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Differential modulation of smooth muscle cell phenotype by next-generation nicotine products and traditional cigarette

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Ci saranno sempre pietre sulla strada davanti a noi.
Saranno ostacoli o trampolini di lancio; tutto dipende da come le usiamo.

Friedrich Nietzsche

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Summary

Abbreviations

AE	Aqueous extract
AhR	*Aryl hydrocarbon receptor*
ACTA2	α-smooth muscle actin
α- BTX	α-Bungarotoxin
CNN1	Calponin
CO	Carbon monoxide
CVD	Cardiovascular disease
CS	Cigarette smoke
E-CIG	Electronic cigarette
ECM	Extracellular matrix
EC	Endothelial cell
ER	Endoplasmic reticulum
ICAM-1	Intracellular adhesion molecule-1
IL	Interleukin
MCP-1	Monocyte chemoattractant protein-1
MMP	Matrix metalloproteinase
MYH11	Myosin 11
NGP	Next generation product
nAChR	Nicotinic acetylcholine receptor
NO	Nitric oxide
PAH	Polycyclic aromatic hydrocarbons
PCNA	Proliferating cell nuclear antigen
PDGF	Platelet-derived growth factor

RAC1	Ras-related C3 botulinum toxin substrate 1
SMC	Smooth muscle cell
TC	Traditional cigarette
THP	Tobacco heating product
TPM	Total particulate matter
TNF- α	Tumor necrosis factor α
UPR	Unfolded Protein Response
VCAM-1	Vascular-cell adhesion molecule-1
TGF-ß	Transforming-growth factor beta

1. ABSTRACT

1.1 ENGLISH VERSION

Cigarette smoke is one of the most important modifiable risk factors for cardiovascular diseases (CVDs) since smoking is responsible for 20% of deaths from coronary heart disease. Cigarette smoke contains 7.000 different components, many of which are known to be cytotoxic, mutagenic or carcinogenic. In the effort to reduce the harm associated with conventional cigarettes caused by the toxicants in the cigarette smoke produced by the burning of tobacco, alternative next generation nicotine products (NGPs, such as E-cigarettes (E-CIG) designed to deliver nicotine through an aerosol state), have been developed.

Our research group has previously demonstrated that cigarette smoke condensate affects the plasticity of smooth muscle cells (SMCs). The aim of my project was to evaluate the effects of cigarette smoke aqueous extracts (AEs) on SMC phenotypic switch potential, a key step in the atherogenic process, and to get more information on the potentially toxic effects of the new NGPs.

The *in vitro* studies in human aortic SMCs, showed that AEs, obtained from a traditional (TC) and E-CIG, stimulated the expression of extracellular matrix genes (ECM) and at the same time, E-CIG showed a potent and faster induction of SMC proliferation and migratory activity, while TC slowed down these processes compared to control and E-CIG. Moreover, we observed that the stimulatory effects exert by E-CIG are probably due to its nicotine content. In addition, α-BTX a potent and selective antagonist of alpha7 nicotinic acetylcholine receptor (α7nAChR) activation counteracted nicotine-induced cell migration and proliferation. Furthermore, the incubation with TC and E-CIG AEs also affected cell morphology, with the extension of lamellipodia, and increased F-actin and RAC1 levels. Interestingly, E-CIG showed a suppressive effect on inflammatory markers that was reversed by blocking α7nAChR with α-BTX. Overall, these results suggested that E-CIG promotes a phenotypic modulation in SMCs. In fact, E-CIG extract induces changes in cytoskeleton

organization enhancing a proliferative and promigratory behavior. Moreover, we can speculate that these effects are mediated by nicotine through α7nAChR activation.

On the contrary, TC increases ECM gene expression and induces changes in cell morphology but it might probably contain some other chemical compounds that overwhelmed the stimulatory effects exerted by nicotine on proliferation and migration activity. In addition, we demonstrated that TC is the most effective in promoting an inflammatory response by inducing the expression of several cytokines and matrix remodeling genes.

In conclusion, these results demonstrated that E-CIG induce SMC phenotypic modulation by promoting SMC proliferation and migration activity and by inducing an alteration in cytoskeletal organization. E-CIG also exerts a negative modulation of the inflammatory pathway reducing cytokines expression. Further, the inhibitory effect is due to nicotine, one of the major components of cigarette smoke, through the activation of α7nAChR. TC is less effective in inducing a proper phenotypic modulation of SMCs while it is the most effective in increasing markers of inflammation and matrix-degrading metalloproteinases thus, promoting vascular inflammation and the atherogenic process.

1.2 VERSIONE ITALIANA

Il fumo di sigaretta rappresenta uno dei più importanti fattori di rischio cardiovascolare ed è responsabile del 20% di cause di morte per coronaropatia. Il fumo è costituito da circa 7.000 componenti, molti dei quali sono riconosciuti come citotossici, mutagenici e cancerogeni. Negli ultimi anni, sono stati sviluppati dei nuovi dispositivi tra cui annoveriamo le sigarette elettroniche con l'obiettivo sia di ridurre gli effetti dannosi derivanti dalla combustione della sigaretta tradizionale sia come ausilio per cercare di smettere di fumare.

Come precedentemente dimostrato dal nostro gruppo, il condensato del fumo di sigaretta che rappresenta la porzione lipofila del fumo totale, è in grado di indurre un cambiamento fenotipico nelle cellule muscolari lisce vasali (SMCs). Lo scopo del mio progetto di ricerca è stato quindi quello di andare a valutare gli effetti dell'estratto acquoso del fumo di sigaretta sul cambiamento fenotipico delle SMCs, le quali a differenza delle cellule muscolari cardiache e scheletriche sono dotate di un elevato grado di plasticità e in seguito ad una serie di insulti ambientali posso subire una modulazione fenotipica caratterizzata da un aumento di produzione di componenti della matrice extracellulare e aumento della capacità proliferativa e attività migratoria.

I nostri studi in vitro su cellule muscolari di aorta hanno mostrato come gli estratti acquosi ottenuti dalla sigaretta tradizionale (TC) ed elettronica (E-CIG) stimolino l'espressione di geni della matrice extracellulare tra cui collagene e proteoglicani. Al tempo stesso l'E-CIG induce una maggior attività proliferativa e migratoria rispetto al controllo e alla TC che sembra invece ridurre tali parametri.

Inoltre, gli effetti osservati con l'E-CIG sembrano essere correlati con il contenuto nicotinico presente. A tal proposito, il pretrattamento con α-BTX, un potente antagonista del recettore α7 nicotinico è in grado di revertire l'effetto stimolatorio indotto dalla nicotina.

Entrambi gli estratti del fumo promuovono un riarrangiamento citoscheletrico determinato da un aumento dei livelli di F-actina del 40% per la TC e del 50%

per la E-CIG e di RAC1. Inoltre, l'E-CIG mostra un effetto inibitorio sull'espressione di marker infiammatori, questo effetto inibitorio viene revertito pretrattando le cellule con α-BTX. In conclusione, questi risultati ci suggeriscono come l'E-CIG sia in grado di indurre un cambiamento fenotipico nelle cellule muscolari lisce vasali, promuovendo sintesi della matrice, proliferazione migrazione e inducendo un cambiamento nell'organizzazione citoscheletrica.

Contrariamente, La TC stimola l'espressione di componenti della matrice e un cambiamento morfologico ma probabilmente contiene delle altre sostanze chimiche che in qualche modo vanno ad antagonizzare/neutralizzare gli effetti stimolatori promossi dalla nicotina sull'attività migratoria e proliferativa.

Abbiamo inoltre dimostrato che la TC risulta avere un maggior impatto sul meccanismo infiammatorio promuovendo infatti l'espressione di citochine e metalloproteasi.

In conclusione, i risultati di questa ricerca hanno evidenziato come l'E-CIG induca una modulazione fenotipica promuovendo un aumento di proliferazione e migrazione di cellule muscolari vasali e inducendo un'alterazione nell'organizzazione del citoscheletro. L'E-CIG sembra inoltre esercitare una modulazione negativa sul meccanismo infiammatorio riducendo l'espressione di alcune citochine. Questo effetto inibitorio sembra esser dipeso dalla nicotina, uno dei maggior componenti del fumo di sigaretta, mediante attivazione del recettore α7 nicotinico.

La TC invece, risulta essere meno efficace nell'indurre una modulazione fenotipica ma stimola una notevole risposta infiammatoria con un aumento dell'espressione di citochine e geni che degradano la matrice extracellulare promuovendo in questo modo il processo aterogenico.

2. INTRODUCTION

2.1 DEVELOPMENT OF ATHEROSCLEROSIS

Cardiovascular diseases (CVDs) are the major cause of death globally, taking an estimated 17.9 million lives each year [1]. CVDs are a group of disorders that affects heart and blood vessels and include coronary heart disease, cerebrovascular disease, and rheumatic heart disease. The main underlaying cause of CVDs is atherosclerosis, a chronic inflammatory disease marked by the formation of atheromatous plaques within the arterial wall. The etiology of atherosclerosis is multifactorial and the trigger can be either systemic or local factors that induce deterioration in vascular function [2]. Among the several risk factors that may provoke or promote the progression of the atherosclerotic process we may count dyslipidemia, hypertension, diabetes mellitus, obesity and tobacco smoking, which constitute the major modifiable risk factors; age, gender and genetic predisposition instead are to be listed among the non-modifiable risk factors [3].

The earliest feature of atherosclerosis is represented by a modification of low-density lipoproteins (LDL) within the intima layer [4]. LDL particles undergo biochemical modification such as oxidation (OxLDL) which promote a change in endothelial permeability and extracellular matrix components, principally proteoglycans, beneath the endothelium layer, leading to an increased penetration and retention of LDL particles in the artery wall [4]. OxLDL activates endothelial cells (ECs) by promoting the expression of adhesion molecules, like vascular-cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1), which mediate the rolling and adhesion of leukocytes [5]. After adhesion to the endothelium, leukocytes migrate inside the tunica intima, in response to cytokines and chemoattractant mediators like monocyte chemoattractant protein-1 (MCP-1). Once inside the arterial wall, monocytes (the most present white blood cells in the plaque) differentiate into macrophages with concomitant upregulation of scavenger receptors (CD68, CD36, SR-B1, LOX-1) that mediate the uptake of OxLDL leading to the formation of foam cells, term given by microscope evidence of lipid-laden cells [6,7]. Macrophages in atherosclerosis can be broadly divided in two groups. Pro-inflammatory macrophages M1 that play an important role in plaque

progression by secreting pro-inflammatory factors such as tumor necrosis factor-α (TNF- α), interleukin-1ß (IL-1ß) and chemokines CXCL9, CXCL10 that drive the plaque toward an unstable phenotype. In contrast, M2 anti-inflammatory macrophages secrete anti-inflammatory factors such as IL-1 receptor agonist and IL-10 and promote tissue repairing hence favoring plaque stability [8]. Stoger et al. using immunohistochemistry and transcriptomic analysis showed that in human atherosclerotic lesions, macrophages expressing pro-inflammatory markers dominated the rupture prone shoulder regions. In contrast, the presence of M2-like macrophages is pronounced in stable regions of the plaque and in the adventitia [9]. Atherosclerotic plaque formation also involves smooth muscle cells (SMCs), recruited from the tunica media. SMCs migrate to the tunica intima and proliferate in response to several environmental cues (growth factors, lipoproteins, inflammation), also produce elastin and collagen, the main components of extracellular matrix (ECM), and form the fibrous cap that covers the plaque. Under the fibrous cap, lie macrophage-derived foam cells. Some of them undergo apoptosis as a results of prolonged endoplasmic reticulum (ER) stress and other stimuli. Over the last decade, ER stress has emerged as a factor that is relevant to several systemic and arterial-wall factors that promote atherosclerosis. ER stress represents a response by cells to transient or prolonged perturbations in ER functions such as those related to protein synthesis, protein folding and calcium regulation [10]. ER stress signaling is often referred to as the Unfolded Protein Response (UPR) that is triggered by three upstream proteins, IRE1, ATF6, and PERK [11] [10]. The activation of these mechanisms promote the proper protein folding and the degradation of misfolded proteins [12]. Despite the beneficial functions of the UPR during transient ER stress, a prolonged ER stress due to various pathological factors such as hyperlipidemia, oxidative stress and calcium imbalance, causes activation of apoptosis and inflammatory pathways [12,13]. Another important mechanism known as efferocytosis is impaired in advanced atherosclerotic lesion. Efferocytosis is a process that allow a rapid clearance of apoptotic cells thus preventing secondary necrosis and triggers an anti-inflammatory response through the

induction of IL-10 and other anti-inflammatory cytokines. Defective efferocytosis leads to the generation of plaque necrosis associated with an enhanced secretion of pro-inflammatory cytokines. Overall, these features characterize plaques that are vulnerable to rupture, which in turn can trigger acute thrombosis and arterial occlusion [14,15].

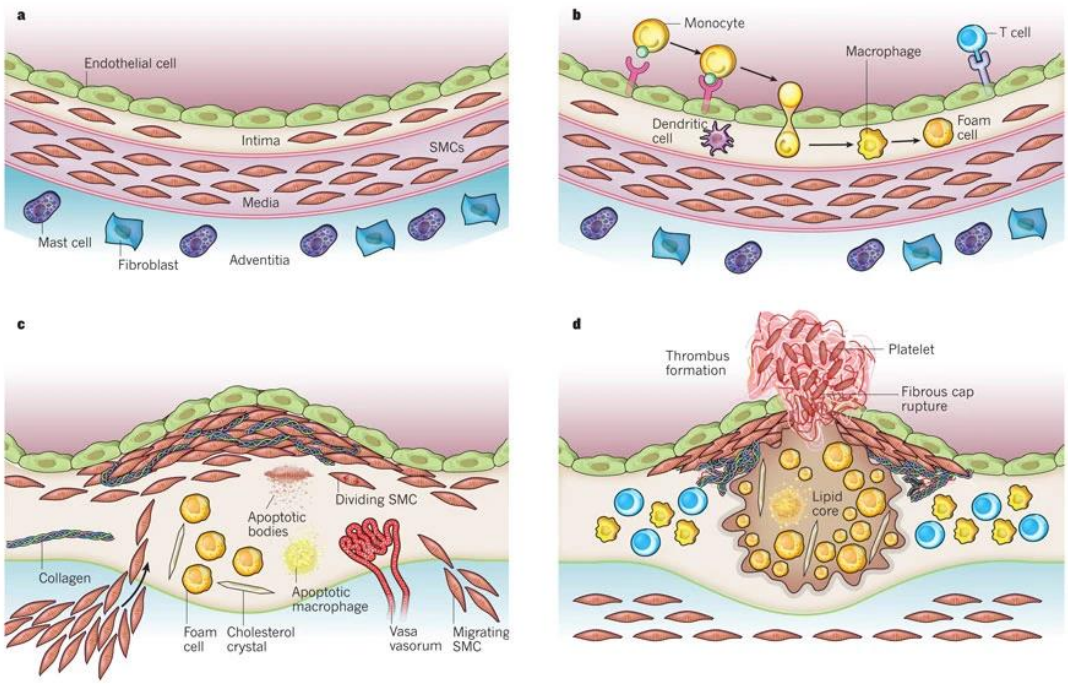


Figure 1. **Atherosclerotic lesion development.** a, the artery is composed by three layers. The tunica intima is the inner layer lined by a monolayer of endothelial cells in contact with blood. The tunica media is the middle layer and contains SMCs and extracellular matrix. The outer one, tunica adventitia, contains mast cells, nerve endings and microvessels. b, the first observable change in the artery wall is represented by an endothelial dysfunction. The endothelium, as a response to shear stress, increases the expression of cell adhesion molecules facilitating the homing (margination and adhesion) of the circulating monocytes to the activated endothelial cells. Monocytes migrate into the intima and in response to inflammatory stimuli differentiate into macrophages with concomitant upregulation of scavenger receptors that mediate the uptake of OxLDL, an event that leads to the formation of foam cells. c, with the progression of the lesion, SMCs migrates from the tunica media to the intima, where they proliferate together with resident SMCs promoting the synthesis of extracellular matrix components. Macrophages and SMCs can die in advancing lesions by apoptosis and extracellular lipid derived from dead cells can accumulate, giving rise to a

necrotic core. d, the ultimate complication of atherosclerotic plaque is represented by thrombosis, a physical disruption of the atherosclerotic plaque [15].

2.2 SMOOTH MUSCLE CELLS

SMCs are specialized contractile cells found in many organs including blood vessels, trachea, stomach, small intestine, and uterus. SMCs play important roles in the formation and function of the cardiovascular, digestive, respiratory, and urinary systems [16]. SMCs, generally present in the tunica media, constitute a large portion of cells in blood vessels [16].

In healthy arteries, SMCs have a fusiform shape and express contractile proteins (including α-smooth muscle actin (ACTA2), myosin 11 (MYH11) and calponin (CNN1) and secrete ECM macromolecules including elastin, collagen and proteoglycans.

The primary functions of SMCs is contraction and regulation of blood vessel tone-diameter, blood pressure, and blood flow distribution [17]. Numerous signaling molecules and factors in the local environment, including cytokines and growth factors, mechanical forces, neuronal stimuli and genetic factors can influence the contractile state of the SMC, which rarely changes under normal physiological conditions [18].

The mature SMC is a cell type which is not terminally differentiated and that retains the ability to change its original features, which distinguish them from myocardial cells and skeletal muscle cells. SMCs are remarkably plastic, in fact they are able to modify their phenotype in response to conditions such as vascular injury, altered blood flow conditions, cell-cell and cell-matrix interactions [18,19].

SMCs are the major producers of extracellular matrix within the vessel wall and in response to atherogenic stimuli can modify the type of matrix proteins produced [20]. SMCs are also capable of performing functions attributed to other cell types. Like macrophages, SMCs can express a variety of scavenger receptors for lipid uptake and can form foam cells [21]. Like endothelial cells, SMCs can also express a variety of adhesion molecules such as VCAM-1 and ICAM-1, allowing monocytes and lymphocytes to adhere and migrate into the

vessel wall [20]. SMCs can adopt a wide range of phenotypes in response to various environmental cues, with different effects on atherogenesis.

2.2.1 SMOOTH MUSCLE CELL PHENOTYPIC MODULATION

After intimal injury, different cell types, including ECs, platelets, and inflammatory cells release mediators, such as growth factors and cytokines that induce multiple effects including phenotypic switch of SMCs from the quiescent “contractile” phenotype state to the active “synthetic” SMCs that can migrate and proliferate from media to the intima, they also increase ECM production, synthetizing types I and III collagen, fibronectin and proteoglycans such as lumican, decorin and byglican [22] [23].

Moreover, SMCs participate in fibrous cap formation [24].The transition toward a synthetic phenotype results in a gradual shifting from a differentiated to a dedifferentiated state, defined by the decrease or loss of several SMC-specific contractile genes [18] and an increased SMC proliferation and migration (Fig. 2) [25] .

The ultrastructure studies of contractile SMCs revealed a lack of endoplasmic reticulum and Golgi’s apparatus, but a relative abundance of contractile filaments. This determines an elongated and spindle-shaped morphology. In contrast, synthetic SMCs exhibit cobblestone morphology which is referred to as epithelioid or rhomboid [26]. On the contrary, the synthetic SMCs contain a high number of organelles involved in protein synthesis as well as extracellular matrix production (Fig. 3).

Moreover, synthetic and contractile SMCs have different proliferative and migratory characteristics [26].

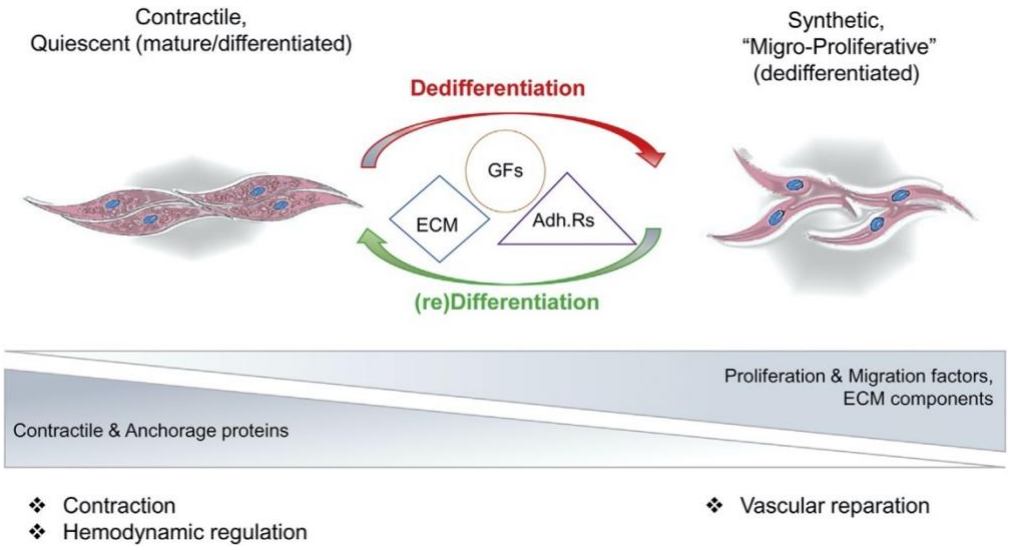


Figure 2. **SMC functional phenotypic plasticity in the normal healthy adult blood vessel**. SMCs primarily exhibit a differentiated, contractile phenotype which is necessary for maintaining normal blood flow and blood pressure. However, in response to vascular injury or alterations in local environmental cues (e.g. cell–cell interactions, cell–matrix interactions and paracrine signals) differentiated SMCs can switch their phenotype to the less differentiated state and acquire functional properties (e.g. migratory, proliferative, synthetic) necessary for tissue reparation [27].

Among the markers used for measuring cell proliferation rate, PCNA (proliferating cell nuclear antigen), a key regulatory factors of cell replication [28], has recently gained attention and holds much promise as it is intricately involved in cell replication processes [29]. Several growth factors such as PDGF-BB (platelet-derived growth factor BB) and TGF-ß (transforming-growth factor beta) are important in the regulation of SMCs proliferation and PCNA expression [30]. Pan et al. demonstrated that PDGF-BB, a potent mitogenic factor, promote the expression of PCNA in SMCs obtained from the aorta of Sprague-Dawley rats by inducing the overexpression of TGF-ß and its signaling cascade [30].

SMC migration from the media to the intima actively participates in the progression of the plaque. These activated cells can migrate in two different areas of the plaque: if they migrate towards the necrotic and rich in lipids core,

they will acquire characteristics typical of macrophage-derived foam cells, incorporating ox-LDL. If, instead, they migrate towards the lumen of the vessel, SMCs will enrich the fibrous cap [27].

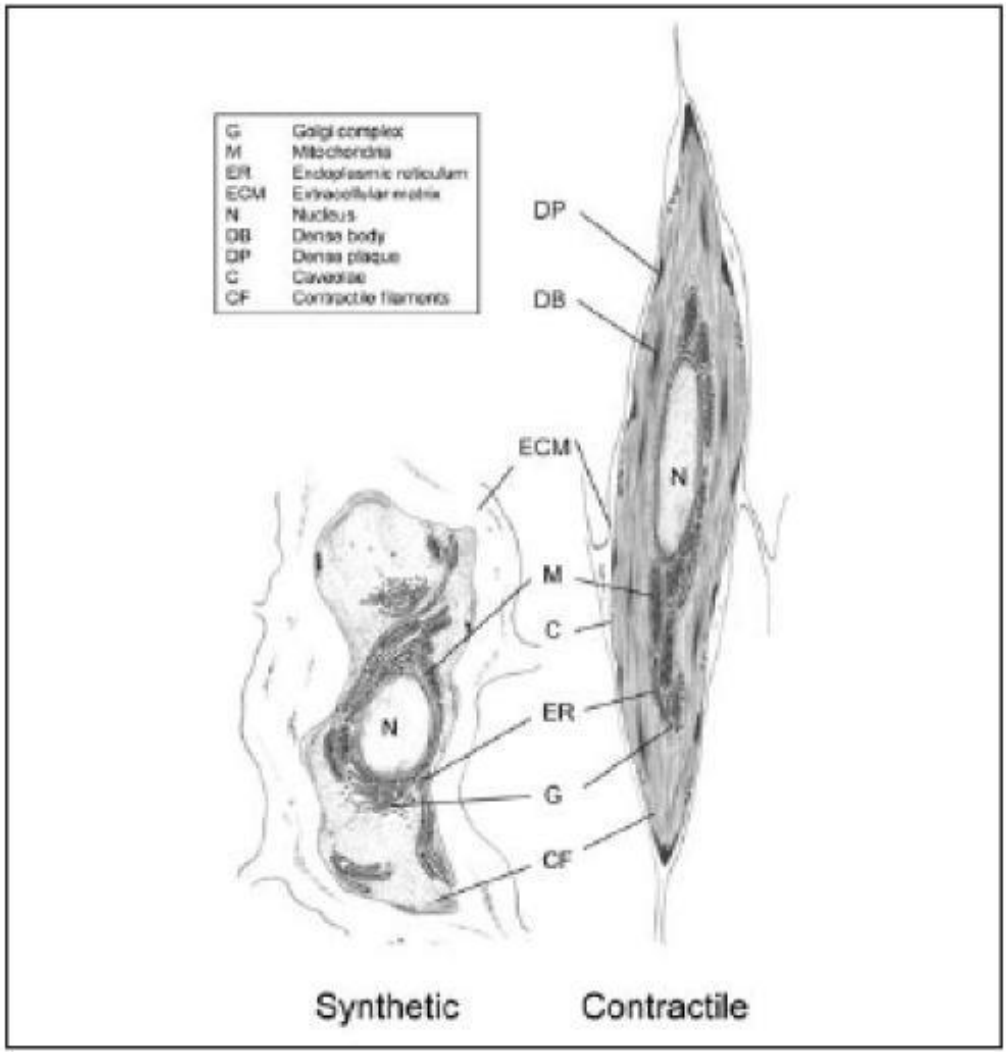


Figure 3. **ultrastructural characteristics of contractile and synthetic SMCs.** N: nucleus, ECM: extracellular matrix, C: caveolae, M: mitochondria, G: Golgi's apparatus, ER: endoplasmic reticulum, DP: dense plaque, DB: dense body, CF: contractile filament [26].

An increasing number of studies have reported that in atherosclerotic lesions, SMCs can acquire different synthetic macrophage-like, fibroblast-like and osteo/chondrogenic-like phenotypes [18][31][32][33].

Allahverdian et al. observed that 40% of cells in advanced lesions of human coronary atherosclerosis express both SM α-actin and CD68 a typical macrophage marker. Consistently, they observed that not all the CD68 positive cells express CD45, a specific myeloid cell lineage marker, suggesting that 40% of CD68 expressing cells are SMCs rather than leukocyte derived [21]. These findings have been confirmed in mouse models by different groups [31][34]. Feil et al. labeled SMCs in the arterial media by tamoxifen treatment in young apoE-deficient mice before the development of atherosclerosis and their fate was monitored in older atherosclerotic animals. Interestingly, they found that medial SMCs can undergo clonal expansion and convert to a macrophage like phenotype [31].

SMCs can also express markers of mesenchymal stem cells and fibroblast as shown in SMC lineage tracing mice [32]. Shankman et al. demonstrated that SMCs within atherosclerotic plaques express macrophage markers (LGALS3), mesenchymal stem cells markers (SCA1) and fibroblast markers (PDGFβR) [32]. Moreover, Wirka et al. demonstrated that SMCs undergoing phenotypic modulation exhibit a shift in gene expression from a contractile to a fibroblast-like phenotype. scRNAseq analysis defined a specific cell cluster with a marked upregulation of genes related to the fibroblast population including fibronectin, collagen 1alpha1 and small leucine-rich proteoglycans like lumican, decorin and biglycan [35].

Similarly, SMCs have been proposed to acquire an osteo/chondrogenic phenotype in the contest of vascular calcifications [33]. The expression of transcription factors like Runx2 and Sox9 determines the osteogenic or the chondrogenic lineage, in particular, Runx2 driving the osteogenic phenotype while Sox9 promote the chondrogenic phenotype [36].

Alver et al. demonstrated that SMCs could transdifferentiate into calcified vascular cells when exposed to osteogenic stimuli. Interestingly, SMCs cultured in osteogenic medium increased the expression of ALP, a relative early marker of osteoblast differentiation. In addition, by genome-wide gene expression analysis they identified 57 ECM genes identically regulated by C-SMCs and osteoblasts [33].

2.2.2 EXTRACELLULAR MATRIX PRODUCTION AND REMODELING BY SMOOTH MUSCLE CELLS

One of the major roles of smooth muscle cells is to produce extracellular matrix (ECM) components both in the healthy and atherosclerotic vessel [37]. In the normal vessel laminin and collagen type IV maintain differentiation of SMCs, while in response to vascular injury SMCs modulate their phenotype and synthesize collagens type I and III, fibronectin and proteoglycans such as decorin lumican and byglican [38] [39], This transition can alter not only the architecture of the vessel, but also the lipid content and the proliferative index. In fact, as atherosclerosis progresses, the presence of many atherogenic cytokines stimulate SMCs to favor the production of proteoglycans and fibronectin as well as enhance the rate of ECM synthesis [40].

Camejo et al. demonstrated that once present in the wall, sulphated proteoglycans entrap LDL via ionic interactions with ApoB100 and ApoE. When bound, LDL undergo biochemical modification such as oxidation, enhancing lipid uptake by macrophage. OxLDL, in turn, stimulates SMC cells to secrete larger amounts of sulphated proteoglycans, which increases their affinity for LDL [41].

Moreover, in vitro work has shown that rat aortic SMC cultured on collagen I and fibronectin substrates can promote SMC phenotypic switch from contractile to synthetic phenotype [42] [43].

It has also been demonstrated that ECM components, including type-1 collagen and fibronectin regulate SMC proliferation [44]. SMC proliferation is suppressed in healthy blood vessels but promoted in atherosclerosis. Cyclin-dependent kinase inhibitors such as $p27^{kip1}$, play a key role in maintaining SMC quiescence and its level depend on the composition of the extracellular matrix [44]. Bond et al. demonstrated that in the atherosclerotic plaque where SMCs are mainly bound to fibronectin and proteoglycans, cdk2 inhibitors, including $p27^{kip1}$ are downregulated in order to promote SMC proliferation [44]. In turn, proliferating cells produce more proteoglycans than quiescent cells, amplifying the effect [41].

In addition to enhanced ECM synthesis, proteolytic degradation of the ECM occurs due to an increased activity of proteases, including matrix metalloproteinases (MMPs) in response to vascular injury and during atherosclerosis [39]. Excessive protease activity and consequent ECM degradation contributes to fibrous cap thinning and collagen content, which are typical features of plaque vulnerability and rupture [45] [39]. MMPs are matrix-degrading enzymes involved in the degradation of collagen, elastin and other components of ECM and thereby play a pivotal role in the regulation of SMC behavior. MMPs are synthetized and secreted as inactive zymogens (proMMPs) requiring extracellular activation by other classes of proteases [46]. Cytokines can increase the production of MMPs and processing of proMMPs to the active form [47]. Bond et al. demonstrated that a synergistic stimulation of human or rabbit fibroblast with IL1-ß or TNFα and PDGF-BB growth factor, promote the SMC synthesis of several MMPs, including MMP-1, MMP-3 and -9, which in turn degrade ECM components [47].

2.3 CARDIOVASCULAR RISK FACTORS

CVD represents the main cause of morbidity and mortality in most countries. The identification of major risk factors through population-based studies and effective control strategies, combining community education and targeted management of high-risk individuals, have contributed to the fall in CVD mortality rates that has been observed in almost all industrialized countries [48].

A risk factor is a condition that increases the chance of developing a certain disease, in this case CVD. This association is almost always done on a statistical base and if a certain person has a certain risk factor, it only increases the probability of developing a certain disease [49].

The cardiovascular risk factors can be subdivided in non-modifiable and modifiable.

The main **non-modifiable** risk factors are:

- **Age**: the cardiovascular risk increases with the advancing age. More than half of those individuals who suffer heart attacks are 65 or older [49].
- **Gender**: statistical data suggest that men are more predisposed than women to the development of coronary heart disease (CHD) or other related disease. In women the cardiovascular event's risk greatly increases in menopause when estrogen levels are reduced. This fact supports the hypothesis that female hormones have protective effects [50].
- **Ethnicity**: a higher incidence of diabetes and moderate and severe hypertension was found among African Americans, conditions that increase the risk of cardiovascular diseases [49].
- **Genetics**: family history of CVD is an important risk factor for premature CHD; this risk increases linearly with increase in number of affected family members [51]. Familial Hypercholesterolemia (FH) is an autosomal-dominant genetic disease responsible for premature coronary heart disease. FH is the result of mutations in the LDL receptor, apolipoprotein B (ApoB), proprotein convertase subtilisin/kexin type 9 (PCSK9) and apolipoprotein E (ApoE) genes, that lead to LDL accumulation, mainly in the plasma [52]. Consequently, individuals with FH have a greater risk of coronary heart disease than normolipidemic individuals [53].

The modifiable risk factors are those on which it is possible to intervene through pharmacological treatments and/or by modifying the individual's lifestyle. The **modifiable** risk factors include:

- **Hypertension**: blood pressure is considered "high" and "uncontrollable" when the systolic blood pressure is ≥ 140 mmHg or diastolic blood pressure is ≥ 90 mmHg[54]. Several studies have shown that raised

blood pressure is among the leading global risk factors for mortality and it is responsible for 9.4 million deaths each year [54].

- **Hypercholesterolemia and hypertriglyceridemia**: lipids are essential for steroid hormones production and bile acids formation; they also provide cell membrane support, but elevated levels of lipids (cholesterol and triglycerides) are the second-leading physiological risk factor for ischemic heart disease, after high blood pressure[54]. Hypercholesterolemia is defined as total cholesterol ≥ 190 mg/dL or ≥ 0.5 mmol/L [54].

  Two kinds of lipoproteins carry cholesterol throughout the body: LDLs, also known as “bad cholesterol”, and high-density lipoproteins (HDLs), called instead “good cholesterol”. A high LDL level often leads to accumulation of cholesterol in the walls of arteries, and this is a critical point for the onset of atherosclerosis [54].

  According to the new guidelines on dyslipidemias by the European Society of Cardiology (ESC) and the European Atherosclerosis Society (EAS)[55], patients are classified according to the absolute risk of incurring a cardiovascular disease:

  - Very very high-risk patients: people who undergo a second cardiovascular event within 2 years from the first, already being treated with the highest tolerated dose, reduction of LDL < 40 mg/dl.
  - Very high-risk patients (10-year risk of cardiovascular mortality > 10%): it is recommended a reduction of LDL cholesterol of at least 50% from baseline and a target LDL < 55 mg/dl.
  - High-risk patients (risk of death from cardiovascular causes between 5 and 10% in 10 years): reduction of LDL cholesterol of at least 50% compared to baseline and a target LDL < 70 mg/dl.
  - Moderate risk patients (risk of death from cardiovascular causes between 1 and 5% in 10 years): the LDL cholesterol target should be < 100 mg/dl.
  - Low-risk patients (risk of death from cardiovascular causes < 1% in 10 years): the LDL cholesterol target < 116 mg/dl is optimal.

- **Obesity**: it can increase cardiovascular disease morbidity and mortality directly and indirectly. The direct effects are due to the structural and functional adaptations of the cardiovascular system to accommodate excess body weight, as well as the effects of adipokines on inflammation and vascular homeostasis. The indirect effects are mediated by coexisting CVD risk factors such as insulin resistance, hyperglycemia, hypertension, and dyslipidemia [56].
- **Diabetes mellitus**: it is a chronic hyperglycemic disease condition attributed to defective insulin secretion or action or both [57]. The metabolic environment of Type 2 Diabetes Mellitus, the most prevalent form of diabetes, including insulin resistance, hyperglycemia, and release of excess free fatty acids, with other metabolic abnormalities affects vascular wall by a series of events including endothelial dysfunction, platelet hyperreactivity, oxidative stress and low-grade inflammation. Activation of these events further enhances vasoconstriction and promotes thrombus formation, resulting in the development of atherosclerosis [57].
- **Cigarette's smoke**: it is a major cause of coronary heart disease, stroke, and peripheral vascular disease; approximately 30-40% of the 500,000 deaths from CHD each year can be attributed to smoking. Individuals who smoke, regardless of family history and any other risk factors, are more likely to develop coronary heart disease[49]. Both active and passive cigarette smoke exposure predispose to cardiovascular events. More than 20 studies have shown a strong correlation between passive smoking and risk of stroke, meta-analysis have documented a 20-30% increased risk of stroke with a likely dose-response relationship [58].

  Cigarette smoking impacts all phases of atherosclerosis, from endothelial dysfunction to acute clinical events, the latter being largely thrombotic; it increases inflammation, thrombosis, and oxidation of LDL cholesterol [59].

2.4 CIGARETTE SMOKE

Cigarette smoking is one of the most powerful and prevalent addiction, influencing the behavior of human beings for over four centuries[60], and causes about 7 million deaths worldwide each year [61].

The well-known health risks of tobacco smoking especially relate to the respiratory tract and the cardiovascular system[60], but an influence on immune system [62] and a relationship with skin diseases and cancers [63] has also been assessed.

Tobacco smoke is generated by a series of combustion pyrolysis, distillation, and sublimation processes. As shown in Fig.4, there are two main regions inside a burning cigarette: a combustion zone and a pyrolysis/distillation zone. Inside the combustion zone temperature ranges from 700 to 950°C, the oxygen from air flow reacts with carbonized tobacco producing carbon dioxide, carbon monoxide and hydrogen. Immediately downstream of the combustion zone is the cooler pyrolysis/distillation zone (200-600°C), where the bulk of the more than 7000 chemicals contained in cigarette smoke are generated [64]. The super-saturated vapor rapidly cools within a few milliseconds in the tobacco rod and condenses forming an aerosol consisting of liquid droplets (the particulate phase) suspended with gases compounds.

When a cigarette is smoked, combustion takes place in two ways: during puffing, air is drawn into the cigarette and mainstream smoke is formed and directly inhaled by the smokers while, between puffs, sidestream smoke is released from the lit end of the cigarette into the atmosphere (Fig 4).

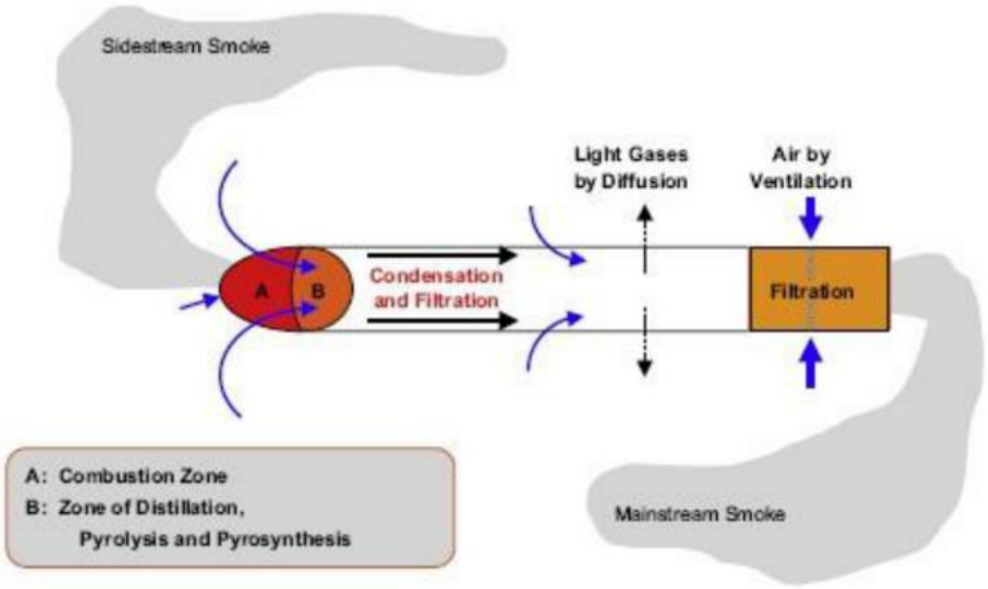


Figure 4. **mainstream and sidestream smoke in a burning cigarette.** Mainstream smoke is smoke directly inhaled into the smoker's mouth while sidestream smoke is smoke that enters the air from a burning cigarette [65].

Burning cigarettes produce as much as 7,000 different components in addition to nicotine, including polycyclic aromatic hydrocarbons (PAH), tobacco glycoproteins and some metals, many of which are known to be antigenic, cytotoxic, mutagenic, or carcinogenic, and most of them are generated by the burning of tobacco [66].

Since 1950s, a lot has been done for understanding smoke composition. Among the numerous toxic and carcinogenic components identified in tobacco smoke, there is a strong focus on the over 40 compounds, called "Hoffmann analytes" [65] (Table 1), a list of analytes drawn up thanks to the efforts of Dietrich Hoffman and collaborators of the American Health Foundation of New York.

Table 1. **Tobacco compounds included in the Hoffmann list.**

CHEMICAL CLASS	COMPOUND	CONTENT
Inorganic compounds	Ammonium	11.02 µg/cigarette
	HCN	109.2 µg/cigarette
	NO	**223.41 µg/cigarette**
	CO	**11.96 mg/cigarette**
Aromatic amines	2-aminonaphthalene	15.1 ng/cigarette
	1-aminonaphthalene	10.3 ng/cigarette
	3-aminobiphenol	3 ng/cigarette
	4-aminobiphenol	1.7 ng/cigarette
PAH	Benzo[a]pyrene	7 ng/cigarette
Carbonyls	Formaldehyde	21.61 µg/cigarette
	Acetaldehyde	560.48 µg/cigarette
	Acetone	264.74 µg/cigarette
	Acrolein	58.77 µg/cigarette
	Propionaldehyde	43.92 µg/cigarette
	Crotonaldehyde	16.8 µg/cigarette
	Methyl ethyl ketone	62.72 µg/cigarette
	Butyraldehyde	29.58 µg/cigarette
Metals	Mercury	3.82 ng/cigarette
	Nickel	5.12 ng/cigarette
	Lead	33 ng/cigarette
	Cadmium	47.8 ng/cigarette
	Chromium	73 ng/cigarette
	Arsenic	10.4 ng/cigarette
	Selenium	34.9 ng/cigarette
TNSA	N'-nitrosonornicotine (NNN)	133.6 ng/cigarette
	4-(methylnitrosoamino)-1-(3-pyridyl)-1-butanone (NNK)	115.6 ng/cigarette
	N'-nitrosoanatabine (NAT)	119 ng/cigarette
	N'-nitrosoanabasine (NAB)	16.3 ng/cigarette
Phenols	Hydroquinone	32.4 µg/cigarette

	Resorcinol	0.91 µg/cigarette
	Catechol	37.9 µg/cigarette
	Phenol	7.32 µg/cigarette
	m-/p-cresol	5.84 µg/cigarette
	o-cresol	1.89 µg/cigarette
Organic compounds	Acrylonitrile	8.28 µg/cigarette
	1,3-butadiene	29.94 µg/cigarette
Volatile hydrocarbons	Isoprene	297.7 µg/cigarette
	Toluene	64.91 µg/cigarette
	Styrene	5.11 µg/cigarette
	Benzene	43.39 µg/cigarette
Nitrogen heterocycles	Quinoline	0.23 µg/cigarette
	Nicotine	**0.75 mg/cigarette**
	Pyridine	7.02 µg/cigarette
Tar	**Tar**	**8.91 mg/cigarette**

Among these compounds, nicotine and carbon monoxide are the most studied [67] and seem to be the main responsible for cigarette toxicity.

Carbon monoxide (CO) is a colorless, odorless, tasteless, and non-irritating gas produced by the incomplete combustion of organic compounds[68]. The endogenous production of CO from the catabolism of hemoglobin is a physiological process. However, CO is toxic at low inhaled concentrations, and lethal at 1% [69]. CO binds with high affinity to hemoglobin (Hb) producing carboxyhemoglobin (HbCO) which has two important adverse effects. First, CO displaces O_2 from Hb, leading to a reduced oxygen content and anemic hypoxia. Second, the complex resulting from oxygen binding to the HbCO has decreased capacity of O_2 carrying and releasing to tissues [69].

CO poisoning causes direct cellular changes involving immunological or inflammatory damage by several mechanisms, such as binding to intracellular

proteins, NO generation leading to peroxynitrite production, lipid peroxidation by neutrophils, mitochondrial oxidative stress, apoptosis, immune-mediated injury, and delayed inflammation [69].
Although everyone has CO in their blood (< 5%), heavy smokers and those in certain occupations may reach 10% saturation [68].

Nicotine is an alkaloid found in the leaves of the tobacco plant *Nicotiana tabacum*. Cigarettes contain 8.4 mg of nicotine on average, of which 1-3 mg is absorbed through smoke inhalation [70]. Smoke particles carry the nicotine distilled from tobacco into the lungs, where the alkaloid is rapidly absorbed into the pulmonary venous circulation. Then nicotine enters the arterial circulation and reaches the brain, where it binds to nicotinic cholinergic receptors (ligand-gated ion channels that normally bind acetylcholine). Stimulation of these receptors leads to the release of a variety of neurotransmitters, most importantly dopamine which signals a pleasurable experience and is critical for the reinforcing effects of nicotine [71]. Therefore, although most of the toxicity of smoking is related to other components of cigarette smoke, it is primarily the pharmacologic effects of nicotine that is responsible for the addiction to tobacco [72].
While nicotine has not been shown to have direct atherogenic effects, through the stimulation of the sympathetic nervous system it could contribute to CVD [73]. Nicotine leads to neuronal and adrenal release of norepinephrine and epinephrine, respectively, and increases heart rate, blood pressure, and myocardial contractility. Nicotine constricts blood vessels, including those in the skin and coronary blood vessels [74].
Several evidence suggest that nicotine has profound immunological effects and inhibits both the innate and adaptive immune responses. Nicotine causes an altered immune response characterized by a reduced inflammatory response, a decreased antibody response and a reduction in T cell-receptor-mediated signaling [75]. Nicotine exposure also affects B lymphocytes development and activation, and alters the differentiation, phenotype, and function of antigen-presenting cells (APCs), including dendritic cells and

macrophages [75]. Some of the immunosuppressive effects of nicotine have been attributed to its effect on the α7-nicotinic acetylcholine receptor (α7nAChR). Nicotinic acetylcholine receptors (nAChRs) are cholinergic receptors that are activated by the neurotransmitter acetylcholine and differently from muscarinic acetylcholine receptors, nAChRs can also respond to nicotine [76]. The α7nAChR consists of 5 homomeric α7 subunits and it was originally discovered in the central nervous system [77]. However it is also expressed in a variety of non-neuronal cells including macrophages, T cells B cells and vascular endothelial and SMCs [78][79][80]. The activation of this receptor reduces the release of the pro-inflammatory cytokines TNF-α, IL-1β, IL-6 and IL-8 [60]. Nizri et al. demonstrated that upon immune activation, $CD4^+$ T cells upregulated α7nAChR expression. In addition, nicotine treatment was associated with a reduced production of Th1 and Th17 cytokines and NF-kB mediated transcription [79]. α7nAChR has also been related to the pathophysiology of atherosclerosis. Several studies using mouse models have described both anti and pro-atherogenic role of this receptor, being this topic still under debate.

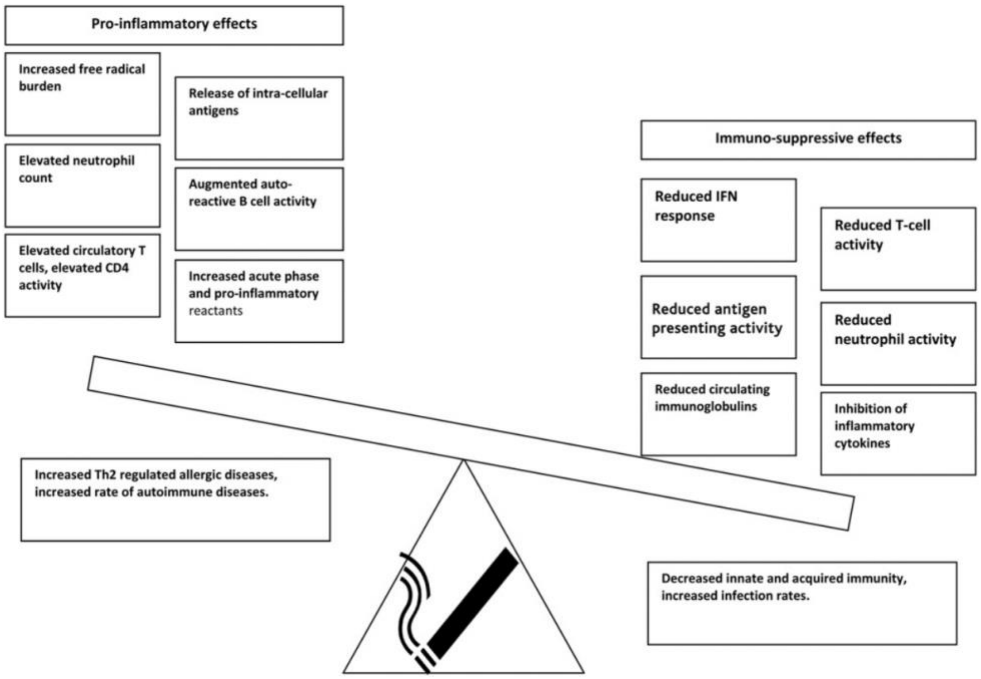


Figure 5. **Cigarette smoke effects on the immune system.** Cigarette smoke affects both the innate and adoptive immune response both increasing inflammatory and autoimmune diseases and decreasing innate and acquired immunity [60].

Chen et al. demonstrated that baroreflex arterial dysfunction promoted atherosclerosis in Apoe$^{-/-}$ mouse, in addition the stimulation of α7nAChR with PNU-282987, a selective agonist of α7-nAChR, attenuated the atherosclerosis process by inhibiting the production of pro-inflammatory molecules such as IL-6 and TNF-α and decreasing ROS level [81]. Similarly, Hashimoto et al. reported that the activation of α7nAChR with AR-R17779, a selective α7nAChR agonist, in Apoe$^{-/-}$ mouse, reduced atherosclerotic plaque area and decreased plasma pro-inflammatory cytokines [82]. Overall, these results suggest that the activation of α7nAChR attenuates the atherosclerotic process by exerting an anti-inflammatory effect. In contrast, Wang et al. observed that nicotine treatment increased the size of the atherosclerotic lesion in Apoe$^{-/-}$ mouse, in addition nicotine activated mast cells (MC) causing cell degranulation histamine release and pro-inflammatory cytokines production [83]. Interestingly, the administration of mecamylamine or α-bungarotoxin (α-BTX), two antagonist of α7nAChR, prevented nicotine-induced MC activation [83]. Due to all these effects, the α7nAChR represents a key element in the

pathophysiology of atherosclerosis and a promising target for the treatment of vascular inflammation and atherogenesis.

2.4.1 HARMFUL EFFECTS OF CIGARETTE SMOKE

The list of diseases related to tobacco use is expanding and a causal relationship has been reported between active smoking and CVDs, respiratory diseases, reproductive disorders, and several types of cancers including cancers of the lung, bladder, cervix, esophagus, kidney, larynx, mouth, pancreas, stomach as well as leukemia [84].

Tobacco use is the main preventable cause of cancer and cancer mortality, responsible for about one-third of all cancer deaths annually. Tobacco smoke contains more than 7,000 chemicals and more than 60 are known or suspected carcinogens, which cause DNA damage [85]. Repeated exposure to tobacco smoke can overwhelm DNA repair mechanisms and results in disrupted normal cellular growth and regulation [86]. More than 7 million people are killed each year owing to tobacco use and exposure to second-hand smoke (also called environmental tobacco smoke) is also a major cause of cancer and a main risk factor for air pollution. A study conducted in 2019 showed how cigarette smoking leads to a massive increase in the total particulate matter (PM) indoors, negatively affecting the quality of air and human health [63].
Lung cancer and chronic obstructive pulmonary disease (COPD) share a common environmental risk factor in cigarette smoke exposure, and COPD is also a major independent risk factor for lung carcinoma, among long-term smokers [87]. COPD is a chronic illness characterized by an abnormal inflammatory response of the lungs to toxic particles and gases, resulting in progressive and not fully reversible airway obstruction [88].
Cigarette smoking is also a well-known health hazard for patients suffering from asthma [89] since it increases exposure to endotoxin (one of the most potent inflammatory agents known) by far, and these high levels could contribute to an elevated IgE and to the development of asthma [60].

The organism's defense capacity is closely related to lung function; in fact, it has been shown that smoking compromises the antibacterial functionality of leukocytes including neutrophils, monocytes, T and B lymphocytes [88]. Cigarette smoking causes an elevation of the polymorphonuclear neutrophils (PMN) count and a reduction of their functionality. Exposure to cigarette smoke stimulates the bone marrow, resulting in the release of leukocytes and platelets into the circulation and thus leading to the increase of PMN count in the circulation of smokers [60]. Macrophages are the main lung cell population serving as first defense line against pollutants due to their antigen-presenting function and phagocytic properties. However, in smokers it has been observed an impaired function of these cells which are less mature and less developed; the phagocytic function is significantly reduced and smokers macrophages have grater inhibitory effects on the proliferation of lymphocytes and natural killer cells [60], thus leading to decreased body defense.

Cigarette smoking (CS) is a recognized risk factor for several chronic systemic diseases with inflammatory components [90]. Recently, evidence has suggested that epigenetic modification mechanisms could explain the link between CS exposure and inflammation. Smoking can activate the enzyme regulating these epigenetic modification (DNA methylation, histone modification, and ncRNAs) and thus mediate the expression of multiple inflammatory genes [91]. CS induces the production of pro-inflammatory cytokines, such as TNF-α, IL-1, IL-6, IL-8, and granulocyte-macrophage colony-stimulating factor (GM-CSF) [90]. Furthermore, it has been shown that smokers have increased susceptibility toward microbial infections and poorer wound healing; there is evidence for cigarette smoke immunosuppressive properties, specifically related to nicotine which decreases the levels of the anti-inflammatory cytokine IL-10 [90].

Lastly, should not be underestimated the effects of exposure to all types of passive smoke, for children. It has been demonstrated that secondhand smoke, especially maternal smoke, causes a statistically significant increase in the risk for infants of developing lower respiratory infections (LRI) in their first two years of life [92].

2.4.2 CARDIOVASCULAR EFFECTS OF CIGARETTE SMOKE

Tobacco smoke remains the leading preventable cause of death, and on average, smokers lose 10 years of life compared with people who never smoke. The CVD risk in low-use smokers is greater than suspected, since even one-cigarette-daily smokers have 40-50% of the increased CVD risk of those who smoke 20 cigarettes daily [93].

Both active and passive tobacco smoking promote all phases of atherosclerosis, from endothelial dysfunction to various types of CVD. Chemicals and toxicants contained in CS cause an increase in heart rate and myocardial contractility, endothelial impairment, prothrombotic state. Smoke is also related to an altered lipid metabolism with decrease of HDL-cholesterol serum levels [93], and hypoxia. Furthermore, over time, smoking enhances insulin resistance and is associated with central fat accumulation, leading to the development of metabolic syndrome and diabetes mellitus. Also related to increased risk for CVD events are arterial stiffness and hypertension, which once again are promoted by tobacco smoking [93].

Experimental and clinical data suggest that cigarette smoke exposure increases oxidative stress as a potential mechanism for initiating cardiovascular dysfunction. One of the earliest manifestations of the atherosclerotic changes in a vessel is impairment of vasodilatory function [59]. In animal and human models, exposure to CS extract has been shown to decrease nitric oxide (NO) synthase-mediated vasodilation [94].

The endothelium continuously synthetizes NO, a soluble gas with a wide range of biological properties aiming to maintain vascular homeostasis including modulation of vascular dilator tone, regulation of local cell growth, and vessel protection from platelets and cell circulating in blood [95]. Several *in vitro* studies have demonstrated that CS is associated with decreased NO availability [59], a condition that may cause coronary arteries constriction during exercise or mental stress and contribute to the development of myocardial ischemia in patients with coronary artery disease. Moreover, a

decrease in NO availability and bioactivity may facilitate vascular inflammation that could lead to oxidation of lipoproteins and foam cell formation. As a result, the impaired endothelium promotes lipoproteins oxidation, SMC proliferation, extracellular matrix deposition, lipid-rich material accumulation, platelet accumulation and thrombus formation [95]. Therefore a diminished bioactivity of NO could have both primary and secondary effects on the initiation and progression of atherosclerosis and on thrombotic events [59].

In addition, inflammation plays a major role in the initiation and progression of atherosclerosis. Several *in vivo* studies demonstrated a strong correlation between cigarette smoke and a higher level of multiple inflammatory markers including C-reactive protein, interleukin-6 and tumor necrosis factor alpha [96][97][98]. Moreover, the association between smoking and these inflammatory markers were similar in males and females [99].

2.4.3 NEXT GENERATION TOBACCO PRODUCTS

Combustion, pyrolysis, and other chemical reactions occur in burning cigarettes, leading to the formation of thousands of chemicals, some of which have been recognized as responsible for most of the damage related to tobacco smoking. For this reason, at the beginning of the 2000s, new devices were developed and appeared on the market allowing to smoke avoiding the tobacco combustion process.

There are two main products, alternative to traditional cigarettes:

- Electronic cigarettes or E-cigarettes
- Tobacco Heating Products or Heat-not-burn tobacco products

Alternative devices use is rapidly increasing among youth and adults with a complex potential impact on public health. These novel products have been advertised as healthier and less expensive than traditional cigarettes (TC) and are sometimes considered as smoking cessation aids. Nevertheless there is

limited knowledge about their long-term health effects, and research suggests that these alternative products may promote initiation and continued use among vulnerable populations, including youth [100].

2.4.4 TOBACCO HEATING PRODUCTS

Tobacco heating products (THP), also known as heat-not-burn products, are a form of nicotine delivery intended to be a less harmful alternative to traditional cigarettes [101]. These devices are composed of battery-powered holders, chargers and plugs or sticks of compressed tobacco, flavors, and other chemicals to produce a nicotine aerosol that imitates cigarette smoking [102]. THP differ from traditional cigarettes because they allow to avoid the combustion process typical of TC, since Heat-not-burn products only heat tobacco and do not burn it. In fact, the chemical complexity of cigarette smoke depends on the heating conditions inside the cigarette. In a conventional cigarette combustion occurs at temperature up to 700-950°C during puffs, and while combustion is limited to the tip of a burning cigarette, in the oxygen deficient distillation zone, in which temperature decreases from 600 to about 200°C, reactions such as pyrolysis and decomposition happen, leading to the generation of most of the smoke toxic compounds (Figure 6).

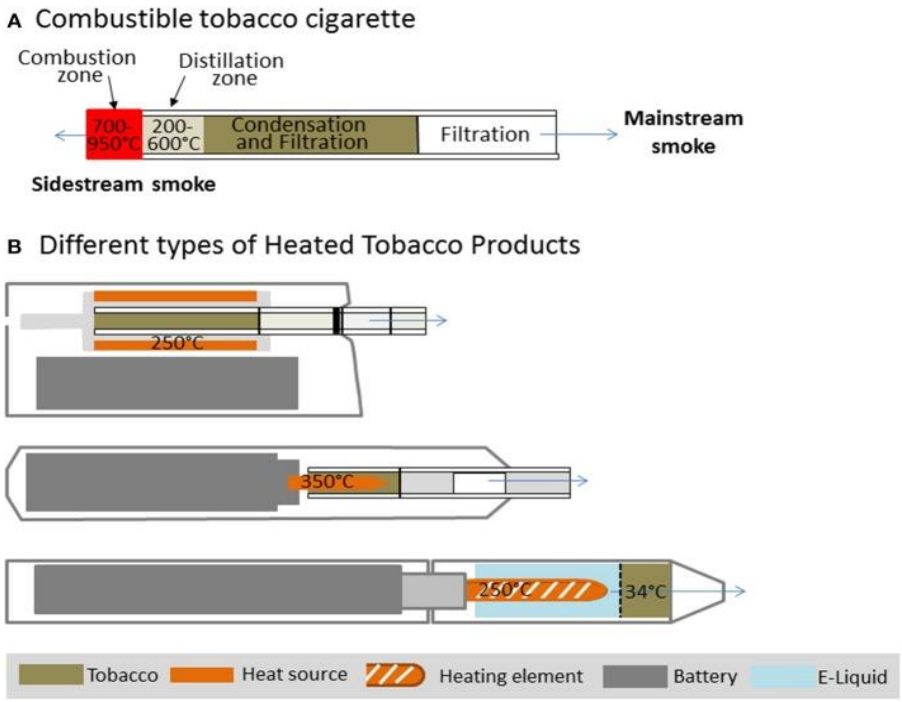


Figure 6. Temperature zones in a combustible cigarette (A) compared to different Tobacco Heating Products (B) [103].

Recently, trying to reduce the exposure to harmful and potentially harmful compounds in mainstream smoke (that is the inhaled and exhaled smoke while taking a puff on a lit cigarette), a new generation of THPs has been developed. These items differ in product design and temperatures applied to tobacco: in some devices tobacco is heated up to 350°C by an electrical heating source, while in other devices vapor is passed through tobacco and extracts compounds such as nicotine and flavors at lower temperatures [103]. GLO is a new device designed by the British American Tobacco (BAT) and comprises a battery-powered device that heats tobacco sticks to approximately 240°C (Figure 7). this process produces a nicotine containing aerosol with a tobacco taste which the user inhales.

The prevalence of THP use has been increasing, especially in highly developed countries such as Japan and Italy and based on this growing market, it is expected that the popularity of these innovative products will continue to increase. Tobacco companies claim that THPs, thanks to their

mechanisms avoiding combustion process, are less harmful than traditional cigarettes. Anyway, the potential impact of THP use on human health has not been fully investigated yet [104]. Moreover, it should be considered that the smoke released by these innovative products contains harmful elements from pyrolysis and thermogenic degradation common to TC smoke, like volatile organic compounds, polycyclic aromatic hydrocarbons, and carbon monoxide [105].

Figure 7. New GLO device owned by British American Tobacco.

2.4.5 E-CIGARETTES

Electronic cigarettes or E-cigarettes (E-CIG) are devices that work by vaporizing a nicotine solution combined with liquid flavors, totally avoiding the combustion process typical of traditional cigarettes. The main components constituting e-cigarettes, contained in a stainless-steel shell, are:

1. cartridge;
2. atomizer;
3. battery.

In the cartridge is stored a liquid mixture typically composed of propylene glycol and/or vegetable glycerin, water, and different types of flavors. The liquid mixture contains nicotine at different concentrations (0-36 mg/mL). The atomizer is activated by a pressure-sensitive circuit when the smoker inhales allowing the passage of air; the activated atomizer heats up the liquid inside the cartridge, thus producing a smoke-like vapor that is drawn through the mouthpiece and then inhaled [106]. However, in December 2018 these devices were improved through Puretech technology, promoted on BAT's iSwitch devices, which uses micro stainless blades to heat the liquid mixture and create the vapor (Figure 8).

Figure 8. I-SWITCH electronic cigarette produced by BAT.

Since E-CIGs do not burn tobacco, nor do they contain it, the composition of the resulting aerosol is significantly different from all other devices in which combustion or heating occurs. For this reason, there is the perception that these devices have fewer risks for human health, or even that they are totally inert; the logical consequence is that the number of E-CIG users has significantly increased over the past few years [107]. The main rationale of use among adult population is the belief that E-CIG are a less harmful choice, which can help with smoking cessation without suddenly stopping nicotine intake. Regarding the E-CIG use among youth, it is mostly induced by curiosity

and experimentation of the various interchangeable flavors of the liquid mixture. However, despite the increasing prevalence of use, there is limited evidence on the general safety and long-term public health impact of e-cigarettes, especially about their efficacy as a smoking quitting method and the potential for their use to lead to subsequent initiation of conventional tobacco product use among youth and young adults [108].

In 2016, FDA finalized a rule extending its authority to cover all tobacco products, including electronic systems. FDA regulates the manufacture, import, packaging, labeling, advertising and distribution. Since there are considerable variation in the chemical constituents and flavorings of various e-liquids and hundreds of different e-cig devices, it is also challenging to determine the potential for toxic health effects [109].

In addition, the chemicals in e-liquids can undergo transformations due to heating; among the most common components there are propylene glycol and glycerol, which when heated, oxidize and develop acrolein, associated with an increased cardiovascular risk, and formaldehyde, which is a known carcinogen[109]. Moreover, flavorings might contain alcohol, aldehydes and chemicals such as diacetyl and acetylpropionyl which are usually used by food manufacture to add creamy flavors but when inhaled they can cause pulmonary injury [110].

Furthermore, according to a report released by the National Academies of Sciences, Engineering and Medicine (NASEM), there is evidence of oxidative stress and development of endothelial cell dysfunction associated with exposure to e-cigarette aerosol [110]. Despite these findings, the NASEM report concluded that there is no evidence whether e-cigarette use is associated with clinical cardiovascular outcomes and subclinical atherosclerosis. For this reason, more research will be needed to determine more confidently the short- and long-term effects of e-cigarette use [109].

2.4.6 CARDIOVASCULAR EFFECTS OF NEXT GENERATION TOBACCO PRODUCTS

Traditional cigarette smoking is a harmful human habit causing several diseases, contributing to significant morbidity and mortality.

Conversely, next generation tobacco products, especially e-cigarettes and THPs, have been strongly advertised and marketed as a safer and healthier alternative to smoking, although these claims are not based on evidence. Data from both humans and animal models consistently demonstrate that use of e-cigarettes and THPs causes health effects both similar and different from those of cigarette smoking[111].

It must be considered that there are several limitations interfering with the full understanding of the cardiovascular impact of next generation tobacco use. On one hand, these new devices have been used as a method to quit smoking, resulting in long-term e-cigarettes/THPs use, rather than in cessation of all nicotine products. Therefore, it is difficult to understand if the effects are due to previous traditional smoking or to the switch to these innovative products[112]. On the other hand, since they only entered the international market in 2007, it may take decades to acquire epidemiological data on health outcomes from long-term use[111].

Several studies have been conducted in animal models, Rao et al. demonstrated that Sprague-Dawley rats exposed to JUUL (a type of E-CIG) for a very short period (5 min), exhibited an impairment in endothelial function comparably to Marlboro red cigarettes [113]. In another study, Espinoza-Derout et al. exposed $Apoe^{-/-}$ mice to e-cigarette aerosol containing 2.4% nicotine for 12 weeks, 12h/day. After treatment, mice developed impaired ventricular systolic function, increased the expression of pro-inflammatory markers and oxidative stress and cardiomyocytes exhibited ultrastructural abnormalities indicative of cardiomyopathy [114]. These studies, demonstrate that acute e-cigarette exposure affects vascular endothelial function and the inflammatory response.

Studies on the acute effects on human subjects have particularly proved that e-cigarette exposure increases cardiac sympathetic responses, heart rate and

blood pressure; arterial stiffness is also increased even after a short exposure to e-cigarettes (Fig.9).

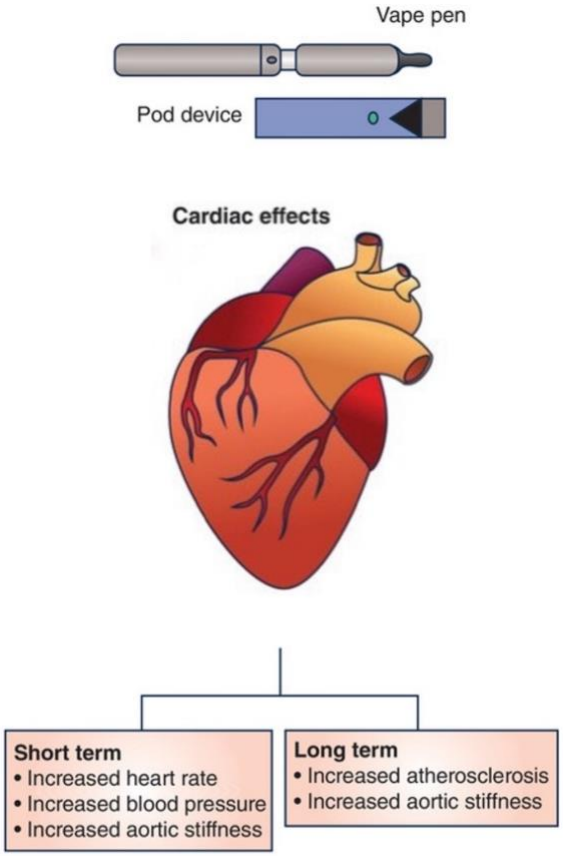


Figure 9. **Effects of e-cigarette on cardiac physiology.** Acute and chronic exposure to e-cigarettes aerosols, increases blood pressure, heart rate arterial stiffness promoting the formation of the atherosclerotic plaque. Adapted from Tsai et al. [115]

Antoniewicz et al. evaluated in seventeen healthy occasional users of tobacco products the effects of e-cigarettes with and without nicotine on vascular function. Blood pressure, heart rate and arterial stiffness were measured at baseline and following 30 min exposure, at different time points. The study showed an acute increase in heart rate and pulse wave velocity (PWV), both arterial stiffness indicators, following exposure with e-cig containing nicotine.

In addition, both e-cig with and without nicotine, increased the systolic and diastolic blood pressure that remained elevated for 10 and 30 min respectively [116]. It is reasonable to expect that these cardiac effects may be caused by nicotine, which is one of the major constituents in e-liquids and is known to have significant effects on cardiovascular system, including activation of the sympathetic system and inducing atherosclerosis by impairing the endothelial function and the inflammatory response. Besides nicotine, e-cigarette aerosols contain a multitude of chemicals and the chronic inhalation of them will lead to many adverse effects on cardiovascular system, with an overall increased risk of myocardial infarct and cerebrovascular accidents [111].

Several research studies have shown that relatively high metals levels, including lead and nickel, which are known to be cardiotoxic, are found in e-cigarette aerosols; arsenic has also been found in some e-liquids.

Although carcinogen levels in e-cigarette emissions may be lower than those present in traditional cigarettes smoke, significant, levels of oxidant species, such as heavy metals and aldehydes, are found in e-cig emissions [112]. In this regard, a crossover, single blind study, was performed by Carnevale et al. in forty healthy subjects (20 smokers and 20 nonsmokers) [117]. They observed that all subjects, both smokers and nonsmokers, smoking both types of cigarettes led to a significant increase in the levels of NOX2-derived peptide and 8-iso-prostaglandin F2α, even though, the effects of the e-cig on these parameters were less pronounced compared to tobacco smoke [117].

Not only e-cigarettes, but also THP are potentially harmful to cardiovascular health, producing emissions of nicotine, particulate matter, benzene, acrolein, and tobacco-specific nitrosamines, even though the levels of these chemicals are lower than those of traditional cigarettes. A study of current smokers demonstrated similar acute effects of THPs and traditional cigarettes on heart rate, blood pressure, and arterial stiffness. Rats exposed to THP had similar vascular endothelial function impairment to those exposed to cigarettes, while cultured macrophages exposed to THP aerosol showed an increased oxidative stress, although less than that induced by cigarette smoke [118].

3. AIM

Numerous studies concerning the risk factors for the development of atherosclerosis are reported in literature [119]. Clinical evidence has defined smoking as a strong risk factor for many pathological conditions including cancers, respiratory and cardiovascular diseases [84]. Many experimental evidences classify cigarette smoking as one of the main "modifiable" risk factors for atherosclerosis [59].

Phenotypic switch that occurs in SMCs in a synthetic state plays a key role in the progression of atherosclerotic disease. SMCs, in presence of pro-atherogenic stimuli, tend to migrate towards the intima, where they undergo a phenotypic modulation [120].

From previous experiments, it was possible to demonstrate how SMCs treated with total particulate matter (TMP or CSC) are subjected to phenotypic modulation. A remarkable plasticity was observed in mature SMCs that allows their rapid adaptation to fluctuating environmental cues, especially during development and progression of vascular diseases such as atherosclerosis [24].

In response to vascular injury, the resident quiescent SMCs undergo transcriptional changes affecting both the downregulation of contractile proteins and concurrent upregulation of protein supporting a proliferative phenotype.

However, the knowledge about how cigarette smoke, especially from e-cigarette, which in the last decade have been placed on the market with the aim of providing consumer an alternative to traditional cigarettes, is able to induce damage to vasculature is limited. For this reason, our research, in collaboration with the British American Tobacco (BAT, Southampton, UK), was focused on observing the effect of aqueous extracts of both traditional cigarette and e-cigarettes, on SMC phenotypic modulation.

In this study we used, aqueous extracts (AEs) that, differently from the particulate matter, contains water-soluble cigarette smoke components from both particulate and vapor phase and represents 90-95% of the total weight of cigarette smoke [121].

The aims of the present study were: 1) To evaluate the potential role of AEs in inducing a phenotypic modulation in cultured vascular SMCs, we investigated several parameters such as expression of extracellular matrix genes, cytoskeletal morphology and SMC proliferative index and migratory activity
2) To better unravel the effects and the differences between traditional cigarette and e-cigarette we measured the expression of pro-inflammatory markers and matrix remodeling genes that play a pivotal role in plaque vulnerability.

4.MATERIAL AND METHODS

4.1 METHODS FOR IN VITRO STUDY OF CIGARETTE SMOKING EFFECTS

The approximately 7,000 components constituting cigarette smoke are distributed between particulate matter and gas phase. Particulate matter, comprises water, nicotine and nicotine-free dry particulate matter (NFDPM or tar) and contributes only 5% to the total weight of the smoke. The remaining 95% is the gas phase, containing other several important chemicals, such as ammonia, carbon monoxide and nitrogen oxides.
British American Tobacco researchers have developed some methods to in vitro evaluate the biological effects of each cigarette smoke fraction. The fraction that can be used are:

Whole smoke: it is the total smoke, including both the particulate matter and the gas phase.

Total particulate matter (TPM): it represents 5% of whole cigarette smoke. The extraction method contemplates the use of a Smoke Machine. TPM is trapped by a special glass fiber filter (Cambridge filter) while the gas phase passes through it[122]. TPM is then eluted with dimethyl sulfoxide for 30 minutes with shaking to make up 20 mg/ml.

Aqueous extract (AE): it accounts for 95% of whole cigarette smoke and consists of all water-soluble components from both particulate and vapor phases. For these reasons it seems to simulate both a real composition of cigarette smoke and the actual passage of the soluble smoke components into body fluids, such as saliva and lung fluids. The obtaining of aqueous extract is based on cigarette smoke bubbling through a phosphate buffer saline (PBS) or through cell culture media, within which the soluble components are trapped.
Both TPM and AE include polycyclic aromatic hydrocarbons, tobacco-specific nitrosamines, phenol and nicotine.

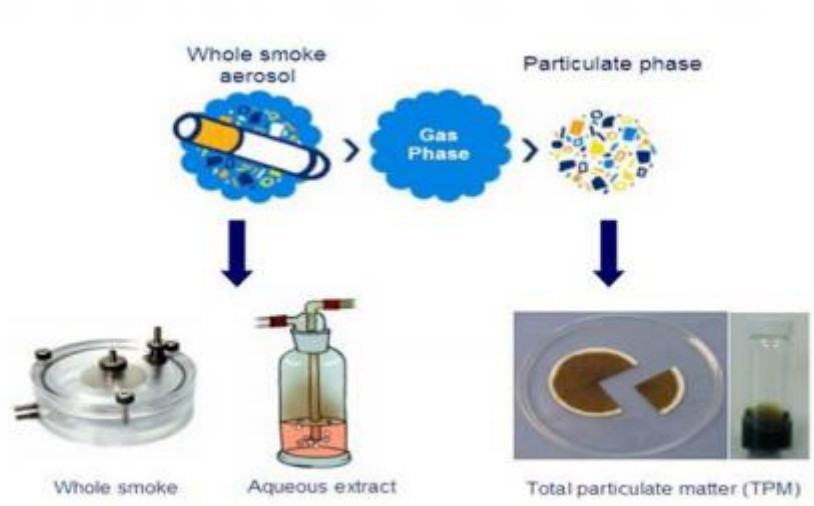


Figure 10. **Cigarette smoke fractions used for analysis:** Whole Smoke, Aqueous extract, Total particulate matter.

4.2 SMOKE EXTRACTS

British American Tobacco (BAT) periodically provides us aqueous extracts of traditional cigarette (TC) and electronic cigarette (E-CIG).

The protocol developed by BAT to generate AEs requires the use of a standard reference cigarette bubbled in 20 ml of cell culture medium at room temperature; this corresponds to about 8 puffs made by their smoking machine (Borgwaldt-KC RM20H rotary smoking machine) and using the Health Canada Intense T-115 regime (Table 2).

Standard reference cigarettes, cataloged with the production batch code 1R6F, are used to prepare traditional cigarette aqueous extracts; these cigarettes are provided by University of Kentucky, by the Tobacco Research and Development Center.

Regarding the preparation of e-cigarette extract, the machines carry out about 24 puffs and using the CORESTA method (CRM81) (Table 2).

Filtration is not applied in any of the two different types of extracts, since BAT purpose is to simulate as much as possible a real situation of use, trying not to avoid any harmful compounds present in the cigarette smoke through filtration.

Aqueous extracts are sent us frozen in 1.5 ml aliquots and kept at -80°C until use.

***Table 2*. Smoking regimens.**

Product	Puff Regimen	Puff Volume (mL)	Puff Interval (s)	Puff Duration (s)	Puff Profile	Vent Blocking
Cigarette	HCI	55	30	2	Bell	100%
THP	HCI_m	55	30	2	Bell	No
Vaping product	CRM No81	55	30	3	Square	N/A

HCI **[1]**
HCI_m same as HCI without vent blocking
CRM No81 **[3]**
N/A = not applicable

4.3 CELL CULTURES

In our experiments we used human aortic smooth muscle cells. Smooth muscle cells were purchased from the American Tissue Culture Collection (ATCC, Manassas, USA) and were used between the fourth and the seventh passage. This type of cells grows in adhesion, with an elongated morphology. The culture medium used is ATCC Vascular Cell Basal Medium, added with 500 µl of ascorbic acid, 500 µl rh EGF, 500 µl rh insulin and rh FGF-b, 25 ml of glutamine, 25 ml of FBS (ATCC Vascular Smooth Muscle Growth Kit) and 5 ml of Penicillin-Streptomycin 100X (Euroclone, Milan, Italy).
Cells were grown in 100 mm dish plates until they have reached confluence. Then, depending on the conditions of the experiment, they were plated in different sizes of plates: 60 mm, 35 mm or smaller.

4.4 CELL VIABILITY (MTT ASSAY)

For the experiment, SMCs were seeded on 48-well plates and treated for 48 hours at 37°C with increasing concentrations of AE (10%; 25%; 50% and

100%). At the end of the incubation, 300 µl of MTT solution (7.5 mg/ml in PBS) was added to each well and incubated for 3 hours at 37 ° C.
Subsequently the medium was aspirated and 100 µl / well of isopropanol / HCl solution (24:1) was added. After a few minutes, with the use of the spectrophotometer (BIO RAD, Berkley, California), the absorbance at 595 nm was evaluated. The following formula was used to calculate vitality:

$$\text{Vitality (\%)} = \mathbf{T/C} \times 100$$

in which **T** corresponds to the treated sample absorbance and **C** to the control absorbance.

4.5 PROLIFERATION ASSAY

SMCs were seeded in 24-well plates at density of 2 x 10^6 cells/well. After 24 hours, media were removed, and cells were incubated for 72 hours with medium containing 0,4% FBS to synchronize cells at G_0 phase of the cell cycle. After 72 hours control dishes were counted with the Coulter Counter (Beckam Coulter, Life Scientific, Milan, Italy) and this was considered the basal number of cells at t_0. Consequently, cells were treated with AE 10% in medium supplemented with 10% of FBS for 24, 48 and 72 hours. Cells were counted and the numbers compared to zero time-point.

4.6 IN VITRO DIRECTIONAL MIGRATION – WOUND HEALING ASSAY

Cells were seeded in 24-well plates and grown to confluence. Cell monolayers were scratched with a sterile 200 µl pipet tip in a straight line, and then washed with growth medium to remove detached cells. At this point, cell monolayer was incubated with 10% AE in medium containing 0.4% FBS to minimize proliferation.

Images of the wounded area were acquired at same spot at different time points (4, 8, 12 and 20 hours) using an inverted microscope (AXIOVERT 200, Carl Zeiss, Oberkochen, Germany, 5X objective lens) equipped with a digital camera.

Quantification of the wound area was performed with ImageJ and cell migration was expressed as a percentage of the wound areas at different time-point compared to initial wound area (t0). The higher the percentage, the higher the migratory activity of the cells following the treatment.

4.7 BOYDEN CHAMBER

The Boyden Chamber assay is a sensitive and useful method for rapidly testing multiple treatments that might alter cell migration[123]. It is based on a chamber of two compartments separated by a microporous membrane. Cultured smooth muscle cells are plated in the upper compartment and are left to migrate through the pores of the membrane into the lower compartment, in which a chemotactic agent is present (Fig. 11)[124].

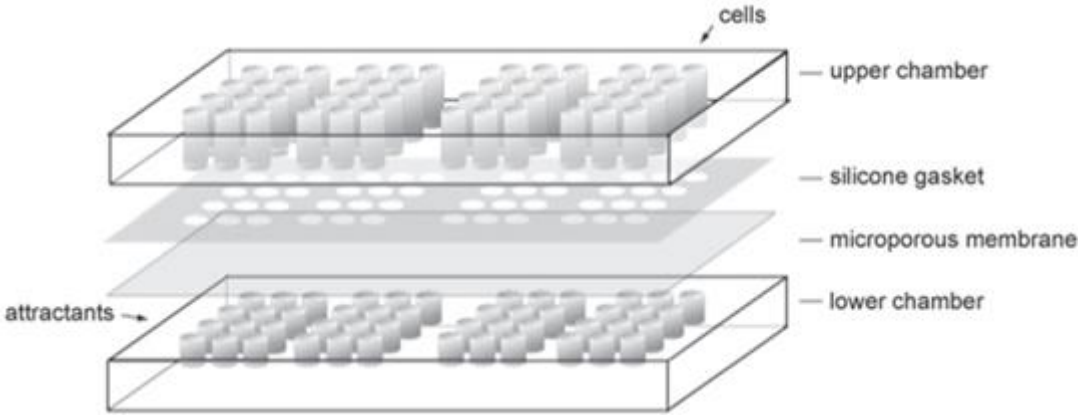


Figure 11. Components of Neuro Probe standard 48-well chemotaxis chamber[124].

Cells were seeded in 35 mm plates and grown to confluence in medium with 5% FBS. Once confluence was reached, medium was aspirated, and cells were incubated with 10% AE for 48 hours.

The Boyden chamber and the polycarbonate membrane were purchased from Biomap (Agrate Brianza, Milan, Italy). The 8 µm microporous membrane was coated with a solution consisting of 0.67 ml collagen, 4 ml acetic acid 0.5 M and 15.33 ml deionized water filtered with 0.22 µm filter. The membrane was left in the solution and incubated at 37°C overnight.

Next day, 26 µl of PDGF 5 ng/ml were added to each well of the lower compartment (the volume should be a slight positive meniscus when the well is filled). The membrane was washed twice with PBS and then, using forceps, placed over the wells of the lower chamber, oriented so that the cut corner corresponds to the Neuro Probe trademark. A silicon gasket was applied over the membrane and the top chamber was placed over the gasket, pushing the top chamber down against the bottom chamber and tightening the thumb nuts. Then cells were washed twice with PBS and detached with trypsin, cell suspension was centrifuged at 1100 rpm for 4 minutes. The pellet was resuspended in medium 0.4% FBS and cells were counted to $5x10^4$ cells/ml. 50 µl of cell suspension were loaded to each well of the upper compartment, avoiding bubbles formation, and the whole chamber was then incubated at 37°C and 5% CO_2 for 6 hours.

After the incubation time, the membrane was lifted with forceps and immediately immersed first in a methanol solution of Fast Green (Diff-Quick Fix) at room temperature for 2.5 minutes, and then in a solution of eosin Y (Diff Quick I) for 2.5 minutes. Lastly, the membrane was transferred in thiazine dye (Diff Quick II) for 1 minute. The side of the membrane with the migrated cells faced down.

The membrane was then rinsed in distilled water, overturned (the migrated cells faced up now), and placed on a glass slide, not to moving the membrane once it encounters the slide. Using a cotton swab, the unmigrated cells were wiped from the top of the membrane.

Images of the migrated cells on the stained membrane were acquired using an inverted microscope (AXIOVERT 200, Carl Zeiss, Oberkochen, Germany, 20X objective lens) equipped with a digital camera. Quantification of the migrated cells was then performed with ImageJ and cell migration was expressed as the number of cells migrating per microscope field, compared to untreated cells.

4.8 RNA SEQUENCING AND DATA ANALYSIS

Total RNA was extracted from cultured SMCs exposed for 24 hours to AEs using a Qiagen miRNeasy Mini Prep Kit. Libraries were made using an Illumina TruSeq Stranded Total RNA Library Prep Kit and sequenced on a HiSeq 2500 Platform. The web tool iDEP.90 (http://bioinformatics.sdstate.edu/idep94/) was used for the analysis using counts data generated from FeatureCounts. GO (gene ontology) analysis and GAGE (Generally Applicable Gene-set Enrichment) pathway analysis were conducted to identify differentially expressed genes (DEGs) at the biologically functional level.

4.9 RNA PREPARATION AND REVERSE TRANSCRIPTION (cDNA synthesis)

Total RNA was extracted with the Direct-zol™ RNA MiniPrep Plus kit (Zymo Research, Irvine, USA) and RNA samples were retrotranscribed using the iScript gDNA Clear cDNA Synthesis kit (BIO RAD, Berkley, USA).
Starting from the RNA concentration detected by NanoDrop® ND-1000 UV/VIS spectrophotometer, we determined the µg of RNA to be retrotranscribed. a mix was prepared, one for each sample, with the following volumes of reagents:

Table 3. Qualitative and quantitative composition of the reagents required for cDNA synthesis.

iScript Reverse Transcription Supermix	RNA	Final volume
4 µl	16 µl	20 µl

The eppendorfs containing the mix were shaken on a vortex, centrifuged, and then loaded into the thermal cycler (MJ Mini, BIO RAD). The program used for the reverse transcription provided the following thermal cycle:

- 25°C for 5 minutes
- 46°C for 20 minutes
- 95°C for 1 minute
- cooling to 4°C

Once the reverse transcription program was finished, the cDNA synthetized was diluted with Water Nuclease Free until a final concentration of 5 ng/µl.

4.10 qRT-PCR

Quantitative real time PCR (qPCR) was performed by using iTaq Universal SYBR Green Supermix and specific primers for selected genes. Primers sequences used for qPCR analysis are shown in Table 4.

Primer	Forward	Reverse
hCOL1alpha1	5'-CACCAATCACCTGCGGTACAGAA-3'	5'-CAGATCACGTCATCGCACAAC-3'
hfibronectin	5'-GCCATGACAATGGTGTGAAC-3'	5'-GCAAATGGCACCGAGATATT-3'
hlumican	5'-GACAAAGGTCTGCTCATCCCA-3'	5'-ATGAAAGGCCGCTGTACCAT-3'
hdecorin	5'-GGGCTGGCAGAGCATAAGTA-3'	5'-CAGAGCGCACGTAGACACAT-3'
hPCNA	5'-AACCTGCAGAGCATGGACTC-3'	5'- TCATTGCCGGCGCATTTTAG-3'
hRAC1	5'-TCCGCAAACAGATGTGTTCTTA-3'	5'-ATGGGAGTGTTGGGACAGTG-3'
hIL-1ß	5'-CCAGGGACAGGACAGGATATGGAGCA-3'	5'-TTCAACACGCAGGACAGGTACAG-3'
hIL-6	5'-CCACTCACCTCTTCAGAACG-3'	5'-CATCTTTGGAAGGTTCAGGTTG-3'
hIL-8	5'-ATACTCCAAACCTTTCCACCC-3'	5'-TCTGCACCCAGTTTTCCTTG-3'
hα7nAChR	5'- GACAGCCGAGACGTGGAG-3'	5'-AGGCAGTGGCTTTACCGTG-3'
hGAPDH	5'-GCACCGTCAAGGCTGAGAAC-3'	5'-TGGTGAAGACGCCAGTGGA-3'
hMMP3	5'-TGTCCCGTTTCCATCTCTCTC-3'	5'-TGGTGATGTCTCAGGTTCCAG-3'
hMMP1	5'-ATGCTGAAACCCTGAAGGTG-3'	5'-GAGCATCCCCTCCAATACCT-3'

The analyses were performed with the CFX CONNECT TM Real Time System (BIO RAD) machine. PCR cycling conditions were as follows: 95°C for 1 min, 40 cycles at 95°C for 10 sec and 60°C for 30 sec.

Data were expressed by using ΔΔCq method. Cq or quantification cycle is the first amplification cycle at which the instrument measures a signal with an intensity that is greater than "threshold line", which distinguishes the background noise from a real amplification signal. Quantification cycle value is inversely proportional to the expression of the investigated gene.

In addition to target genes, a "housekeeping" gene (HSK) was inserted for each plate, allowing the normalization of the data. HSK gene was chosen

among the genes constitutively expressed in the cell and not subject to modulation driven by treatments to which cells were subjected. Several candidates were tested (GAPDH, 18S and ß-actin) to identify the most stable gene for the proposed experimental conditions.
For HSMCs the HSK gene used was the one coding GAPDH.
For the analysis, ΔCq was first calculated as the difference between Cq of target gene and Cq of HSK gene for the same treatment.

$$\Delta Cq = Cq_{sample} - Cq_{HSK}$$

Then the difference between ΔCq of control and ΔCq of treated sample was calculated, obtaining the ΔΔCq.

$$\Delta\Delta Cq = \Delta Cq_{treated\ sample} - \Delta Cq_{control}$$

Finally, the Fold Change (FC) was calculated, allowing to evaluate the expression of the gene of interest compared to control.

$$FC = 2 - \Delta\Delta Cq$$

4.11 WESTERN BLOT ANALYSIS

SMCs were seeded in two separate 35 mm petri dish in complete media. after 24 hours, cells were treated with AE 10% for 48h.
For the preparation of total cell lysates, SMC were washed with ice-cold PBS and lysed with lysis buffer (NaCl 150mM, TRIS 50mM pH 7.6, NONIDET P-40 0.5% and protease inhibitors (Merck, Milan, Italy)). Protein concentration was determined using the Pierce BCA Protein Assay Kit (Pierce, Rockford, USA), accordingly to manufacturer's instructions. Twenty µg of proteins and a molecular mass marker (Euroclone, Milan, Italy) were separated on 7.5/10%

sodium dodecylsulfate-polyacrylamide gel (SDS PAGE) under denaturing and reducing conditions. Proteins were then transferred to a nitrocellulose membrane by electroblotting technique.

Membranes were then placed on a shaking plate and covered with 5% non-fat dried milk in tris-buffered saline containing 0.2% of tween used as blocking buffer for 1 h.

The membranes were incubated overnight at 4°C with a diluted solution (5% BSA or non-fat dried milk) of the following human primary antibodies: anti-IL-1beta (1:2000)(Abcam, Cambridge, UK), anti-Rac1 (1:1000) (Cell Signaling, Danvers, USA), anti-PCNA (1:1000) (Dako, Glostrup, Denmark), anti-MMP3 (1:5000) (Abcam, Cambridge, UK), anti-MMP1(1:5000) (Abcam, Cambridge, UK), anti-GAPDH (1:10000) (Abcam, Cambridge, UK), anti- α Tubulin (1:1500) (Sigma Aldrich, St. Louis, USA)

The blots were washed with PBS-TWEEN and then exposed for 90 min at RT to a diluted solution (5% non-fat dried milk) of the secondary antibodies: goat anti-mouse (1:5000) (Abcam, Cambridge, UK), goat anti-rabbit (1:5000) (Abcam, Cambridge, UK). Immunoreactive bands were detected by exposing the membranes to ClarityTM Western ECL chemiluminescent substrates (Bio-Rad Laboratories) for 5 min and images were acquired with the Odissey FC system (LI-COR, Nebraska, USA). GAPDH and α Tubulin were used as loading control for protein normalization and quantitative densitometric analysis was performed with Image Studio software (LI-COR).

4.12 ELISA ASSAY

SMC were treated with 10% of AE for 48h. Then, cell culture medium was centrifuged at 13000 rpm for 10 min to remove debris. The supernatant was collected, and the levels of IL-6 and IL-8 were assayed using the IL-6 and IL-8 human ELISA kits according to the manufacturer’s instructions. The values were normalized with total cell protein contents, extracted from the cell

monolayer, determined by BCA assay (Thermo Fischer Scientific, Waltham, USA).

4.13 CONFOCAL MICROSCOPY

SMC were seeded onto sterile microscopy slides in 24 well-plates until 30% confluent and left to attach overnight.

The day after, growth medium was aspirated, and cells were incubated for additional 48 hours with 10% smoke aqueous extracts.

six slides were prepared in total, two for each condition:

- (1,2) CTRL
- (3,4) TC
- (5,6) E-CIG

After two days of incubation, cells were washed with PBS, fixed with 4% paraformaldehyde and then permeabilized with 0.1% Triton (100X) in PBS. Cells were washed three times, 10 minutes each with PBS and subsequently saturated with 5% BSA for 1 hour on a shaking plate.

Actin filaments were stained with fluorescent phalloidin (Alexa Fluor 488 phalloidin, (ThermoFisher Scientific, Waltham, USA) for 1h at room temperature and nuclei were stained with DAPI.

Images were captured with confocal microscope (FRET FLIM, 40x objective lens) and the fluorescence intensity measurement was executed using the Fiji/ImageJ software.

4.14 DATA AND STATISTICAL ANALYSIS

Data were presented as the mean ± SD (standard deviation) of three experiments carried out in triplicates.

When three groups were compared, data were checked for equality of group variances with Brown-Forsythe test and analyzed by one-way ANOVA test

followed by Dunnett's or Tukey's correction for multiple comparisons. Results were considered statistically significant for p-values < 0.05. Statistical analysis and graphical presentations were performed using GraphPad Prism 8 (GraphPad Software Inc., USA).

5. RESULTS

5.1 EFFECTS OF AEs ON SMCs VIABILITY

The first step in our experiments was to evaluate the cytotoxicity of AEs, with the aim of identifying a range of non-toxic concentrations that could be used in subsequent experiments.

For this purpose, human SMCs were incubated for 48 h with the extracts and cell viability was assessed by the MTT assay as described in “Materials and Methods”. Each experiment was performed at least three times and conducted in triplicate for each condition.

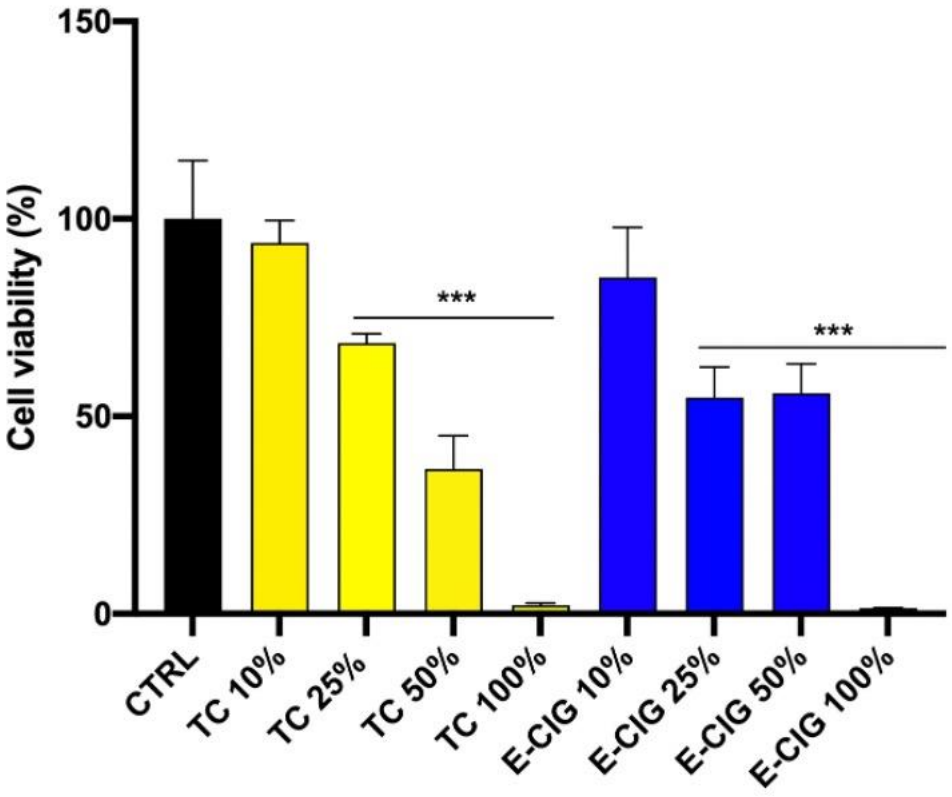


Figure 12. **In vitro cell viability assay.** Human SMCs were incubated with increasing concentrations of AEs (10%, 25%, 50% and 100%). The cytotoxicity was measured using the MTT assay after 48 hours. Data are expressed as mean ± SD of experiments conducted in triplicate. The p-value was determined by one-way ANOVA followed by Dunnett’s post hoc test and considered significant for *** $p < 0.001$ vs CTRL.

As shown in Figure 12, the addition of increasing concentrations of cigarette extracts caused a concentration-dependent cell toxicity, starting already from the 25% concentration and with a 100% cell death at the highest concentration tested (100%).
The concentration that did not cause any cell toxicity for all the extracts was at 10%. Therefore, this is the concentration that we used in the following experiments.

5.2 EFFECTS OF AES ON SMCs TRANSCRIPTOME PROFILE

In order to evaluate the transcriptional profile of SMCs, cells were exposed to TC and E-CIG extracts for 24 hours and RNA sequencing was performed using Hiseq 2500 Platform. Using a threshold of false discovery rate (FDR) less than 0.05 and a fold change greater than 1.3, we identified 281 upregulated genes and 184 downregulated genes upon TC treatment (Panel A1) and 216 upregulated and 147 downregulated genes following E-CIG treatment (Panel A2) Figure 13.

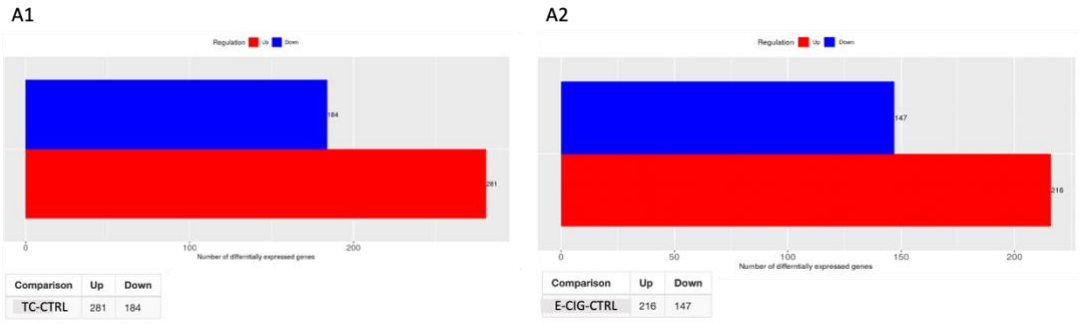


Figure 13. **Statistics of differential expressed genes (DEGs)identified by DESeq2**. Panel A1 and A2 show the numbers of differentially expressed genes upon treatment with TC and E-CIG vs CTRL, red and blue indicate genes upregulated or downregulated by TC and E-CIG treatment, respectively.

The up and down-regulated genes were then subjected to Gene Ontology (GO) biological process enrichment analysis, As shown in Figure 14, upon TC treatment, upregulated genes are related to ECM organization, locomotion and cellular response to chemical stimuli while down-regulated genes are related to regulation of cell migration, cell motility and response to lipids (Figure 14, Panel A1 and A2). In particular, we detected significant enrichment of CYP1A1, CYP2S1, CYP1B1, ADAMTS1, ADAMTS14, MMP-1, COL5A3 target genes among the upregulated genes. CYP1A1, CYP2S1 and CYP1B1 are a class of cytochrome P450 enzymes that are regulated by the aryl hydrocarbon receptor (AhR) and dioxin.

While ADAMTS1, ADAMTS14 and MMP-1 belongs to the MMP family and are involved in the degradation of ECM thus playing an important role in tissue remodeling. Moreover, COL5A3 belongs to the collagen gene family and is the main component of the ECM.

On the other hand, E-CIG treatment caused the downregulation of cholesterol and steroid biosynthetic processes while the upregulated genes are overwhelmingly involved in cell cycle, cell division and microtubule cytoskeleton organization processes (Figure 15, Panel A1 and A2).

A1

Enriched pathways in DEGs for the selected comparison:

Direction	adj.Pval	nGenes	Pathways
Down regulated	1.3e-14	18	Cholesterol metabolic process
	3.5e-14	18	Sterol metabolic process
	1.6e-13	13	Cholesterol biosynthetic process
	9.3e-13	13	Sterol biosynthetic process
	8.6e-11	19	Steroid metabolic process
	8.9e-10	20	Alcohol metabolic process
	1.4e-09	14	Alcohol biosynthetic process
	5.0e-09	14	Steroid biosynthetic process
	6.0e-09	22	Organic hydroxy compound metabolic process
	1.7e-08	35	Lipid metabolic process
	3.3e-08	15	Organic hydroxy compound biosynthetic process
	1.0e-07	19	Extracellular matrix organization
Up regulated	2.1e-71	112	Mitotic cell cycle
	5.9e-68	132	Cell cycle
	9.6e-68	104	Mitotic cell cycle process
	2.9e-66	118	Cell cycle process
	1.9e-52	77	Cell division
	5.0e-51	61	Chromosome segregation
	1.0e-50	68	Nuclear division
	4.1e-49	55	Nuclear chromosome segregation
	6.7e-48	60	Mitotic nuclear division
	1.7e-47	48	Mitotic sister chromatid segregation
	2.1e-47	50	Sister chromatid segregation
	1.5e-37	47	Spindle organization
	4.9e-37	44	Microtubule cytoskeleton organization involved in mitosis
	1.2e-34	41	Mitotic spindle organization

A2

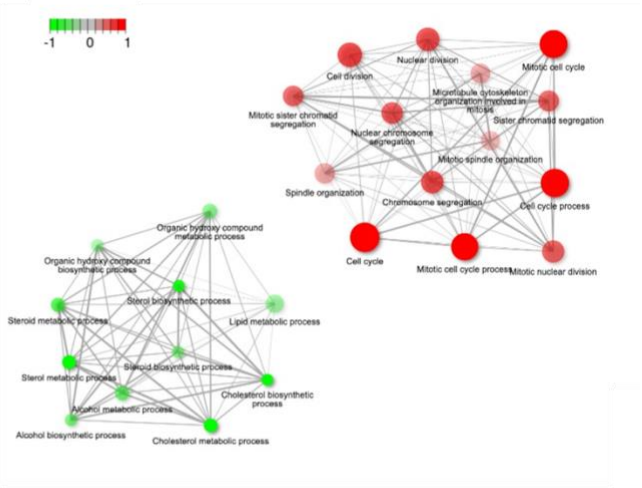


Figure 14. **Enriched GO terms in up and down-regulated genes upon TC treatment**. Panel A1 table list of enriched pathways, Panel A2 hierarchical clustering network enrichment, red and green indicate genes induced or suppressed by TC treatment, respectively.

A1

Enriched pathways in DEGs for the selected comparison:

Direction	adj.Pval	nGenes	Pathways
Down regulated	7.5e-06	57	Anatomical structure morphogenesis
	2.0e-05	44	Tissue development
	2.9e-05	33	Circulatory system development
	4.2e-05	22	Negative regulation of cell differentiation
	4.2e-05	61	Animal organ development
	4.2e-05	31	Anatomical structure formation involved in morphogenesis
	4.2e-05	12	Muscle cell development
	5.3e-05	23	Negative regulation of cell population proliferation
	5.3e-05	61	Response to organic substance
	5.3e-05	36	Cell migration
	5.3e-05	26	Response to lipid
	5.3e-05	22	Muscle structure development
	4.6e-07	65	Cell motility
	7.0e-05	44	Movement of cell or subcellular component
	7.5e-05	29	Regulation of cellular component movement
Up regulated	1.2e-09	86	Anatomical structure morphogenesis
	1.0e-08	80	Regulation of multicellular organismal process
	1.2e-08	105	Regulation of biological quality
	2.6e-07	90	Cellular response to chemical stimulus
	2.9e-07	42	Negative regulation of multicellular organismal process
	7.4e-07	89	Animal organ development
	7.9e-07	61	Cell population proliferation
	9.2e-07	33	Negative regulation of cell population proliferation
	1.2e-06	54	Regulation of cell population proliferation
	2.8e-06	59	Tissue development
	3.4e-06	72	Response to external stimulus
	3.4e-06	57	Locomotion
	7.7e-06	24	Extracellular matrix organization
	7.7e-06	41	Tube development

A2

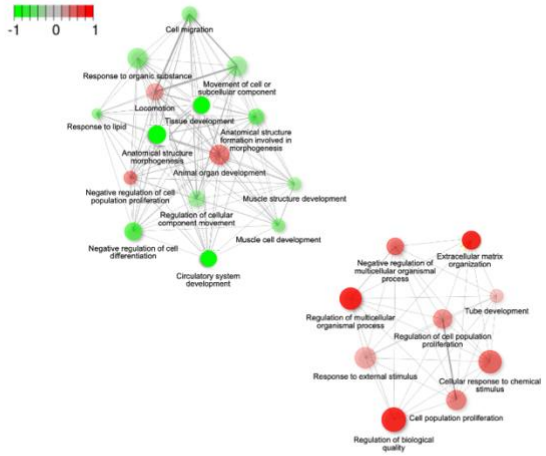
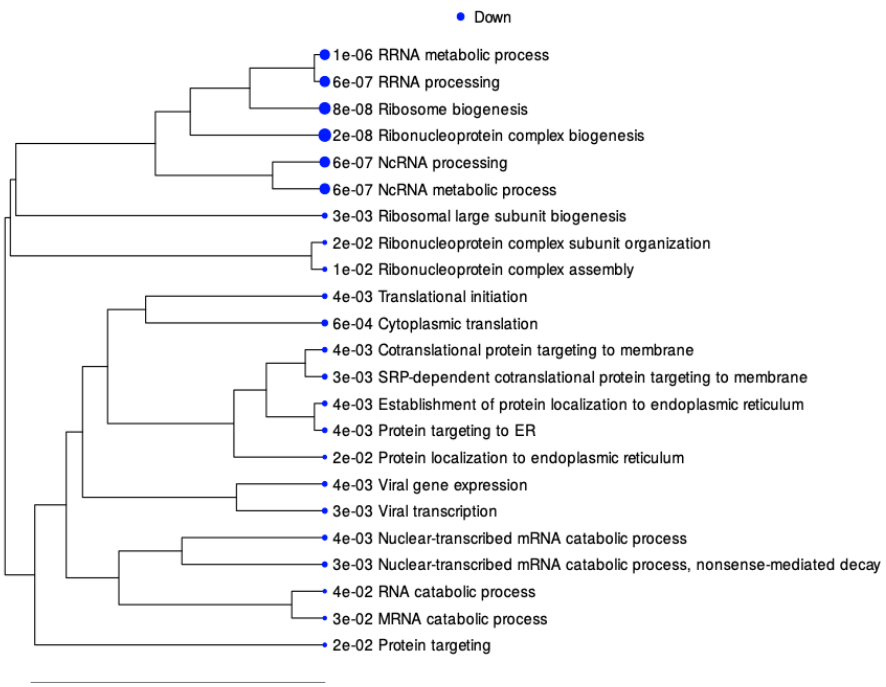


Figure 15**. Enriched GO terms in up and down-regulated genes upon E-CIG treatment**. Panel A1 table list of enriched pathways, Panel A2 hierarchical clustering network enrichment, red and green indicate genes induced or suppressed by E-CIG treatment, respectively.

GAGE pathway analysis on SMCs exposed to TC extract gave us additional information. In fact, as shown in Figure 16 Panel A1 and A2, TC treatment seems to negatively affect DEGs highly associated with pathways related to the biogenesis of ribosome biogenesis and ribonucleoprotein complex suggesting that TC may perturb ribosomal functions and protein biogenesis. Ribosome biogenesis and protein translation are finely coordinated with and play a central role in cell growth and proliferation [125]. Impairment of any of these pathways can perturb cell growth.

A1



A2

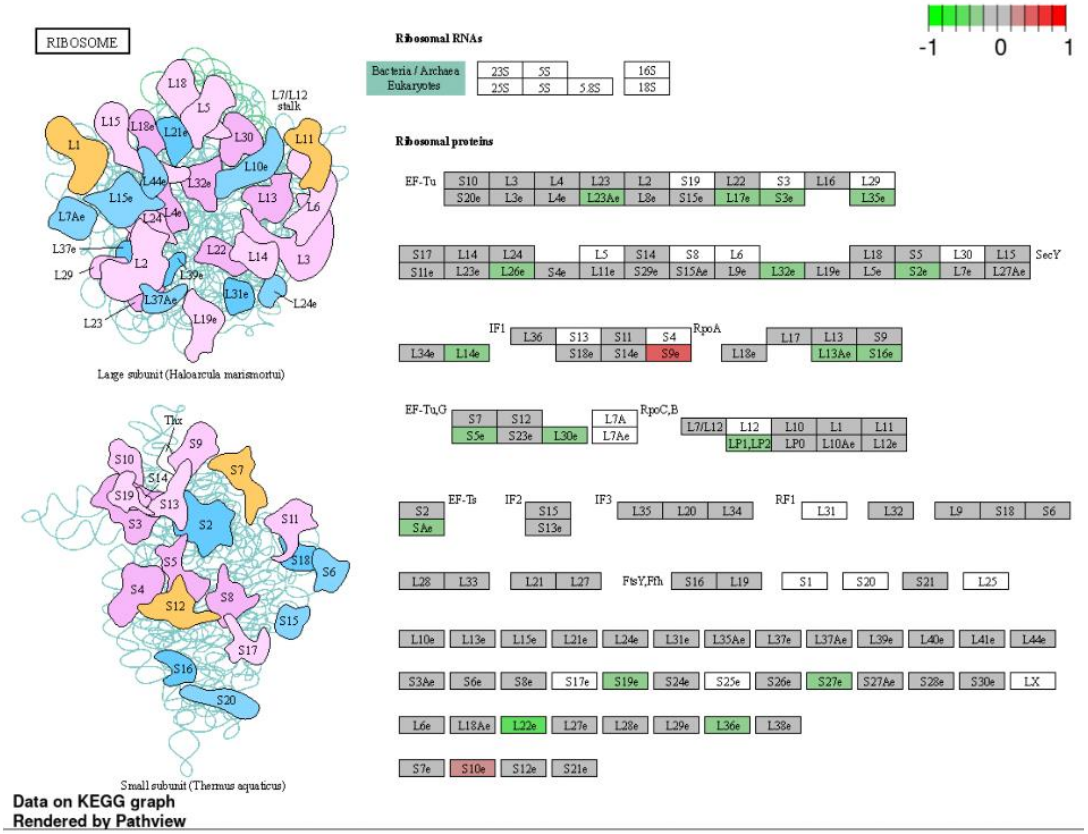


Figure 16. **Pathway analysis upon TC treatment.** Panel A1 GAGE pathway analysis on KEGG gene set, Panel A2 Expression profiles of ribosome related genes visualized on an KEGG pathway diagram, red and green indicate genes induced or suppressed by TC treatment respectively.

Conversely, PGSEA pathway analysis for E-CIG extract, gave us similar results that agree with our enrichment analysis based on DEGs. In fact, as shown in Figure.17 (Panel A1) the upregulated DEGs were highly associated with pathways including DNA replication and cell cycle processes. In addition, as shown in Figure.17 (Panel A2) among the most upregulated genes related to cell-cycle pathway we can found cell division cycle 20 (CDC20), cyclin dependent kinases (CDK1, CDK2) and E2F1,2,3, transcription factors. all these factors tightly control the progression of cell cycle thus regulating cell proliferation [126]. Notably, higher expression of CDC20 plays an important

role in tumorigenesis and progression of several cancers [127] [128]. Shang et al. demonstrated that overexpression of CDC20 promoted cell proliferation and cell invasion in osteosarcoma cells, while its deletion inhibited cell growth and induced cell cycle arrest [126].

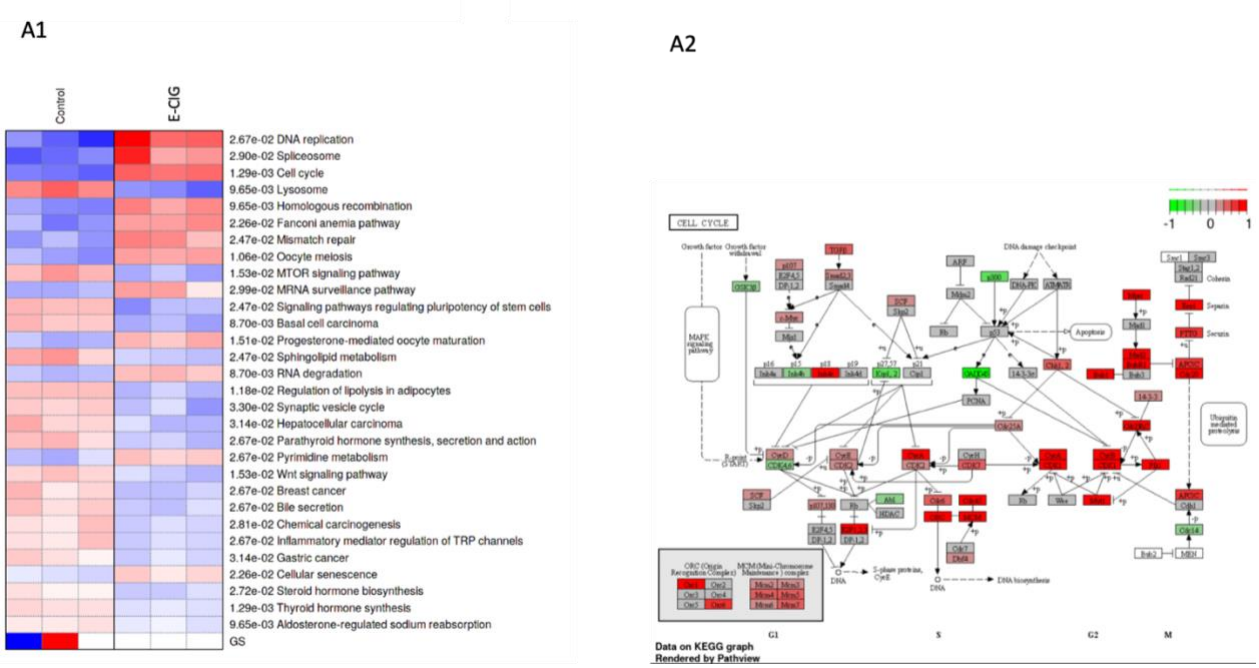


Figure 17. **Pathway analysis upon E-CIG treatment.** Panel A1 PGSEA pathway analysis on KEGG gene set, red and blue indicate pathways induced or suppressed by E-CIG treatment, respectively.

Panel A2 Expression profiles of cell-cycle related genes visualized on an KEGG pathway diagram, red and green indicate genes induced or suppressed by E-CIG treatment, respectively.

5.3 EFFECTS OF AEs ON EXTRACELLULAR MATRIX GENE EXPRESSION

In response to environmental cues or vascular injury, SMCs may modulate their phenotype, promoting the secretion of ECM components including collagen type I proteoglycans and fibronectin. Based on RNA-seq data,

we evaluated the effects of our AE on the expression of extracellular matrix components such as collagen type 1 alpha 1 (COL1alpha1), fibronectin, lumican and decorin.

Gene expression evaluation was carried out after RNA extraction and the subsequent reverse transcription and qRT-PCR analysis, as described in the dedicated section in “Materials and Methods”.

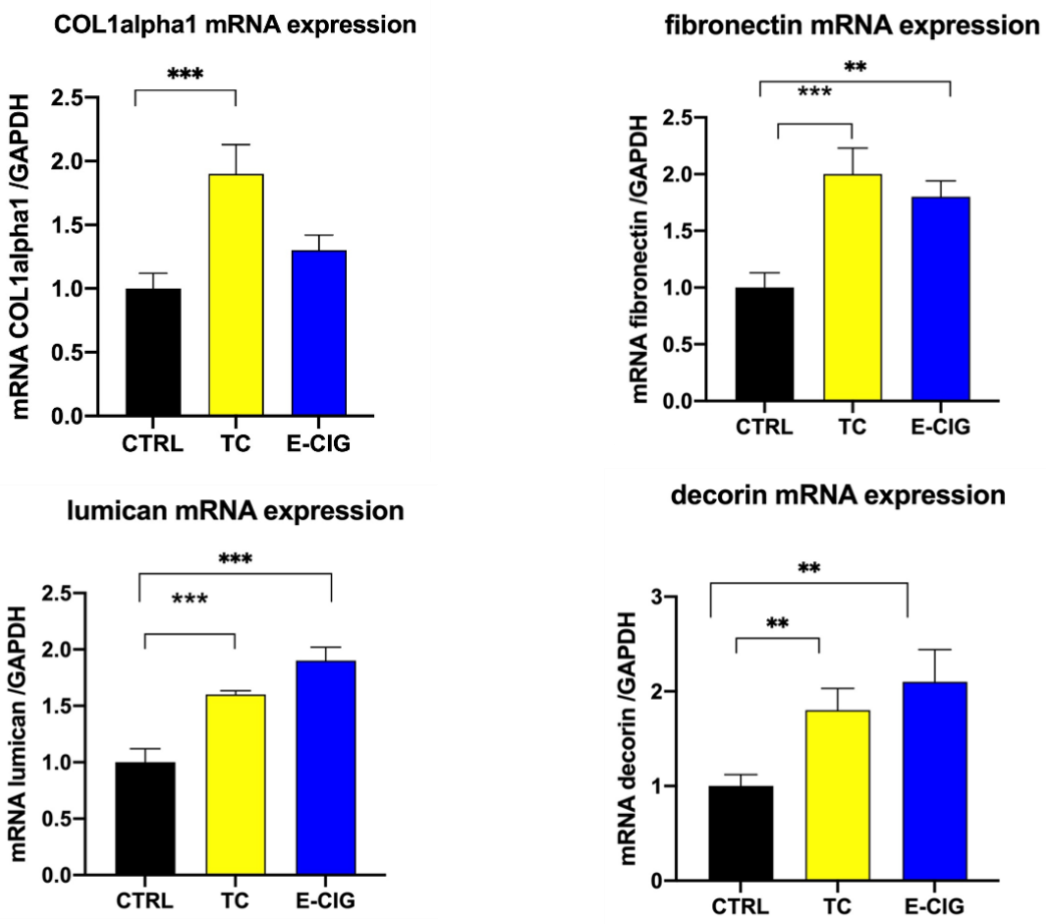


Figure 18. **Real time qPCR on extracellular matrix gene expression levels.** Cells were treated with AEs for 48 hours. RNA was then extracted, ECM markers expression levels evaluated by real time PCR. All experiments were performed at least three times. Data are presented as mean ± SD. The p-value was determined by one-way ANOVA followed by Dunnett’s post hoc test and considered significant for ** $p < 0.01$, *** $p < 0.001$ vs CTRL.

Figure 18 shows that both TC and E-CIG aqueous extract increase the expression of four ECM genes such as COL1alpha1(by about 1.9-fold and 1.3-

fold respectively), fibronectin and decorin (by 1.9-fold and 2-fold respectively) and lumican by 1.6-fold and 1.9-fold respectively compared to control (CTRL).

5.4 EFFECTS OF AEs ON SMC PROLIFERATION

SMC phenotypic modulation, occurring in the progression of atherosclerosis, is characterized by an increased proliferative capacity. For this reason, we measured the effects of aqueous extracts on SMC proliferation. Cell proliferation rate was assessed by cell counting after 24, 48 and 72 hours of incubation.

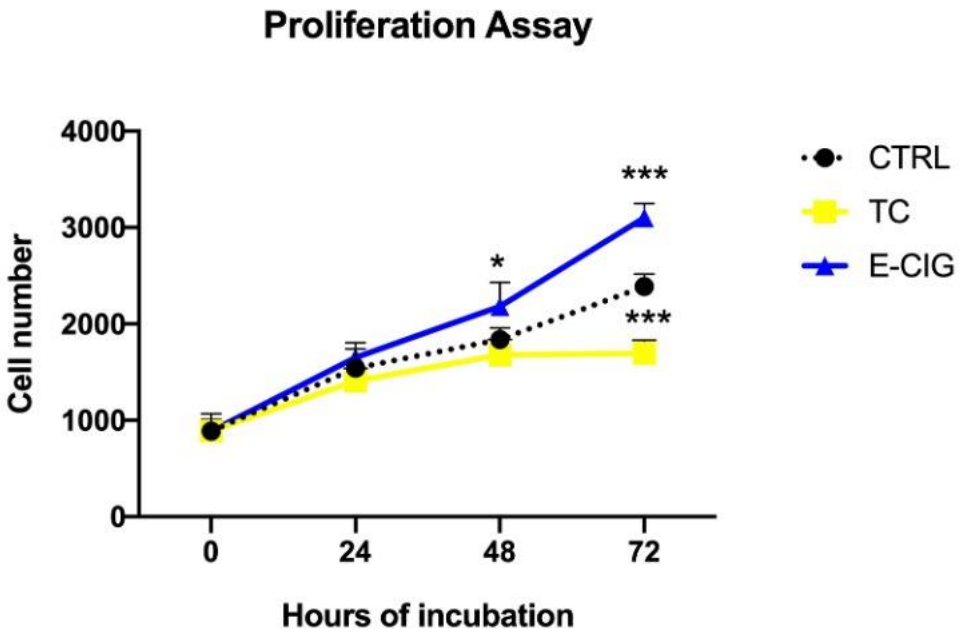


Figure 19. **Cell proliferation rate of SMCs.** SMCs were treated with 10% AEs for 24, 48 and 72 hours, cell counting was assessed by coulter counter apparatus. All experiments were performed at least three times and data are presented as mean ± SD. The p-value was determined by one-way ANOVA followed by Dunnett's post hoc test and considered significant for * $p < 0.05$ vs CTRL, *** $p < 0.001$ vs CTRL.

As shown in Figure 19, we observed a time dependent stimulation of SMC proliferation in the samples incubated with E-CIG. The effect started after 24 hours of incubation and reached a 30% increase after 72 hours.
The addition of TC extract instead reduced the proliferative rate compared to control, especially after 72 hours of treatment, where we observed a 40% decrease in the proliferative index.

5.5 EFFECTS OF AEs ON PROLIFERATING CELL NUCLEAR ANTIGEN GENE AND PROTEIN EXPRESSION

Proliferating cell nuclear antigen (PCNA) is an important molecular marker for cell proliferation. For this reason, we evaluated if smoke extracts modulate its expression both at gene and protein level.
RNA extraction and reverse transcription were performed after 48 hours-treatment of SMCs with TC and E-CIG extracts or 5% FBS growth medium (CTRL). Then gene expression was evaluated by q-RT-PCR.

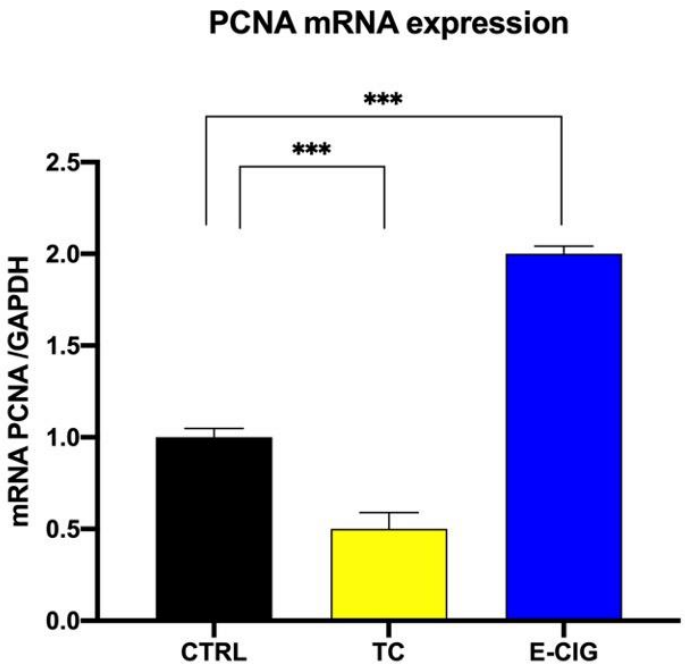


Figure 20. **Real time qPCR on PCNA expression levels.** Cells were treated with AEs for 48 hours. RNA was then extracted, and smooth muscle markers expression levels evaluated by real time PCR.
All experiments were performed at least three times and data are presented as mean ± SD. The p-value was determined by one-way ANOVA followed by Dunnett post hoc test and considered significant for *** $p < 0.001$ vs CTRL.

Figure 20 shows that, consistently with the proliferation experiment, E-CIG treatment doubled PCNA expression, while the addition of TC halved it.
PCNA protein expression was then evaluated by Western Blotting analysis, which confirmed the trend observed for gene expression, as shown in Figure 21.

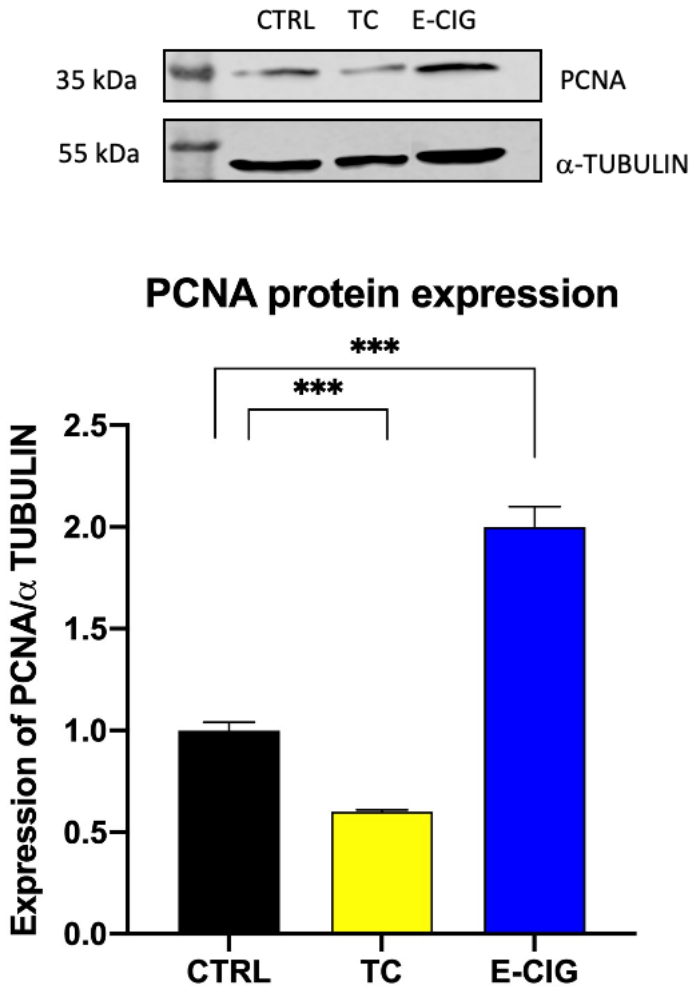


Figure 21. **Western Blotting analysis of PCNA expression**. SMCs were treated with 10% AEs. Cells were harvested after 48 hours and protein concentration evaluated with BCA kit. α-tubulin was used as loading control. All experiments were performed at least three times. Data are presented as mean ± SD. The p-value was determined by one-way ANOVA followed by Dunnett's post hoc test and considered significant for *** $p < 0.001$ vs CTRL.

5.6 EFFECTS OF NICOTINE ON SMC PROLIFERATION

Cigarette smoke, as mentioned before, is made up of over 7,000 different chemicals distributed between the particulate and gas phases. Due to the chemical complexity of cigarette smoke, is quite difficult to state which components might be responsible for the effects we have observed in the previous experiments.

The aqueous smoke extracts we used have at least one common chemical component, which is nicotine: although the concentration of nicotine is slightly different in the two samples.

- TC nicotine content = 4.3 µM
- E-CIG nicotine content = 6.1 µM

To understand whether the different behavior of traditional cigarette and the electronic cigarette on cell proliferation was due to nicotine, a proliferation assay was performed incubating SMCs with increasing concentrations of nicotine (from 1 µM to 10 µM), and at different time points (for 24, 48 and 72 hours). In addition, to verify the role of α7nAChR in nicotine-induced cell proliferation, α-bungarotoxin (α-BTX), a potent and selective antagonist of α7nAChR, was added to the culture media 30 min before nicotine treatment in order to evaluate whether α-BTX may counteract the effects due to nicotine.

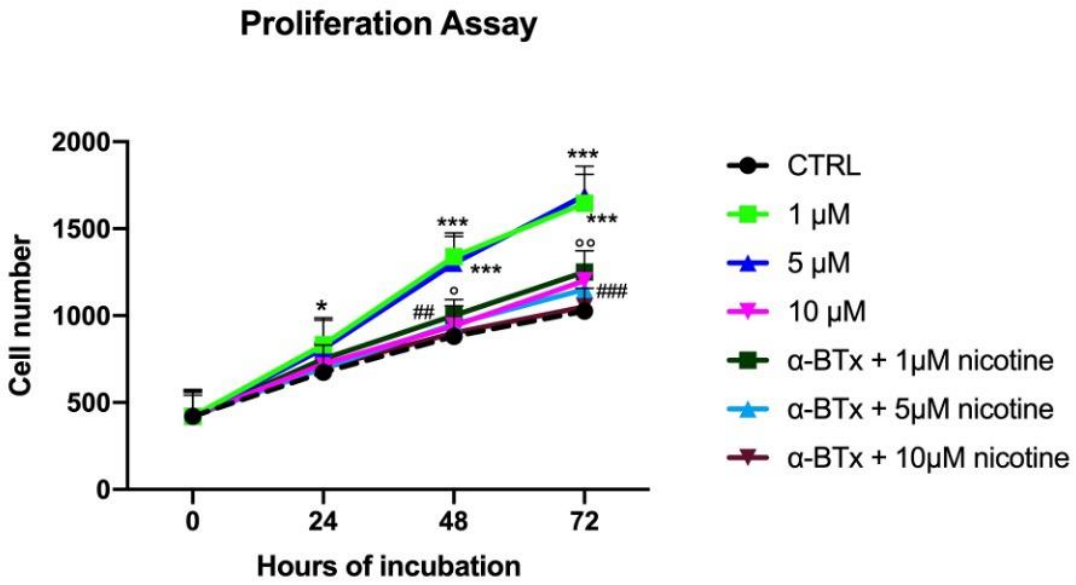


***Figure 22*. Cell proliferation rate of SMCs treated with nicotine.** SMCs were treated with different nicotine concentrations treatments: 1 µM, 5 µM and 10 µM for 24, 48 and 72 hours, with/without pretreatment with 1 µM α-BTX for 30 min. Cell counting was assessed by coulter counter apparatus. The p-value was determined by one-way ANOVA followed by Tukey's post hoc test and considered significant for * p <0.05 vs CTRL, *** p <0.001 vs CTRL, ° p <0.05 vs 1 µM, °° p <0.01 vs 1 µM, ## p <0.01 vs 5 µM, ### p <0.001 vs 5 µM.

As shown in Figure 22, after 24-hours of treatment, all the three different nicotine concentrations did not seem to affect cell proliferation, compared to control.

Conversely, after 48- and 72-hours the addition of nicotine at 1 µM and 5 µM stimulated SMC proliferation (+ 40%), and up to 50% after 72 hours, while the incubation with 10 µM nicotine is pretty much comparable with the control group, and only after 72 hours we observed a slightly increase. Conversely, the enhancement of nicotine in cell proliferation was partially reversed by pretreatment with α-BTX.

In fact, after 48 and 72 hours, α-BTX showed about 70-90% reversal of nicotine-induced cell proliferation at a concentration of 1 µM and 5 µM. These results, suggest that nicotine may promote SMC proliferation through the activation of the alpha7 nicotinic receptor.

5.7 EFFECTS OF AEs ON SMC MIGRATION

Another important feature that characterizes the phenotypic switch is represented by SMC migration, which was evaluated by the scratch test, or wound healing assay, and by the Boyden Chamber, as indicated in the “Materials and Methods” paragraph.

Cells were incubated with the different treatments and then cell monolayers were wounded with a sterile pipette tip. Images of three to four random fields of the wounded area were captured at time 0 (T0, start) and subsequently at different time-points (T4, T8, T10 and T20).

A stimulation of the migratory activity would result in a faster reclosure of the monolayer lesion.

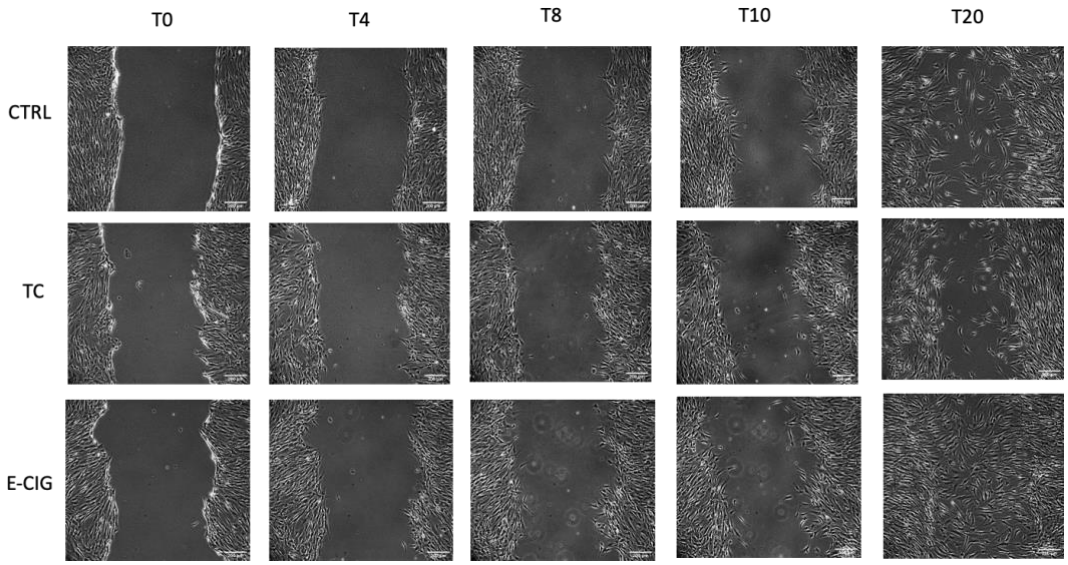


Figure 23. **Cell migration capability assessed by wound healing assay.** SMC were treated with 10% AEs and cell monolayers were scratched with 200 µl tips. Images were taken 0,4, 8, 10,20 hours after wounding. Quantification of the migrated cells was then performed with ImageJ. Scale bar, 200 µm.

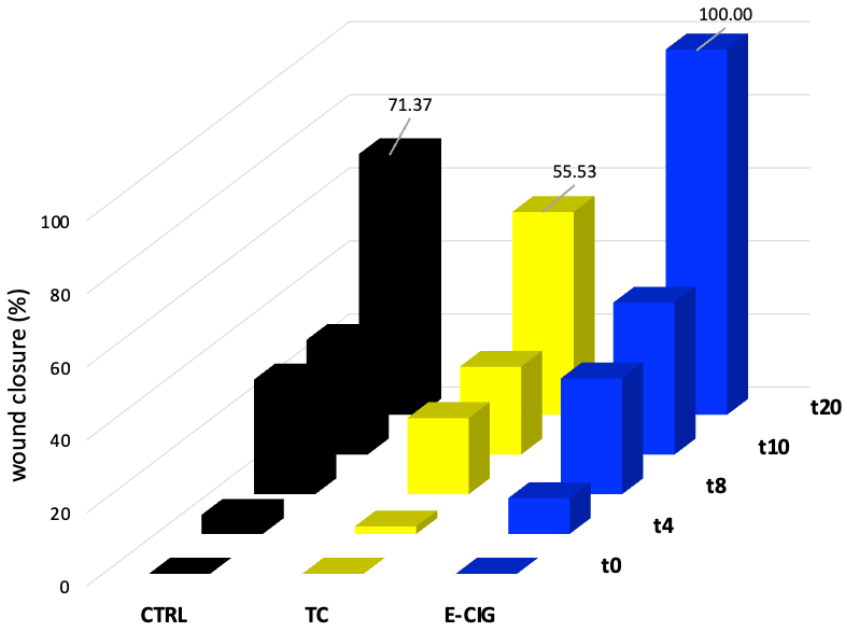


Figure 24. **Graphical quantitation of the % of wound healing at different time-points.** Cells were incubated as described in Figure 23.

As shown in Figure 23, E-CIG is the most effective in inducing a faster wound reclosure leading to a complete reclosure of the wounded area after 20 hours. It is important to note that the maximum effect obtained in control cells was only a 72% reclosure after 20 hours (Figure 24).

In the same experimental conditions, TC extract was less effective in stimulating SMC migratory activity (about 55% reclosure versus 72% in control after 20 hours).

Next, we measured the effects of the extracts using a different experimental approach such as the Boyden chamber.

The assay was carried out as described in “Materials and Methods”. Images of the migrated cells were acquired using an inverted microscope and cell migration was expressed as the percentage of migrated cells per microscope field, compared to untreated cells (CTRL).

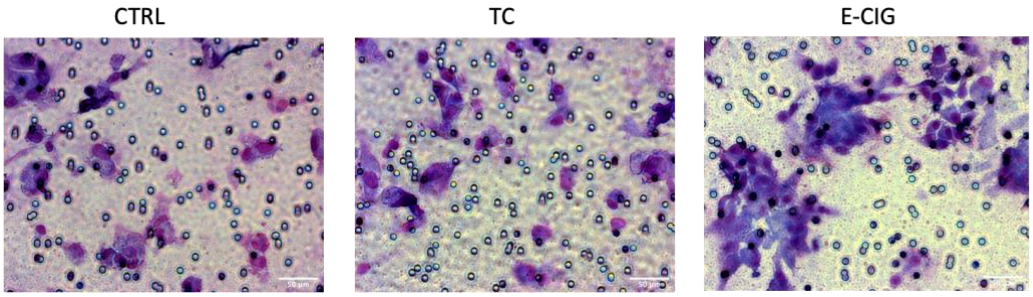


Figure 25. **Cell migration assessed by Boyden chamber assay.** SMCs were treated for 48 hours with 10% AEs, added to the upper chamber and allowed to migrate through the 8 µm pore size membrane into the lower chamber containing PDGF 5 ng/ml. Transmigrated cells were counted using an inverted microscope (objective lens 20X), Scale bar, 50 µm.

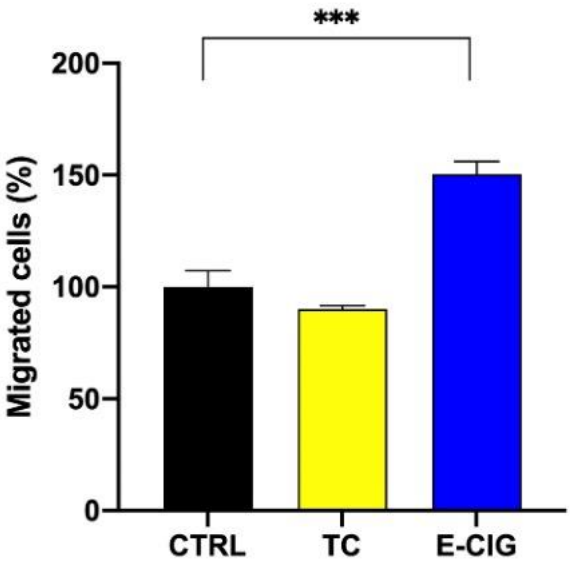


Figure 26. **Histogram graph of the % migrated cells assessed by Boyden chamber assay.** Data are presented as the percentage of the migrated cells ± SD. The p-value was determined by one-way ANOVA followed by Dunnett's post hoc test and considered significant for *** p <0.001 vs CTRL.

The result obtained in the previous assay was confirmed by the Boyden Chamber assay. As shown in Figure 25 and 26 E-CIG is the more potent in stimulating cell migration with a 50% increase compared to control and TC extract. Similarly to the data obtained with the scratch test where we observed

a 15% decrease following TC exposure, TC treatment slightly decreased (-10%) the number of migrated cells compared to control.

5.8 EFFECTS OF NICOTINE ON SMC MIGRATION

Several groups demonstrated that nicotine promotes SMC migration via nicotinic acetylcholine receptors (nAChRs) and G protein-coupled receptors [129] [130].

To determine whether the migratory effects observed with our extracts were due to their nicotine content, a wound healing assay was performed incubating SMCs with increasing concentrations of nicotine (from 1 μM to 10 μM) in a range similar to what is present in the 10% of cigarette smoke AEs we added to SMCs in our experiments. To further verify the role of α7nAChR in nicotine-induced cell migration, α-BTX was added to the culture media 30 min before nicotine treatment in order to evaluate whether it may counteract the effects due to nicotine.

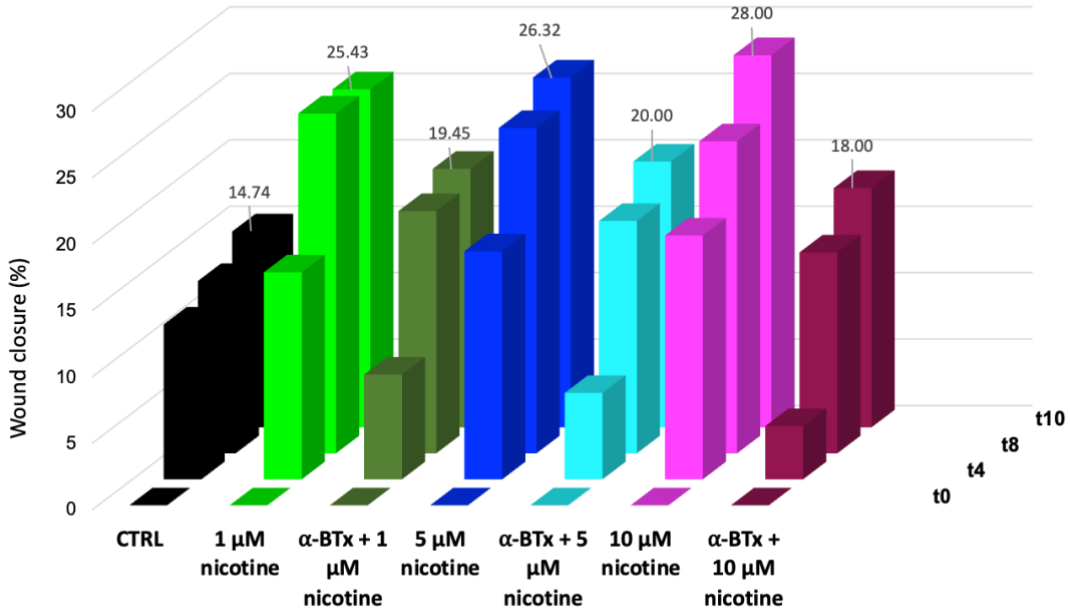


Figure 27. **Graphical quantitation of the % of wound healing at different time-points.** Cells were treated with increasing concentrations of nicotine (from 1 µM to 10 µM) for 4,8 and 10 hours, with/without pretreatment with 1 µM α-BTX for 30 min.

As shown in Figure 27, 10 hours of nicotine exposure caused a significant increase of cell migration in a concentration-dependent manner compared to the control group (about 70-90% increased reclosure for 1 µM, 5 µM and 10 µM of nicotine versus control). After 20 hours, wounds were completely healed which made it impossible to measure the wounded area at this time-point.
This effect was partially reversed/reduced by pretreatment with α-BTX. In fact, α-BTX consistently reduced nicotine-induced cell migration at all concentrations tested. These results suggest that nicotine may promote SMC migration through the activation of the alpha7 nicotinic receptor.

5.9 EFFECTS OF AEs ON F-ACTIN PROTEIN EXPRESSION, EVALUATION BY CONFOCAL MICROSCOPY

Cell migration in vivo or in vitro begins with stimulation of cell surface receptors that transduce the external signal to a series of coordinated remodeling events that alter the structure of the cytoskeleton.

Cell migration is initiated by an actin-dependent protrusion of the cell's leading edge which is composed of structures called lamellipodia and filopodia that contain actin filaments [123].

By confocal microscopy technique, we observed F-actin protein expression after SMC treatment with smoke aqueous extracts. Staining with phalloidin, that binds with high-affinity to filamentous actin, revealed a difference in the morphology and in fluorescence intensity following the incubation with TC and E-CIG compared to untreated cells. As shown in Figure 28, cells treated with TC and especially with E-CIG formed a protrusive structure termed lamellipodia compared to control where F-actin filaments are loosely arranged with the same homogeneous feature.

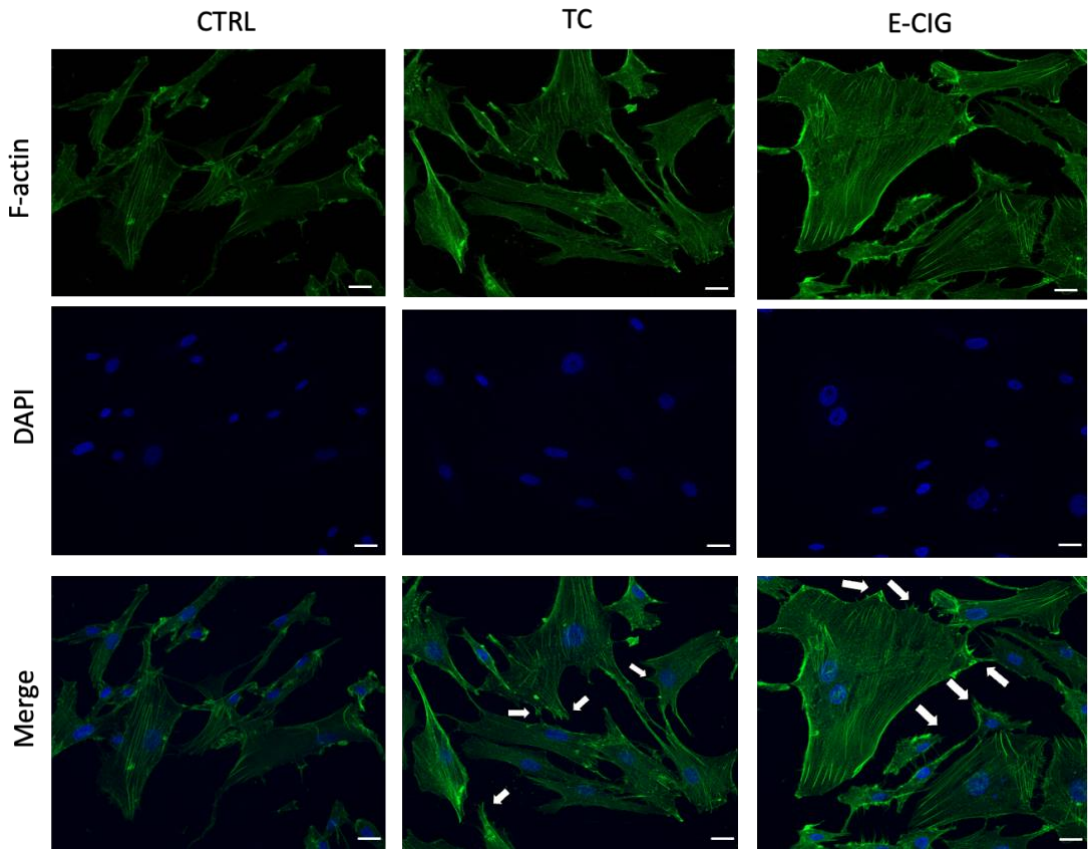


Figure 28. **Cytoskeletal organization of SMCs assessed by Confocal microscopy analysis**. SMCs were treated for 48 hours with 10% AE and F-actin expression was evaluated by confocal microscopy. Cells were stained for F-actin (phalloidin, green) and nuclei (DAPI, blue), white arrows represent filopodia, scale bar 5 µm.

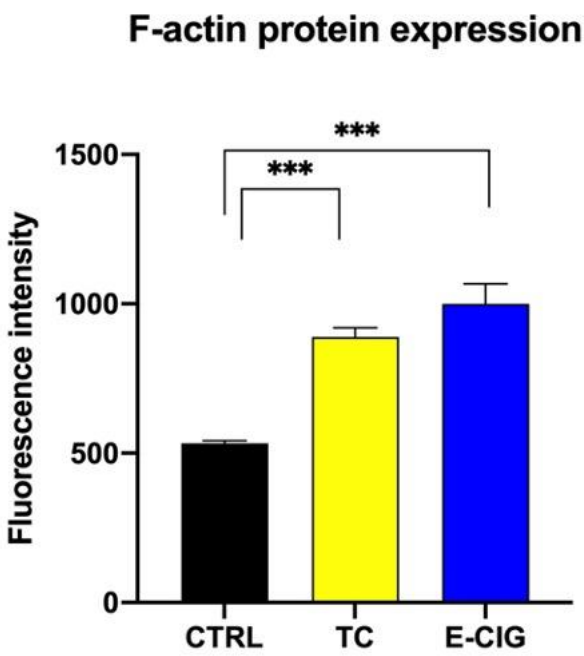


Figure 29. **Histogram graph of the % migrated cells assessed by Confocal microscopy.** All experiments were performed at least three times. Data are presented as the mean ± SD. The p-value was determined by one-way ANOVA followed by Dunnett's post hoc test and considered significant for *** p <0.001 vs CTRL.

In addition, as shown in Figure 29, E-CIG aqueous extract is the most effective in inducing F-actin expression, in fact, fluorescence intensity doubled compared to the untreated cells (CTRL).

On the other hand, a significant increase induced by TC extract was also observed compared to the control.

5.10 EFFECTS OF AEs ON RAC1 EXPRESSION IN SMCs

Rho family of the small GTPases mediates actin dynamics in SMCs. Particularly, Rac1 regulate F-actin polymerization in lamellipodia by activating the Arp2/3 complex [131].

For that reason, we observed whether smoke AEs affected Rac1 expression levels; the evaluation was performed by qRT-PCR and Western Blotting analysis, after incubating SMCs with TC and E-CIG extracts for 48 hours.

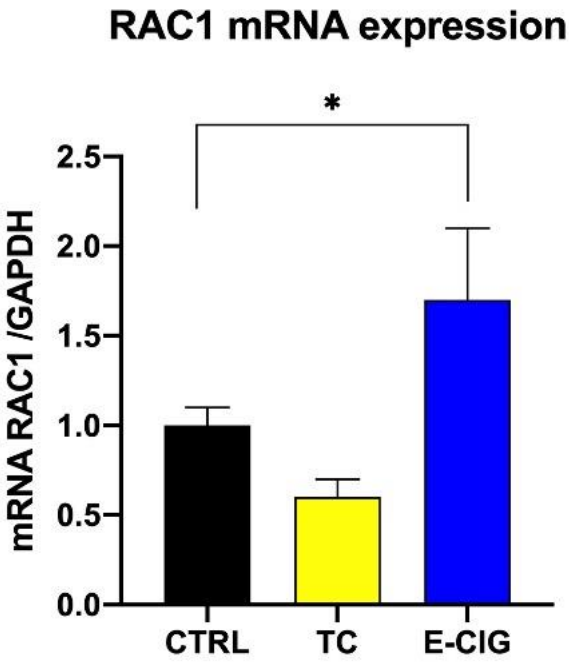


***Figure 30*. Real time qPCR on RAC1 expression levels.** Cells were treated with AEs for 48 hours. RNA was then extracted and RAC1 expression levels evaluated by real time PCR. All experiments were performed at least three times and data are presented as mean ± SD. The p-value was determined by one-way ANOVA followed by Dunnett's post hoc test and considered significant for * $p < 0.05$ vs CTRL.

As shown in Figure 30, E-CIG exposure significantly stimulated the expression of Rac1 (up 1.7-fold), while TC extract decreased its expression (40% reduction) although the result is not statistically significant.

Next, we measured the effect at the protein level by western blot analysis. As shown in Figure 31, consistently with the mRNA data, E-CIG extract significantly increased Rac1 levels. We also observed a significant increase of Rac1 with the TC, although the same extract decreased mRNA level.

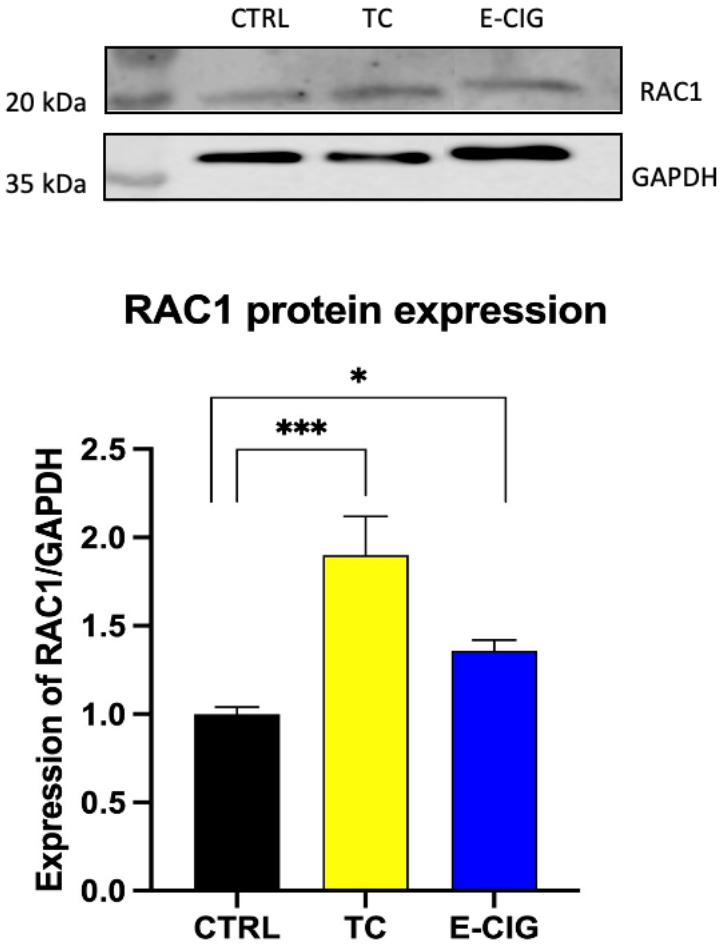


Figure 31. **Western Blotting analysis of RAC1 expression**. SMCs were treated with 10% AEs. Cells were harvested after 48 hours and protein concentration evaluated with BCA kit. GAPDH was used as loading control. All experiments were performed at least three times. Data are presented as mean ± SD. The p-value was determined by one-way ANOVA followed by Dunnett's post hoc test and considered significant for * $p < 0.05$ vs CTRL, *** $p < 0.001$ vs CTRL.

5.11 EFFECTS OF AEs ON PRO-INFLAMMATORY AND MATRIX REMODELING GENES AND PROTEINS IN SMCs

Secretion of pro-inflammatory cytokines, alterations of vascular ECM and tissue remodeling processes in general are central elements in atherogenesis and plaque stability. Therefore, we further analyzed the effects of cigarette smoke aqueous extracts on pro-inflammatory and matrix remodeling genes (MMPs). We measured the levels of several markers such as IL1-β, IL-6, IL-8, MMP-1, MMP-2, MMP-3 and MMP-9.

SMCs were incubated for 48 hours with smoke extracts, then gene and protein expression were evaluated by qRT-PCR Western Blotting and ELISA analysis.

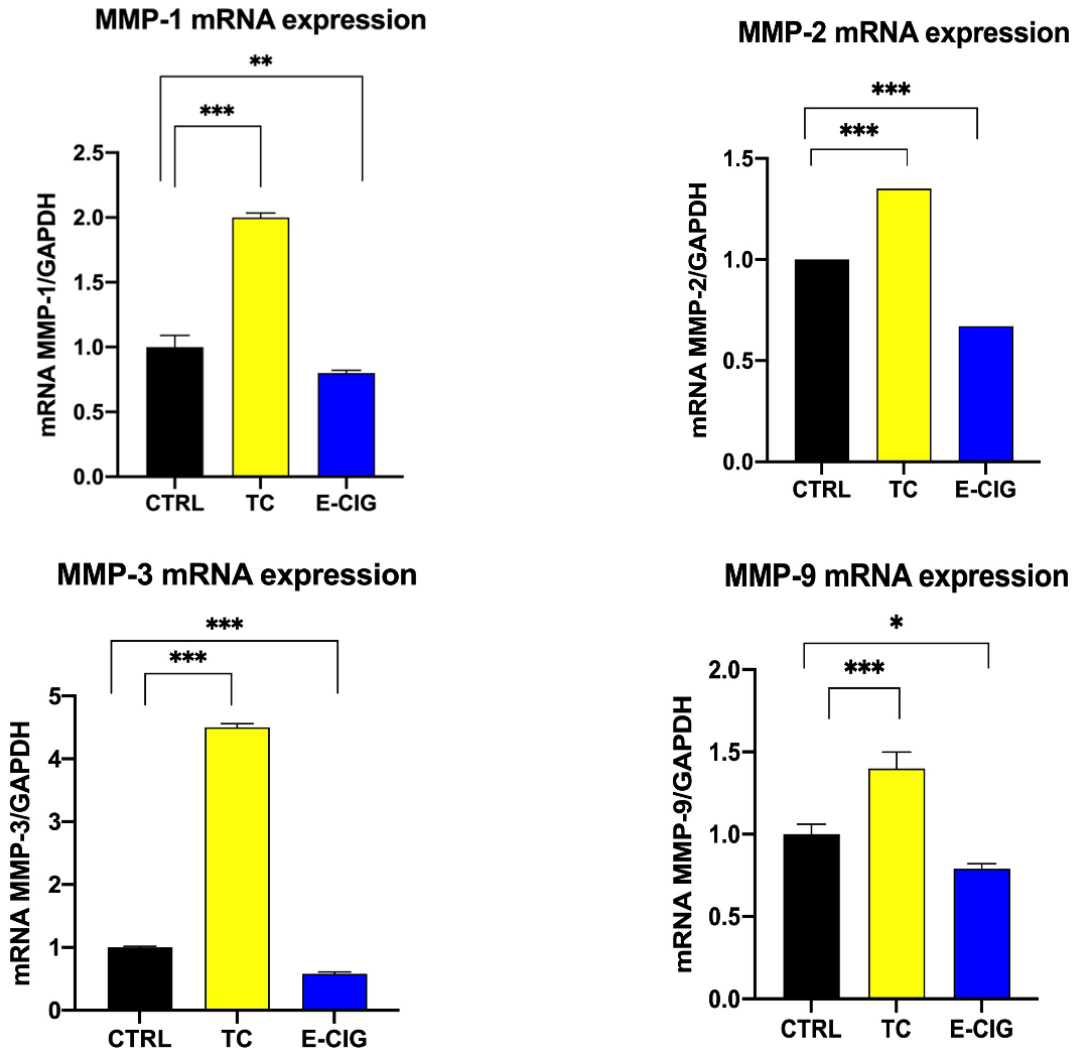


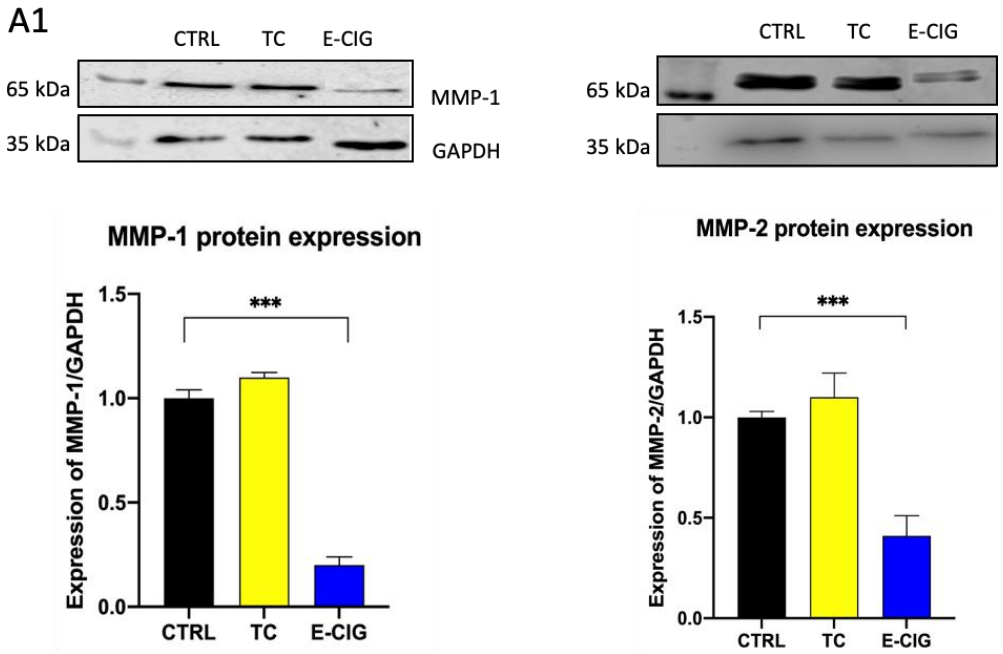
***Figure 32*. Real time qPCR on matrix metalloproteinases expression levels.** Cells were treated with AEs for 48 hours. RNA was then extracted and MMP-1, MMP-2, MMP-3 and MMP-9 expression levels evaluated by real time PCR.

All experiments were performed at least three times and data are presented as mean ± SD. The p-value was determined by one-way ANOVA followed by Dunnett's post hoc test and considered significant for ** $p < 0.01$, *** $p < 0.001$ vs CTRL.

.

As shown in Figure 32, the addition of TC and E-CIG affected the expression of MMP genes. TC increased the expression of MMP-1 and -3 (up to 2-fold and 4-fold respectively) and of MMP-2 and -9 (up to 1.4-fold). On the contrary, E-CIG significantly reduced the expression of MMPs, especially MMP-3 with a 40% decrease. We also analyzed the effects at the protein level by western

blot analysis. As shown in Figure 33 (Panel A1 and A2), TC stimulated the expression of MMP-3 but did not significantly affect MMP-1 and MMP-2, while E-CIG reduced the expression of all MMPs evaluated, and in particular of MMP-3 that was almost completely abolished (up to 94% reduction)



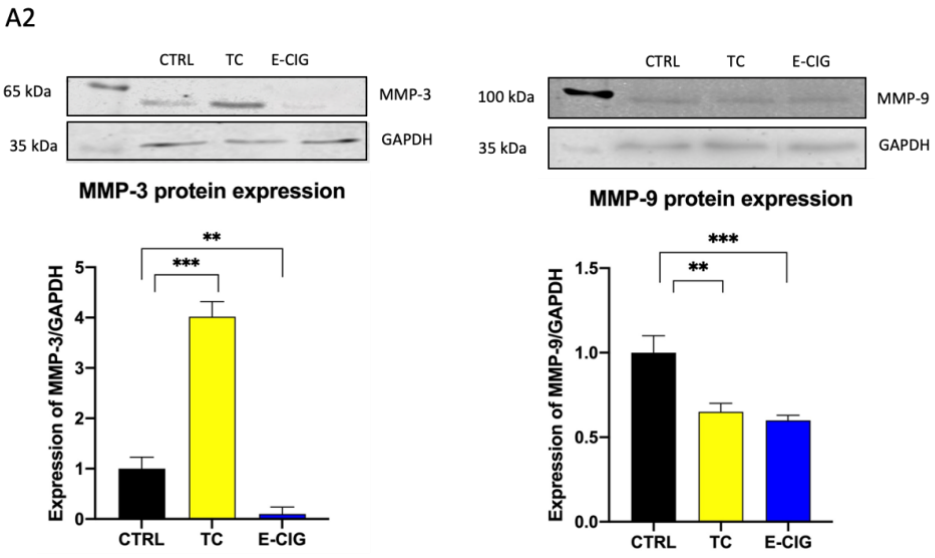


Figure 33. **Western Blotting analysis of MMP expression**. SMCs were treated with 10% AEs. Cells were harvested after 48 hours and protein concentration evaluated with BCA kit. GAPDH was used as loading control. All experiments were performed at least three times. Data are presented as mean ± SD. The p-value was determined by one-way ANOVA followed by Dunnett's post hoc test and considered significant for ** $p < 0.01$, *** $p < 0.001$ vs CTRL.

To further analyze the effects of cigarette smoke aqueous extracts on inflammation-related markers, we also measured the effects of the cigarette extracts on the expression of interleukins IL1-β, 6 and 8. As shown in Figure 34, the incubation of SMCs with TC caused a statistically significant increased expression of IL1-β, IL-6 and IL-8 genes. On the contrary, E-CIG reduced the expression of the three interleukins compared to both control and TC-treated cells.

Next, we measured the effect at the protein level by western blot for IL-1β and by ELISA assay for IL-6 and IL-8. As shown in Figure 35, consistently with the mRNA data, TC significantly increased interleukins levels. while, E-CIG maintained the inhibitory effect on IL1-β expression (up to 60% reduction) and on IL-8 but increased the concentration of IL-6 as determined by ELISA assay.

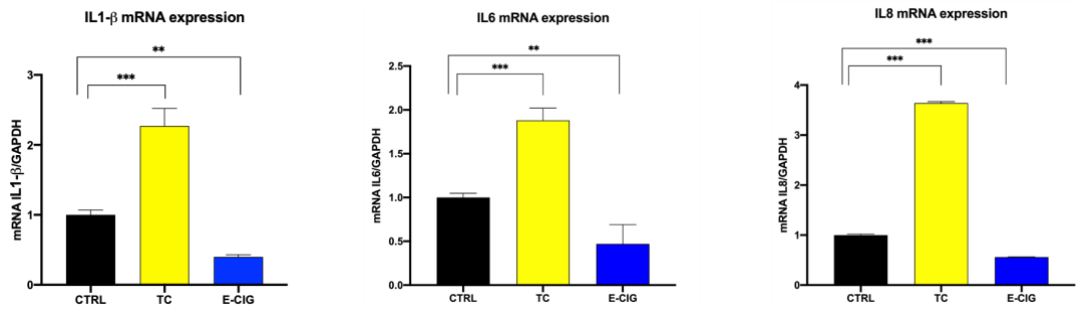


***Figure 34*. Real time qPCR on interleukins expression levels.** Cells were treated with AEs for 48 hours. RNA was then extracted and interleukins expression levels evaluated by real time PCR.

All experiments were performed at least three times and data are presented as mean ± SD. The p-value was determined by one-way ANOVA followed by Dunnett's post hoc test and considered significant for ** $p < 0.01$, *** $p < 0.001$ vs CTRL

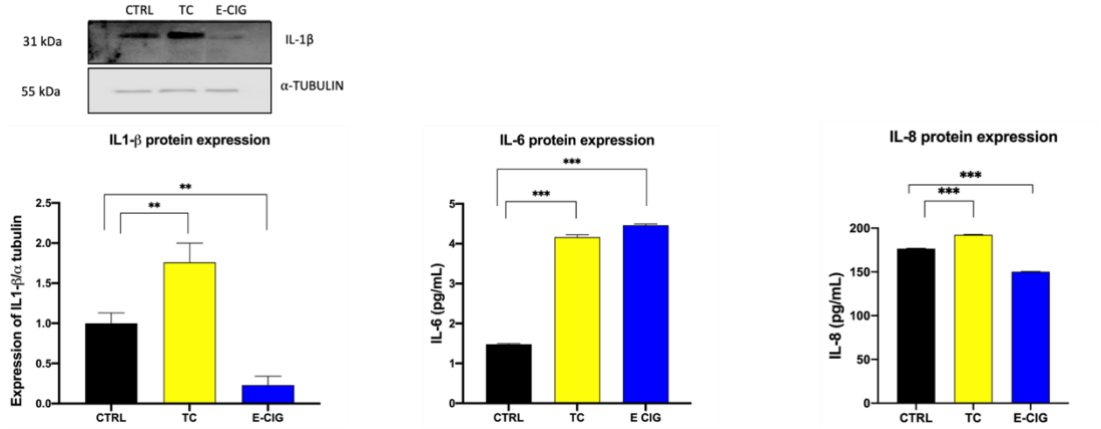


***Figure 35.* Western Blotting and ELISA analysis of IL1-β, IL-6 and IL-8 expression.** IL-1β protein expression was evaluated by Western Blotting analysis on collected media. α-tubulin was used as loading control.IL-6 and IL-8 protein concentration was assessed through ELISA analysis on collected media. Results were expressed as pg of protein for ml of media. All experiments were performed at least three times. Data are presented as mean ± SD. The p-value was determined by one-way ANOVA followed by Dunnett's post hoc test and considered significant for ** $p < 0.01$, *** $p < 0.001$ vs CTRL.

5.12 α7nAChR ACTIVATION RESULTS IN INHIBITION OF THE INFLAMMATORY PATHWAY IN SMC

Since several groups reported that the activation of α7nAChR exerts anti-inflammatory and immune modulatory reactions [81] [82] [132]. We investigated whether the E-CIG-induced the inhibition of the inflammatory pathway is related to the activation of α7nAChR.

First, we measured the expression of α7nAChR in SMCs and whether our extracts modulate it.

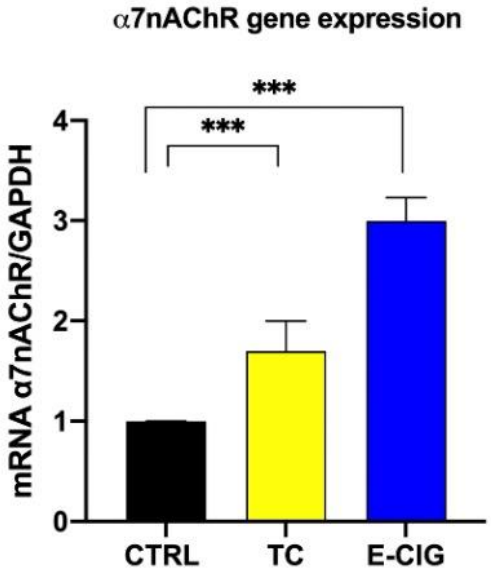


Figure 36. **Real time qPCR on α7nAChR expression levels.** Cells were treated with AEs for 48 hours. RNA was then extracted and α7nAChR expression levels evaluated by real time PCR.

All experiments were performed at least three times and data are presented as mean ± SD. The p-value was determined by one-way ANOVA followed by Dunnett's post hoc test and considered significant for *** $p < 0.001$ vs CTRL.

Figure 36 shows that E-CIG significantly increased the expression of α7nAChR (about 3-fold increase vs control). TC stimulated its expression although at a lower level about 1.7-fold increase).

Moreover, to ascertain whether the anti-inflammatory effects observed following E-CIG exposure was related to α7nAChR activation, SMCs were pretreated for 30 min with α-BTX. Then, smoke extracts were added for 48 hours, and IL1-β, IL-6 and IL-8 gene expression was measured by Real time qPCR.

As expected, SMCs treated with E-CIG extract, significantly reduced IL-1β, IL-6 and IL-8 expression (Up to 30% vs CTRL) (Figure 37). This effect was reversed by the α7nAChR antagonist α-BTX. In fact, pretreatment with α-BTX restored to basal levels interleukins expression. While pretreatment with α-BTX followed by TC exposure further stimulated cytokine expression.

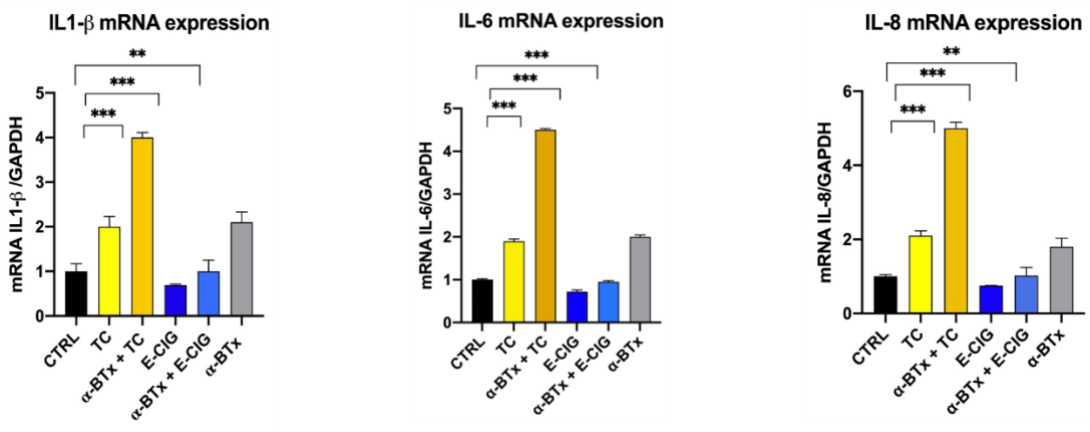


Figure 37. **Real time qPCR on IL-1β, IL-6 and IL-8 expression levels.** Cells were treated with smoke extracts for 48h with/without pretreatment with 1 μM α-BTX for 30 min. RNA was then extracted and IL-1β, IL-6 and IL-8 expression levels evaluated by real time PCR. All experiments were performed at least three times and data are presented as mean ± SD. The p-value was determined by one-way ANOVA followed by Dunnett's post hoc test and considered significant for ** $p < 0.01$, *** $p < 0.001$ vs CTRL.

6. DISCUSSION

Atherosclerosis is a chronic inflammatory disease, involving mostly the intima of large and medium size arteries [133]. It is responsible for several important vascular events which are the leading causes of most of the cardiovascular morbidity and mortality in the Western World today, including coronary artery disease (CAD), stroke, and peripheral arterial disease[134]. Its multifactorial etiology is characterized by either systemic or local factors that induce deterioration in vascular function[135].

The pathologic condition can be described as a progressive deposition of lipids in the vessel's intima, which is accompanied by an inflammatory response[136]. In addition to this, SMC proliferation occurs, gradually developing into the formation of an atherosclerotic plaque[137].

Vascular SMCs, mostly located within the medial layer, play a fundamental role in the development of atherosclerotic plaque, since this cell type undergoes phenotypic changes induced by several factors, like cytokines and growth factors, mechanical forces, neuronal stimuli, and genetic factors[138]. SMCs tend to modulate their phenotype, from a "contractile" phenotype to a "synthetic" one, leading to an increased proliferation and migration towards the intima[22].

For some years, our research group has been dealing with cigarette smoking, which is well known to be one of the most important modifiable risk factor for cardiovascular disease[139].

Cigarette smoke is constituted by approximately 7,000 different components, distributed between particulate matter and gas phase, which contain lipophilic and water-soluble compounds respectively. Many of these chemicals are known to be antigenic, cytotoxic, mutagenic, or carcinogenic [66].

We developed our research in collaboration with British American Tobacco (Southampton, UK), who provided us with traditional cigarette (TC) and electronic cigarette (E-CIG) aqueous extracts (AEs). Differently to TPM that includes lipophilic components of the particulate phase of cigarette smoke, AE contains water-soluble components of both the particulate and vapor phases.

Moreover, AE seems to better mimic both the *in vivo* exposure and the actual passage of water soluble components into body fluids (saliva, bronchial and alveolar fluids) [140].

Alternative cigarette devices use is rapidly increasing among youth and adults, since they were advertised as safer, healthier, and less expensive than traditional cigarettes, and are sometimes considered as smoking cessation aids. Nevertheless, these claims are not based on evidence, and because of their recent appearance on the market, there is still limited knowledge about the long-term effects of these products.

Next generation tobacco products, unlike traditional cigarettes, allow to smoke avoiding the tobacco combustion process at very high temperatures. This characteristic is very important because most of the toxic components of cigarette smoke are formed through combustion process.

E-CIG does not contain tobacco but heats up a liquid mixture typically composed of propylene glycol, water, and different types of flavors, besides nicotine at different concentrations.

Our experimental research aimed at evaluating the effects of aqueous extracts of both traditional cigarette and electronic cigarette on SMC phenotypic modulation. The study was particularly focused onto better unravel the effects of cigarette smoke extracts on SMC phenotypic switch potential, a key step in the atherogenic process, and to get more information on the potentially toxic effects of the electronic cigarettes.

SMC phenotypic modulation is generally associated with a marked increase in ECM synthesis and SMC proliferation and migratory activity [23].

In order to evaluate the transcriptional pathways regulated by *AEs*, we treated SMCs with TC and E-CIG extracts for 24 hours and performed bulk RNA sequencing (RNA-Seq) in collaboration with Stanford University under the supervision of Professor Juyong Brian Kim and Professor Thomas Quertermous. As shown in Figure 14 TC treatment increased the expression of several cytochrome P450 enzymes that are responsible for both

metabolically activating and detoxifying polycyclic aromatic hydrocarbons (PAHs) and aromatic amines present in cigarette smoke.

Many substrates for CYP1 enzymes are aryl hydrocarbon receptor (AhR) ligands, thus the induction of CYP1A1 is regulated by aryl hydrocarbon receptor (AhR) and dioxin.

Anttila et al. demonstrated that CYP1AI is upregulated up to hundred-fold in lung tissue of tobacco smokers. [141]

Moreover, several groups demonstrated that high inducibility of CYP1A1, due to genetic polymorphisms, is considered to be a risk factor for lung cancer in smokers [142]. In addition, we observed that TC increased the expression of collagen, one of the main components of extracellular matrix and of several MMPs that are responsible for tissue remodeling and degradation of ECM proteins.

Therefore, we evaluated the effects of AEs on ECM gene expression. As shown in Figure 18. SMCs increased the expression of COL1alpha1, decorin, lumican and fibronectin. All these genes are usually upregulated during SMC phenotypic modulation [35]. Orr and Forsberg groups demonstrated that culturing SMC on collagen I and fibronectin substrates can promote SMC phenotypic switch from contractile to synthetic phenotype. In particular, they observed an enhanced expression of the inflammatory protein vascular cell adhesion molecule (VCAM) and synthesis of ECM components like collagen elastin and proteoglycans [42] [43].Previous studies have shown that exposure of airway SMCs to TC extract resulted in increased proliferation and deposition of ECM components such as collagen I, collagen III and fibronectin [143]. Here we reported for the first time that not only TC but also E-CIG may affect the expression of ECM genes thus promoting SMC phenotypic modulation.

Furthermore, we observed from RNAseq data that E-CIG upregulated several genes involved in cell cycle and cell division processes promoting in this way cell proliferation. Thus, we evaluated SMC proliferative index that is another important parameter that characterizes SMC phenotypic modulation. As shown in Figure 19. E-CIG has the maximum effect on cell proliferation causing

a 30% increased proliferation rate after 72 h of treatment and it also doubled the expression of PCNA (proliferating cell nuclear antigen), an important proliferative marker (Figure 20 and 21). On the contrary, TC addition seems to slow down SMCs proliferation.

As already mentioned, since cigarette smoke is composed by several thousands of different chemicals distributed between both the particulate and the gas phases, it is particularly difficult to ascertain the component responsible for the effects we have observed in our experiments.

One common component of the extracts is nicotine. Several findings have been reported regarding the biologic effects of this alkaloid. Nicotine may contribute to atherosclerosis onset and progression by many different mechanisms. Some studies have shown that nicotine induces a phenotypic switch in SMCs, from a contractile to a synthetic phenotype [144], suggesting the important role of this alkaloid in vascular disease, since recruitment and proliferation of synthetic SMCs within the intima of injured vessels are key events in the pathogenesis of vascular occlusive diseases. Furthermore, different groups, demonstrated that nicotine induces SMC proliferation through the activation of p38 MAPK and ERK1/2 signaling in human and primary SMCs isolated from calves aorta [145][144].

To understand whether the different behavior of traditional cigarette and electronic cigarette on cell proliferation was caused by nicotine content, a proliferation assay was performed incubating SMCs with increasing concentration of nicotine alone. Nicotine was added in a range like that present in 10% AEs, in particular TC contains 4.3 µM and E-CIG 6.1 µM of nicotine. As shown in Figure 22, a significant increase in proliferation rate was observed after 48 and 72 hours of incubation, and this trend is particularly evident in cells incubated with 1 µM and 5 µM of nicotine, where proliferation is stimulated by about 50% compared to control. Conversely, the proliferation rate of SMCs treated with 10 µM nicotine seems to be comparable to the control, only after 72 hours we observed a slightly increase.

On the contrary, TC reduces SMC proliferation by about 40% as shown in Figure 19, although its nicotine content is 4.3 µM, a concentration of nicotine

that when added alone increases the proliferation rate (Fig.22). Our hypothesis is therefore that the stimulatory proliferative effect exerted by E-CIG is probably due to its nicotine content. In fact, pretreatment with α-BTX a potent antagonist of α7nAChR, showed about 70-90% reversal of nicotine-induced cell proliferation at a concentration of 1 µM and 5 µM (Figure 22).

On the other end TC might contain some other components that are able to inhibit cell proliferation bypassing the stimulatory effect of nicotine. In this regard, Horton et al. described the potential role of acrolein in inhibiting cell proliferation [146]. Acrolein is an unsaturated α,β-aldehyde generated by the pyrolysis and combustion of tobacco products, it can also be released during the combustion of petroleum fuels, biodiesel and plastic. Acrolein has been suggested to play a role in several diseases including Alzheimer's disease, cardiovascular disease and diabetes mellitus [147]. Low doses of acrolein decrease the proliferation rate of human lung carcinoma cell lines, probably due to changes in gene expression mediated by changes in glutathione (GSH) levels[146]. This hypothesis is still under evaluation.

We also evaluated the effects of AEs on SMC migratory activity. Once again, E-CIG is the most effective in promoting cell migration. As shown in Figure 23, E-CIG speeded up the complete reclosure of a lesion caused in cell monolayer after 20 hours (Wound Healing assay), at a much faster rate than what observed in control or TC-treated cells. The Boyden Chamber assay confirmed this trend: E-CIG treatment increases cell migration by about 50% (Figure 26). On the contrary, TC treatment slightly decreased the number of migrated cells compared to control. On the same line, Taylor et al. demonstrated by scratch assay that exposure of endothelial cells to TC extract decreased the migration rate in a concentration-dependent manner [148]. Additionally, they demonstrated that cells treated with nicotine alone did not show any changes in migratory activity, supporting the idea that nicotine is not responsible for the inhibition of endothelial cell migration [148]. These findings are in line with recent studies that have demonstrated that nicotine may induce migration and proliferation of endothelial cells and vascular SMCs. Park et al., showed that nicotine dose-dependently enhanced HUVEC proliferation and migration

compared to untreated cells [149]. As reported by Heeschen, the stimulatory effect induced by nicotine on endothelial cell growth and tube formation is mediated through nicotine acetylcholine receptors activation [150].

In order to better define E-CIG-induced SMC migration, we also investigated the effects of smoke extracts on SMC cytoskeleton and in particular we focused our attention on F-actin expression. In fact, to migrate SMCs need to extend lamellipodia toward the stimulus via actin polymerization, which is coordinated by many actin-binding proteins regulated also by small G proteins, such as Rac1[123]. Once again, as shown in Figure 28-29, E-CIG has the major effect on F-actin expression, which is more than doubled compared to the untreated cells. In addition, cells treated with E-CIG formed a protrusive structure termed lamellipodia compared to control where F-actin filaments are loosely arranged with the same homogeneous feature. The formation of lamellipodia is usually related to the activation of small G proteins like Rac1 and RhoA, which regulate the organization of cytoskeletal actin [131]. We measured the expression of Rac1 both at gene and protein level. As shown in Figure 30 and 31, E-CIG significantly increased the expression of Rac1 both at gene and protein level, while TC exposure increase Rac1 protein expression but decreased mRNA levels. Recently, Liang et al. demonstrated that exposing vascular SMCs to nicotine, induced the expression of cytoskeleton-related proteins (α-actin, β-actin, F-actin calponin and caldesmon) and the activation of Rho GTPase pathway (RAC, CDC42 and RhoA) which in turn activates downstream targets such as MYPT1, PAK1 AND PI3K [151]. In addition, they demonstrated that nicotine exposure increased SMC migration and proliferation rate. This increase was suppressed by treatment with α-BTX or Y27632 (an α7-nAchR specific antagonist and inhibitor of Rho-Kinase activation) suggesting that nicotine can increase migration and proliferation of SMCs in a Rho GTPase-dependent manner [151]. Taking everything into account, we can speculate that E-CIG induces changes in cytoskeleton organization by increasing the expression of F-actin and of the Rho GTSase Rac1 as shown in Figure 29,30,31. As a result, migration and proliferation rate of SMC is enhanced. We can also speculate that these effects could be mainly

due to nicotine presence since we demonstrated that nicotine, at the same concentration present in E-CIG AE, stimulate SMC proliferation and migration (Fig.22 and Fig.27). Moreover, as shown in Figure 27, the increased migration rate was partially blocked following treatment with α-BTX. Overall, these results indicate that E-CIG can stimulate migration and proliferation of SMCs through the activation of α7nAChR. On the contrary, TC extract seems to stimulate F-actin and Rac1 protein expressions, suggesting a modulation of cell morphology without affecting cell proliferation and migration. In this regard, further studies are needed in order to better reveal whether the inhibitory effects we observed on cell proliferation and migration, were due to a specific chemical compound present in cigarette smoke besides nicotine (acrolein?). In fact, some studies have demonstrated an antagonistic effect when multiple known cigarette smoke compounds are mixed together [152]. Conversely, synergism between two or more compounds may be necessary to induce toxic or harmful effects [152]. Thus, compounds found in cigarette smoke extracts may oppose the effects of other compounds, thereby neutralizing a known deleterious effect of a single one.

Atherosclerosis is chronic vascular inflammation associated with the secretion of several cytokines, which are produced by T cells, macrophages, endothelial cells, and smooth muscle cells. SMCs in particular produce cytokines that attract and activate leukocytes, induce proliferation of SMCs, promote endothelial cell dysfunction, and stimulate production of extracellular matrix components [20].

It is well established that TC contributes to cardiovascular disease by promotion of inflammatory and oxidative pathways [153], less is known about the inflammatory effects of E-CIG on vascular cells. Our attempt was to understand the potential effects on the expression of proinflammatory and matrix remodeling-related markers after exposing SMCs to smoke extracts.

In our experimental settings, smoke extracts displayed different behaviors. As shown in Figure 32,33,34,35, TC significantly increased the expression of all pro-inflammatory and matrix remodelling-related markers such as IL-1β, IL-6, IL-8, MMP-1, MMP-2 and MMP-3 both at gene and protein levels. These data

are in line with RNAseq results where we observed an upregulation of genes related to extracellular matrix organization.

Surprisingly, E-CIG reduced all these markers at gene and protein level while increased the concentration of IL-6 as determined by ELISA assay (Figure 35). Some of the immunosuppressive effects of nicotine have been attributed to its effect on the α7-nicotinic acetylcholine receptor. Activation of this receptor has been shown to reduce the production of pro-inflammatory cytokines such as TNF-a, IL-1β, IL-6 and IL-8[60]. In our project, we raised the hypothesis that the activation of α7nAChR may inhibit the inflammatory pathway. First, we measured gene expression of α7nAChR, that resulted to be up-regulated following E-CIG and TC treatments (Figure 36). Then, we tested whether blocking α7nAChR with α-BTX could reverse the blockade of interleukins induced by E-CIG treatment. As shown in Figure 37, this effect was counteracted by the α7nAChR antagonist α-BTX, while, pretreatment with α-BTX followed by TC exposure further increased the expression of all interleukins tested.

As shown in table 5, our data demonstrate that TC is less effective in inducing a proper phenotypic modulation of SMCs even though it affects cell morphology. However, TC is the most effective in increasing markers of inflammation and matrix-degrading metalloproteinases thus, promoting vascular inflammation and enhancing the atherogenic process. The aqueous extract from a TC stimulates the expression of matrix metalloproteinases and cytokines expression. Even when we blocked α7nAChR, we observed a further increase in cytokine expression. Therefore, TC might contain some other ingredients that have a stimulating effect on the inflammatory response, overwhelming the inhibitory effects exerted by α7nAChR. These data are consistent with results from other studies indicating that traditional cigarette-induced proinflammatory alterations in vascular cells [154] [155] and increase the expression and activity of MMPs [156]. Lemaître et al. demonstrated that exposure of vascular endothelial cells to cigarette smoke and acrolein induce the expression of MMP-1 through the downregulation of the mTOR/p70S6K pathway [156]. Cigarette smoke may also increase MMP expression via

activation of inflammatory transcription factors. The expression of the AP-1 transcription factor complex is positively associated with MMP expression [157].

On the other end, we observed that E-CIG inhibits the expression of inflammatory markers through the activation of α7nAChR. In fact, blocking this receptor with a specific antagonist, restored to basal levels interleukins expression as shown in Figure 37. From a certain point of view, the inhibition of the inflammatory response by E-CIG could be a positive and beneficial effect. However, Madison et al. prompted that E-CIG smoke aberrantly alters the physiology of lung epithelial cells and resident immune cells and promotes poor response to infectious challenges [158]. On the same line, Han et al. warned that the destabilization of NLRP3 and caspase-1 in monocytes may provide a mechanistic insight on immunosuppression in smokers by blunting their immune response to toxicants [159]. Moreover, mice exposed to E-CIG vapor reduced antibacterial host defense responses, associated with decreased levels of several cytokines/chemokines like IL-3, IL-27,GM-CSF in bronchoalveolar lavage [160].

Table 5. **Summary table**

	ECM secretion	Proliferation rate	Migratory activity	Cytoskeletal organization	Proinflammatory markers
TC AE	⇑	⇓	⇓	⇑	⇑
E-CIG AE	⇑	⇑	⇑	⇑	⇓

In conclusion, it is still not clear which of the various chemicals contained in cigarette smoke mixture should be regarded as predominantly responsible for the effects we observed. Further studies are needed to understand which of the tobacco toxicants actually affects/drives the inflammatory process and to determine what impact this may have on pathological development. Currently, studies are ongoing aimed at investigating the role of acrolein, one of the major

toxic components of smoke present in both traditional and electronic cigarettes, on SMC phenotypic modulation. In particular, we are focused on studying the effects of acrolein on SMC proliferation/migration rate and inflammatory response. Acrolein has been shown to induce alterations in inflammatory signaling and gene expression. In fact, as reported by Valacchi et al., acrolein inhibits IL-8 expression both at gene and protein level by suppressing NFkB in airway epithelial cells [161] suggesting an important role in the anti-inflammatory response.

In addition, in collaboration with UNITECH and University of Siena, we are also evaluating how cigarette smoke alters the proteomic profile of smooth muscle cells to identify differentially expressed proteins in SMC after exposure to TC and E-CIG.

Based on these *in vitro* results, we can conclude that acute exposure to electronic cigarette induces vascular dysfunction, by promoting SMC phenotypic modulation and by impairing the inflammatory response. Further *in vivo* studies are needed to better reveal the short/long-term exposure effects to E-CIG in atherosclerosis-prone apolipoprotein E-deficient ($ApoE^{-/-}$) mouse (study ongoing).

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PhD activity

Congress participation

06/2021	virtual edition	International conference on Biomedicine “In vitro characterization of an anti-HER2 affibody-monomethyl auristatin E conjugate in HER2-positive breast cancer cells”
05/ 2021	virtual edition	Spring meeting giovani ricercatori SISA “Effects of next generation tobacco and nicotine products on smooth muscle cell phenotypic modulation”
11/2020	MILANO	34°National Congress SISA virtual edition “Effects of next generation tobacco and nicotine products on the inflammatory response of aortic vascular smooth muscle cells”
06/2020	CHIESA IN VALMALENCO	Spring school, oral report of the project “Effects of next generation tobacco and nicotine products on smooth muscle cell phenotypic modulation”
04/2019	CHIESA IN VALMALENCO	Spring school, oral report of the project “Effects of next generation tobacco and nicotine products on smooth muscle cell phenotypic switch, a key process in atherogenesis”
11/2018	BOLOGNA	32°National Congress SISA “Role of HDL3 in phenotypic modulations induced by cholesterol and cigarette smoke in vascular smooth muscle cells”
10/2018	MILANO	Regional Congress SISA Lombardia “Role of HDL3 in phenotypic modulations induced by cholesterol and cigarette smoke in vascular smooth muscle cells”
07/2018	MILANO	NEXT STEP IX- la giovane ricerca avanza “In vitro evaluation of an anti-her-2 affibody-monomethyl Auristatin E conjugate in HER2-positive cancer cells”.

Tutoring

- Tutor of five experimental master thesis
- Tutor of two compilation master thesis

Secondment

Erasmus+ Traineeship at University of Wroclaw, Poland, (01/02-01/09/2016)

Project Title:" Purification *and in vitro evaluation of an anti-HER2 Affibody-Monomethyl Auristatin E Conjugate, in HER2 positive breast cancer cells*"

Visiting research student at University of Stanford, USA, (01/11/2021-present)

Publications

- Potential statin drug interactions in elderly patients: a review
  Damiani I., Corsini A., Bellosta S.
  Expert Opinion on Drug Metabolism and Toxicology, 2020, *16*(12), 1133–1145

- Angiogenesis inhibition with selective compounds targeting the key glycolytic enzyme PFKFB3: identification of PFKFB-mediated angiogenesis inhibitors
  Abdali, A., Baci, D., Damiani, I., Belloni, F., De Dominicis, C., Gelmi, M. L., Bellosta, S.
  Pharmacological Research, 2021, *168*, 105592

- Purification and in vitro evaluation of an Anti-HER2 Affibody-Monomethyl Auristatin E conjugate, in HER2-positive cancer cells
  Damiani I., Castiglioni S., Sochaj-Gregorczyk A.M., Otlewski J., Corsini A., Bellosta S.
  Cancer Biology, 2021, 7;10(8):758.