochondrial fluorescence proportional to mitochondrial pore opening); or calcein, $CoCl_2$ and calcium ionophore ionomycine (maximal quenching of mitochondrial fluorescence). The percentage of mitoprobe positive cells were therefore arbitrarily set in the untreated control population to 25%. These settings were then maintained for all subsequent experiments.

DETECTION OF ACTIVATED CASPASE-1

Activation of caspase-1 was assayed with FAM-FLICA probes (ImmunoChemistry Technologies, USA). FLICA is a cell permeant that binds covalently the active caspases and is revealed by a green fluorescent signal. Cells were stained following the manufacturer's instructions. Briefly, cells were detached, washed, and stained with 1x FLICA solution in the dark for 50 min at 37°C, counterstained with propidium iodide, and immediately analyzed. The fluorescence intensity from the two channels was analyzed together.

SENESCENCE-ASSOCIATED β-GALACTOSIDASE STAINING

Senescence-associated β-galactosidase activity (SA-β-gal), a common molecular marker of cellular aging *in vitro*, was determined using Senescence Detection Kit (ab65351, Abcam). This marker is a lysosomal enzyme active at pH acids also close to neutrality. Lysosomal activity increases throughout aging, but also with cellular confluence, therefore it is appropriate that the cells do not go to confluence during the *in vitro* growth process. Therefore non-confluent HUVECs cultured in 24-well plates were fixed for 15 min at room temperature, then washed twice in PBS. Cells were incubated overnight at 37°C with Staining Solution Mix (containing X-Gal). SA-β-Gal was assessed by light microscopy. The percentage of positive cells was determined by counting at least 500 cells/well.

RNA EXTRACTION and QUANTITATIVE REAL TIME PCR (qRT-PCR)

Total RNA was isolated from HUVECs, using the NucleoSpin RNA kit (Macherey-Nagel) according to the manufacturer's instructions. The RNA purity and concentration were measured on a Nanodrop Spectrophotometer. Approximately 400 ng of RNA from each sample were converted to cDNA using the iScript™ cDNA Synthesis Kit (Bio-Rad) according to the manufacturer's instructions.

qRT-PCR reactions were conducted on a MyiQ Single Color Real-Time PCR Detection System (Bio-Rad) in a 15 µL total reaction volume, using the iQ^{TM} SYBR Green Supermix (Bio-Rad). The primers sequences for the genes of interest [written 5'-3'] were:

- p16 Fw: CATAGATGCCGCGGAAGGT, Rv: CTAAGTTTCCCGAGGTTTCTCAGA;
- GAPDH Fw: TCCACTGGCGTCTTCACC, Rv: GGCAGAGATGATGACCCTTTT.

All primers were used at a concentration of 400 nM. Each reaction was run in duplicate and for each gene a no-template control was included. The qPCR was programmed to start with a 3 min of denaturation step at 95°C for polymerase activation, followed by 40 cycles of 15 sec denaturation at 95°C and 30 sec of annealing/extension at 60°C, during which fluorescence was measured. Next, a melting curve was constructed by increasing the temperature from 55 to 95°C in sequential steps of 0,5°C for 6 sec while continuously monitoring fluorescence. The mRNA expression of the genes of interest was calculated according to the delta-delta Ct method ($2^{-\Delta\Delta Ct}$) using GAPDH as reference gene for normalization.

DATA ANALYSIS

Each experiment was performed at least in three biological replicates conducted in different experimental sessions. Statistical significance between the groups was evaluated by Students' t-Test and P value less than 0,05 was considered statistically significant and represented as: "*" p≤0,05, "**" p<0,01 or "***" p<0,001. Data are represented as mean±SD.

RESULTS

CIGARETTE SMOKE TOXICITY AND AGING

CSE REPRODUCIBILITY, STABILITY AND OPTIMAL CONCENTRATION IDENTIFICATION

Cigarette smoke, one of the main risk factors for cardiovascular diseases, creates a strongly oxidizing environment which causes important changes at the cellular level and premature aging, especially on the endothelium.

In the present study, cigarette smoke extract (CSE) exposure of endothelial cells was optimized in order to develop a model of cellular stress and premature aging. 5 batches of CSE were produced by bubbling 3000 mL of tobacco smoke from 10 cigarettes in 50 mL complete medium that was referred as 100%, as described. Effect on cells in terms of viability and oxidative status was tested immediately and after 6 months of storage of the 100% CSE-medium at 4°C in the dark. In particular, at each time HDFs were treated with freshly eight-times diluted CSE (12,5%) for 24h and subsequently viability and ROS cellular content were evaluated.

As shown in Figure 13 no differences were observed between different CSE batches and following different storage time in relation to cellular viability, apoptosis induction and cytosolic ROS content. Treatment with each CSE batch resulted significantly different from the untreated control for all parameters evaluated. In particular, the exposure to CSE always produced a significant decrease in live cells, which however is not reflected in a significant increase in the mortality rate, but rather in a significant increase in the amount of apoptotic cells. Also the cytosolic ROS content increased after the CSE treatment.

The cigarette smoke extract prepared according to the described method, produced reproducible results suggesting that the 100% CSE-medium solution is stable at least up to six months of storage at 4°C in the dark.

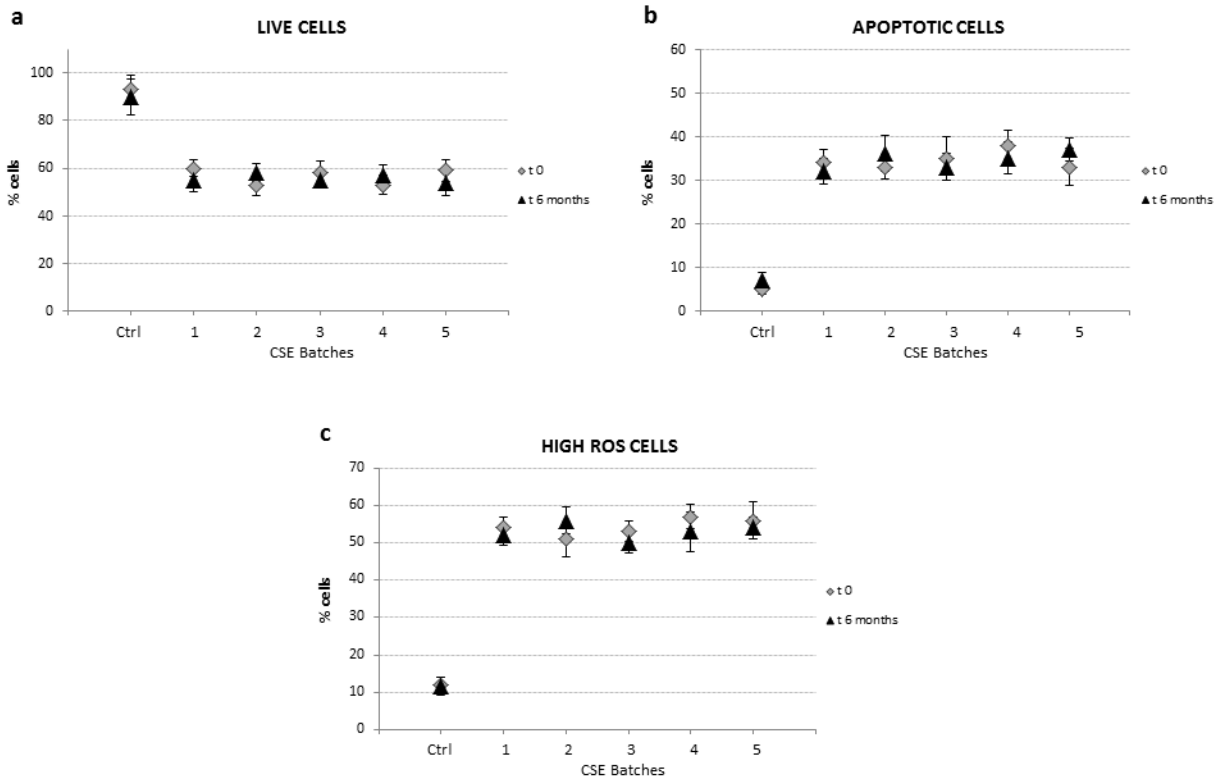

Figure 13: Variability between 5 different batches of CSE freshly prepared (t0) and after 6 months (t6 months) of storage at 4°C, evaluating their effects in live (a), apoptotic (b) and high ROS content cells (c) after HDFs exposure to 12,5% CSE for 24h compared with untreated HDFs (Ctrl).

After cigarette smoke extraction optimization, young HUVECs were exposed to a range of CSE-medium concentrations (0; 3,125; 6,25; 12,5; 25; 50%) for 24h. In Figure 14 it is possible to observe a significant viability decrease already in cells incubated with the lowest CSE-medium concentration. However mortality increased only after exposure to the two highest concentrations of CSE-medium. This effect could be associated with the observed increased opening of mitochondrial Permeability Transition Pore (mPTP) compatible with the early apoptotic phases. In addition at all the tested CSE-medium concentrations, a gradual and very significant increase in ROS production was observed. These data suggest that cigarette smoke was able to induce a significant oxidative damage in endothelial cells associated with a significant decrease in viability.

From the previous data, in order to identify an optimal compromise between lack of mortality and significant increase in oxidative stress, the intermediate concentration of CSE-medium tested (12,5%), was chosen for subsequent experiments aimed at evaluating both aging-inducing activity of CSE and quinones protective effects.

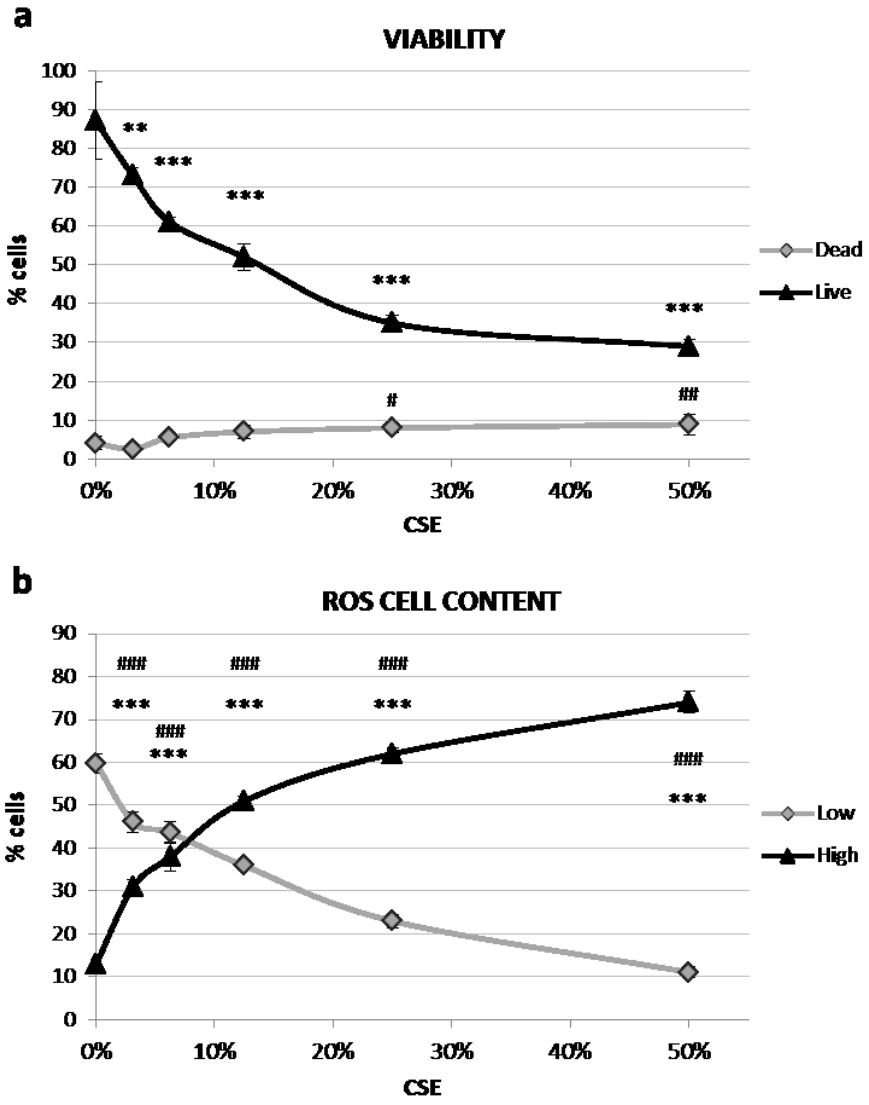

Figure 14: Effects of the 24h treatment of young HUVECs with CSE. Viability (a) and ROS cellular content (b) were evaluated. Significant of variation was calculated vs. untreated cells (0% CSE) and are represented as "*" for Live and Low ROS, while "#" for Dead and High ROS.

CSE INDUCES PREMATURE SENESCENCE

Numerous *in vivo* and *in vitro* studies support the association between cigarette smoke exposure and early cellular aging, in particular in the vasculature being a major cause of atherosclerotic lesion development. In the present study it was verified whether a sub-lethal CSE-medium exposure was able to induce the cellular phenotypic change comparable to the senescent one. Accordingly, markers of oxidative imbalance, mitochondrial dysfunction and senescence markers were quantified in young endothelial cells either 12,5% CSE-medium treated for 24 or 48h or untreated controls as well as replicative senescent HUVECs, that were used as positive controls.

Treatment with CSE in this experimental model significantly increased the percentage of cells showing a high cytosolic ROS content cells from 12±2% in unexposed control to 54±3% in CSE exposed young cells ($p<0,001$). Also the cellular content of superoxide anion ($O_2^{-\bullet}$), a highly reactive oxygen radical deriving mainly from the mitochondrial metabolism, increases very significantly following CSE treatment (Young: 8±1%; CSE: 53±2%; $p<0,001$), even higher to what was observed in senescent HUVECs (Senesc: 46±3%; $p_{CSE\text{-}senesc}<0,05$).This could suggest that the CSE has an important mitochondrial action. In parallel with the increase of $O_2^{-\bullet}$, a significant opening of mPTP was observed (Young: 26±3%; CSE: 39±3%; $p<0,05$), suggesting that incubation in presence of CSE-medium could trigger mitochondrial dysfunction. Moreover, an increased activation of caspase-1 (Young: 29±2%; CSE: 37±3%; $p<0,01$), a relevant marker of inflammation, was also observed.

In summary, as reported in Figures 15 and 16, CSE-medium exposure of young cells was able to increase both oxidative stress and inflammation markers, although these indices, with the exception of superoxide anion, are significantly lower than that observed in senescent cells.

The same behavior was observed analyzing specific senescence markers: the activity of SA-β-galactosidase (Young: 16±4%; CSE: 39±5%; $p<0,001$)and the expression of p16 (Young: 1 fold; CSE: 4,1±1,1 fold; $p<0,01$) (Fig. 17).

These data suggest that the exposure of young endothelial cells to 12,5% CSE is able to induce biochemical changes in the same direction as those that occur in replicative senescence.

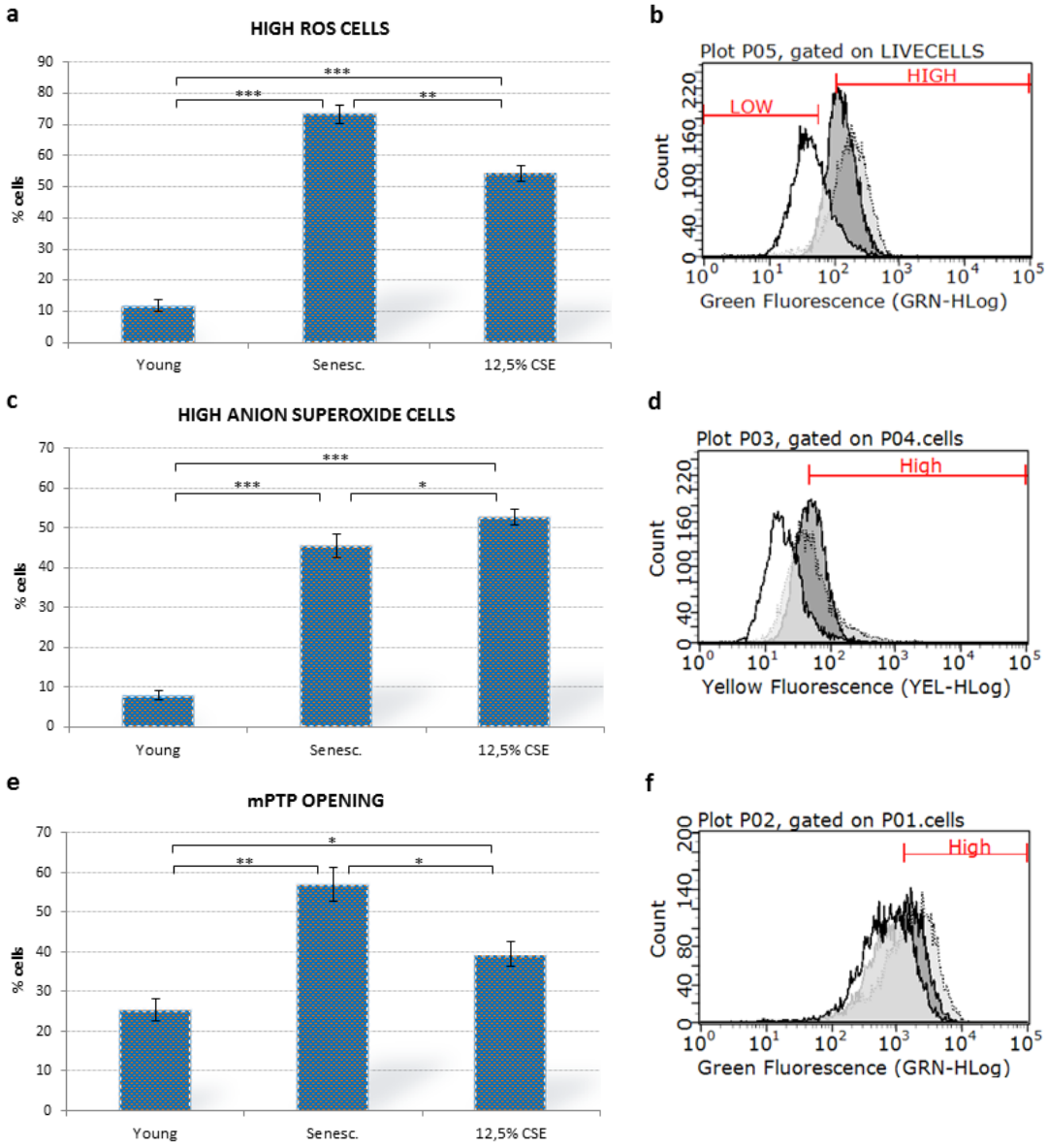

Figure 15: High ROS (a) and High superoxide anion (c) cells and mPTP opening (e) in 12,5% 24h CSE-treated young HUVECs; untreated young and senescent HUVECs. On the right panels are represented their respective fluorescence distribution (b, d, f) where the white curve represents young HUVECs, the dark grey CSE treated cells, the light grey senescent HUVECs."*" p<0,05; "**" p<0,01; "***" p<0,001.

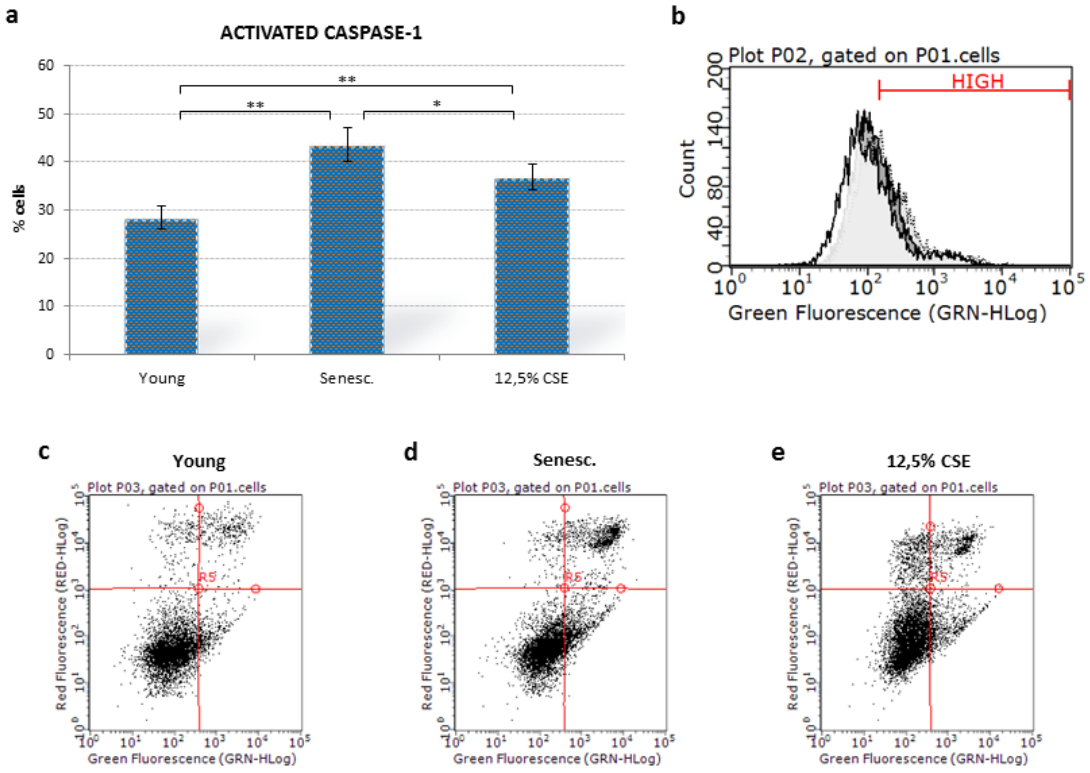

Figure 16: Modulation of caspase-1 activation by 12,5% CSE treatment (for 24h) of young HUVECs compared to untreated young and senescent HUVECs. (a) Percentage of cells with activated caspase-1; (b) representative distribution of green fluorescence proportional to activated caspase-1: white curve young HUVECs, dark grey CSE treated cells, light grey senescent HUVECs. (c=young; d=senescent; e=12,5% CSE) Dot-plot representing the green (caspase-1) and red (viability) fluorescent distribution: LL: unstained live cells; LR: live cells with activated caspase-1; UR: apoptotic or death cells with activated caspase-1; UL: apoptotic or death cells without activated caspase-1. "*" p<0,05; "**" p<0,01.

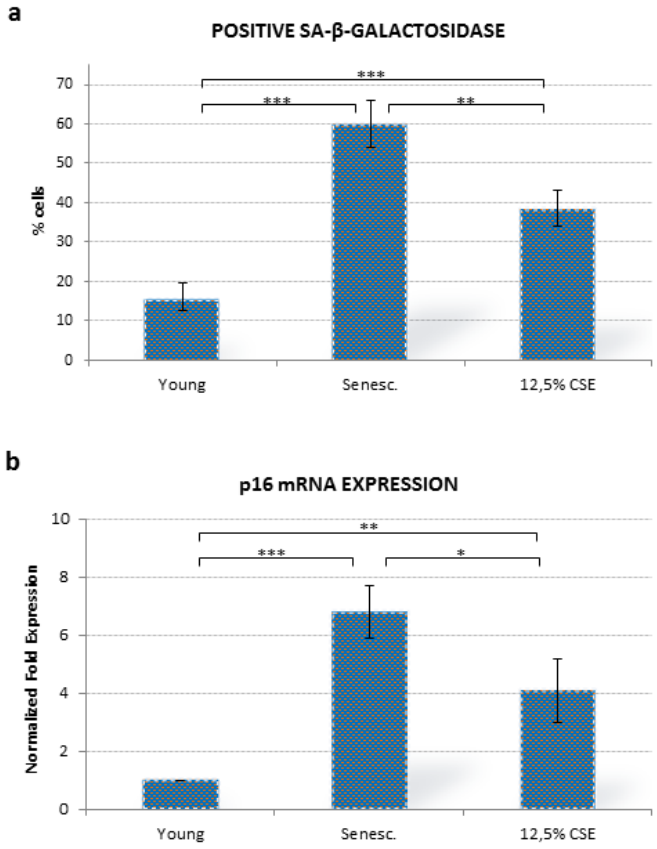

Figure 17: Effects of 12,5% CSE exposure (for 48h) of young HUVECs on the senescence markers: SA-β-galactosidase activity (a) and p16 mRNA (b), compared to untreated young and senescent HUVECs. "*" $p<0,05$; "**" $p<0,01$; "***" $p<0,001$.

QUINONES EFFECTS ON CSE STRESSED CELLS

EFFECTS OF UBIQUINOLAND VITAMIN K

Coenzyme Q_{10} and vitamin K are two classes of quinones which play an important role in cardiovascular health. The first acts as an antioxidant and as a bioenergetic cofactor, while the latter activates dependent vitamin K proteins (VKDP) involved both in coagulation processes and in maintaining the elasticity of blood vessels, however these processes are not well known. Since cigarette smoking is one of the main risk factors of atherosclerosis, it is interesting to investigate the role of both these quinones in a cellular model of endothelial cells exposed to CSE.

To assess the effect of vitamin K and ubiquinol supplementation in preventing damage associated to cigarette smoke exposure, the bioavailability of these lipophilic molecules was first verified by testing different carriers both *in vivo* and *in vitro*. Identification of optimal carriers or solvent is essential in light of the scarce solubility of the compounds in aqueous medium. Furthermore, the potential interference of the CSE on CoQ_{10} and vitamin K bioavailability was tested. Subsequently, to tests their biological effects, ubiquinol and K vitamers were supplemented to young HUVECs for 24h, and subsequently the same cells were co-incubated with quinones in complete medium containing 12,5% CSE for another 24 or 48 hours.

BIOAVAILABILITY

Ubiquinol and K vitamers are lipophilic molecules with a similar chemical structure, with different lipophilicity mainly influenced by the nature and the length of isoprenoid side chain. Slightly different chemical structures are associated with different physical-chemical characteristics, including different solubility. Several organic carriers are used in the literature in studies aimed at verifying their biological effects. These differences could be one of the cause of some discrepancies in the results of these studies. In fact the bioavailability, tested both *in vivo* and *in vitro*, is very variable depending on the carrier.

To test this hypothesis an *in vivo* pharmacokinetic analysis of MK7 was evaluated in seven healthy subjects by measuring plasma levels for 8 hours, after a single bolus administration of 1 mg MK7 in 100 mL of milk. In particular, in two separate days MK7 was tested either as a direct solubilization of the powder or added as oil/water emulsion to the milk. Comparable vitamin K plasma levels were recorded at basal time both among the subjects and between the experimental days. As shown in

Figure 18, MK7 added as a powder caused an increase in the vitamin K plasma concentration between 2 and 4 hours much lower than the emulsion. Moreover MK7 plasma levels from emulsion administration remained constantly higher overall the observation time (8h) compared with the powder. In fact, although after 4 hours on average plasma levels of the seven subjects resulted comparable, significantly higher levels were detected both at 2h and at the end of the observation (8h). This suggests that the menaquinone-7-emulsion is associated with improved bioavailability and faster absorption (peak at 2h) compared to MK7 provided as powder solution which has the absorption peak at 4 hours. In particular MK7 plasma concentration after 2h increased more than 2000% with the emulsion, while only 600% with powder compared to baseline (Fig. 18b). The result was reinforced by the analysis of the area under the curve (AUC) (Fig. 18c), a relevant summarizing parameter in pharmacokinetic studies.

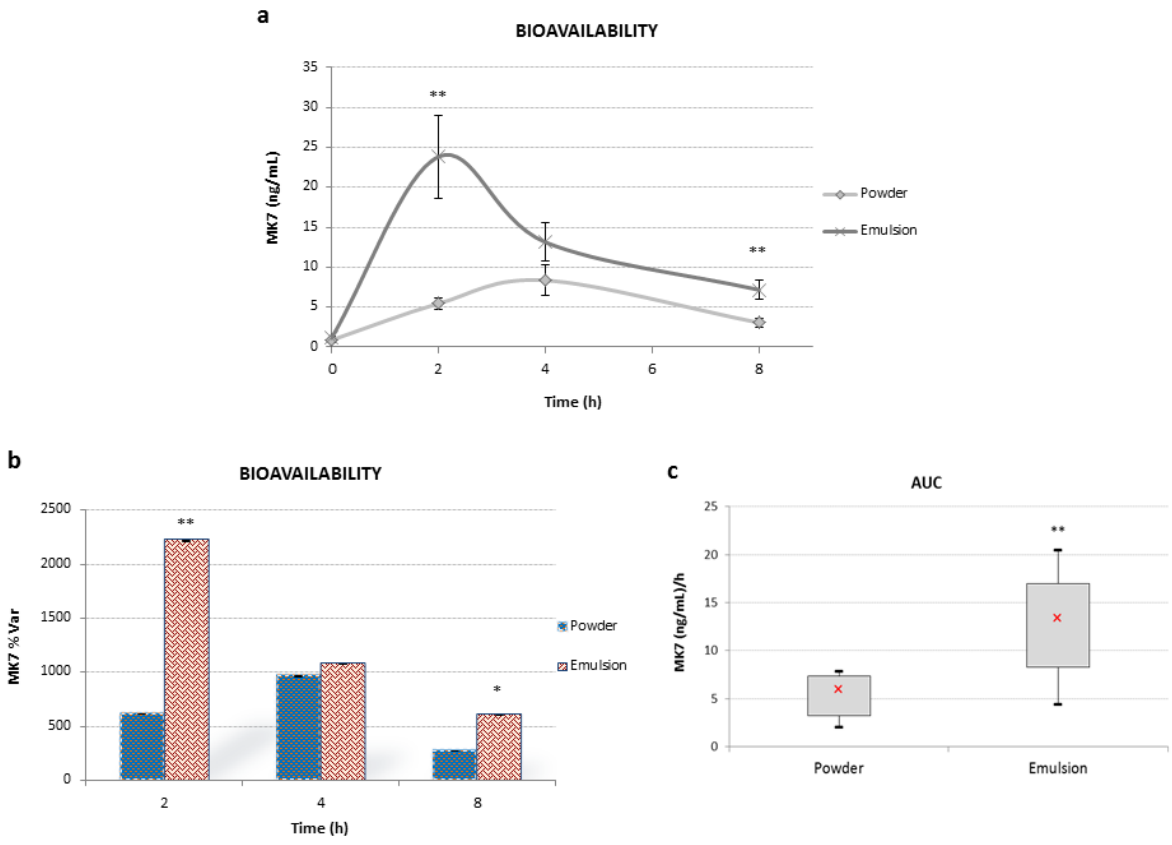

Figure 18: MK7 *in vivo* bioavailability. (a) Plasma MK7 levels before (time 0) and after 2, 4 and 8 hours the administration of 1 mg MK7 as powder or emulsion both dissolved in 100 mL of milk, (b) percentage of variation at 2, 4 and 8 hours compared to time 0. (c) Summary of pharmacokinetic data reported as distribution of area under curve data. n= 7; significance calculated vs. powder administration at each time point. "*" p<0,05; "**" p<0,01.

Similar results were obtained from *in vitro* experiments, when K vitamers bioavailability was tested in human dermal fibroblasts (HDFs) supplemented for 24h with K vitamers either solubilized in different organic solvents or as micellar preparation using hydrogenated castor oil particles (Cremophor).

From the Figure 19 the difference between the various carriers used is clearly visible. That was, the bioavailability of menaquinone-7 (MK7) and phylloquinone (K1) with Et-OH and THF was significantly lower than when they were solubilized with the Cremophor in a micellar suspension. The result was similar for menachinone-4 (MK4) when it was solubilized in Et-OH, while its bioavailability with THF was comparable to MK4 in Cremophor. Summarizing, Cremophor was the optimal carrier associated with enhanced cellular bioavailability of K vitamers. Moreover, by comparing the bioavailability of the K vitamers solubilized in Cremophor, it was observed that MK7 was absorbed by HDFs with greater efficiency than the other K vitamers, since its cellular content was 4 and 2 times greater than MK4 and K1 respectively. On the contrary MK4 was the K vitamer showing the lowest bioavailability. In the non-supplemented cells, the content of K vitamers was no-detectable.

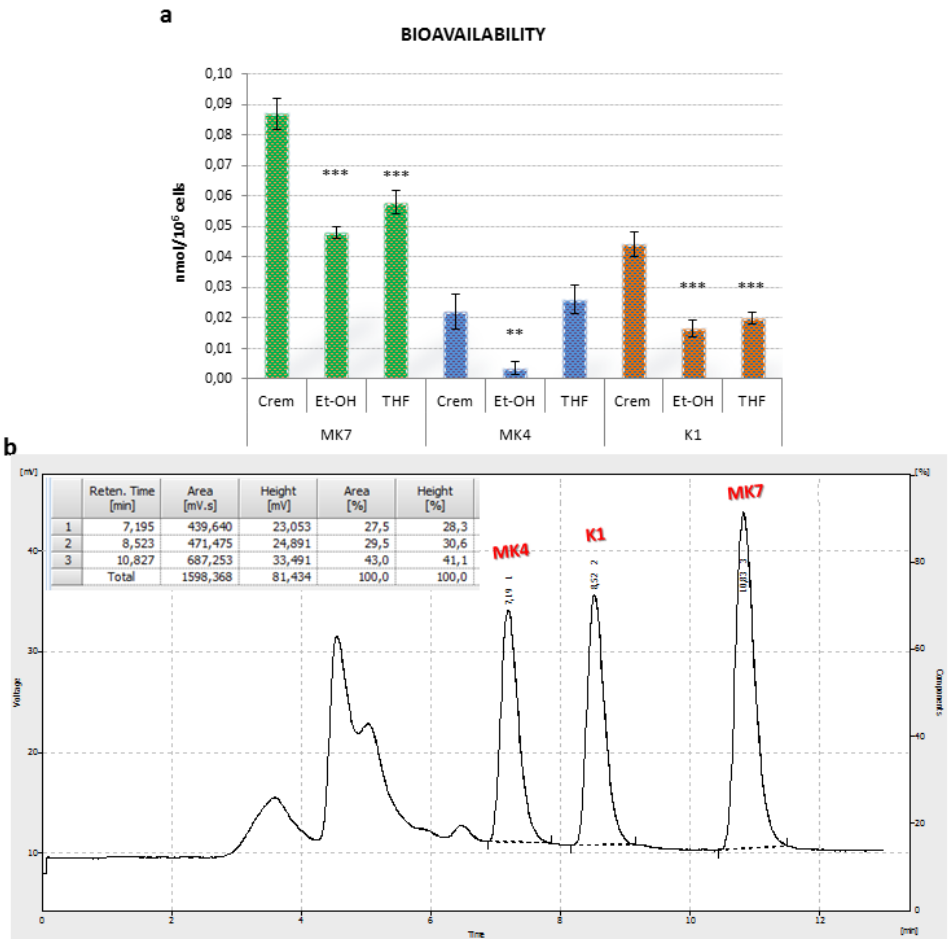

Figure 19: K vitamers cellular uptake using different carriers. Cellular K vitamers content after HDFs exposure for 24h to 10 µM K vitamers solubilized in ethanol (Et-OH), tetrahydrofuran (THF) or in a micellar preparation using Cremophor (Crem).K vitamers are not detectable in untreated cells. (b) An example of K vitamers chromatogram obtained by HPLC system. Significance calculated for each K vitamer vs. data obtained using Cremophor. "**" p<0,01; "***" p<0,001.

Subsequently, it was verified whether CSE could interfere with the absorption and oxidative status of the tested quinones. Therefore, young HUVECs were incubated for 24h with 10 µM of Cremophor-solubilzed molecules in the standard culture medium or in the medium containing 12,5% CSE. Untreated HUVECs were used as a control.

Incubation of HUVECs in presence of 10 µM ubiquinol (QH), the antioxidant active form of CoQ_{10}, resulted in the total CoQ_{10} cellular content increase of about 235 times and a significant decrease in the percentage of oxidized CoQ_{10} from 56±3% to 14±2% (Fig. 20a, b). CSE exposure did not affect either the cellular content or the oxidative status of CoQ_{10} in control cells and QH supplemented cells.

Similarly, CSE did not interfere with the bioavailability of K vitamers (Fig. 20c), which were more efficiently absorbed by HUVECs than HDFs, as the K vitamers cellular content after supplementation in HUVECs appears to be 21 times higher than in HDFs.

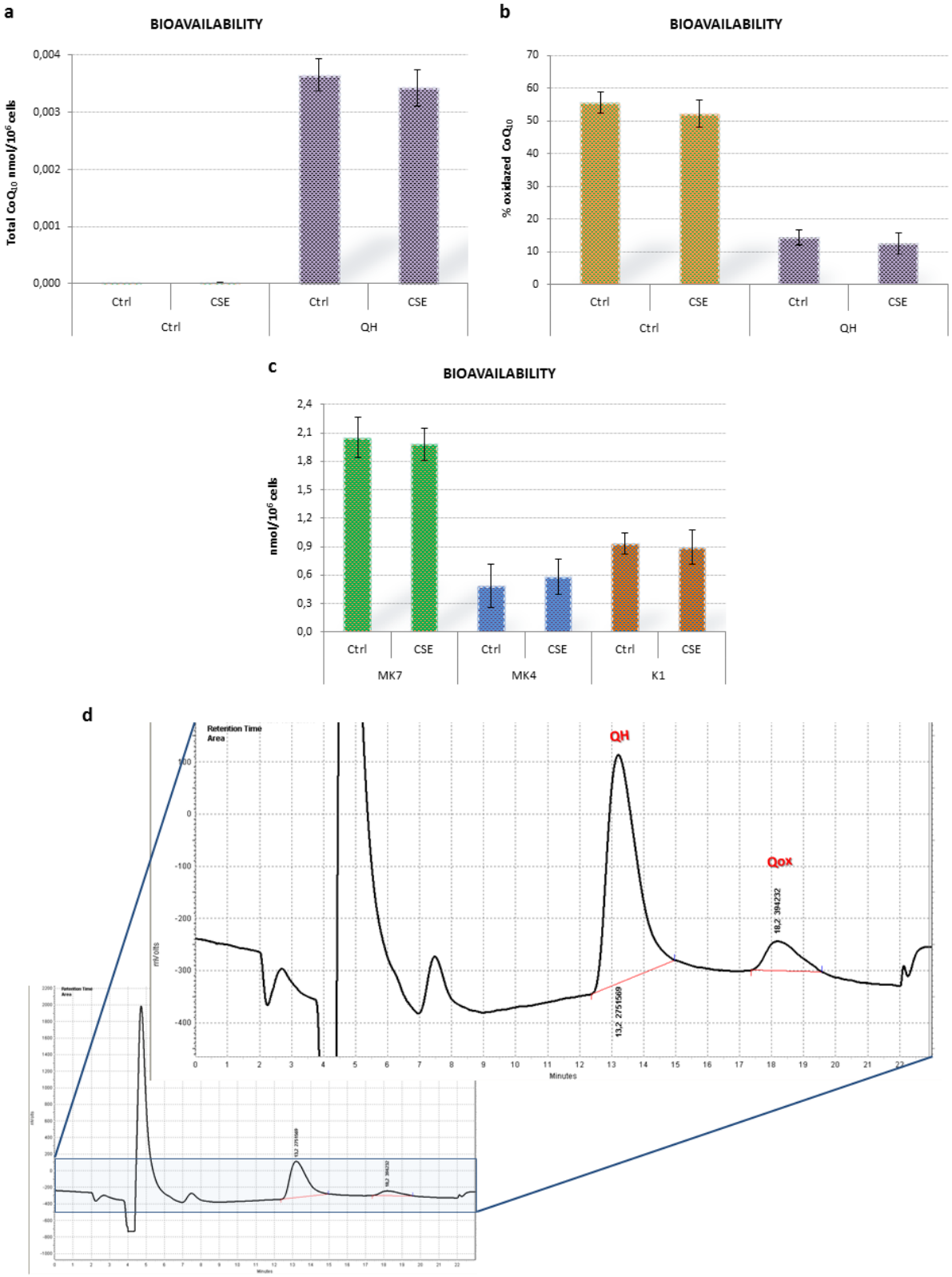

Figure 20: CSE doesn't affect quinones bioavailability. QH (a) and K vitamers (c) cellular content and the CoQ_{10} oxidative status (b) were evaluated in young HUVECs treated with 10 µM of quinones alone or in association with 12,5% CSE medium for 24h. Untreated and CSE exposed cells were used as controls. K vitamers were not detectable in non-supplemented cells. In the panel "d" was reported an example of CoQ_{10} chromatogram obtained by HPLC system.

VIABILITY

Ubiquinol and K vitamers supplementation efficiently counteracted the loss of viability associated to cigarette smoke extract exposure. In particular, a significant increase in the percentage of live cells simultaneously to a significant decrease in the percentage of apoptotic cells was observed for all tested molecules at the highest concentration (10 µM) (Fig. 21a, c). However, a significant effect of MK7 in enhancing viability and lowering apoptotic rate was observed already at 1 µM concentration, the lowest tested.

The detailed analysis of the results obtained with 10 µM (Fig. 21b, d) emphasized that the protective effect of QH and MK7 was the most effective since CSE treated samples in presence of these molecules was not statistically different from the negative control. In other words, both compounds have shown a complete protection from CSE-induced loss in viability. Moreover, only in MK7 treated cells, CSE induction of apoptosis was completely counteracted. Although, also MK4 and K1 were found to have a good protective effect, even if to a minor extent.

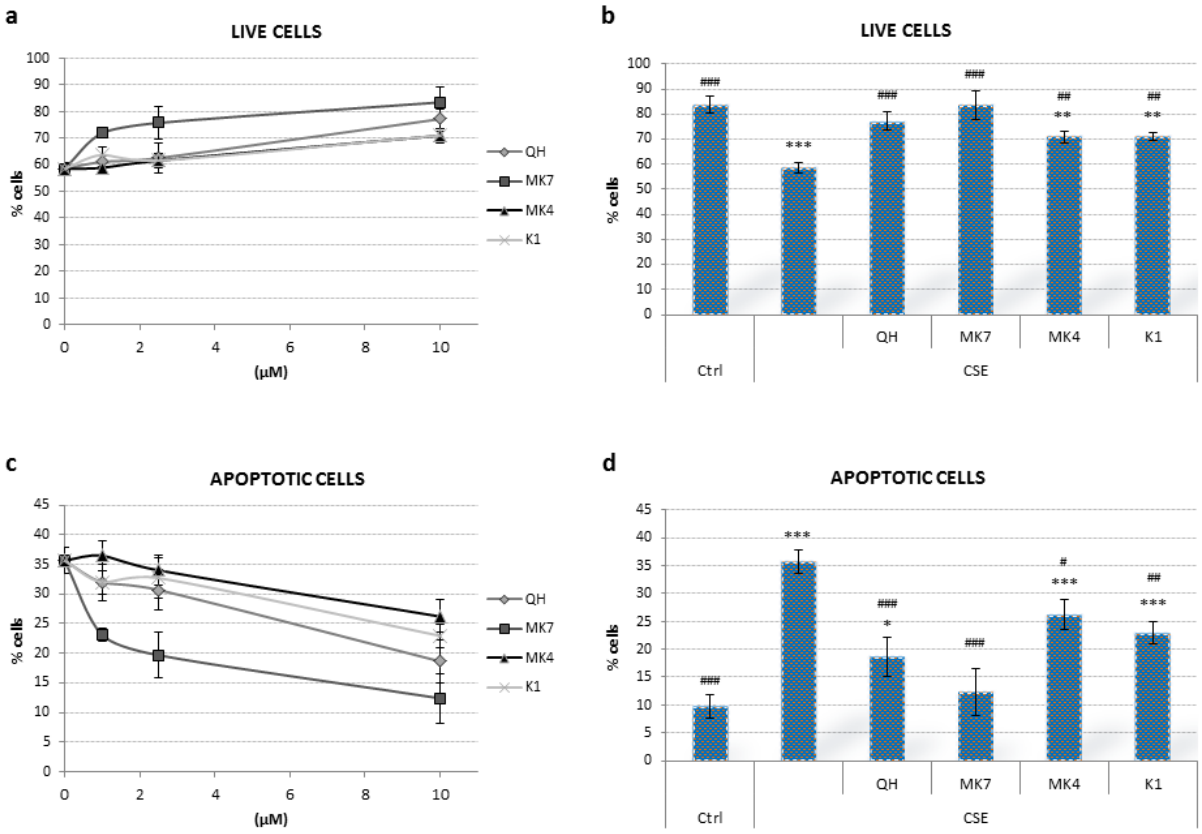

Figure 21: Effects of quinones supplementation on CSE-induced loss viability. Young HUVECs were supplemented with a range of quinones concentration. After 24h the medium was replaced with 12,5%CSE medium containing the same molecule for 24h and live (a) and apoptotic (c) cells were evaluated. In the panels "b" and "d" were reported live and apoptotic cells respectively in presence of 10 µM quinones. Significance was calculated vs. untreated cells (Ctrl) ("*" p vs. Ctrl) or treated only with 12,5% CSE ("#" p vs. CSE). "*" p<0,05; "**" p<0,01; "***" p<0,001.

ROS AND SUPEROXIDE ANION PRODUCTION AND mPTP OPENING

In order to verify whether the antioxidant and anti-inflammatory activity of quinones could underlie the protective effects on viability, oxidative status and mitochondrial functionality was investigated in the proposed model of CSE-exposed HUVECs.

Figure 22a, b shows that as the concentration of the K vitamers increases, the percentage of cells characterized by a high cytosolic ROS content was decreased with respect to the CSE treated control. In particular, the extent of the decrease was significant with all quinones at the highest tested concentrations (10 µM). However, different levels of efficacy among the molecules was evident, since ubiquinol and menaquinone-7 produced a significant reduction of ROS already at the lowest concentration (1 µM). On the contrary, to achieve a significant protective effects, MK4 and K1 had to be used at a 10 times higher concentration. Overall, any quinones at none of the tested concentrations was able to completely counteract CSE-induced oxidative stress since in all quinone-treated samples the percentage of cell with an high ROS content was significantly higher than the negative control.

MK7 was found to be the most effective molecule also in preventing the increase of the mitochondria-derived superoxide anion content ($O_2^{-\bullet}$) caused by exposure to CSE (CSE: 54±5%; MK7+CSE: 29±3%; $p<0,01$), already at the lowest concentration (1 µM) in analogy with what was observed for total ROS content. On the contrary, QH and K1 again were effective only at the highest concentration (10 µM) and to a lower level of significance (CSE: 54±5%;QH+CSE: 41±3%; K1+CSE: 46±3%; $p<0,05$) (Fig. 22c, d). Interestingly, differently from the other quinines tested, MK4 significantly promoted the formation of superoxide anion, i.e. it amplified oxidative-stress induced by cigarette smoke, despite the fact that in relation to other, not strictly mitochondrial parameters, a generally protective effect of this compound was observed.

Furthermore, while QH, MK7 and K1 showed a significant protective effect also in relation to mitochondria generation of ROS; surprisingly those were not associated to a decrease of the mitochondrial membrane pore opening; while, as happened with superoxide anion, MK4 promoted the opening of mPTP, i.e. it amplifies the damage induced by CSE (CSE: 38±3%; MK4+CSE: 53±3%; $p<0,01$) (Fig. 22e).

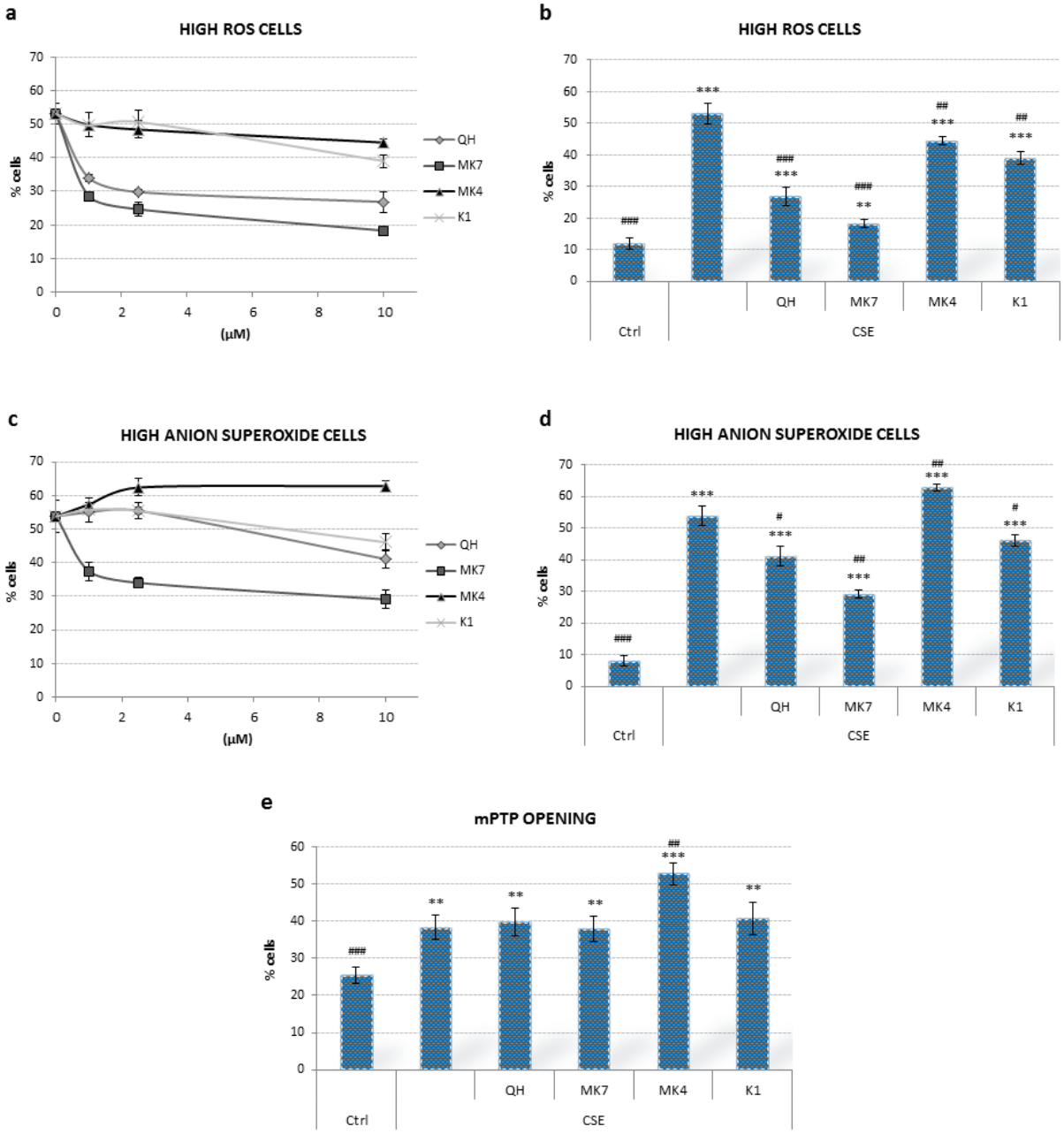

Figure 22: Effects of quinones supplementation on CSE-induced oxidative stress and mitochondrial dysfunction. Young HUVECs were supplemented with quinones and after 24h the medium was replaced with 12,5% CSE medium containing the same molecule for 24h. A range of quinones concentration was used to measuring high ROS (a) and high superoxide anion (c) cell content, while only 10 µM was used to evaluate mPTP opening. In the panels "b" and "d" were reported high ROS and high superoxide anion cells respectively in presence of 10 µM quinones. Significance of variation was calculated vs. untreated cells (Ctrl) ("*" p vs. Ctrl) or treated only with 12,5% CSE ("#" p vs. CSE). "*" p<0,05; "**" p<0,01; "***" p<0,001.

CASPASE-1

CSE pro-inflammatory effect on young endothelial cells, likely associated to the observed pro-oxidant effect observed, was characterized by an increased activity of caspase-1 (casp-1). Since QH and MK7 were the most effective quinones in the prevention of CSE-induced oxidative stress, their activity was also tested toward the inhibition of casp-1 activation. In fact, as shown in Figure 23, supplementation with 10 µM QH or MK7 was able to completely counteract the pro-inflammatory effect of CSE on the activation of caspase-1. In that the percentage of quinone+CSE cells showing presence of activated caspase-1 were significantly lower in comparison to CSE-challenged cells (CSE: 37±1%; QH+CSE: 30±2%; MK7+CSE: 32±2%; $p<0,05$). Moreover, the percentage of activated casp-1 cells resulted comparable both in quinone+CSE treated cells and in negative control cells (Ctrl: 29±3%).

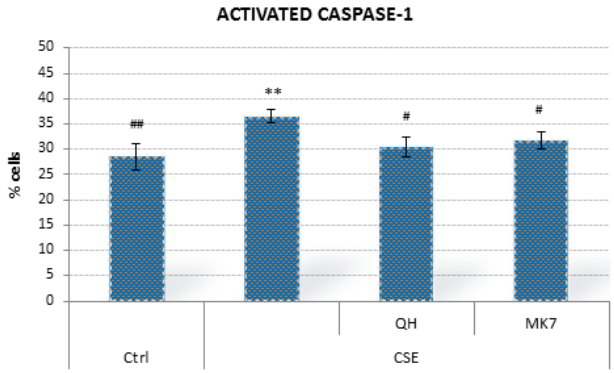

Figure 23: Effects of quinones supplementation on caspase-1 activation induced by CSE. Active casp-1 was evaluated in young HUVECs after supplementation with 10 µM QH or MK7 for 24h and other 24h of incubation with 12,5% CSE medium containing the same molecule. Significance of variation was calculated vs. untreated cells (Ctrl) ("*" p vs. Ctrl) or treated only with 12,5% CSE ("#" p vs. CSE). "*" $p<0,05$; "**" $p<0,01$.

SENESCENCE MARKERS

Oxidative stress, mitochondrial dysfunction and chronic inflammation are characteristic features of the senescent phenotype. Accordingly CSE exposure, in the proposed experimental setting, was able to induce specific markers of cellular aging such as SA-β-galactosidase (SA-β-gal) enzyme and p16 expression. Also in this case tested quinones showed a protective effect toward CSE-induced stress and senescence phenotype. In fact, CSE-induced increase in the percentage of SA-β-gal positive cells (CSE: 38±5%) was found to be effectively counteracted by supplementation with 10 µM QH (QH+CSE: 25±3%; p<0,05) and MK7 (MK7+CSE: 26±4%; p<0,05) (Fig. 24a).These values were slightly higher than the negative control not exposed to CSE (Ctrl: 16±4%) although not in a significant manner. On the contrary, tested quinones were not able to significantly decrease the expression of p16 mRNA levels, another accredited marker of cellular senescence, although in the co-incubation of quinones and CSE a tendency toward protection was observed, although the extent of this decrease in expression was not significant (Fig. 24b).

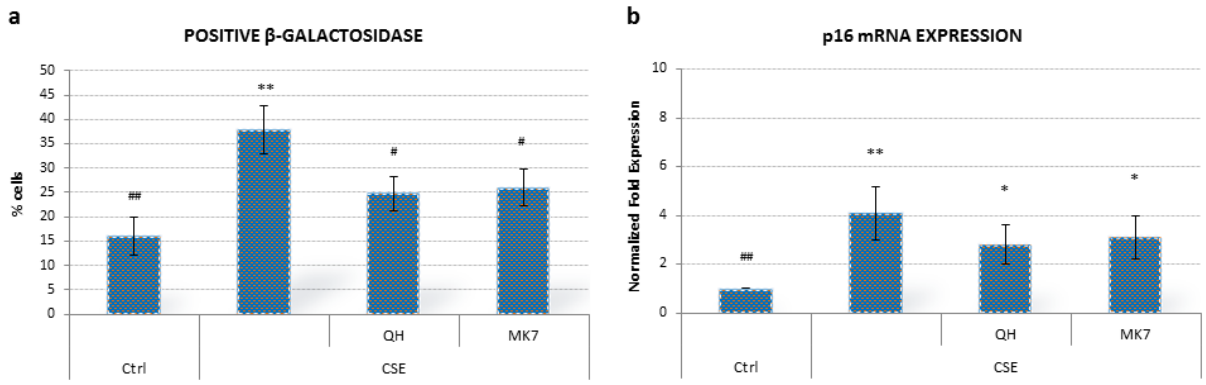

Figure 24: Effects of quinones supplementation on senescence markers induced by CSE. SA-β-galactosidase activity (a) and p16 mRNA (b) were evaluated in young HUVECs after supplementation with 10 µM QH or MK7 for 24h and other 48h of incubation with 12,5% CSE medium containing the same molecule. Significance of variation was calculated vs. untreated cells (Ctrl) ("*" p vs. Ctrl) or treated only with 12,5% CSE ("#" p vs. CSE). "*" p<0,05; "**" p<0,01.

EFFECTS OF VITAMIN K ANTAGONIST

In order to get a better insight on the molecular mechanism underlying K vitamers effect it was decided to use a selective antagonist of vitamin K epoxide reductase (Warfarin). In fact, since quinone reduction is an essential step in vitamin K activation and turnover in the so called vitamin K cycle, inhibition of this process leads to a substantial inactivation of all vitamin K dependent proteins.

This broad approach was deemed necessary since, differently from ubiquinol, whose activity as an antioxidant and bioenergetic cofactor is well described, K vitamers are mainly known for their function as cofactor of carboxylases in activating a set of 17 vitamin K dependent proteins. These vitamins are mainly known for their calcium binding ability and cover essential functions in the coagulative process by activating calcium dependent enzymes involved in the coagulative cascade.

More recently their role has been associated to the activation of calcium binding proteins important for cardiovascular and bone health, two aspects strictly connected. The optimal activation of calcium dependent proteins in both districts enables accumulation in bones to prevent osteoporosis and fractures and inhibit calcium deposition in arteries, or other ectopic calcification that could promote tissue dysfunctionality and damage. Although these process are both linked with aging, their association with the proposed CSE model of stress could be mediated by different proteins, hence the decision to test the hypothesis that a decreased activity of such proteins could lead to enhanced susceptibility toward tobacco smoke compounds.

In order to identify the optimal concentration of Warfarin, a range of concentrations was initially tested, based on the data reported in the literature. In particular young HUVECs were pre-incubated with Warfarin for 48h followed by another 24 or 48h of co-incubation Warfarin and 12,5% CSE.

VIABILITY

Unexpectedly, Warfarin contrasted the loss of cell viability caused by CSE in a dose-dependent manner. In particular, Warfarin+CSE exposure produced a significant increase in the percentage of live cells and a simultaneously significant decrease in the percentage of apoptotic cells. The extent of such variations was significant only at the highest concentrations of Warfarin tested (100 and 200 µM) (Fig. 25a). However, also at these concentrations Warfarin pretreatment was unable to counteract completely the effect of CSE but rather a significant decrease in live cells compared to unexposed controls was observed.

Warfarin 100 µM was the lowest concentration significantly improving cell viability; increasing Warfarin dosage a further increase was observed in live cells due to a significant decrease in apoptotic cells, while the percentage of dead cells remained unchanged through all the concentration tested. In particular, the detailed analysis of the viability in presence of Warfarin 100 µM showed that both the amount of live and apoptotic cells were improved significantly compared to the CSE treated, however they were not comparable to untreated young HUVECs (negative control) (Fig. 25b).

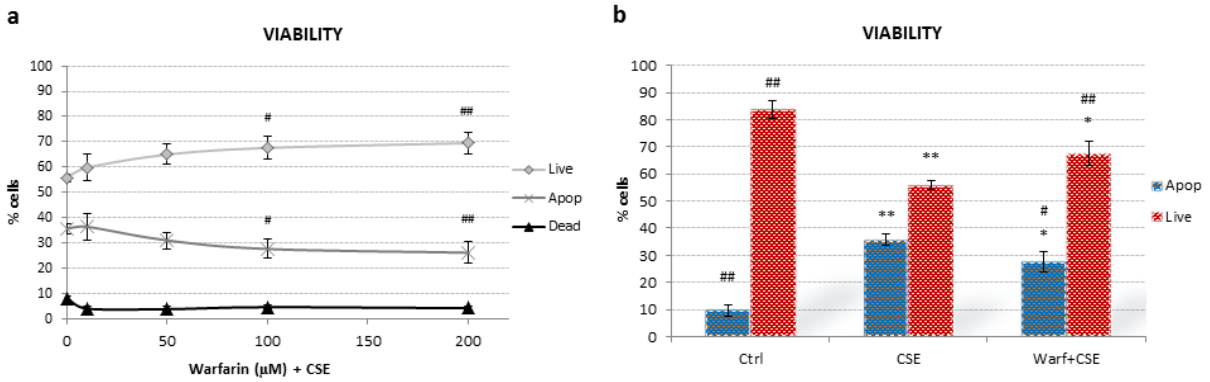

Figure 25: Effects of Warfarin supplementation on CSE-induced loss of viability. Young HUVECs were supplemented with a range of Warfarin concentration. After 48h the medium was replaced with 12,5% CSE medium containing the same molecule for 24h and the viability were evaluated (a). In the panels "b" was reported the viability in presence of 100 µM Warfarin. Significance of variation was calculated vs. untreated cells (Ctrl) ("*" p vs. Ctrl) or treated only with 12,5% CSE ("#" p vs. CSE). "*" p<0,05; "**" p<0,01.

ROS AND SUPEROXIDE ANION PRODUCTION AND mPTP OPENING

Experiments presented in relation to the effect of CSE on endothelial cells have shown that treatment with tobacco extract was able to induce a remarkable cellular stress characterized by an increased production of cytosolic ROS and superoxide anion and a consequent alteration of the mitochondrial permeability transition pore opening (mPTP) indicating mitochondrial dysfunction. Since K vitamers have shown a protective effect on this model, we aimed at further investigating molecular mechanisms involved by inhibiting vitamin K dependent proteins.

Also in this occasion, rather than producing an increased susceptibility, Warfarin, at all the tested concentrations, showed a significant antioxidant effect by significantly counteracting the increase in cytosolic ROS and mitochondrial $O_2^{-\bullet}$ accumulated in cells starting from the concentration of 50 µM (Fig. 26a, c). This effect seems to reach a maximum at 100 µM with no significant further improvements at higher concentrations. Notably, although Warfarin provided a significant protective effect, it was not able to completely inhibit ROS formation, in a similar fashion to what was observed

in viability studies. Moreover, the improvement of the oxidative status and lowered mitochondrial $O_2^{-\bullet}$ production were not associated to a decrease in the mPTP opening (Fig. 26e). This suggest that beneficial effect of Warfarin 100 µM are likely linked to an antioxidant effect probably indirectly triggered by this drug, while no apparent interference with mitochondrial function was present.

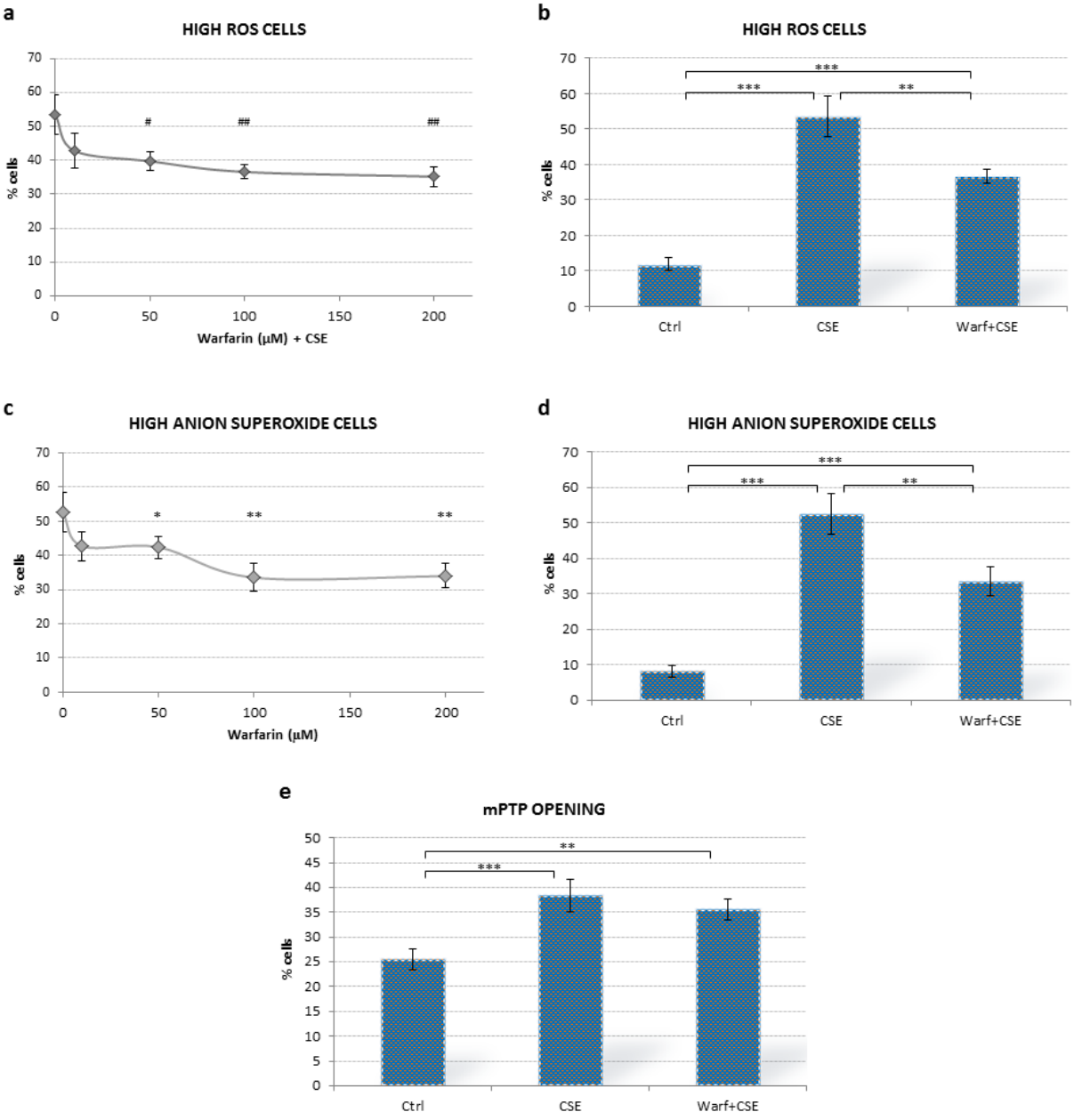

Figure 26: Effects of Warfarin supplementation on oxidative stress and mitochondrial dysfunction CSE-induced. Young HUVECs were supplemented with Warfarin and after 48h the medium was replaced with 12,5% CSE medium+Warfarin for 24h. A range of Warfarin concentration was used to measuring high ROS (a) and high superoxide anion (c) cell content, while only 100µM was used to evaluate mPTP opening (e). In the panels "b" and "d" were reported high ROS and high superoxide anion cells respectively in presence of 100µM Warfarin. Significance was calculated vs. untreated cells (Ctrl) ("*" p vs. Ctrl) or treated only with 12,5% CSE ("#" p vs. CSE). "*" p<0,05; "**" p<0,01; "***" p<0,001.

CASPASE-1

In relation to pro-inflammatory effect of CSE on young endothelial cells represented by the increased activation of caspase-1, treatment with Warfarin 100 µM was able to completely counteract the increase of caspase-1 activation as the percentage of cells showing activated caspase-1 (Warf+CSE: 31±1%) was significantly lower than the CSE treated HUVECs (CSE: 37±2%), and comparable with the unexposed negative control (Ctrl: 29±3%) (Fig. 27).

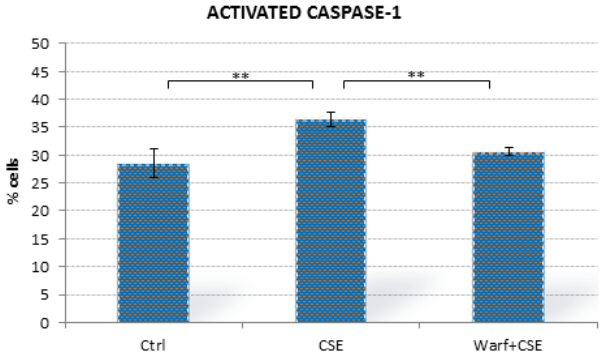

Figure 27: Effects of Warfarin supplementation on caspase-1 activation induced by CSE. Active casp-1 was evaluated in young HUVECs after supplementation with Warfarin 100µM for 48h and other 24h of incubation with 12,5% CSE medium+Warfarin. Significance of variation was calculated vs. untreated cells (Ctrl) and cells treated only with 12,5% CSE. "**" p<0,01.

SENESCENCE MARKERS

Although Warfarin pretreatment has shown a protective effect toward oxidative damage caused by CSE in young endothelial cells, it didn't seem to have any protective effect toward pro-senescence effect of CSE. As reported in Figure 28, Warfarin exposure produced only a slight and non-significant decrease in the percentage of SA-β-galactosidase positive cells (CSE: 38±5%; Warf+CSE: 29±4%) and the levels of expression of p16 mRNA (CSE: 4,1±1,1 fold; Warf+CSE: 3,2±1,3 fold).

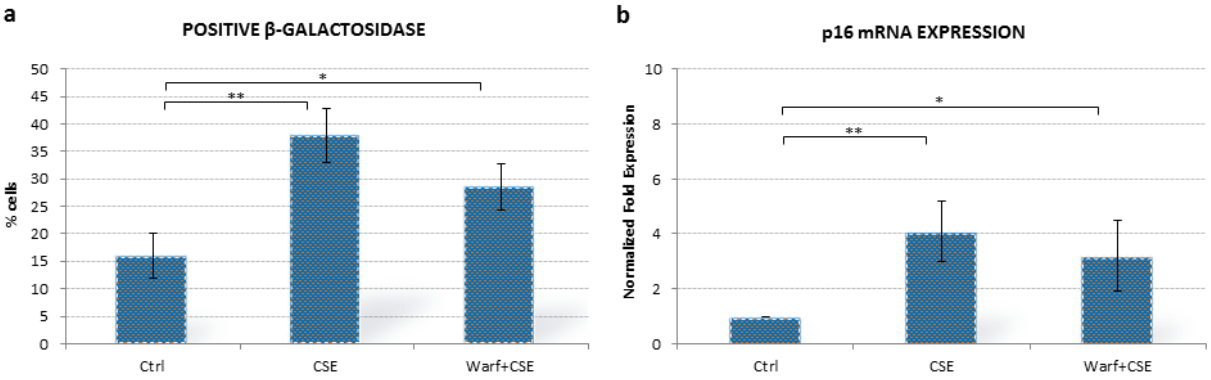

Figure 28: Effects of Warfarin supplementation on senescence markers induced by CSE. SA-β-galactosidase activity (a) and p16 mRNA (b)were evaluated in young HUVECs after supplementation with Warfarin 100 µM for 48h and other 48h of incubation with 12,5% CSE medium+ Warfarin. Significance of variation was calculated vs. untreated cells (Ctrl) and cells treated only with 12,5% CSE. "*" $p<0,05$; "**" $p<0,01$.

DISCUSSION

Cardiovascular disease (CVD) is the major cause of death and disability globally. The principals modifiable CVD risk factors are hypertension, dyslipidemia, obesity and diabetes which are largely related to lifestyle habits such as cigarette smoke, unhealthy diet and physical inactivity. In particular, cigarette smoke, comprising a mixture of multiple toxic chemical compounds, causes a strongly oxidizing vascular environment that leads to vascular stiffening and promotes the development and progression of atherosclerotic lesions.

The aging is another CVD risk factor, but this is an unavoidable physiological process which could be delayed following a healthy lifestyle, characterized by a balanced diet and adequate physical activity. The first provides essential nutrients and natural antioxidant molecules, while the second one stimulates the endogenous antioxidant defenses. However, aging process could be accelerated by external factors, such as cigarette smoke, that promote oxidative stress and cell damages. Therefore CVD, aging and cigarette smoke result closely related to each other.

Vascular endothelium is a crucial element with regards to cardiovascular function and its dysfunction is an early manifestation of atherosclerotic disease. Therefore, clarifying the mechanism of cigarette smoke action is essential to identify potential protective molecules. In the present study, a model of young endothelial cells (HUVECs) was used to investigate the toxicity and the pro-senescent effects of Cigarette Smoke Extract (CSE) comparing CSE exposed young cells with untreated cells in replicative senescence.

Dose- and time-dependent reduction in the number of viable cells was reported in dermal and lung fibroblasts after CSE exposure [*Yang 2013; Carnevali 2003*]. Moreover, Carnevali et al., after 3h of CSE exposure, observed an increase of phosphatidylserine residues exposure, which are normally located in the internal phospholipid layer and are actively translocated to the external layer becoming detectable by Annexin V which is a characteristic feature of early phases of apoptosis [*Carnevali 2003*]. Accordingly with these data, we found in young HUVECs incubated for 24h with CSE a significant viability decrease already at the lowest concentration tested (3,125%). Decrease in the percentage of live cells was not associated with a parallel increase in the percentage of dead cells, but rather with a significant increase in the percentage of apoptotic-like cell, established on the base of differential permeabilities of two DNA-binding dyes. On the contrary, Csordas et al., suggested that protein damage caused by CSE activates autophagy, ultimately leading to necrotic death of HUVECs [*Csordas 2011*]. In this respect in the literature it was also shown that modality of CSE-induced cell death signaling could also be influenced by the CSE concentration used: lower

concentrations induce caspase-independent apoptosis-like programmed cell death trough the recruitment of the Apoptosis Inducing Factor (AIF); whereas incubation with higher concentrations interrupts apoptotic signaling and induces necrosis because the cell damage become too extensive [*Messner 2012*]. In addition, Csizar et al., found an increase of Caspase 3/7 activity after CSE treatment, suggesting a caspase-dependent apoptosis [*Csiszar 2008*].

Apoptosis occurs under several physiological and pathological situations, and it represents a common mechanism of cell replacement, tissue remodeling, and removal of damaged cells. It is characterized by cell shrinkage, chromatin condensation, inter nucleosomal DNA fragmentation, and formation of apoptotic bodies. Apoptosis may occur spontaneously or in response to specific stimuli such as heat stress, radiation, steroids, and oxidative stress [*Chandra 2000*].

Chemicals contained in cigarette smoke are known to trigger an imbalance between reactive oxygen species (ROS) production and antioxidant system resulting in oxidative stress. There are evidences that cigarette smoke constituents impair mitochondrial function and elicit mitochondrial oxidative stress in various cell types. *In vitro* treatment with CSE caused mitochondrial impairment associated with loss of cellular ATP, rapid depolarization of mitochondrial membrane potential and activation of permeability transition pore followed by apoptotic cell death [*Messner 2012; Slebos 2007*]. In smokers a higher level of oxidative mtDNA damage has been observed [*Ballinger 1996*]. These data support the hypothesis that cigarette smoke may contribute to increase a risk for cardiovascular disease also by promoting mitochondrial dysfunction and damage.

In fact, in our model, CSE treatment of young HUVECs produced a high significant increase in the cellular content of both cytosolic ROS and superoxide anion ($O_2^{-\bullet}$), a highly reactive oxygen radical deriving mainly from the mitochondrial metabolism, in addition to increase mPTP opening.

Increased levels of $O_2^{-\bullet}$ can be implicated in pro-atherogenic vascular phenotypic alterations, including induction of pro-inflammatory gene expression [*Csiszar 2009*]. In particular CSE in human coronary arterial endothelial cells was shown to induce NF-κB activation which promote the transcription of a large range of genes implicated in inflammation, including cytokines (IL-6, IL-1β and TNFα), chemokines and adhesion molecules [*Orosz 2007*]. Importantly, pro-inflammatory IL-1 family activates the transcription factors NF-kB which activates protein-1, increases the expression of vascular adhesion molecules (e.g. ICAM-1 and VCAM-1) and induces chemokines (e.g. IL-8, a neutrophil chemoattractant) that together promote inflammatory cell infiltration from the circulation into the affected tissues [*Dinarello 2009*]. In particular IL-1β is activated by active caspase-1. Pro-caspase-1 can itself be activated by the inflammasome, a multiprotein platform activated upon non-microbial and stress-associated signals, including reactive oxygen species and extracellular

adenosine triphosphate [*Martinon 2009*]. Pauwels et al., have shown that IL-1β is an important molecule involved in the pulmonary inflammation CSE-induced in mice model and human subjects [*Pauwels 2011*]. We confirmed that the CSE exposure can lead to inflammation by increasing the caspase-1 activation in young HUVECs.

The increase in the imbalance between radicals production and antioxidant defenses resulting in an increase in oxidative stress that promotes mitochondrial dysfunction and inflammation is also observed in senescent cells. This analogy between young HUVECs CSE exposed and senescent HUVECs is confirmed by estimating the specific senescence markers: SA-β-galactosidase and p16 expression, which are increased following exposure of young cells to 12,5% CSE for 48h.

SA-β-gal is a hydrolase located in the lysosomes. Lysosomal β-gal splits β-linked terminal galactosyl residues from some substrates, such as gangliosides. Whereas p16 it has the ability to block cell progression from G1 to S phase and is regarded as a major dominant senescence gene [*Campisi 2013*].

In conclusion our data point out that the proposed model of CSE-exposed endothelial cells, in addition to inducing relevant oxidative stress condition, followed by an increased inflammatory state and altered mitochondrial function, is also able to induce a premature senescence. All these changes are critical to the development of endothelial dysfunction and may promote the onset and the progression of atherosclerotic lesions.

Growing evidences indicate that the CSE induced oxidative stress and inflammation may be prevented by antioxidant molecules, such as alpha-tocopherol (vitamin E) and ascorbic acid (vitamin C) [*Hossain 2011*], Resveratrol [*Csiszar 2008*], Glutathione, Melatonin, Lipoic acid and Coenzyme Q_{10} (CoQ_{10}) [*Kaisar 2015*].

Ubiquinol (QH), the CoQ_{10} active form, and vitamin K play a fundamental role in vascular health in physiological conditions by protecting the endothelium from oxidative damage, inflammation and ensuring the functionality of important vitamin K dependent proteins involved in vascular health. Therefore it is possible that QH and K vitamers could also counteract cigarette-induced damage in endothelial cells.

Kaisar et al. have found that 2 hours 10 μg/mL oxidized CoQ_{10} supplementation followed by CSE treatment (12h) was able to reduce the pro-inflammatory cytokines (IL-6, IL-8) release, whereas it weakly prevented the up-regulation of PECAM-1, VCAM-1 and E-selectin [*Kaisar 2015*]. On the contrary, Gairola et al. showed that theCoQ_{10} supplementation in apoE-deficient mice was not able

to counteract the CSE induced atherosclerotic lesions progression [*Gairola 2010*]. There are not many data in the literature regarding the effect of CoQ_{10} supplementation in response to cigarette smoke stress. However, some studies suggested the protective role of CoQ_{10} supplementation in both *in vitro* H_2O_2-treated HUVECs [*Huo 2018*] and *in vivo* model: human with coronary artery disease [*Lee 2013*] and in senescence-accelerated mice [*Tian 2014*]. While there are few studies regarding the role of K vitamers in aging and in stress conditions.

In the present study we have found a strong protective effect of QH and MK7 supplementation counteracting the viability loss, the oxidative stress and inflammation increase, and, finally, aging progression. In particular, at 10 μM, these molecules improved the oxidative stress (cytosolic ROS and mitochondrial superoxide anion) but are not able to prevent mitochondrial dysfunction characterized by increasing mPTP opening. However QH and MK7 showed also an inhibition of caspase-1 activation induced by CSE.

The antioxidant and anti-inflammatory effects of QH and MK7 resulted in a significant improvement in viability counteracting the apoptotic shift and in the prevention of SA-β-galactosidase increase. However neither quinones were able to counteract CSE-induced expression of p16.

In particular, in relation to viability and oxidative status of the CSE-treated cells, MK7 was the most active molecules among the quinones tested and its anti-apoptotic and $O_2^{-\bullet}$ curbing activity was already highly significant at concentrations 1 μM, ten times lower than QH.

The suppression of oxidative stress could be the main beneficial function of QH and MK7 in the proposed model, leading to general improvement in cellular functionality. This effect is not necessarily associated to a direct radical scavenging role of these molecules. In fact, recent data show that Nrf-2 (nuclear factor erythroid 2-related factor 2), the main mediator of cellular adaption to oxidative stress, and its target genes were strongly increased by CSE in a dose-dependent manner [*Muller and Hengstermann 2012; Giebe 2017*]. Following oxidative insult, Nrf-2 dislodged from Keap-1 (Kelch-like erythroid cell-derived protein 1) binding domain and translocates from the cytoplasm into the nucleus binding to the corresponding Antioxidant Response Element (ARE). This ultimately leads to overexpression of detoxifying agents like heme oxygenase (decycling) 1 (HMOX1) and NAD(P)H quinone dehydrogenase 1 (NQO1). An elevated level of NQO1 is an indication of the activation of a cellular defense mechanism in response to oxidative stimuli such as CSE exposure. Antioxidants capable of acting intracellularly by stimulating ARE pathways are able to increase this antioxidative response.

On the contrary, Fratta Pasini et al. found that in HUVECs exposed to smokers' serum, the expression of Nrf-2, HMOX1 and of glutamate-cysteine ligase catalytic (GCLC) subunit, the enzyme that catalyzes

the rate-limiting step of GSH synthesis, were decreased [*Fratta Pasini 2012*]. These implicate a reduction of antioxidant defense, therefore in this scenario the antioxidants supplementation could counteract the oxidative damage caused by CSE.

Furthermore, the great efficiency of vitamin K in preventing the damage caused by smoking could be mediated by NQO1 whose expression is also influenced by Nrf-2 nuclear translocation. NQO1 is known to increase the vitamin K recycling efficiency making it more available for carboxylases responsible for vitamin K dependent proteins (VKDP) activation.

However, incubation with Warfarin, a vitamin K antagonist by inhibiting vitamin K recycling, not amplified the damage induced by CSE, on the contrary, showed a protective effect.

This, on the one hand, suggests that vitamin K could act through VKDP-independent mechanisms as supported by Ohsaki et al. which demonstrated that MK4 inhibit LPS-induced inflammation in cultured monocyte via the inactivation of the NF-κB signaling pathway independently of its carboxylative effect [*Ohsaki 2010*]. On the other hand, our finding could suggest compensatory mechanisms triggered by the cell to by-pass Warfarin-associated K-cycle inhibition that could involve induction of alternative reductase. In this case adaptative response might have masked the negative effect of the VKDP activation deficiency in our experimental model.

In the present study, the other K vitamers tested, MK4 and K1, showed only a slight improvement in viability and cytosolic ROS content. In addition, MK4 amplified the CSE-induced increase of anion superoxide and consequently increase the mPTP opening, causing the amplification in mitochondrial dysfunction.

The minor protective effect of K1 and MK4 could be the consequence of their lower bioavailability reported in the literature. In fact, Sato et al. showed that in healthy women, MK7 was found to have greater bioavailability compared to MK4 [*Sato 2012*]. In addition, Shea and Holden reviewed the relation between dietary vitamin K intake and vascular calcification and they concluded that dietary menaquinone intake may be more likely to protect against vascular calcification than phylloquinone. [*Shea and Holden 2012*]. Moreover, different tissues seem to be able to convert K1 into menaquinone to act as a carboxylase cofactor, highlighting a specific role of menaquinone in extra-hepatic tissues [*Okano 2008*]. In this respect MK7, due to its improved bioavailability compared to other dietary menaquinones, could be more effective than the other forms of vitamin K.

In support to that, Nakamura et al. suggested that the optimal dose of MK4 to decrease inactive osteocalcin was 600 μg/day or more [*Nakamura 2014*], which is much higher than the required dose

for MK7 (180 µg/day) [*Knapen 2013*]. In particular, bioavialability is also influenced by the carriers/solvent used. As shown in the present study, the solvent play an important role in the bioavailability of the K vitamers both *in vitro* and *in vivo*. In particular all K vitamers tested showed the best bioavailability when dissolved in a micellar suspension (with oil and arabic gum for *in vivo* assays, and with cremophor and glycerol for *in vitro* tests). Lower cellular bioavailability of MK4 and K1 compared to MK7 was verified in our experimental system both for human dermal fibroblast and endothelial cells, and may underlie the lower efficacy of these molecules.

SUMMARY AND CONCLUSIONS

The aim of the present study was to investigate the damage induced by cigarette smoke (CSE) in a cellular model of human endothelium and to verify whether bioactive compounds active in cardiovascular health, namely coenzyme Q10 and vitamin K, could counteract the adverse effects of cigarette smoke, and slow down process associated with the development of atherosclerotic lesions.

It was found that the CSE exposure was capable to induce significant oxidative stress responsible for the alteration of mitochondrial function. In addition, CSE induced the increase of caspase-1, a marker of inflammation, which could be induced either directly, by the molecules present in the CSE, or indirectly, through the altered radicals production. Probably these changes were the main causes of the observed early cellular senescence.

Supplementation with phylloquinone and menaquinone-4 was not effective in preventing changes induced by CSE. In particular MK4 amplified CSE-induced endothelial dysfunction.

On the contrary, optimistic results were obtained with the use of ubiquinol and menaquinone-7 which have effectively counteracted the viability loss, oxidative stress and inflammation in a slowing of premature aging CSE-induced.

Surprisingly also Warfarin showed a protective action against CSE, in particular by contrasting the oxidative stress and the inflammatory process.

This suggests that the inhibition of vitamin K does not cause an amplification of the CSE-induced damage and Warfarin could induce antioxidant and anti-inflammatory pathways, probably triggered by alternative activation of reductases involved in vitamin K recycling. This also suggests that vitamin K could act not only as activator of the VKDP, but also through carboxylases-independent pathways.

Further investigations are necessary to understand the mechanisms of action of these molecules, in particular of MK7 and Warfarin.

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SCIENTIFIC PAPER

1. Orlando P, Silvestri S, Galeazzi R, Antonicelli R, Marcheggiani F, **Cirilli I**, Bacchetti T, Tiano L. *"Effect of ubiquinol supplementation on biochemical and oxidative stress indexes after intense exercise in young athletes"*. **Redox Rep**. 2018 Dec;23(1):136-145. doi: 10.1080/13510002.2018.1472924.

2. Damiani E, Brugè F, **Cirilli I**, Marcheggiani F, Olivieri F, Armeni T, Cianfruglia L, Giuliani A, Orlando P, Tiano L. *"Modulation of oxidative status by normoxia and hypoxia on cultures of human dermal fibroblasts: how does it affect cell aging?*". **Oxidative Medicine and Cellular Longevity**. 2018 Sep 23;2018:5469159. doi: 10.1155/2018/5469159.

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4. Giannubilo SR, Orlando P, Silvestri S, **Cirilli I**, Marcheggiani F, Ciavattini A, Tiano L. *"CoQ10 Supplementation in Patients Undergoing IVF-ET: The Relationship with Follicular Fluid Content and Oocyte Maturity"*. **Antioxidants (Basel)**. 2018 Oct 13;7(10). pii: E141. doi: 10.3390/antiox7100141.

5. **Cirilli I**, Silvestri S, Marcheggiani F, Olivieri F, Galeazzi R, Antonicelli R, Recchioni R, Marcheselli F, Bacchetti T, Tiano L, Orlando P. "*Three Months Monitored Metabolic Fitness Modulates Cardiovascular Risk Factors In Diabetic Patients*". **Diabetes Metab J**. Accepted.

6. Marcheggiani F, **Cirilli I**, Orlando P, Silvestri S, Vogelsang A, Knott A, Blatt T, Weise JM, Tiano L. "*Mitochondrial dysfunction in a senescence model of Coenzyme Q10 deprived human dermal fibroblasts*". Submitted.

7. Orlando P, Silvestri S, Marcheggiani F, **Cirilli I**, Tiano L. "*Menaquinone 7 stability of formulations and its relationship with purity profile*". Submitted.