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EFFECTS OF CIGARETTES, E-CIGARETTES AND SWEDISH SNUS ON VASCULAR FUNCTION

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THESIS FOR DOCTORAL DEGREE (Ph.D.)

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“Giving up smoking is the easiest thing in the world. I know because I've done it thousands of times”

Mark Twain (1835-1910)

ABSTRACT

Background

Cigarette smoking is considered one of the leading causes of preventable morbidity and premature death worldwide. A large portion of this is attributable to cardiovascular disease such as ischemic heart disease and stroke. However, with the public's increasing awareness of the harm and diseases related directly to cigarette smoke, alternative combustion-free nicotine delivery products have gained in popularity. Two of the products which have seen substantial market growths during the last few years are Swedish snus and electronic cigarettes. In recent years, an increasing number of studies have demonstrated a correlation between snus usage and increased mortality in coronary heart disease and stroke. Although there are studies with contradictory results, regarding the general underlying increase of cardiovascular risk. So far, there is limited data available on the potential negative health effects of e-cigarette smoking.

The overall aim of this thesis was to investigate vascular health effects caused by acute cigarette and e-cigarette inhalation as well as chronic snus usage.

Methods and Results

In Paper I, twelve healthy volunteers were subjected to cigarette smoking or not-smoking in a cross-over study. Microvesicles and endothelial progenitor cells were analyzed in collected blood samples at baseline and 1h, 4h and 24h following exposure. Cigarette smoking caused an acute increase in endothelial progenitor cells and in microvesicles of platelet, leukocyte and endothelial origin.

Paper II was a randomized, cross-over study where 16 healthy volunteers were exposed to electronic cigarette inhalation with nicotine vs non-inhalation. Biomarkers were analyzed in the same approach as in Paper I. E-cigarette inhalation caused an acute increase in endothelial progenitor cells. Microvesicles, with the exception of endothelial derived microvesicles, were unaffected.

In Paper III, seventeen healthy volunteers inhaled e-cigarette vapor with and without nicotine. In this double-blinded, randomized, cross-over study arterial stiffness was analyzed at baseline and at predetermined intervals for 4 hours following exposure. E-cigarette vapor inhalation containing nicotine caused a transient acute increase in heart rate and arterial stiffness.

Paper IV was a cross-sectional study investigating healthy long-term snus users (mean age 44.8 years) and age-matched controls. Arterial stiffness, forearm blood flow as well as fibrinolytic function and endothelial progenitor cells in blood samples were analyzed. Snus users had significantly higher arterial stiffness as well as impaired endothelial function, i.e. decreased forearm blood flow upon glyceryl tri-nitrate infusion. There was no difference in fibrinolytic function and endothelial progenitor cells between the two groups.

Discussion

Smoking a single cigarette causes a rapid activation of endothelial cells, platelets and leukocytes. As cigarette smoking has known detrimental effects on vascular health, our results were not unexpected. However, e-cigarette inhalation as well as smoking a conventional cigarette, causes a similar swift mobilization of endothelial progenitor cells. This may be interpreted as an acute endothelial stress caused by nicotine. Furthermore, inhaled nicotine is linked to increases in arterial stiffness, which is chronically altered in daily snus users. This elucidates that nicotine alone may alter endothelial function, both upon acute and chronic exposure. Snus users also display an attenuated effect of glyceryl tri-nitrate which further strengthens the findings that snus use is associated with increased mortality following myocardial infarction.

In summary, we demonstrate that the nicotine content in combustion-free nicotine delivering products may alter vascular function. E-cigarettes and snus should therefore not be considered as harmless recreational products.

LIST OF SCIENTIFIC PAPERS

I. Mobarrez F, **Antoniewicz L**, Bosson JA, Kuhl J, Pisetsky DS, Lundbäck M. The effects of smoking on levels of endothelial progenitor cells and microparticles in the blood of healthy volunteers. PloS one. 2014;9(2):e90314.

II. **Antoniewicz L**, Bosson JA, Kuhl J, Abdel-Halim S, Kiessling A, Mobarrez F, Lundbäck M. Electronic cigarettes increase endothelial progenitor cells in the blood of healthy volunteers. Atherosclerosis. 2016;255:179-85

III. **Antoniewicz L**, Brynedal A, Hedman L, Lundbäck M, Bosson JA. Acute effects of electronic cigarette vapor on the vasculature and the conducting airways. *Manuscript*

IV. **Antoniewicz L**, Kabele M, Bosson JA, Lundbäck M. Chronic snus use in healthy males increases arterial stiffness and alters endothelial function. *Manuscript*

The articles will be referred to as **Papers I-IV** and are reproduced in full as appendices. The published papers have been reprinted with kind permission of the publishers.

Note: Although pulmonary measurements were also conducted in Paper II and III and the obtained results are discussed in the respective papers, they have not been included in the dissertation text, as its focus is on vascular effects.

CONTENTS

LIST OF ABBREVIATIONS

ACh	Acetylcholine
AIx	Augmentation index
AIx75	Augmentation index corrected for a heart rate at 75bpm
AP	Augmentation pressure
BK	Bradykinin
CD	Cluster of differentiation
CO	Carbon monoxide
CS	Cigarette smoke
cGMP	cyclic Guanosine monophosphate
CVD	Cardiovascular disease
DBP	Diastolic blood pressure
ECV	Electronic cigarette vapor
EDV	Endothelial dependent vasodilation
EIDV	Endothelial independent vasodilation
EMVs	Endothelial derived microvesicles
eNOS	Endothelial nitric oxide synthase
EPCs	Endothelial progenitor cells
FBF	Forearm blood flow
FMD	Forearm mediated dilation
GC	Guanylate Cyclase
GTN	Glyceryl tri-nitrate (Nitroglycerine)
GTP	Guanosine Triphosphate
HMGB1	High mobility group box 1
HR	Heart rate
KDR	Kinase domain insert receptor

LMVs	Leukocyte derived microvesicles
MMVs	Monocyte derived microvesicles
MVs	Microvesicles
NO	Nitric oxide
NRT	Nicotine replacement therapy
PAHs	Polycyclic aromatic hydrocarbons
PAI	Plasminogen activator inhibitor
PECAM1	Platelet endothelial cell-adhesion molecule 1
PKG	Protein Kinase G (cGMP dependent protein kinase)
PMVs	Platelet derived microvesicles
PM	Particulate matter
PS	Phosphatidylserine
PWV	Pulse wave velocity
ROS	Reactive oxygen species
SBP	Systolic blood pressure
SDF-1	Stromal derived factor 1
tPA	tissue-Plasminogen activator
TSNAs	Tobacco specific nitrosamines
VEGF	Vascular endothelial growth factor
VOCs	Volatile organic compounds
VOP	Venous occlusion plethysmography

1 INTRODUCTION

When browsing the internet, one can easily stumble upon social media discussions and news headlines such as “Tobacco industry: Smoking isn't bad for your health”, “Swedish snus is good for public health” or “E-cigarettes should be on sale in hospital shops, health body says” [1-3]. In an era of easily accessible information with overwhelming amounts of newspaper articles, scientific reports mixed with personal opinions on blogs, social media or forums, it may be difficult to distinguish verified facts from biased speculation on this topic. The present thesis will certainly not answer all the questions raised by these headlines, but it will try to present an unbiased account of the effects of these products with an emphasis on vascular health.

1.1 CIGARETTES

The World Health Organization estimates that 6 million people die every year due to the negative health effects of cigarette smoking [4]. These preventable deaths are mostly attributed to the development of cancer, respiratory and cardiovascular disease (CVD) [5]. Even though cigarette smoke (CS) and its effects on human health have been studied extensively over the years, the underlying pathways through which CS exhibits its negative effects on the vasculature are still not fully elucidated. CS is a complex mixture of combustion derived compounds that cause DNA damage, inflammation and oxidative stress [6]. With the surge of anti-smoking campaigns, increased taxes on tobacco products and further smoking bans and restrictions, the global sales of cigarettes have consistently decreased over the last decade [7]. Therefore, the major multinational tobacco companies started to recognize the need to invest in alternative tobacco and nicotine products such as Swedish snus or electronic cigarettes (e-cigarettes) [8, 9].

1.2 SWEDISH SNUS

The sale and commercial distribution of Swedish snus is prohibited within the European Union (EU) since 1992. Sweden was granted an exemption from this rule as a condition to becoming a member state in 1995, arguing a historically long tradition of snus use that dates back to the 18th century [10]. In spite of strong lobbying efforts by Swedish Match towards the European Commission of Health, this ban still applies for the remaining member countries [8, 11]. In 2011 several leading tobacco companies, including Swedish Match, Altria (Marlboro) and R.J. Reynolds (Camel), made a push to expand their marketing in the United States. Snus was initially sold in predetermined test market cities in the US, however it quickly became widely available and continues to be a highly competitive market. The marketing campaigns are set up to appeal to current smokers as a discreet alternative in situations where smoking is not possible, yet they also noticeably target non-tobacco users, predominantly aimed at women and adolescents [12, 13].

Cigarettes and snus are both tobacco products, however during the burning process of combustible tobacco, carcinogenic and pro-inflammatory substances are produced and

inhaled, including tobacco-specific nitrosamines (TSNAs), volatile organic compounds (VOCs) such as aldehydes and benzene, polycyclic aromatic hydrocarbons (PAHs) and metals [14]. Snus, which is a smokeless product, does contain many of these same substances, but in significantly lower amounts compared to cigarettes [15]. It is also important to distinguish between Swedish snus and American oral moist snuff, also known as chewing tobacco or 'dip'. Due to different temperatures during manufacturing as well as fermentation process, American oral moist snuff contains much higher amounts of aldehydes, TSNAs and PAHs than Swedish snus [15, 16]. Another key difference between these two products is that the saliva while using chewing tobacco needs to be spit out. For many Americans, this conjures up old-fashioned images of Western spittoons or baseball players, however the reality of today is that most users of chewing tobacco will carry an empty plastic bottle or a 'mud jug' which is often seen as inappropriate for the workplace and many other public situations. This difference is often heavily emphasized in snus advertising in the US, with phrases such as 'spitless', 'discreet' and 'break free' [12, 13].

In Sweden, 22.2% of men and 3.9% of women use snus on a daily basis [17]. Cigarette smoking is less common with only 10.6 % of men and 11.2 % of women classified as daily smokers [17]. There is currently a debate whether snus should be introduced and actively suggested as a smoking cessation aid. Sweden is frequently mentioned as a favorable example considering the low habitual smoking rate compared to other developed countries. Tobacco Companies refer to the low prevalence of cigarette smoking in Sweden as "The Swedish Experience" and claim that it is largely attributed to the widespread use of snus [18, 19]. Survey studies have suggested that Swedish men have used snus as a smoking cessation aid [20, 21]. However, three placebo-controlled studies testing snus as a smoking-replacement therapy failed to demonstrate that snus is superior to established nicotine replacement therapy (NRT) [22-24]. NRT does not contain TSNAs or other potential health hazardous compounds found in snus, and has established quit plans, therefore making it a more feasible approach to smoking cessation [24].

Those who advocate that chronic snus use is still an acceptable substitute for smoking refer to the great difficulties to link snus to increased morbidity [18, 19, 25, 26]. However, in recent years an increasing number of studies have demonstrated a correlation between snus use and increased mortality in myocardial infarction and stroke [27-30]. Snus use has also been associated with an increased risk of type 2 diabetes and heart failure [31-33]. Arefalk et al. examined mortality after myocardial infarction among smokers and snus users and found that both snus users and smokers who quit their tobacco use halved their risk of dying within the next two years [34]. However, several other studies have seen no increased risk of cardiovascular events directly associated with snus use [35-37]. As snus has long been limited to a Scandinavian market, few studies have been conducted until recently. The contradicting and many times inadequate data, has led to considerable media attention where it has been questioned whether snus should really be regarded as a healthy and safe alternative to smoking [2].

The acute effects of snus on human vasculature are still not very well known. Snus does increase blood pressure and heart rate and has been linked to endothelial dysfunction measured by flow-mediated dilatation in the brachial artery (FMD) [38, 39]. Snus is also known to have a negative impact on cardiac diastolic function [40]. Beyond this, the health effects of snus have been relatively unexplored.

1.3 ELECTRONIC CIGARETTES

In recent years, the e-cigarette has been introduced as an alternative to conventional cigarette smoking. First patented by a Chinese pharmacist in 2003, the e-cigarette quickly found its way onto the global market. It consists of a tank filled with liquid which is transported through a cotton or silica wick into a battery driven atomizer which heats the liquid and produces an aerosol (Fig. 1) [41]. This aerosol is inhaled much in the same way as ordinary cigarette smoke. It is important to note that no combustion occurs during this process and e-cigarette users refer to the inhalation as "vaping" rather than smoking. Thus far, several models of e-cigarettes have been launched through its relatively short history. The first-generation model ('cigalikes') resembles a conventional cigarette and is usually not refillable or rechargeable. The second generation, which started to diverge from the resemblance to traditional cigarettes, also introduced refillable tanks as well as rechargeable batteries. Third/fourth generation devices include a user interface that allows in-detail control of several heating parameters (Fig. 1). The e-cigarette liquid (e-liquid) can be purchased with or without nicotine and is available in a huge variety of flavors such as candy, desserts, tobacco, menthol, fruits as well as spices and herbs [42]. It can also be purchased as individual components, so the user can create their own mixtures.

Initially distinctive e-cigarette-focused companies produced and sold the products. However with decreasing cigarette sales in the western world, in addition to observing the rapid growth of the e-cigarette market share, international tobacco industries have gradually acquired already established e-cigarette companies to include in their portfolios [7, 9]. Today, e-cigarettes constitute a large and exponentially growing market, particularly in the US, EU and parts of Asia. Due to quick changeovers in this market, it is often difficult to estimate total global numbers of e-cigarette users. However, in the US it is estimated that 3.2% of adults and in the EU 2 % use e-cigarettes on a regular basis [43, 44].

E-cigarettes are currently marketed towards smokers as a healthier alternative to cigarettes and towards non-smokers as a new and trendy product with a large variety of designs and flavors [42, 45]. Current cigarette smokers as well as smoking-naïve individuals are both known to be consumers of e-cigarettes [46]. Recently, some researchers have warned that e-cigarettes may be a gateway to conventional cigarettes [46, 47]. Several studies have also examined the validity of e-cigarettes as a smoking cessation aid. Certain show positive quit results, whereas others have found an increased risk of double use, i.e. both cigarettes and e-cigarettes [48-51]. Despite great uncertainty about the safety of the e-cigarette, it is widely considered by the public to be a healthier alternative to smoking and a feasible tool for smoking cessation.

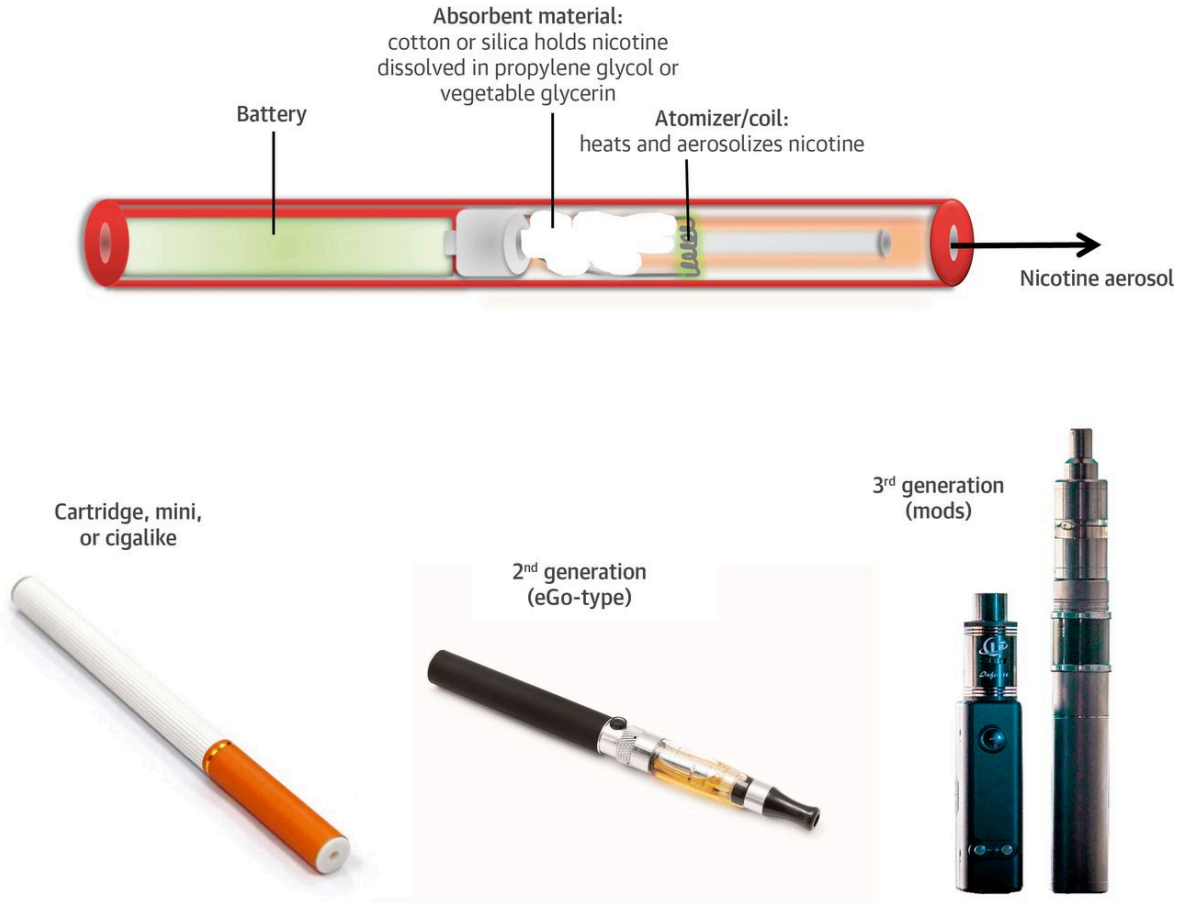

Fig. 1. E-cigarette schematic and pictures of different e-cigarette generations. Adopted from Morris, P.B., et al., Cardiovascular Effects of Exposure to Cigarette Smoke and Electronic Cigarettes: Clinical Perspectives From the Prevention of Cardiovascular Disease Section Leadership Council and Early Career Councils of the American College of Cardiology. J Am Coll Cardiol, 2015. **66**(12): p. 1378-91.

The e-liquid is composed of a base mixture of varying mixtures of vegetable glycerin (VG) and propylene glycol (PG) and may also contain nicotine and various flavoring agents [52, 53]. VG is a common food-additive and also found in industrial, commercial and pharmaceutical products. PG is best known for its use in fog-machines and exposure is known to cause eye and airway irritation [54]. The effects of VG when heated and inhaled are not known. In addition to nicotine, several toxins have been found in the e-cigarette vapor and e-liquid including nitrosamines, PAHs, volatile organic compounds (VOCs) and metals. However, the concentrations of these compounds are usually much lower compared to CS [52, 53]. On the other hand, one research group found reactive oxygen species (ROS) in similar quantities as detected in CS, and 6 times higher levels of copper in the e-cigarette aerosol [55]. Jensen et al. found that the amount of formaldehyde was significantly higher when e-liquid was vaporized at higher temperatures and estimated a 5-15 times higher risk of cancer compared to conventional cigarettes [56]. On the other hand, a study comparing carcinogens in urine of cigarette smokers and e-cigarette users showed significantly lower levels in the latter group [57]. Thousands of different flavorings are offered and for most of

these, the potential health effects are unknown. One flavor-additive, diacetyl, which can be found in buttery or custard flavorings can cause pulmonary damage (bronchiolitis obliterans) [58]. Furthermore, menthol and cinnamon flavorings have been associated to cytotoxicity and inflammation in vitro [59, 60].

So far, in vitro studies have been contradictory [52]. Several adverse effects of e-cigarette vapor or e-liquid were found in different cell lines (endothelial, epithelial and fibroblastic cells) such as increased cytotoxicity and gene expression, decreased cell viability or reduced antioxidant defenses [61-64]. Other studies have shown little or no cytotoxicity [65-67]. There are only a few studies that compare the concentration of particles in the e-cigarette vapor to conventional CS. Real-time measurement of particulate matter revealed lower levels in e-cigarette vapor compared to CS [68]. Pellegrino et al. showed that ECV contained lower levels of fine and ultrafine particles than in CS [69]. Though, there are two studies that have displayed a similar number and size distribution of particles in e-cigarette vapor as in regular CS [70, 71].

There is currently a debate about the possible health effects of e-cigarette use and authorities are struggling with regulations regarding sales, advertisement and use of this new product. Initially, the Swedish Medical Products Agency passed a decision that classified e-cigarettes as a drug because of its nicotine content. Following several appeals to the Swedish Supreme Administrative Court this decision was finally overturned in February 2016. Other European countries are also conflicted about regulations as there is no common legislation within the EU. The European Commission tried to pass harder regulations on the distribution of e-cigarettes, however strong lobbying efforts by the e-cigarette industry caused the European Commission to amend its directive on tobacco products and thus allow the trade of e-cigarettes in the EU [72].

1.4 MEASUREMENTS OF VASCULAR HEALTH

Most cardiovascular disease (CVD) is attributed to the development and progress of atherosclerosis. Endothelial dysfunction is defined as the impairment of vasodilating, anti-inflammatory and anti-thrombotic properties of the endothelium and is considered to be the first step in the onset and advancement of atherosclerosis [73]. Many methods have been developed to assess vascular health and endothelial dysfunction which has resulted in deeper insights surrounding the physiological and pathophysiological processes.

1.4.1 Arterial Stiffness

With increasing age, changes naturally develop in the vascular wall, though these can be further accelerated by risk factors for CVD. These modifications involve not only the endothelium, but also the surrounding smooth muscle cells and elastic membranes, which result in endothelial dysfunction, loss of elastic properties and arterial thickening [74]. This pathophysiological process leads to increased arterial stiffness which can be assessed utilizing various methods [75].

1.4.1.1 Pulse wave analysis

During a cardiac cycle, a forward moving pulse pressure wave is generated in systole (Fig. 2). This wave travels through the aorto-arterial bed and is partly reflected at points of impedance mismatch, i.e. branching points in the periphery of the arterial tree, mainly at arteriole level. A retrograde traveling reflection wave is created which physiologically arrives back at the heart during diastole, enhancing coronary artery filling. With increased central aortic stiffness and peripheral resistance, the pulse wave travels faster and is reflected earlier, instead arriving at the heart during end-systole. This results in an increase of the central pulse pressure which is defined as augmentation pressure (AP). The pulse pressure waveform (overlap of the forward-travelling and reflected wave) can be recorded by applanation tonometry at the carotid or the radial artery. When recorded at the radial artery, a transfer function is applied to estimate the central pulse pressure waveform. The percentage of the pulse pressure which is attributed to AP is reported as the augmentation index (AIx). AIx is sensitive to changes in heart rate (HR), so it is normally reported standardized for a heart rate at 75bpm (AIx75) [76].

1.4.1.2 Pulse wave velocity

Another method of assessing arterial stiffness is the estimation of the forward travelling pulse pressure transit-time by determining arterial pulse wave velocity (PWV) [77]. The arterial pulse wave is commonly registered at the carotid artery and the femoral artery. The distance between these two points is then divided by the time it takes for the pulse wave to travel this length, resulting in PWV. Increased PWV is associated with an increase in central arterial stiffness.

The current recommendations for measuring arterial stiffness is to employ both methods, since PWV and AIx75 provides slightly different information and are not considered completely interchangeable [78]. PWV may be seen as a “direct” assessment of central aortic stiffening, whereas pulse wave analysis provides additional, systemic information on arterial stiffness and is seen as an indirect marker for endothelial function. Several studies have shown that increased arterial stiffness is a blood-pressure independent predictor for CVD risk and that it may serve as a biomarker for pharmacological and non-pharmacological interventions [79, 80].

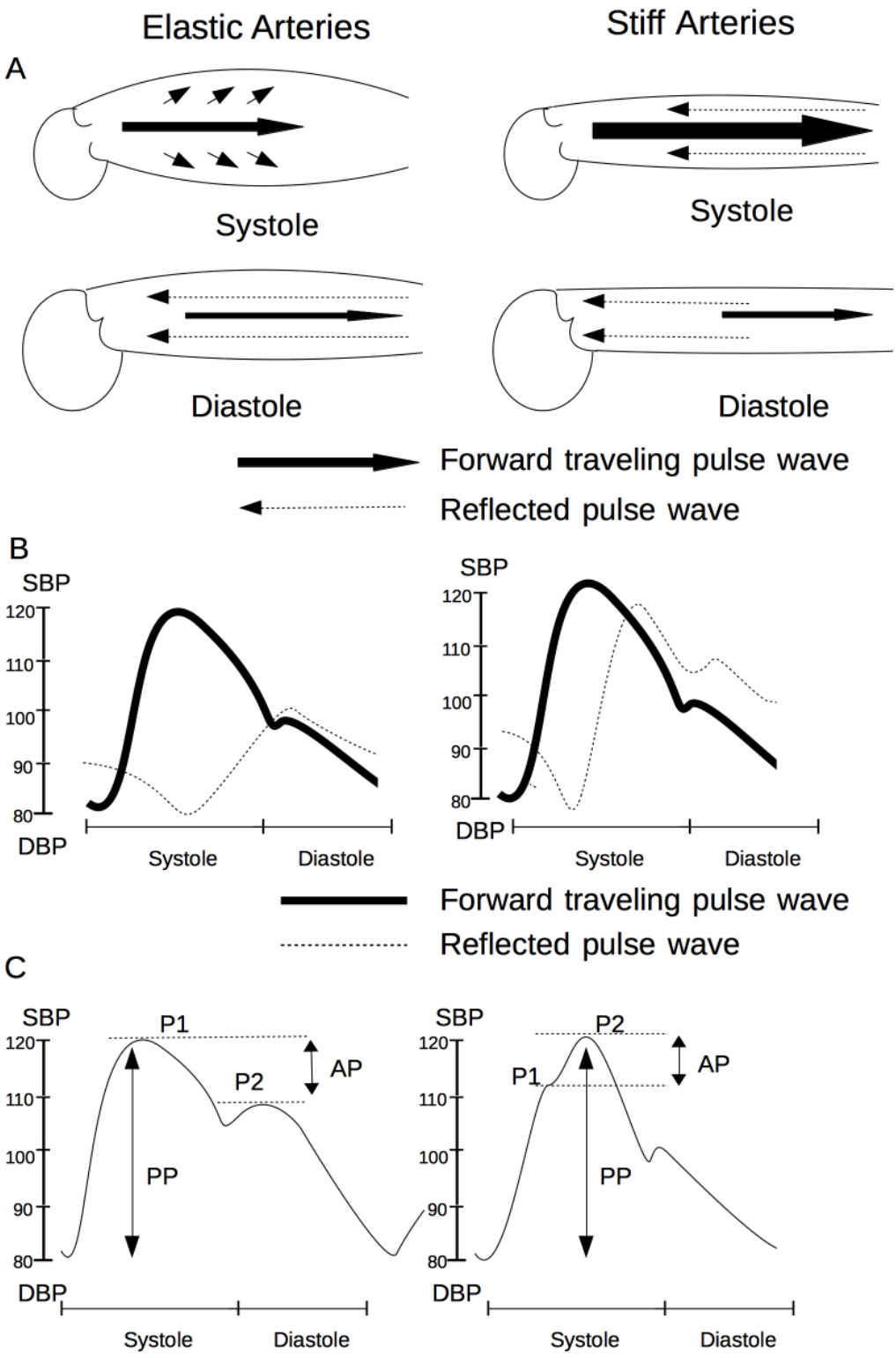

Fig. 2. A) and B) Schematic over the forward travelling and reflected pulse-pressure wave in the aorta in elastic and stiff arteries. C) Conjugate pulse-pressure waveform. The schematic on the left shows no augmentation of the pulse pressure (PP). Peak 1 (P1) is not increased by the reflected pulse pressure wave represented as peak 2 (P2). In stiff arteries, P2 is increased. P2-P1 is defined as augmentation pressure (AP). Augmentation index (AIx) is reported as (AP/PP)x100. On the left: PP = 40mmHg, AP = -10mmHg, AIx = -25%. On the right: PP = 40mmHg, AP = 10mmHg, AIx = 25%.

1.4.2 Venous occlusion plethysmography

Healthy arteries dilate in response to numerous stimuli, such as reactive hyperemia, pharmacological vasodilators, the sympathetic nervous system and endogenous substances. Different vascular beds can be assessed by a variety of methods [81]. One of the first approaches to estimate endothelial function was the measurement of forearm blood flow (FBF) using venous occlusion plethysmography (VOP) which also allows for the assessment of fibrinolytic function at the same time [82, 83].

1.4.2.1 Forearm blood flow

In this method, a cuff is placed around the upper arm and inflated to a pressure that occludes venous outflow yet allows for arterial inflow in the lower arm (Fig. 3). A mercury strain gauge is placed around the lower arm in order to record changes in the circumference of the arm. The tissue volume change is proportional to the arterial blood flow, i.e. the faster the tissue volume change, the faster the FBF. To exclude the complex vascular bed in the hand a second cuff is placed around the wrist and inflated to the pressure that briefly occludes both venous and arterial flow. Endothelial function is measured as the response of FBF to various vasodilating drugs infused through a brachial intra-arterial cannula. Typically, different drugs are used to assess divergent vasodilating pathways. Acetylcholine (ACh), methacholine and bradykinin (BK) bind to the endothelium and cause vasodilation through different pathways, but primarily through the local release of nitric oxide (endothelium dependent or NO-dependent pathway). Sodium nitroprusside (SNP) and glyceryl trinitrate (GTN or nitroglycerin) are direct NO-donors, therefore these drugs allow for the assessment of NO-independent pathways. As a result of the cuffs and the doses the infused drugs mainly generate local effects and simultaneous VOP of the non-infused arm serves as the control [81]. This method is highly reproducible and even though endothelial function is assessed in the forearm it is well correlated to cardiovascular risk factors and CVD [84-87]. The main disadvantages to this gold standard method is that it is time-consuming and semi-invasive.

1.4.2.2 Fibrinolytic function

The endothelium has the ability to release fibrinolytic tissue-plasminogen activator (tPA) and pro-coagulant plasminogen activator inhibitor (PAI), which can both be stimulated upon infusion of bradykinin or substance P [88]. By analyzing these complexes in the infused as well as the non-infused arm, the fibrinolytic capacity of the endothelium can be estimated. This validated process has previously been employed to assess fibrinolytic function both in the forearm and in coronary arteries [89-91].

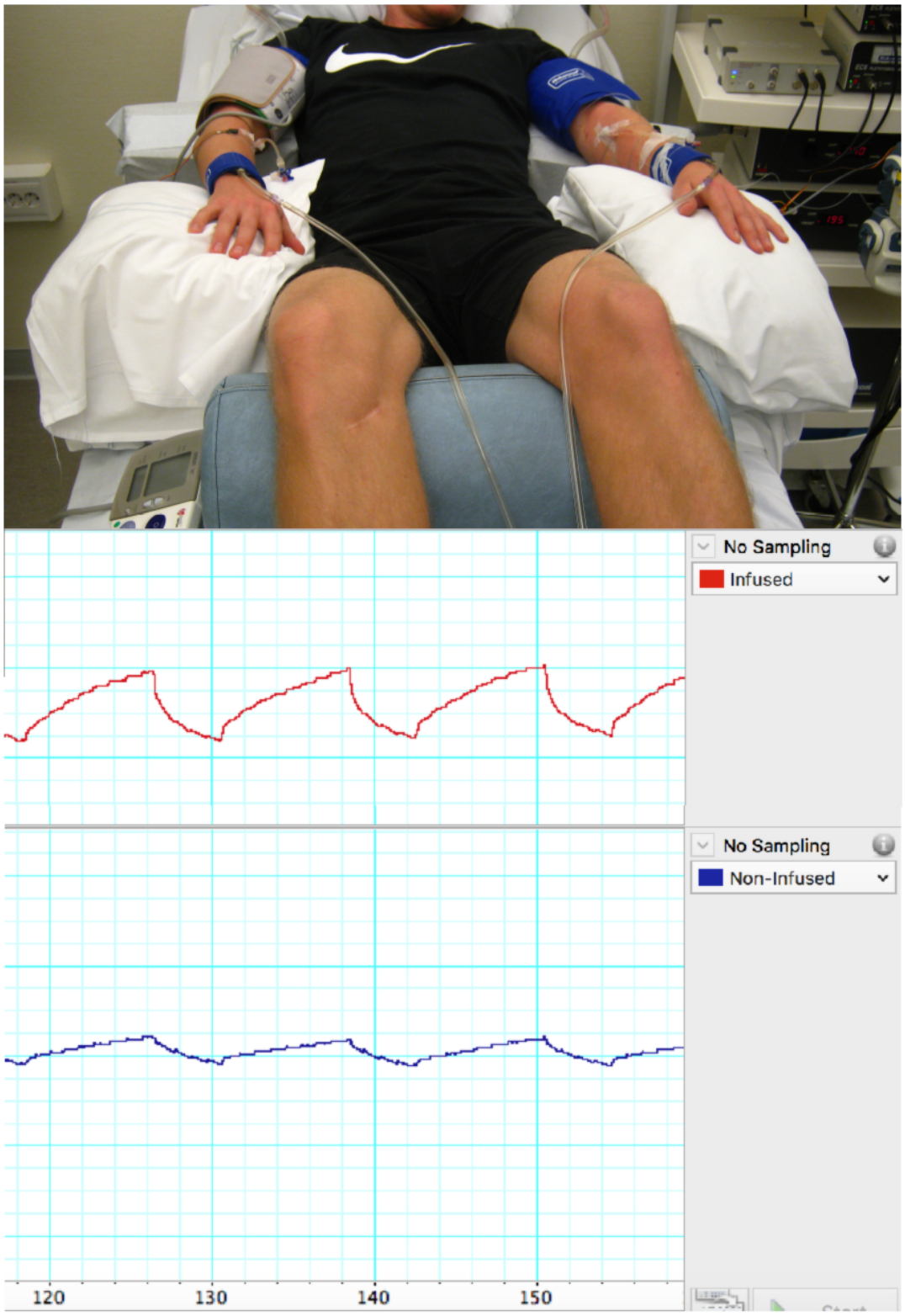

Fig 3. Venous occlusion plethysmography. Top picture shows a volunteer in a semi-supine position with inflatable cuffs, around the upper arms and the wrists. An intra-brachial artery needle is placed on the left arm for infusion of vasodilators. Bilateral strain-gauges are positioned around the forearm to detect changes in arm circumference. Venous cannulas are in place on both arms for tPA/PAI-blood sampling. The bottom picture demonstrates the change in forearm volume over time [seconds] upon administration of bradykinin in the infused (top) and non-infused arm (bottom).

1.4.3 Biomarkers for vascular health

Several biomarkers have been proposed for the measurement of vascular health [92]. Two biomarkers have emerged during the last couple of years that are of particular interest in this area: Microvesicles (MVs) and endothelial progenitor cells (EPCs).

1.4.3.1 Microvesicles

Microvesicles were first described in 1967 by Peter Wolf as "platelet-dust". Since then, researchers have further characterized this new discovery and renamed them microvesicles or microparticles [93]. MVs are small vesicles (100nm to 1µm in diameter) that can be released from the membrane of all cells and platelets upon their activation or apoptosis [94-96]. Like the cell membrane, MVs consist of phospholipids and receptors, depending on the cell of origin and may also carry nucleotide information like DNA or RNA. Upon cell activation, extracellular calcium flows into the cell causing a subsequent calpain and kinase activation and phosphatase inhibition. The resulting cytoskeleton disruption allows for the formation or "blebbing" of MVs from the cell membrane (Fig. 4). Several triggers, which are related to cell activation, inflammation and thrombosis may induce MV mobilization, i.e. reactive oxygen species (ROS), collagen or thrombin [94-96].

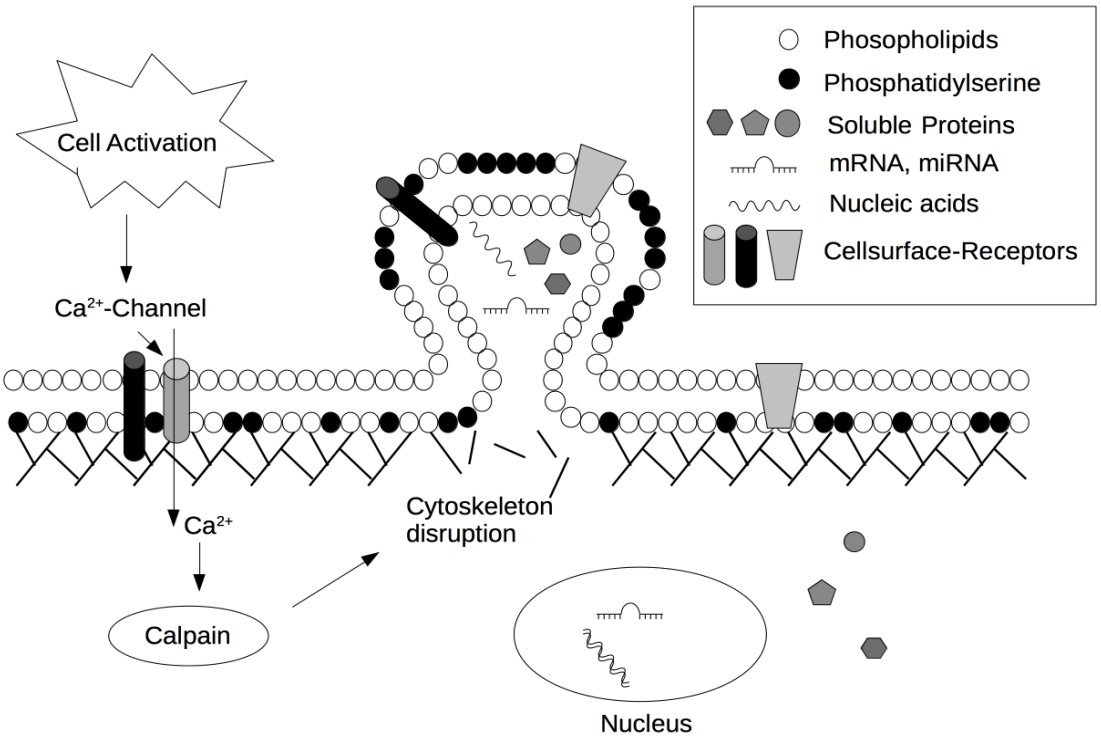

Fig. 4. Schematic over the formation of a microvesicle and its composition.

The physiological explanation why MVs are released is not yet fully known. MVs can be incorporated by other cells and therefore be involved in intracellular communication [97]. MVs are also involved in hemostasis, as they exhibit pro-coagulant properties through extra-

cytoplasmic phosphatidyl-serine and expression of tissue factor, but also induce fibrinolysis through tissue plasminogen activator (tPA) [98, 99]. Furthermore, MVs may trigger endothelial homing of leukocytes through expression of cell-adhesion molecules in endothelial cells and are therefore involved in inflammatory processes [100]. Furthermore, it has been suggested that MVs participate in the regulation of vascular tone by modulating NO production [101, 102]. Taken together, MVs play a crucial role in maintaining an equilibrium of endothelial function.

MVs may serve as biomarkers that reflect different pathophysiological states. Of particular interest are MVs originating from endothelial cells, leukocytes, platelets and erythrocytes, as they can be measured in the blood stream. Most MVs (40-90%) originate from platelets, followed by endothelial and red-blood cell origin [94-96]. Elevated levels of MVs are found in patients with myocardial infarction, ischemic stroke, hypertension or type 2 diabetes [103-106]. MVs may also serve as biomarkers in other pro-inflammatory states such as infectious or rheumatic diseases [107, 108]. More studies are needed in order to investigate the reproducibility of MVs as biomarkers for specific diseases. However, it is quite possible that in a near future MVs may become available as biomarkers utilized in routine clinical practice.

1.4.3.2 Endothelial Progenitor Cells

Endothelial progenitor cells derive from hematopoietic stem cells in the bone marrow and are a heterogeneous population of pluripotent cells. First discovered by Asahara et al., EPCs have been found to play an important role in vascular repair and neoangiogenesis [109-112]. However, characterization is still debated as surface markers of EPCs may differ in different states of maturation, often classified into ‘early’ and ‘late’. Several surface antigens, which are co-expressed on hematopoietic stem cells, have been proposed in order to categorize EPCs, such as CD34, CD31, CD133, CD117, CD105, CD184 and CD309 [110-112]. Even though it is still debated which markers truly define EPCs and in which state of maturity, some markers have become more common in the identification of EPCs, namely CD34 and CD133 (co-expressed on hematopoietic stem cells) and CD309, also called vascular endothelial growth factor receptor 2 (VEGFR2) or kinase insert domain receptor (KDR).

Early EPCs mainly reside in the bone marrow whereas late EPCs are found in the circulation as well as within the vascular wall (Fig. 5) [113]. The main cytokines initiating EPC mobilization and homing to the site of vascular activation and/or damage are vascular endothelial growth factor (VEGF) and stromal cell-derived factor 1 (SDF-1) [114]. Therefore, elevated levels of EPCs are found in states of acute vascular injury such as myocardial infarction, unstable angina or ischemic stroke [115-117]. The number of circulating EPCs decreases with age and lower levels are also found in states associated with chronic vascular impairment, for example hypertensive disease, dyslipidemia and type 2 diabetes [118-121]. Reduced levels have also been demonstrated in chronic cigarette smokers with stable coronary artery disease (CAD), as compared to controls and non-smokers with CAD [122]. Hence, it has been suggested that chronic mobilization of EPCs as a consequence of recurring vascular damage/activation leads to a depletion of EPC reserves. Levels of

circulating EPCs are an independent and inversely correlated risk factor for the development of CVD [123, 124]. EPCs have also been investigated as possible therapeutics in CVD [114].

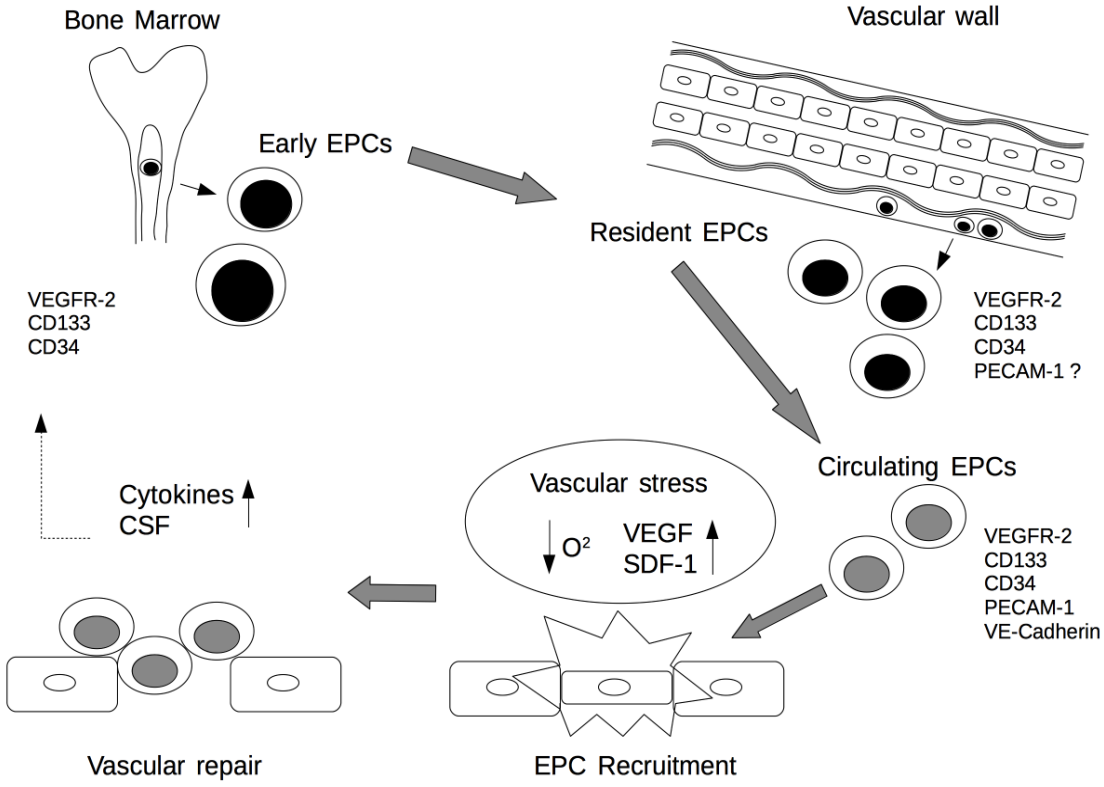

Fig. 5. Schematic over mobilization of endothelial progenitor cells and cell-surface markers in different states of maturation. CSF: Colony stimulating factors.

2 AIMS

To assess acute effects of cigarette inhalation on circulating microvesicles and endothelial progenitor cells (Paper I)

To assess acute effects of e-cigarette inhalation on circulating microvesicles and endothelial progenitor cells (Paper II)

To assess the acute effects of e-cigarette inhalation on arterial stiffness (Paper III)

To assess the effects of chronic snus use on arterial stiffness and endothelial function as measured by venous occlusion plethysmography (Paper IV)

To assess the effects of chronic snus use on endothelial progenitor cells (Paper IV)

3 MATERIALS AND METHODS

Paper I was a randomized, cross-over study where healthy volunteers were exposed to cigarette smoke inhalation vs non-smoking. MVs and EPCs in blood samples were analyzed.

Paper II was a randomized, cross-over study where healthy volunteers were subjected to electronic cigarette inhalation with nicotine vs non-inhalation. MVs and EPCs in blood samples were analyzed.

Paper III was a double-blind, randomized, cross-over study where healthy volunteers were exposed to electronic cigarette inhalation with nicotine vs without nicotine. Arterial stiffness was analyzed.

Paper IV was a cross-sectional study investigating healthy, long-term, daily snus users and age-matched controls. Arterial stiffness and forearm blood flow as well as fibrinolytic function and EPCs in blood samples were analyzed.

3.1 STUDY DESIGN

3.1.1 Paper I

Twelve healthy intermittent smokers (max 10 cigarettes per month) were recruited in this randomized cross-over study (mean age 26 ± 5 years, 6 females and 6 males) (Fig. 6). We recruited sporadic smokers due to their ability to inhale cigarette smoke, as well as not exposing nicotine-naïve individuals to nicotine. Exclusion criteria were CVD, diabetes mellitus, asthma, allergy as well as respiratory infection 4 weeks prior to the study. Volunteers had to refrain from heavy exercise, caffeine and alcohol for 24 hours prior to exposure. Any form of nicotine usage was not allowed within 7 days prior to exposure. Participants were randomized to no-exposure (control) or smoking a whole cigarette (John Silver®) during approximately 5 minutes, with one-week washout period between exposures. Exposure and blood sampling were performed in a temperature-controlled room (22 °C) with adequate ventilation to promptly remove exhaled smoke. Volunteers rested at least 15 minutes in a semi-supine position before blood was obtained from an antecubital vein at baseline, and at 1, 4, 24 hours after exposure.

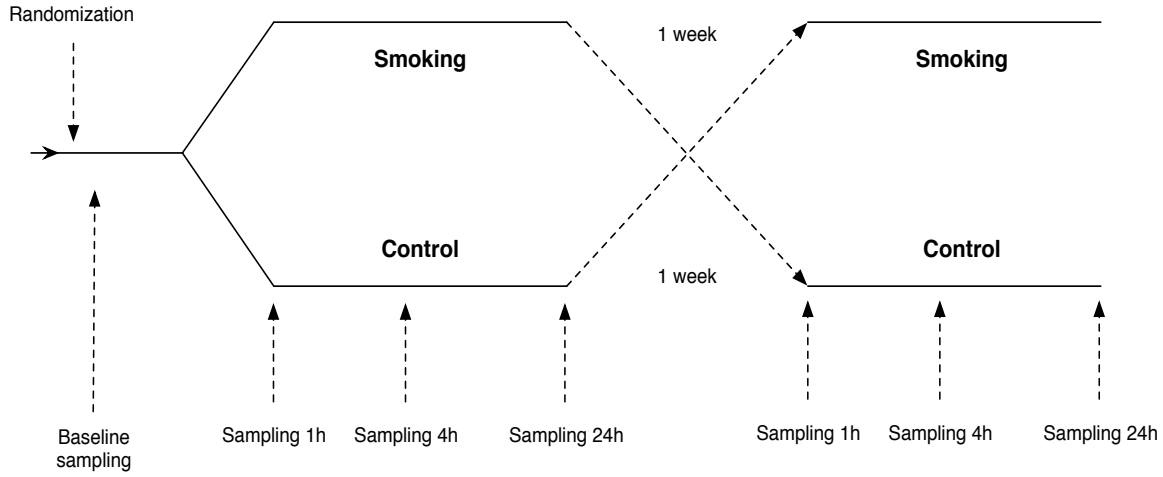

Fig. 6. Study-design, Paper I.

3.1.2 Paper II

Sixteen healthy sporadic smokers (max 10 cigarettes per month) were recruited in a randomized cross-over study (11 males and 5 females, mean age 27 ± 5 years) (Fig. 7). Exclusion criteria were the same as in the study described in Paper I. All volunteers completed a normal health declaration. Prior to exposure, all volunteers had to refrain from caffeine, alcohol and heavy exercise for 24 hours and nicotine usage for 7 days. Volunteers were randomized into inhaling ECV or no-exposure in a cross-over fashion. Washout period between the exposures was minimum of one week. Unflavored e-liquid with a nicotine concentration of 12mg/ml was used and participants inhaled 10 puffs of ECV during 10 minutes. Inhalation frequency and nicotine concentration of the e-liquid were chosen to achieve a comparable nicotine exposure to a combustible cigarette with 1mg of nicotine [125]. Blood samples were drawn from an antecubital vein at baseline, and at 1, 4 and 24 hours after exposure. All samples were collected following 15 minutes of rest, with volunteers resting in a semi-supine position. Exposures and blood sampling were performed in a temperature-controlled room (22 °C) with adequate ventilation to remove exhaled ECV.

E-liquid without added flavorings and with a nicotine content of 12mg/ml was used: propylene glycol 49.4%, glycerin 44.4%, 5% ethanol (Valeo laboratories GmbH, Germany) [126]. A second-generation, e-cigarette with a dual-coil CE5 atomizer was used (eGo XL, operating at 3,7V, 1100mAh,).

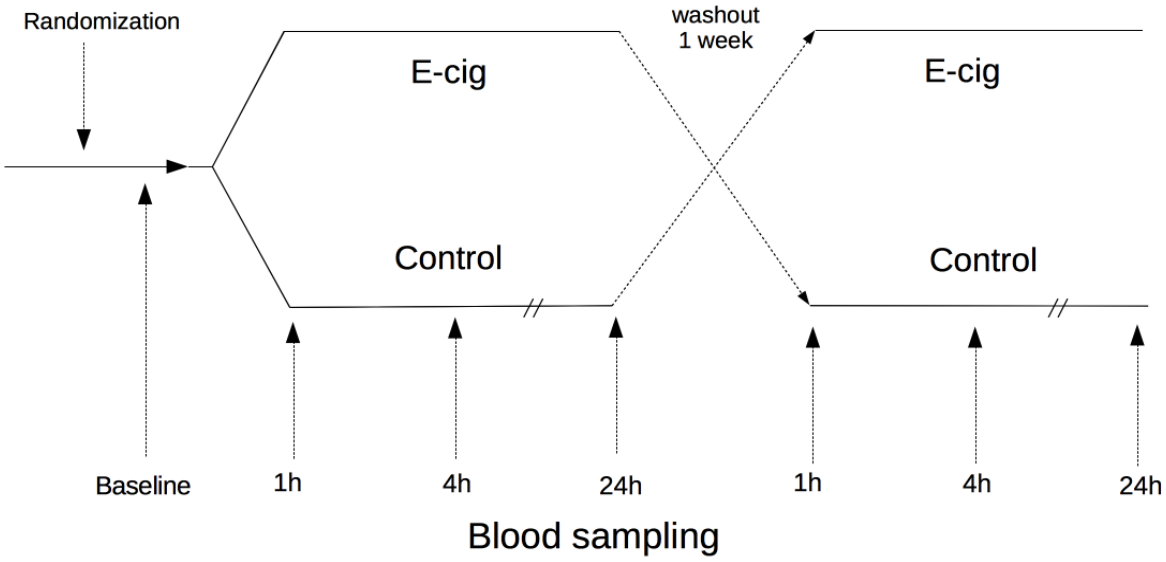

Fig. 7. Study design, Paper II.

3.1.3 Paper III

Seventeen healthy seldom smokers (max 10 cigarettes per month) were included in a randomized, double-blind, cross-over study (10 females, 7 males, mean age 26 ± 3 years). Study participants were randomized to inhaling ECV with or without nicotine with a minimum of one week between exposures (Fig. 8). Upon exposure, participants inhaled 30 puffs of ECV with or without nicotine for 30 minutes. Prior to the study days, volunteers had to abstain from alcohol and caffeine for 12 hours and from heavy exercise for 24 hours and from other tobacco and nicotine-containing products for 14 days. Exclusion criteria included any form of cardiovascular, respiratory, systemic or chronic disease, symptoms of infection or inflammation within 2 weeks prior to study start, BMI≥30 or pregnancy. All volunteers underwent a preliminary clinical examination including ECG, dynamic spirometry, pregnancy test and routine blood tests including full blood count, electrolytes, creatinine, apolipoproteins, HbA1c, APTT and INR. All exposures were performed in a well ventilated, temperature-controlled room. Measurements included heart rate (HR), systolic and diastolic blood pressure (SBP, DBP) and arterial stiffness, these were performed at baseline and every 10 minutes for 30 minutes at 0 h, 2 h and 4 h following exposures.

E-liquid consisted of 49.4% propylene glycol, 44.4%, glycerin and 5% ethanol without any added flavorings (Valeo laboratories GmbH, Germany) [126]. Premixed e-liquids with and without added nicotine were used (0mg/ml and 19 mg/ml resp.).

A variable mod third-generation e-cigarette was used (eVic-VT, Shenzhen Joyetech Co., Ltd., China). The same settings were used for all exposures (temperature 230°C, effect 32 W, resistance 0,20 Ω). A dual coil nickel atomizer was used.

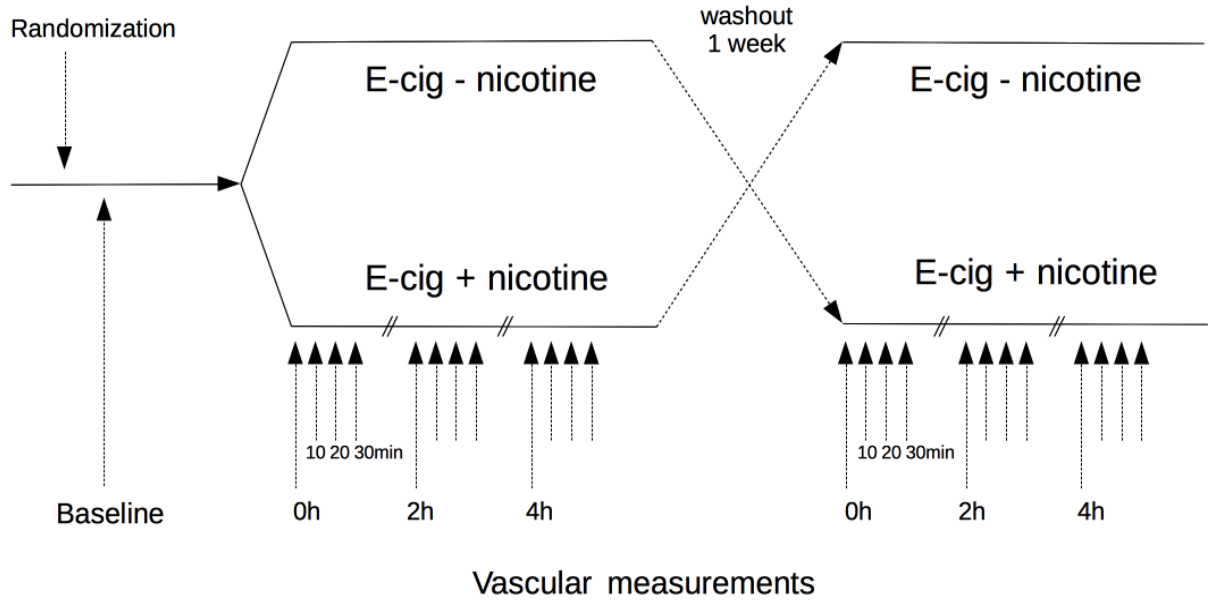

Fig. 8. Study design, Paper III.

3.1.4 Paper IV

Twenty-four healthy, male, chronic snus users (≥15 years of snus use) and 26 age-matched male controls between the age of 30 to 65 years were included into the study. Exclusion criteria were hypertension, any form of cardiovascular, metabolic or respiratory disease, BMI >30 and active allergy or inflammation within four weeks prior to the study. Upon enrollment, study participants had to complete a health form and were investigated with ECG, dynamic spirometry, blood pressure control and blood tests (total blood count, white blood count, Na, K, creatinine, Apolipoprotein A and B, HbA1C, INR, APTT). Height, weight and waist circumference were ascertained upon enrollment. Self-reported tobacco as well as alcohol consumption and level of physical activity was assessed. Prior to measurements, study participants had to abstain from all forms of nicotine, alcohol and caffeine for 24 hours and from vigorous physical activity for 48 hours. Measurements of arterial stiffness and FBF were performed in a quiet, temperature-controlled room. All volunteers were resting comfortably in a semi-supine position.

3.2 METHODS

3.2.1 Microvesicles (Paper I and II)

Blood samples were drawn from an antecubital vein into EDTA containing test tubes and centrifuged at 2000*g* for 20 minutes at room temperature (RT) to obtain platelet free plasma. Until analysis, samples were stored at -70°C.

Prior to analysis, samples were thawed and centrifuged at 2000*g* (20 minutes at RT). Supernatant was centrifuged at 13 000*g* for 2 minutes at RT. Subsequently, 20 µL of sample was incubated for 20 minutes in the dark with phalloidin-Alexa 660 (Invitrogen, Paisley, UK), lactadherin-FITC (Haematologic Technologies, Vermont, USA), CD41-PAC or CD41-PE (Platelet-MVs (PMVs), Beckman Coulter, Brea, CA, USA), CD144-APC (Endothelial-MVs (EMVs), AH diagnostics, Stockholm, Sweden), CD62E-APC ((EMVs), Beckman Coulter, Brea, CA, USA), CD45-PC7 (Leukocyte-MVs (LMVs), Beckman Coulter, Brea, CA, USA) and CD14-PC7 (Monocyte-MVs (MMVs), Beckman Coulter, Brea, CA, USA). In addition, PMVs were labeled with CD154-PE (CD40 Ligand, Abcam, Cambridge, UK) and in Paper II also with CD62P-PE (P-selectin, Beckman Coulter, Brea, CA, USA). MMVs were also labeled with anti-HMGB1-PE (R&D Systems, Minneapolis, USA). Nuclear staining was performed with SYTO13 (Invitrogen, Paisley, UK) alone or double stained with CD144-APC, CD45-PC7, CD41-APC or anti-HMGB1-PE. MVs were measured by flow cytometry (Beckman Gallios, Beckman Coulter, Brea, CA, USA). The MV-gate was determined using Megamix beads (BioCytex, Marseille, France), with diameters of 0.5 µm, 0.9 µm and 3.0 µm, respectively. Particles smaller than 1.0µm in size and negative to phalloidin were considered as MVs. Phalloidin was used in order to exclude cell membrane fragments [127]. MV-counts were calculated as: (MVs counted x standard beads/L)/standard beads counted (FlowCount, Beckman Coulter, Brea, CA, USA). As negative control, conjugate isotype-matched immunoglobulin with no reactivity against human antigens was used (IgG1- PC7, IgG1-APC, IgG1-PE and IgG1-FITC).

3.2.2 Endothelial progenitor cells

3.2.2.1 Paper I and II

Blood samples were drawn from an antecubital vein into sodium citrate containing test tubes and analyzed within one hour. EPCs were measured by flow cytometry (Beckman Gallios, Brea, CA, USA). Whole blood (20 µl) was incubated with CD309 (Becton Dickinson, Franklin Lakes, New Jersey, USA) and CD34-FITC (Beckman Coulter, Brea, CA, USA). Conjugated isotype-matched immunoglobulin with no reactivity against human antigens (IgG1-PE, IgG1-FITC) was used. Cell-fix (Becton Dickinson, San Jose CA, USA) was added after 30 min incubation in dark. Based on size and complexity characteristics of leukocytes, 20 000 leukocyte events were collected, and EPC events were counted in the leukocyte gate.

3.2.2.2 Paper IV

EPCs were measured by flow cytometry (FACSCalibur™, Becton Dickinson, San Jose CA, USA). Whole blood (100 µl) was incubated with CD309-APC (R&D Systems, Abingdon UK), CD34-FITC (Becton Dickinson, San Jose CA, USA), and CD133-PE (Bergisch Gladbach, Germany). Conjugated isotype-matched immunoglobulin (IgG1-PE, IgG1-FITC, and IgG1-APC) with no reactivity against human antigens was used as negative control. After 30 min incubation at 4°C in darkness, red blood cells were lysed with FACS Lysing solution (Becton Dickinson, San Jose CA, USA) for 10 minutes at RT. The remaining cells were then

washed by adding PBS and centrifuged for 10 minutes (twice) and cells were then fixed by adding cell-fix (Becton Dickinson, San Jose CA, USA). 100 000 - 200 000 leukocyte events (based on size and complexity characteristics) were collected and presented as number of EPC events.

3.2.3 Blood pressure and Arterial stiffness (Paper III and IV)

All assessments were performed with volunteers in a semi-supine position in a quiet, temperature-controlled room. Blood pressure and heart rate were measured using a validated semi-automatic oscillometric sphygmomanometer; Paper III: Omron M7 (Omron Healthcare Europe B.V., Hoofddorp, NL) and Paper IV: Boso-Medicus (Boso, Jungingen, Germany). Arterial stiffness was assessed using pulse wave analysis (PWA) and pulse wave velocity (PWV).

PWV was determined by the Vicorder™ system (Skidmore Medical, Bristol, UK). This system measures the pulse transit time between two inflatable cuffs; one placed around the neck and the other around the thigh in order to register the pulse waves in the carotid and femoral arteries. The distance between these two points is manually measured and recorded PWV can then be.

Pulse wave analysis was assessed by micromanometer applanation tonometry (Millar Instruments, Texas, USA) on the right radial artery and analyzed with SphygmoCor™ software (AtCor Medical, Sydney, Australia), which evaluates the aortic pulse pressure waveform via a validated mathematical transfer function. Augmentation index (AIx) and augmentation pressure (AP) were calculated from this waveform. Since AIx is inversely proportional to HR it was normalized for a heart rate at 75 bpm (AIx75). All measurements were in acceptance with the SphygmoCor™ quality control criteria [128].

3.2.4 Forearm blood flow and fibrinolytic function (Paper IV)

All participants underwent cannulation of the brachial artery using a 27-standard wire-gauge steel needle. Following 30 minutes of saline infusion, acetylcholine (ACh) at 5, 10 and 20 µg/min, glyceryl tri-nitrate (GTN) at 4, 8 and 16 µg/min and bradykinin at 100, 300 and 1000 pmol/min were infused for 6 minutes at each dose. All infused vasodilators were separated by 20 minutes of saline infusion and given in a randomized order, maintained at an infusion rate of 1mL/min. Forearm blood flow (FBF) was assessed in both arms (infused and non-infused) by venous occlusion plethysmography using a mercury-in-silicone gauge as describe previously [83]. Briefly, inflatable cuffs were placed around the upper arm and around the wrists. The strain gauge was placed around the widest circumference at the lower arm. The upper cuff was inflated to 40 mmHg for 8 seconds and deflated for 4 seconds. The cuff around the wrist was inflated to 200 mmHg to exclude the circulatory bed in the hand. During inflation of the upper cuff, venous backflow was temporary cut off and arterial inflow caused an increase in lower arm circumference which was registered by the strain gauges and continuously recorded in Lab Chart (AD Instruments, Dunedin, NZ). One measurement cycle

lasted for 3 minutes. Usually, the last 5 recordings were used and averaged. FBF was expressed as ml/s per 100ml of forearm volume.

Periphery venous cannulas (17 gauge) were inserted into large ante-cubital veins on both arms. Ten milliliters of blood were drawn simultaneously (Stabilyte tubes, Biopool Int., Ventura, CA, US) from both sites at baseline and during infusion of bradykinin at each incremental dose. Samples were kept on ice before centrifuged at 2000*g* for 30 minutes at 4°C and stored at -80°C before analyses. Plasma PAI- and tPA- antigen concentrations were analyzed by enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Abingdon UK). Plasma flow was calculated as the FBF multiplied by 1-hematocrit. TPA and PAI net release were calculated by subtracting the plasma levels of tPA and PAI in the infused and the non-infused arm, multiplied by forearm plasma flow in the infused arm.

3.2.5 Cotinine analysis (Paper I-III)

Levels of plasma cotinine were analyzed with an ELISA test kit, following manufacturer instructions (Calbiotech, Spring valley, CA, USA).

3.3 STATISTICAL ANALYSIS

Blinded investigators performed all analysis.

3.3.1 Paper I

Statistical analysis were performed using GraphPad Prism software (5.0c, GraphPad Software Inc., CA, US). Two-way, repeated measures ANOVA was applied, and skewed variables were logarithmically transformed prior to analysis. P-values of <0.05 were considered as statistically significant.

3.3.2 Paper II

GraphPad Prism software (5.0b, GraphPad Software Inc., CA, US) and SPSS Statistics (22.0, IBM Corporation, NY, US) were used for statistical analysis. Due to skewness of data, medians and interquartile ranges (IQR) are reported. A two-way repeated measures ANOVA was applied. Data was logarithmically transformed prior to analysis. If sphericity was violated, Greenhouse-Geisser corrected results were presented. P-values of <0.05 were considered to be statistically significant.

3.3.3 Paper III

The statistical analyses were performed with GraphPad Prism 7.0 (GraphPad Software Inc., CA, US) and SPSS 24.0 (IBM Corporation, NY, US). Data was checked for normality by Shapiro-Wilk test. Two-way, repeated measures ANOVA was used. If Mauchly's test for sphericity was violated, Greenhouse-Geisser corrected results were presented. Within subject contrasts were analyzed to compare baseline values to all other time-points. P-values of <0.05 were considered statistically significant.

3.3.4 Paper IV

Statistical calculations were performed with GraphPad Prism software (7.0, GraphPad Software Inc., CA, US) and SPSS Statistics (24.0, IBM Corporation, NY, US). Data was checked for normality applying Shapiro-Wilk test. Skewed variables were checked for outliers and were analyzed applying a non-parametric test (Mann-Whitney U Test) and normally distributed variables were compared with independent samples T-test. Two-way ANOVA for repeated measures was performed on measurements of FBF. If sphericity was violated, Greenhouse-Geisser corrected results were presented. Skewed variables in multiple measures ANOVA were analyzed following logarithmic transformation. Multiple regression analysis (Method: Stepwise Enter) was applied for arterial stiffness measurements. Prior to analysis, independent variables were checked for collinearity. P-values of <0.05 were considered to be statistically significant.

3.4 ETHICAL APPROVALS

In accordance with the Declaration of Helsinki, all volunteers, in all studies have accepted and signed a written informed consent. Paper I and II were approved of by the regional Ethics Review Board in Stockholm and Paper III and IV were approved by the regional Ethics Review Board in Umeå.

4 RESULTS

4.1 PAPER I

4.1.1 Endothelial progenitor cells

Endothelial progenitor cells increased following inhalation of CS and peaked at 4h (Fig. 9, Table 1). This change over time was significant in multiple measures ANOVA for the interaction variable of 'time*exposure' (p=0.011).

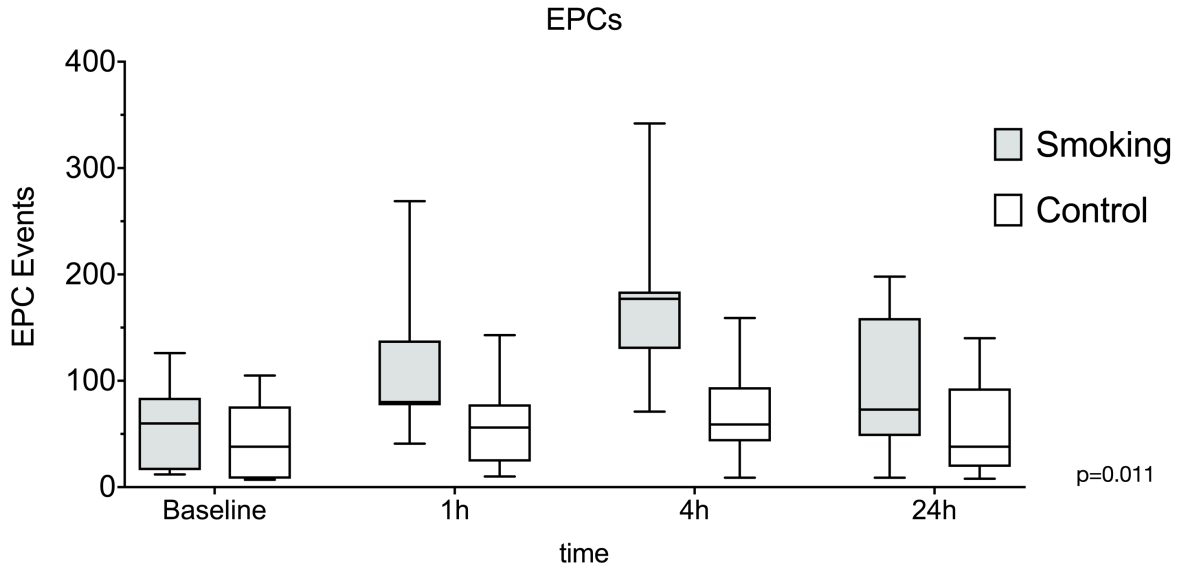

Fig. 9. Endothelial progenitor cells (EPCs) during cigarette inhalation and control.

4.1.2 Microvesicles

Microvesicles and most of MV-subtypes (EMVs, PMVs, LMVs) increased significantly following cigarette smoking (Fig. 10, Table 1). MVs and LMVs containing nuclear molecules increased significantly but not EMVs. No significant change was observed for VE-cadherin positive EMVs and HMGB1 exposure.

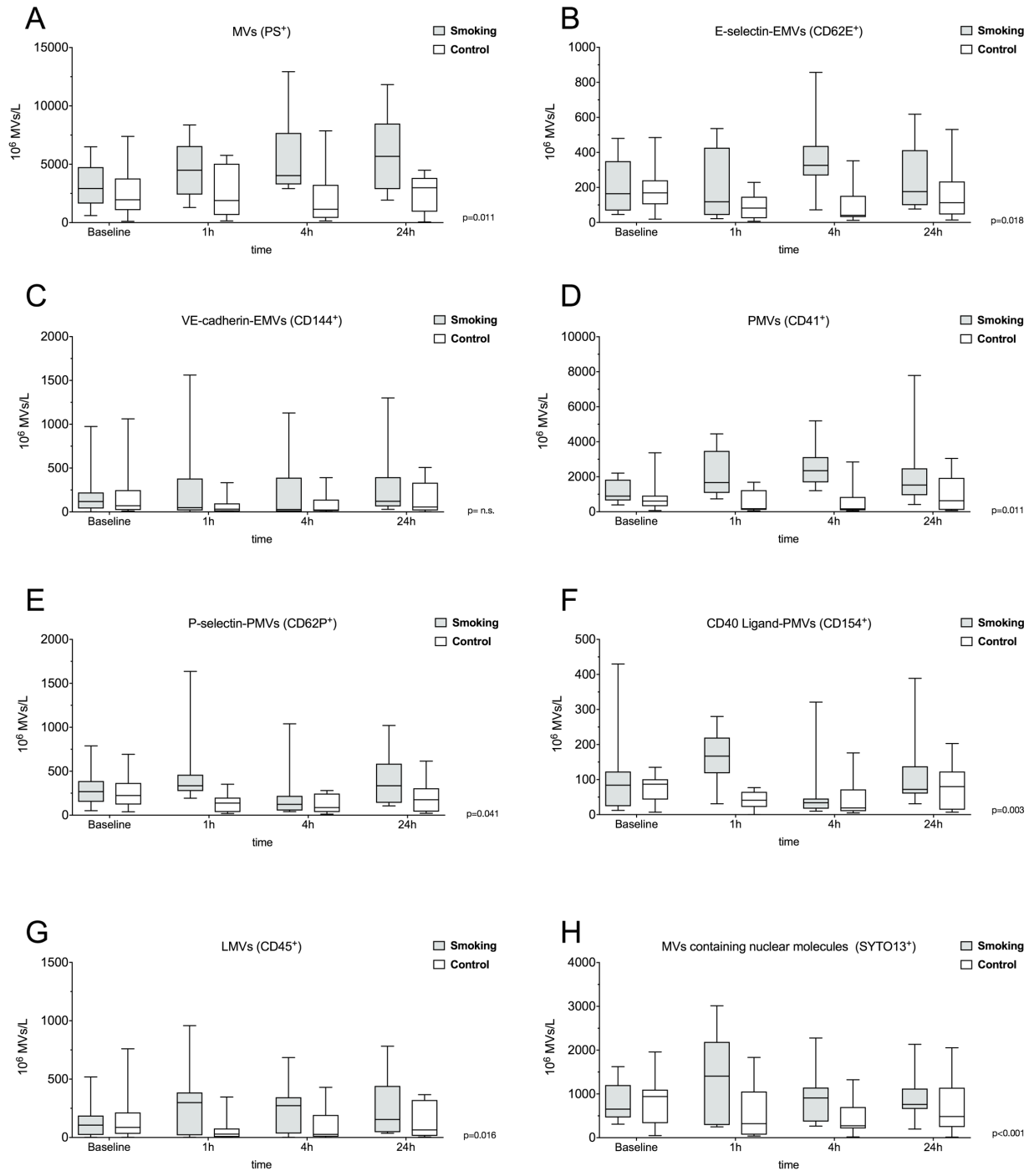

Fig. 10. Microvesicles during smoking and control. (A) total microvesicles (MVs), B) E-selectin positive endothelial-MVs (EMVs), C) VE-cadherin positive EMVs, D) platelet-MVs (PMVs), E) P-selectin positive PMVs, F) CD40 Ligand positive PMVs, G) leukocyte-MVs (LMVs), H) nuclear molecules containing MVs. P-values are given for multiple measures ANOVA and the interaction variable of 'time*exposure'.

		Baseline	1h	4h	24h	p-value
EPCs	smoking	60 [12:126]	80 [41:269]	177 [71:342]	73 [9:198]	0.011
	control	38 [7:105]	56 [10:143]	59 [9:159]	38 [8:140]	
MVs	smoking	2922 [608:6494]	4491 [1296:8369]	4032 [2917:12934]	5678 [1931:11827]	0.011
(PS)	control	1962 [111:7389]	1894 [150:5763]	1144 [145:7860]	2987 [58:4493]	
LMVs	smoking	106 [2:519]	299 [2:958]	273 [2:685]	154 [36:782]	0.016
(CD45)	control	87 [2:760]	29 [0:347]	27 [0:430]	65 [5:367]	
PMVs	smoking	893 [393:2206]	1667 [734:4452]	2348 [1207:5195]	1525 [415:7790]	0.011
(CD41)	control	611 [68:3366]	174 [56:1687]	167 [51:2843]	632 [72:3045]	
PMVs + P-selectin	smoking	268 [51:789]	335 [193:1636]	123 [41:1040]	335 [106:1021]	0.041
(CD41+CD62P)	control	224 [39:693]	138 [19:352]	87 [12:280]	176 [22:615]	
PMVs + CD40Ligand	smoking	84 [12:430]	167 [31:280]	34 [10:321]	72 [31:389]	0.003
(CD41+CD154)	control	87 [7:135]	41 [0:77]	19 [5:176]	80 [7:203]	
E-selectin EMVs	smoking	164 [46:480]	118 [22:536]	326 [72:857]	176 [77:618]	0.018
(CD62E)	control	169 [19:485]	82 [7:229]	41 [12:352]	113 [14:531]	
VE-cadherin EMVs	smoking	118 [0:975]	48 [0:1561]	27 [0:1129]	121 [29:1301]	0.297
(CD144)	control	70 [10:1062]	31 [2:333]	22 [2:391]	56 [2:507]	
MVs + NM	smoking	652 [309:1624]	1407 [249:3014]	907 [265:2278]	760 [198:2133]	<0.001
(PS + SYTO13)	control	941 [48:1959]	321 [39:1834]	273 [19:1325]	485 [14:2054]	
PMVs + NM	smoking	138 [36:536]	331 [12:1441]	43 [2:531]	222 [152:912]	0.003
(CD41 + SYTO13)	control	130 [43:492]	56 [22:478]	31 [10:929]	70 [10:594]	
MMVs + NM	smoking	80 [10:536]	188 [12:878]	19 [0:212]	92 [48:425]	0.014
(CD14 + SYTO13)	control	63 [14:176]	22 [5:169]	5 [0:145]	22 [2:241]	
VE-cadherin EMVs + NM	smoking	7 [0:97]	14 [0:75]	2 [0:46]	24 [7:65]	0.364
(CD144 + SYTO13)	control	17 [2:58]	5 [0:46]	2 [0:51]	5 [0:43]	
E-selectin EMVs + NM	smoking	664 [157:2333]	471 [14:2109]	297 [80:1588]	997 [280:2017]	0.412
(CD62E + SYTO13)	control	1014 [154:2901]	425 [34:1339]	167 [46:1747]	229 [65:3159]	
PMVs + HMGB1	smoking	261 [10:936]	236 [5:813]	65 [14:849]	292 [29:806]	0.808
(CD41 + HMGB1)	control	188 [17:644]	128 [5:767]	97 [12:688]	121 [7:521]	
MMVs + HMGB1	smoking	41 [0:106]	22 [0:150]	12 [0:97]	31 [2:101]	0.818
(CD14 + HMGB1)	control	19 [2:101]	22 [0:118]	14 [0:191]	31 [0:92]	
HMGB1 + NM	smoking	919 [398:1815]	1047 [157:1863]	652 [186:1670]	1115 [246:2464]	0.218
(HMGB1 + SYTO13)	control	1127 [84:2307]	410 [58:2008]	340 [34:1561]	842 [24:2273]	

Table 1. Median number and [total range] of endothelial progenitor cells (EPCs) and microvesicles (MVs) at baseline and 1h, 4h and 24h following cigarette inhalation and control. P-values from multiple measures ANOVA ('time*exposure') are presented. PS: Phosphatidylserine, LMVs: Leukocyte-MVs, EMVs: Endothelial-MVs, MMVs: Monocyte-MVs, PMVs: Platelet-MVs, NM: Nuclear molecules, HMGB1: High mobility group protein B1.

4.2 PAPER II

Cotinine was measured at baseline in all study participants to ensure nicotine abstinence prior to the study. Despite instructions, elevated levels of cotinine were found in two participants. Both were excluded, and 14 participants were analyzed (9 males and 5 females, mean age 28.4 ± 5.1 years).

4.2.1 Endothelial progenitor cells

Levels of EPCs were increased 1h and 4h following inhalation of ECV (Fig. 11, Table 2). This was statistically significant in repeated measures ANOVA for the interaction-variable 'time*exposure' (p=0.002) as well as for separate time point analysis.

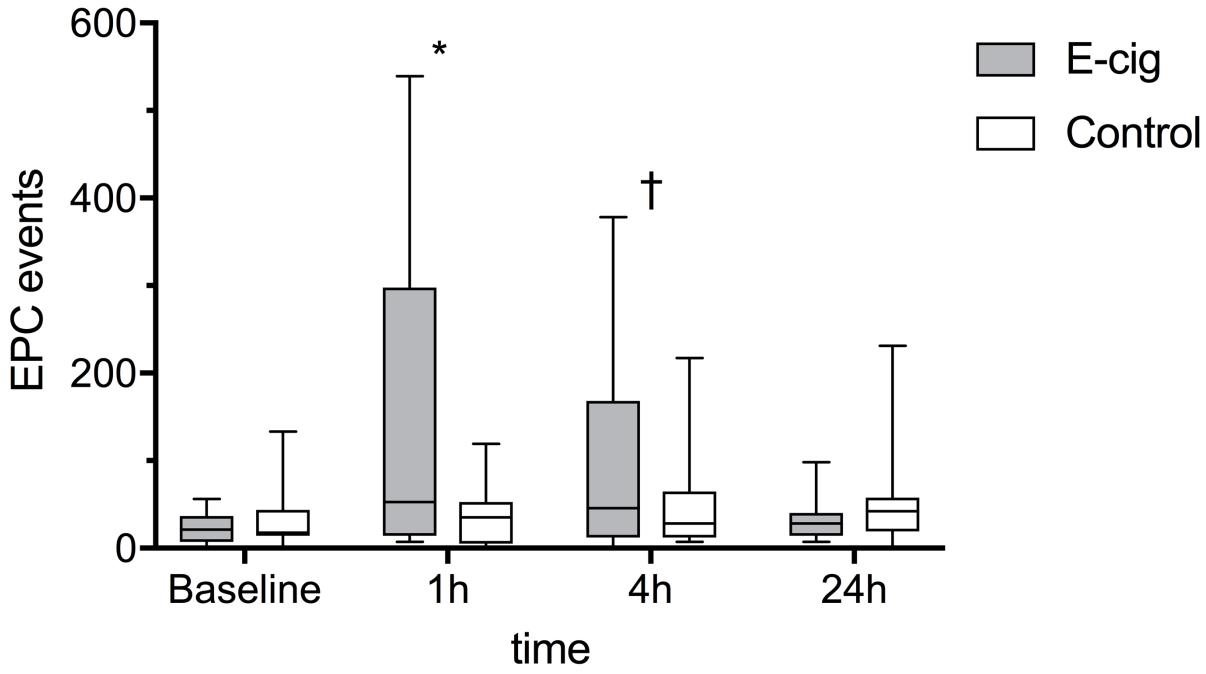

Figure 11. Endothelial progenitor cells (EPCs) during e-cigarette inhalation and control. Separate time point analyses: * 1h vs baseline: p=0.003 and † 4h vs baseline: p=0.036.

4.2.2 Microvesicles

There were no significant differences in MVs and MV-subgroups between the two exposures with the exception of $CD62E^+$ EMVs (Table 1). Further analysis of inflammation markers (HMGB1) and nuclear molecules (SYTO13) showed the same pattern.

		Baseline	1h	4h	24h	p-value
EPCs	e-cig	21 [7:35]	52.5 [14:287]	45.5 [14:147]	28 [14:39]	0.002
	control	17.5 [14:42]	35 [7:49]	28 [14:63]	42 [21:56]	
MVs	e-cig	1725 [731:4012]	2600 [1264:7668]	5102 [2164:7858]	5731 [1402:7176]	0.683
(PS)	control	1557 [1020:4997]	3277 [2038:4987]	3700 [2545:4494]	2724 [2012:4858]	
LMVs	e-cig	4 [2:17]	27 [15:42]	50 [15:61]	32 [21:55]	0.501
(CD45)	control	8 [3:15]	12 [10:23]	31 [14:46]	35 [15:45]	
MMVs	e-cig	63 [32:117]	52 [18:89]	63 [41:102]	79 [45:123]	0.138
(CD14)	control	26 [7:97]	15 [7:80]	13 [4:36]	21 [13:69]	
MMVs + HMGB1	e-cig	37 [16:72]	32 [11:69]	29 [2:40]	45 [30:81]	0.964
(CD14 + HMGB1)	control	12 [7:69]	8 [5:38]	8 [3:20]	11 [8:38]	
PMVs	e-cig	551 [207:976]	576 [159:1493]	255 [155:694]	271 [52:463]	0.605
(CD41)	control	758 [329:952]	438 [201:849]	311 [239:478]	222 [85:346]	
PMVs + HMGB1	e-cig	76 [34:135]	30 [5:80]	43 [23:120]	81 [35:155]	0.970
(CD41 + HMGB1)	control	32 [14:127]	23 [3:58]	31 [8:87]	39 [16:68]	
PMVs + P-selectin	e-cig	24 [20:68]	136 [36:304]	156 [109:318]	76 [50:182]	0.191
(CD41 + CD62P)	control	22 [8:49]	90 [38:189]	92 [42:179]	84 [57:108]	
PMVs + CD40Ligand	e-cig	15 [10:31]	62 [27:122]	94 [36:172]	50 [25:94]	0.168
(CD41 + CD154)	control	15 [4:39]	46 [27:113]	54 [34:82]	49 [41:70]	
VE-cadherin EMVs	e-cig	19 [12:42]	10 [7:16]	20 [9:25]	20 [11:30]	0.944
(CD144)	control	19 [8:28]	8 [4:23]	10 [7:20]	10 [6:19]	
E-selectin EMVs	e-cig	8 [2:17]	14 [8:43]	28 [17:65]	20 [15:40]	0.038
(CD62E)	control	9 [4:22]	19 [12:40]	23 [14:42]	23 [11:37]	
MVs + NM	e-cig	1120 [763:2233]	1427 [706:4664]	1262 [422:4068]	593 [252:1013]	0.232
(PS + SYTO13)	control	1489 [566:3481]	1572 [972:3500]	2103 [1340:4011]	1005 [363:1692]	
NM + HMGB1	e-cig	164 [56:448]	188 [62:459]	215 [32:613]	46 [14:198]	0.266
(SYTO13 + HMGB1)	control	132 [46:997]	231 [112:297]	371 [74:983]	96 [11:211]	

Table 2. Median number and interquartile ranges [IQR] of endothelial progenitor cells (EPCs) and microvesicles (MVs) at baseline and 1h, 4h and 24h following e-cigarette inhalation and control. P-values from multiple measures ANOVA ('time*exposure') are presented. PS: Phosphatidylserine, LMVs: Leukocyte-MVs, EMVs: Endothelial-MVs, MMVs: Monocyte-MVs, PMVs: Platelet-MVs, NM: Nuclear molecules, HMGB1: High mobility group protein B1.

We noticed that data for MVs and EPCs was significantly right-skewed and several participants had concomitantly high EPC and MV values. We analyzed those five participants who displayed high EPC-values after one hour following ECV inhalation and observed a trend towards increased EMVs ($CD62E^+$, p=0.091), PMVs ($CD62P^+$, p=0.081) and CD40 Ligand positive PMVs (CD154, p=0.066) following 4 hours of e-cigarette exposure (Table 3).

		Baseline	1h	4h	24h	p-value
EPCs	e-cig	35 [14:42]	329 [287:420]	98 [70:147]	14 [14:28]	0.017
	control	21 [14:42]	21 [7:35]	21 [21:49]	49 [42:63]	
MVs	e-cig	1290 [969:2159]	1977 [799:3223]	2048 [1757:4838]	2794 [1402:5862]	0.722
(PS)	control	1290 [319:5488]	3353 [2470:4987]	2833 [2092:4494]	2102 [700:3928]	
LMVs	e-cig	3 [2:17]	18 [15:39]	51 [14:57]	21 [5:39]	0.451
(CD45)	control	5 [4:9]	12 [10:19]	14 [14:26]	35 [24:46]	
MMVs	e-cig	67 [50:69]	49 [22:55]	115 [93:431]	45 [17:200]	0.278
(CD14)	control	19 [15:40]	7 [7:47]	11 [4:14]	14 [10:21]	
MMVs + HMGB1	e-cig	37 [32:44]	28 [12:36]	3 [2:7]	30 [9:94]	0.455
(CD14 + HMGB1)	control	9 [9:10]	5 [3:24]	6 [4:9]	8 [6:11]	
PMVs	e-cig	310 [144:375]	278 [153:1742]	234 [155:236]	323 [26:350]	0.409
(CD41)	control	589 [195:758]	215 [74:1086]	303 [239:303]	85 [26:346]	
PMVs + HMGB1	e-cig	55 [42:61]	8 [5:13]	30 [23:61]	56 [10:175]	0.757
(CD41 + HMGB1)	control	14 [13:20]	7 [3:19]	8 [8:30]	28 [14:67]	
PMVs + P-selectin	e-cig	10 [10:68]	140 [15:236]	159 [50:372]	61 [56:198]	0.081
(CD41 + CD62P)	control	12 [6:19]	68 [38:103]	45 [40:94]	57 [47:105]	
PMVs + CD40 Ligand	e-cig	10 [6:31]	61 [15:98]	129 [29:172]	46 [35:94]	0.066
(CD41 + CD154)	control	6 [3:14]	39 [33:91]	36 [18:57]	41 [32:56]	
EMVs	e-cig	18 [12:21]	9 [7:14]	13 [4:25]	13 [10:15]	0.550
(CD144)	control	16 [8:20]	11 [1:18]	7 [3:8]	10 [8:13]	
EMVs	e-cig	17 [7:23]	20 [10:43]	49 [14:65]	22 [9:40]	0.091
(CD62E)	control	22 [7:22]	26 [17:40]	23 [17:56]	20 [17:24]	
NM	e-cig	1080 [1047:1159]	706 [563:4664]	1000 [422:1524]	237 [188:395]	0.521
(SYTO13)	control	1539 [1045:2574]	972 [944:1600]	2068 [1340:2393]	445 [363:1692]	
NM + HMGB1	e-cig	177 [92:224]	126 [91:242]	122 [90:308]	29 [17:44]	0.801
(SYTO13 + HMGB1)	control	150 [39:378]	150 [28:261]	224 [74:518]	87 [10:96]	

Table 3. Median number and interquartile ranges [IQR] of endothelial progenitor cells (EPCs) and microvesicles (MVs) at baseline and 1h, 4h and 24h following e-cigarette inhalation and control of the five5 volunteers with the highest EPC increases following e-cigarette inhalation. P-values from multiple measures ANOVA ('time*exposure') are presented. PS: Phosphatidylserine, LMVs: Leukocyte-MVs, EMVs: Endothelial-MVs, MMVs: Monocyte-MVs, PMVs: Platelet-MVs, NM: Nuclear molecules, HMGB1: High mobility group protein B1.

4.2.3 Cotinine

Plasma cotinine levels following inhalation of ECV (Paper II) were compared to cotinine levels from smoking a conventional cigarette (Paper I). Both samples were obtained at 4h after inhalation. Median cotinine levels were significantly lower (p=0.017) following ECV inhalation (4.1 ng/ml, IQR: 3.5-4.7) than following smoking of a conventional cigarette (7.8 ng/mL, IQR: 4.6-14.2).

4.3 PAPER III

Cotinine was measured at baseline in all study participants to ensure nicotine abstinence prior to the study. Two volunteers were excluded due to elevated cotinine levels. Fifteen volunteers (9 females, 6 males, mean age 26 ± 3 years) were included into the analysis.

4.3.1 Arterial stiffness

Results from all vascular measurements are shown in Fig. 12 and Table 4. Following both ECV exposures, there was a significant increase in SBP and DBP that remained elevated for 10 and 30 minutes respectively. Heart rate (HR), PWV and AIx75 increased significantly following inhalation of ECV with nicotine and remained elevated for 20 minutes as compared to after inhalation of ECV without nicotine.

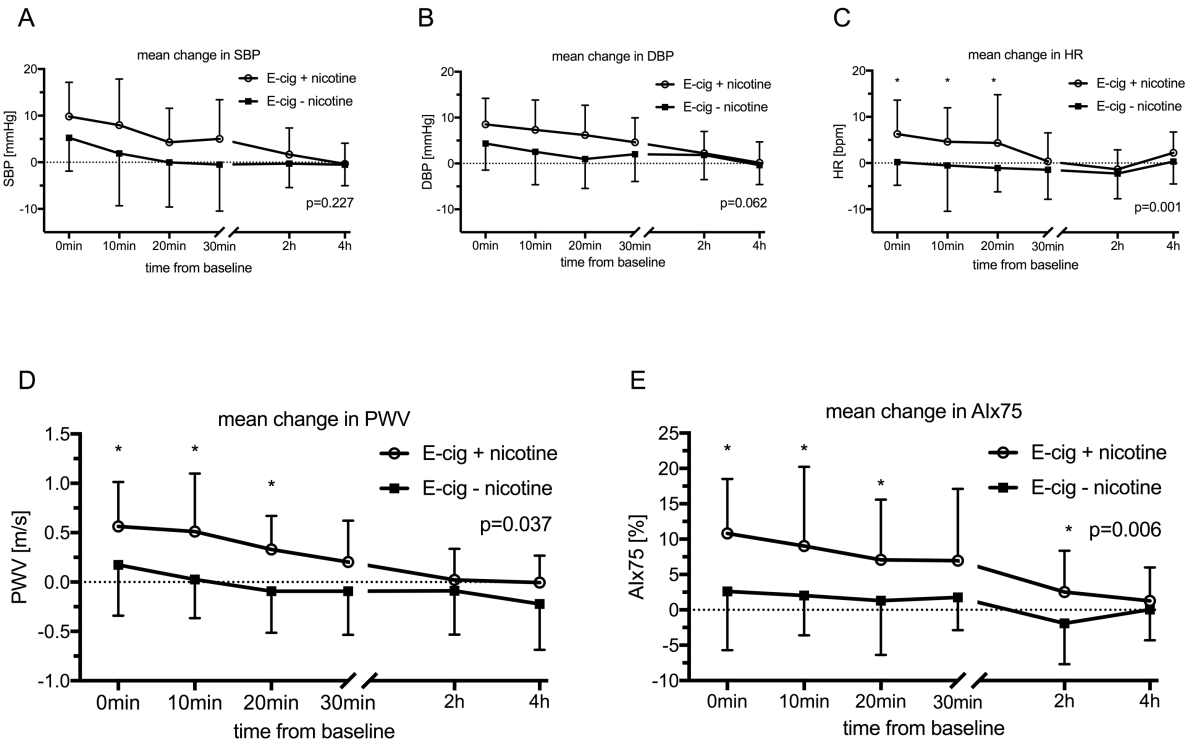

Fig. 12. Mean change in vascular measurements with standard deviations from baseline following exposure to e-cigarette vapor, with and without nicotine. A) systolic and B) diastolic blood pressure (SBP, DBP), C) heart rate (HR) D) pulse wave velocity (PWV), E) heart-rate corrected augmentation index (AIx75). P-values from multiple measures ANOVA ('time*exposure') are presented.
* denotes significant contrast for 'time*exposure' from baseline.

	ECV	baseline	0min	10min	20min	30min	2h	4h	p-values time	p-values time* exp.
SBP	+ nic.	109.4±9.5	119.3±9.5†	117.4±13†	113.7±10.3	114.5±12	111.1±10.1	109.1±9.5	<0.001	0.227
	- nic.	109.3±10.3	114.5±13.2†	111.2±16.1†	109.3±15.5	108.8±15.4	109±10.2	108.8±11.7		
DBP	+ nic.	70.3±5.7	78.9±5.9†	77.7±6.6†	76.5±6.6†	74.9±5.8†	72.6±5.4	70.5±6.6	<0.001	0.062
	- nic.	70.2±5.8	74.5±6.9†	72.7±8.2†	71.1±8.1†	72.2±8†	72±6.5	69.8±6.6		
HR	+ nic.	65.4±8.5	71.7±11.3*	70±12.4*	69.7±12.9*	65.7±10.7	64±9.9	67.6±10.9	0.015	0.001
	- nic.	63.8±9.7	64±10.7	63.3±12.2	62.7±8.4	62.3±9.2	61.5±9.4	64.1±9.9		
PWV	+ nic.	5.8±0.8	6.4±0.8*	6.3±0.9*	6.1±0.9*	6±0.8	5.8±0.8	5.8±0.9	<0.001	0.037
	- nic.	6.2±0.9	6.4±1	6.2±0.9	6.1±0.8	6.1±0.9	6.1±0.8	6±0.8		
AIx75	+ nic.	-5.1±9.5	5.7±11*	3.9±13.2*	2±11.1*	1.9±10.1	-2.6±11*	-3.8±10.4	<0.001	0.006
	- nic.	-2±9.2	0.6±12.8	0±10.7	-0.7±12.9	-0.3±10.7	-3.9±10.7	-2±9.5		

Table 4. Vascular measurements at baseline and following exposure to electronic cigarette vapor (ECV) with and without nicotine (+nic./-nic.). Values represent means ± SD. Systolic blood pressure (SBP), diastolic blood pressure (DBP), heart rate (HR), pulse wave velocity (PWV) and heart-rate corrected augmentation index (AIx75). P-values from multiple measures ANOVA are presented.
* denotes significant contrast for 'time*exposure' from baseline.
† denotes significant contrast for 'time' from baseline

4.4 PAPER IV

4.4.1 Baseline characteristics

Twenty-four male chronic snus users and 26 age and gender-matched controls were included into the study. For baseline characteristics see Table 5. The loose type of snus was used by 13 subjects and the portion/pouch type by the other 11 subjects (54% vs 56% resp.). Snus users had significantly higher alcohol consumption as compared to controls.

	Snus users (n=24)	Controls (n=26)	p-values
age [years]	44.8±8.5	43.4±8.6	n.s.
BMI [kg/m^2]	25.5±2.4	25±3.3	n.s.
waist circumference [cm]	91.8±7.7	90.3±8.6	n.s.
alcohol consumption [ml/week]	62.3±57.3	29.3±30.3	0.017
strenuous physical activity [h/week]	2.4±1.5	2.5±1.7	n.s.
hemoglobin [g/L]	152.8±7.5	147.3±10.3	n.s.
leukocyte count [x10^9/L]	5.7±1.2	5.4±1.1	n.s.
platelet count [x10^9/L]	225±29.4	219.3±45.7	n.s.
creatinine [µmol/L]	85.8±10.2	87.7±10	n.s.
apolipoprotein A [g/L]	1.5±0.2	1.5±0.2	n.s.
apolipoprotein B [g/L]	1.1±0.3	0.9±0.2	n.s.
apolipoprotein B/A ratio	0.7±0.2	0.6±0.2	n.s.
Hba1C [mmol/mol]	35.6±3.7	36.2±2.6	n.s.
snus use [years]	29.3±8.5	0±0	
snus use [cans/week]	5.8±2.4	0±0	

Table 5. Baseline characteristics in Paper IV, reported as mean values ± SD.

4.4.2 Arterial stiffness

There was no difference in SBP, DBP or HR between chronic snus users and controls (Table 5). Pulse wave velocity (PWV) and heart rate corrected augmentation index (AIx75) were significantly higher in chronic snus users compared to controls (Table 5, Fig. 13).

	Snus users	Controls	p-values
SBP [mmHg]	123.9±9.7	123.7±10.7	n.s.
DBP [mmHg]	78.5±7	76.1±8.1	n.s.
HR [bpm]	54.9±8.5	55.5±7.5	n.s.
PWV [m/s]	7.1±0.9	6.6±0.8	0.026
AIx75 [%]	7.3±7.8	0.1±13.2	0.023

Table 5. Mean values ± SD for systolic and diastolic blood pressure (SBP, DBP), heart rate (HR), pulse wave velocity (PWV) and augmentation index corrected for a heart rate at 75bpm (AIx75).

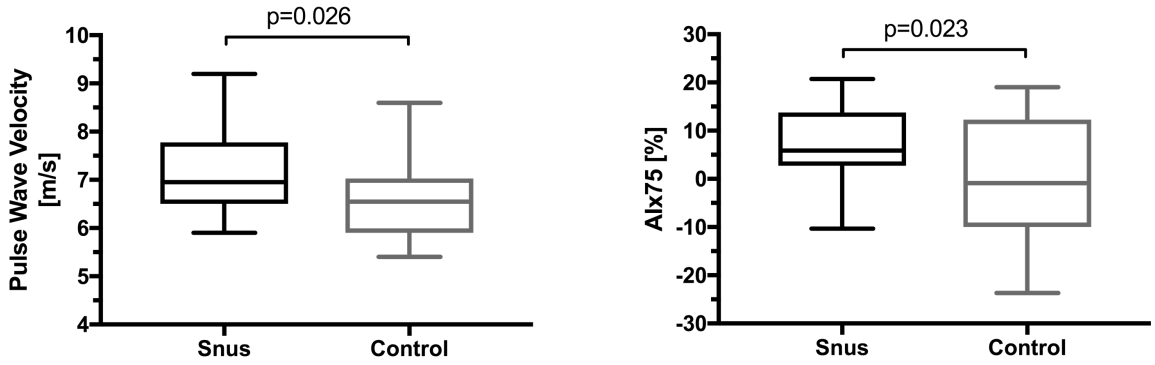

Fig. 13. Boxplots for arterial stiffness measurements in chronic snus users and controls.

There was a significant correlation between waist circumference and BMI, apolipoprotein ratio and strenuous physical activity. Age was significantly correlated with HbA1c. Multiple regression analysis was performed with snus usage, age, alcohol consumption and waist circumference to predict PWV and AIx75. The only independent variables that significantly predicted PWV and AIx75 were age and snus consumption:

PWV: 5.378 + (age x 0.028) + (snus use: 0.491); $F(2, 47) = 4.968$, $p=0.011$, $R^2=0.175$.

AIx75: -29.47 + (age x 0.681) + (snus use: 6.244): $F(2, 47) = 12.888$, $p<0.001$, $R^2=0.354$

4.4.3 Forearm blood flow and fibrinolytic function

All vasoactive drugs (ACh, GTN, BK) caused a dose-dependent increase in FBF (repeated measures ANOVA: $p<0.001$). There was a significant difference in FBF between chronic snus users and controls when administering GTN (Fig. 14). At the highest concentration (GTN: 16µg/min) chronic snus users had significantly lower FBF compared to controls. There was no significant difference in bradykinin induced tPA and PAI release between chronic snus users and controls (Table 6).

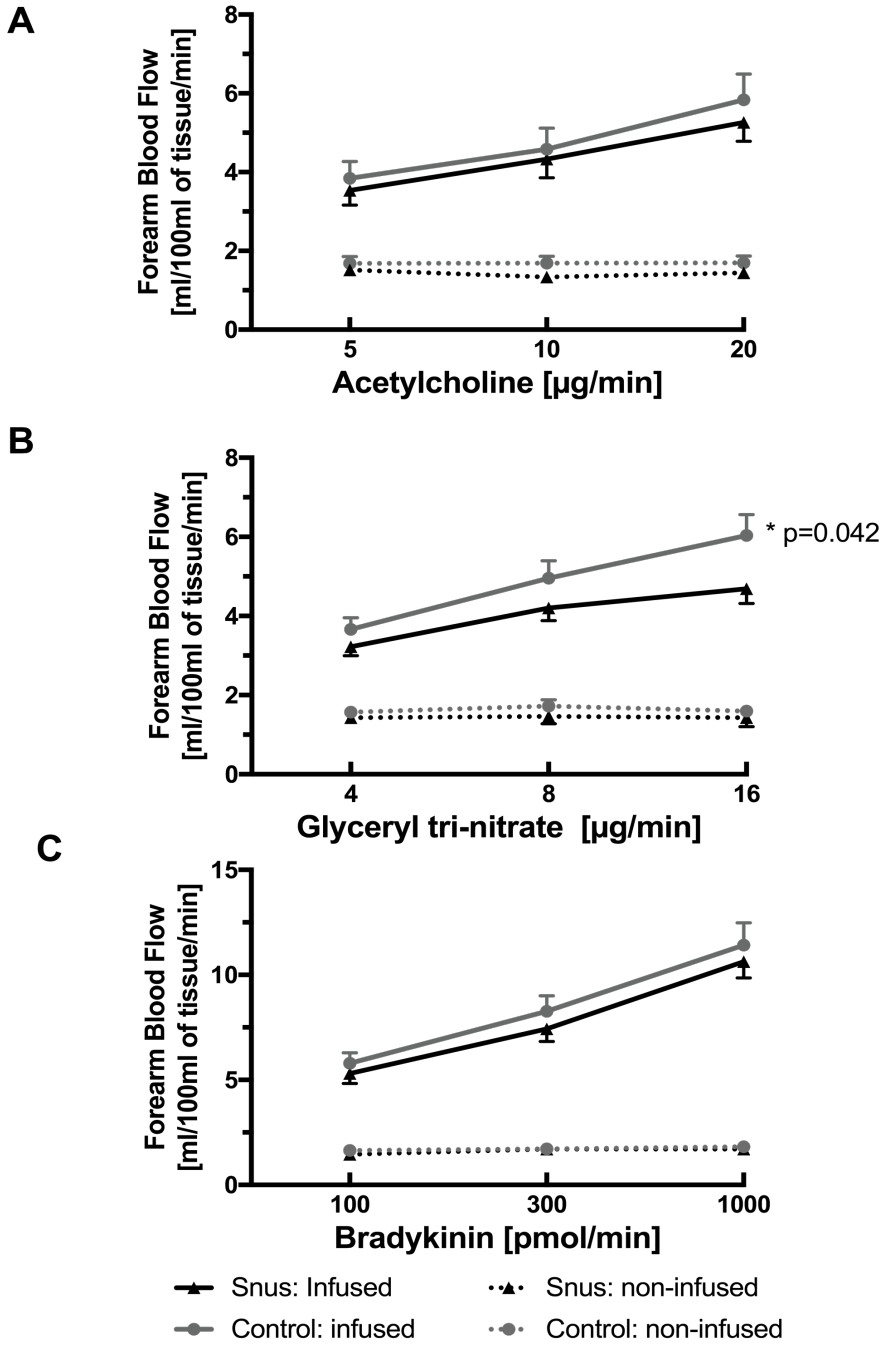

Fig. 14. Forearm blood flow (mean values ± SEM) in chronic snus users and controls during unilateral, intra-brachial infusion of vasoactive drugs in infused (solid line) and non-infused (dotted line) arms. * demarks a significant difference in blood flow at 16µg/min Glyceryl tri-nitrate infusion.

			Bradykinin infusion		
		baseline	100 pmol/min	300 pmol/min	1000 pmol/min
Snus	tPA	0.04 [0.08]	0.63 [1.04]	1.68 [2.36]	4.02 [3.93]
	PAI	-0.04 [0.16]	-0.4 [1.21]	-1.46 [3.19]	-1.58 [3.04]
Controls	tPA	0.03 [0.11]	0.34 [0.72]	1.19 [1.41]	3.59 [7.91]
	PAI	-0.08 [0.17]	-0.6 [0.93]	-1.27 [2.31]	-1.71 [4.09]

Table 6. Tissue plasminogen activator (tPA) and plasminogen activator inhibitor (PAI) antigen net release [ng/100 ml of tissue/min] in chronic snus users and controls. Values represent medians and [IQR]. There were no significant differences between chronic snus users and controls.

4.4.4 Endothelial progenitor cells

There was no significant difference in EPCs ($CD34^+$ KDR^+ cells or $CD34^+$ KDR^+ $CD133^+$ cells) between chronic snus users and controls (Table 7).

	Snus	Control	p-value
CD34 + KDR [x10^6/L]	1 [1.26]	0.89 [0.93]	0.951
CD34 + KDR + CD133 [x10^6/L]	0.61 [1.45]	0.64 [0.47]	0.836

Table 7. Levels of endothelial progenitor cells ($CD34^+$ KDR^+ and $CD34^+$ KDR^+ $CD133^+$) in chronic snus users and controls. Values represent medians and [interquartile ranges].

5 DISCUSSION

The present thesis investigated the effects of brief cigarette and e-cigarette inhalation on the release of microvesicles and endothelial progenitor cells in peripheral blood. We assessed the acute effects of e-cigarette inhalation and chronic snus use on arterial stiffness. Additionally, forearm blood flow and endothelial progenitor cells were measured in chronic snus users.

5.1 CIGARETTES (PAPER I)

The long-term effects of both active and passive cigarette smoke (CS) on vascular health have been studied extensively during the last decades. Cigarette smoking is a well-known strong independent risk factor for the development of CVD, although the pathways through which CS exhibits these effects are not entirely understood. Paper I demonstrated an immediate increase in MVs of leukocyte, platelet and endothelial origin and an acute mobilization in circulating EPCs following smoking of one single conventional cigarette (Fig. 15).

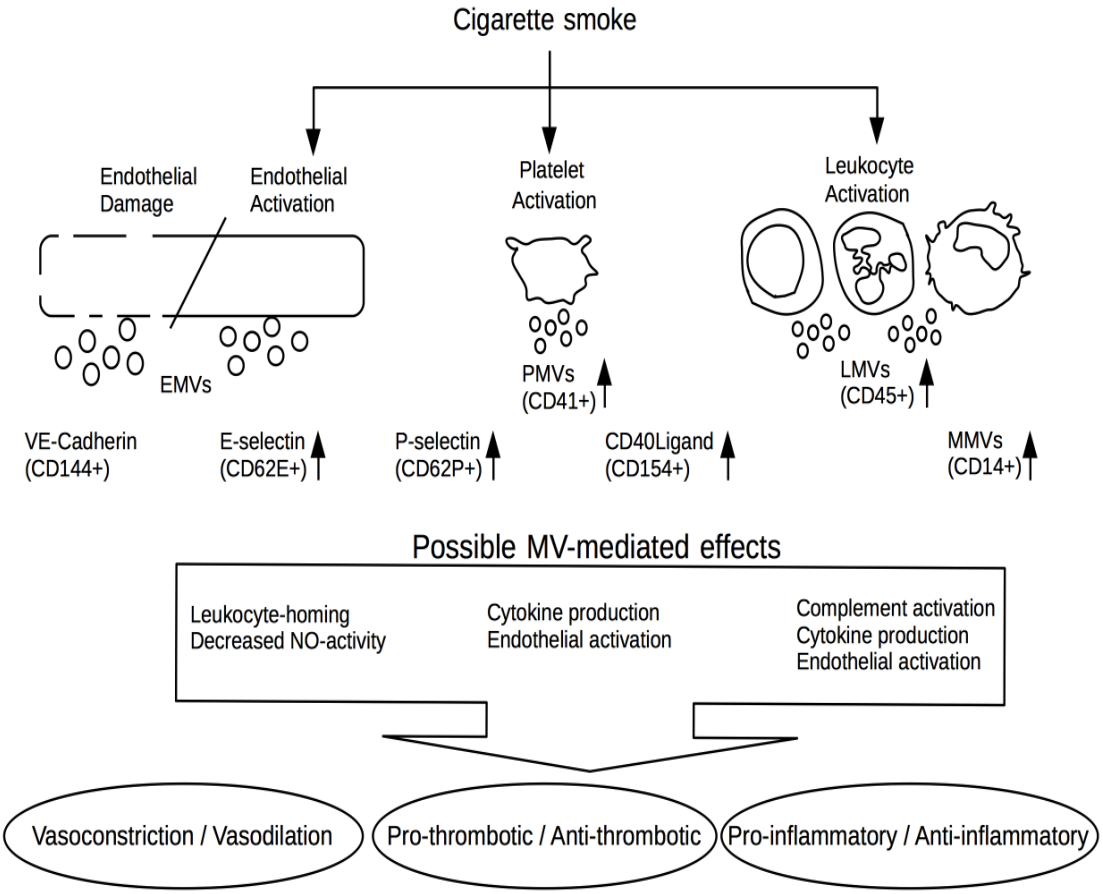

Fig. 15. Effect of cigarette smoke on microvesicles, Paper I.

5.1.1 Microvesicles

5.1.1.1 Platelet derived microvesicles

Platelet derived MVs (PMVs) positive for CD62P (P-selectin) and CD154 (CD40 Ligand) increased significantly following inhalation of CS. This is particularly interesting as PMVs have a coagulation capacity which is 50 to 100 times higher than activated platelets [129]. P-selectin, which is commonly expressed on endothelial cells and platelets, is an important marker for leukocyte homing, inflammation and thrombosis [130]. CD40-Ligand is found on activated platelets, leukocytes and endothelial cells and is a powerful mediator of thrombosis and inflammation [131]. Indeed, patients with stable carotid plaques, manifest ischemic stroke and myocardial infarction, all exhibit elevated levels of PMVs [132-134]. This indicates that endothelial damage is a key cause for PMV mobilization and it has been suggested that activated PMVs seem to play an important role in thrombus formation and in the pathogenesis of atherosclerosis [135].

Smokers have a persistent pro-coagulant state compared to non-smokers [136]. Hence, it could be expected that PMVs would be chronically elevated in smokers. However, healthy daily smokers have been shown to have decreased numbers of PMVs compared to non-smoking controls, which raises issues with the theory of PMVs being a simple mediator of thrombosis [137, 138]. It has even been postulated that circulating PMVs may exhibit anti-thrombotic properties [139, 140]. A decreased amount of circulating PMVs may in this case be associated with a pro-thrombotic state. It may be possible that repeated exposures to CS accelerates PMV budding, which ultimately leads to a depletion of circulating PMVs. Therefore, chronically decreased levels of PMVs in otherwise healthy individuals may constitute a risk factor for future thrombotic events. This however would have to be investigated in future studies.

5.1.1.2 Endothelial microvesicles

Levels of endothelial MVs (EMVs) positive for CD62E (E-selectin) were elevated after CS inhalation. E-selectin is a cell adhesion molecule found on activated endothelial cells. It plays an important role in leukocyte homing and is a marker for inflammation and atherogenesis [141]. In vitro studies suggest that EMVs participate in maintaining vascular hemostasis through regulation of the inflammatory and pro-thrombotic response [142]. Furthermore, EMVs have the ability to decrease NO-production in endothelial cells [143]. Consequently, EMVs can be seen as a marker for endothelial dysfunction. Indeed, increased levels of $CD62E^+$ EMVs have been demonstrated to be associated with worsened clinical outcomes in patients with pulmonary hypertension and stroke [144, 145].

VE-cadherin positive EMVs (CD144) were not affected following CS inhalation. VE-cadherin is found in the intercellular junctions of endothelial cells and plays a key role in endothelial integrity and permeability [146]. VE-cadherin positive EMVs originate from a disruption in the endothelial layer, i.e. apoptotic endothelial cells. Indeed, $CD144^+$ EMVs

levels are increased in states of severe vascular damage such as myocardial infarction or ischemic stroke [147, 148].

Similar to our results, Schwarz et al. demonstrated an increase of activated EMVs (i.e. $CD62E^+$ EMVs), but not apoptotic EMVs [149]. On the other hand, Heiss et al. showed an increase in both, activated and apoptotic EMVs following 30 minutes of secondhand smoke exposure in never-smoking subjects [150]. It is quite possible that endothelial apoptosis occurs following a longer exposure to CS. The duration of the exposure in our study may have been too short to demonstrate this effect (only 5 minutes).

The duration of CS inhalation may be an important factor for the development of endothelial apoptosis. Strulovici-Barel et al. investigated the impact of smoking cessation in healthy smokers and smokers with COPD compared to non-smokers on EMVs from apoptotic and activated endothelial cells over the time course of one year [151]. They demonstrated that initially, the levels of EMVs from activated as well as apoptotic endothelial cells in healthy smokers where increased. However, smoking cessation was associated with a decline, to comparable levels of the non-smoking group. On the other hand, this effect on EMVs was not observed in patients with established COPD, suggesting that the endothelial layer is already markedly damaged in this patient group.

Badrnya et al. showed that levels of $CD144^+$ EMVs in young daily smokers (mean age 24 years) were not elevated compared to age-matched non-smokers [137]. This further demonstrates that chronic endothelial disruptive properties are achieved over years of exposure to CS. We speculate that smoking one cigarette causes the activation of endothelial cells, but not acute endothelial apoptosis in young healthy volunteers. However, repeated exposure to CS appears to cause endothelial apoptosis over the course of time.

5.1.1.3 Leukocyte derived microvesicles

Leukocyte derived MVs (LMVs) have not yet been studied as extensively as EMVs or PMVs. We observed that LMVs increased significantly following acute exposure to CS. LMVs can be shed from neutrophils, monocytes and lymphocytes and are further differentiated by several markers [152]. LMVs exhibit both pro- and anti-inflammatory properties. They participate in hemostasis, in the maintenance of endothelial function and are also involved in the development of atherosclerosis [152]. When analyzing atherosclerotic plaques for subpopulations of MVs the highest fraction compose LMVs, whereas no PMVs are found [153].

LMVs are increased in states of acute vascular injury such as severe systemic infections and ischemic stroke, as well as after acute exposure to combustible air pollution [154-156]. Chronic vascular injury also results in increased levels of LMVs, for example in patients with hyperlipidemia and subclinical atherosclerosis [157, 158]. On the other hand, LMVs seem not to be chronically increased in patients with stable type 2 diabetes, a known risk factor for vascular disease [159]. This highlights the importance of investigating and understanding the acute and chronic effects of different risk factors on all subtypes of MVs.

5.1.1.4 Nuclear content of microvesicles

In order to evaluate if MVs were derived from apoptotic cells (in contrast to MVs triggered by activation), nucleic content of MVs was analyzed, i.e. nucleic acids (SYTO13) and the nuclear protein high mobility group box protein 1 (HMGB1). We speculated that $SYTO13^+$ and $HMGB1^+$ MVs would derive from the nucleus of apoptotic cells. LMVs and PMVs positive for SYTO13 increased following CS exposure. However, the nuclear protein HMGB1 remained unaffected which does not support that apoptosis is the main cause for the increase in MVs. SYTO13 may bind mitochondrial DNA and miRNA, normally present in MVs [160]. Therefore, a sole increase in $SYTO13^+$ MVs may reflect activation rather than apoptosis of cells.

5.1.2 Endothelial progenitor cells

EPCs increased significantly following CS inhalation and had returned to baseline values at 24 hours. The main trigger for EPC mobilization is the release of VEGF and SDF-1 due to vascular activation and hypoxia [111, 114]. A general inflammation in itself does not seem to mobilize EPCs [161]. In conditions of acute vascular damage such as following stroke or myocardial infarction, elevated levels are found in the circulation up to 30 days afterwards [115, 116]. EPCs are recruited from vascular niches and the bone marrow to the site of the lesion and stimulate vascular repair through the excretion of pro-angiogenic cytokines and growth factors [162, 163].

While acute vascular injury increases levels of circulating EPCs, states of chronic vascular injury such as stable type 2 diabetes, hyperlipidemia, hypertension or chronic smoking are associated with reduced amounts of EPCs [119-121, 164]. These effects seem reversible as antihypertensive, lipid lowering and anti-diabetic therapy as well as smoking cessation increase amounts of circulating EPCs [165-169]. This suggests that chronic vascular injury ultimately leads to a depletion of EPC reserves and therefore chronically decreased levels of EPCs may be seen as a risk factor for CVD [123, 124].

5.2 ELECTRONIC CIGARETTES (PAPER II-III)

Due to the novelty of e-cigarettes no data exists thus far on long-term health effects. Therefore, studies investigating potential e-cigarette health impacts on vascular integrity are crucial. Paper II demonstrated an acute increase of EPCs following brief inhalation of electronic cigarette vapor (ECV) with nicotine. No significant increase in microvesicles, with the exception of EMVs positive for E-selectin, was detected. In Paper III, inhalation of ECV with nicotine increased arterial stiffness, yet not ECV without nicotine.

5.2.1 Endothelial progenitor cells

Ten puffs of nicotine containing ECV was demonstrated to mobilize EPCs. As discussed before, the main trigger for EPC mobilization is the endothelial expulsion of VEGF-2 and SDF-1 – two cytokines responsible for the homing of EPCs to the vascular wall [114]. Li et al. demonstrated in mice that daily oral administration of nicotine caused an initial increase of

EPC proliferation in vitro following one month of exposure and then a subsequent decrease of EPC proliferation at both, 3 and 6 months [170]. In addition, they also showed that these EPCs showed signs of dysfunction. In vitro exposure of EPCs to nicotine results in augmented proliferative, adhesive and migratory capacity [171-173]. Hence, it is plausible that the main trigger for EPC mobilization is nicotine, as inhalation of ECV contains no combustion derived products.

As paper II did not include inhalation of nicotine-free e-liquid, we cannot establish in this study whether other compounds in ECV could also have effects on the endothelial cells. Animal models have shown that the pulmonary vascular bed may be an important site for resident EPCs [163, 174]. It is possible that the inhalation of ECV per se and not nicotine specifically mobilizes EPCs. The vapor produced by e-cigarettes contains fine, ultrafine and nano-particles, which can penetrate deep into the lungs [70, 71, 175]. However, the composition of these particles as well as their effect on human health needs to be further investigated.

5.2.2 Microvesicles

Compared to smoking a conventional cigarette we did not observe the same increase after ECV inhalation in MVs, with the exception of E-selectin positive EMVs. On one hand, combustion derived constituents may play a crucial role in the activation of the endothelium, platelets and leukocytes. On the other hand, it is possible that the exposure of 10 puffs of ECV may be too little to observe effects on these cell populations. On an average day, the typical e-cigarette user inhales around 230 puffs, so the exposure in paper II was minor compared to average daily use [176]. This may further be supported by a sub-analysis of the five subjects with the highest increase in EPCs (Table 3). We noticed that these individuals had a non-significant trend towards higher PMVs and E-selectin positive EMVs as compared to controls. Consequently, a longer ECV exposure would be advantageous in upcoming studies.

5.2.3 Arterial stiffness

In paper III, ECV with nicotine caused a transitory increase in arterial stiffness as measured by PWV and AIx75. This further highlights that nicotine itself may generate an acute impact on vascular function. Intake of one, 2 mg nicotine tablet acutely increases PWV in healthy non-smokers [177]. It has been previously demonstrated that acute exposure to CS increases arterial stiffness and that healthy chronic smokers in general have increased arterial stiffness compared to non-smokers [178]. Smoking cessation for 12 months decreases arterial stiffness [179]. Hence it is possible that chronic exposure to ECV with nicotine also results in a chronically altered AS, which is an independent risk factor for CVD [79].

5.3 SNUS (PAPER IV)

Healthy chronic snus users displayed increased arterial stiffness and impaired endothelial function as measured by venous occlusion plethysmography. There was no significant

difference in EPC counts and fibrinolytic function as measured by tPA/PAI release between the two groups.

5.3.1 Arterial stiffness

Snus is often advocated as a safer alternative to cigarette smoking due to the low amounts of tobacco specific nitrosamines (TSNAs) and that no combustion derived products are inhaled into the lungs [15]. Some studies and meta-analyses suggest that there is no association of snus usage to CVD, including myocardial infarction and stroke [25, 26, 180]. However, several epidemiological studies demonstrated that daily snus users have higher mortality rates when they have developed CVD [28, 30, 34]. Therefore, the finding that daily snus use may chronically alter AS is of great interest. Increased arterial stiffness is an independent risk-factor for the future onset of CVD and strengthens the case that snus use may indeed play a role in the development of CVD [79, 80].

Nicotine is one of the main constituents in snus which could alter AS. Argacha et al. demonstrated that acute exposure to secondhand CS increases arterial stiffness but not inhalation of non-nicotine herbal cigarettes [181]. This indicates that the nicotine content alone may have a crucial role in the stiffening of the aorta. Long-term effects of nicotine on arterial stiffness are not yet well investigated. The only products that would allow the investigation of long-term nicotine effects on vascular health are different types of nicotine replacement therapy (NRT). However, individuals that use NRT as a cessation aid have normally used a tobacco product for a lengthy period of time, which will have affected arterial stiffness. Furthermore, cessation studies are normally conducted during a rather short period of time, ranging from 3 to 18 months [182, 183]. Nevertheless, one study investigating AIx75 following smoking cessation with NRT compared to varenicline (CHAMPIX^{TM}) found a greater reduction of AS in the nicotine-free varenicline group [182]. This supports the theory that nicotine may chronically alter aortic distensibility. Indeed, in vitro models have shown that nicotine alone has aortic-remodeling properties and may alter smooth muscle cells from contractile to synthetic type [184, 185].

5.3.2 Forearm blood flow

Endothelial independent, but not dependent, vasodilation (EIDV, EDV respectively) was altered in chronic snus users. This suggests that snus use does not primarily affect eNOS, the principal enzyme for the formation of endogenous NO [186]. However, it is possible that snus and/or nicotine may attenuate vasodilation further downstream of eNOS, i.e. the GC/PKG pathway. Two animal models have demonstrated endothelial dysfunction in nicotine exposed rats [187, 188]. These studies found an attenuated effect of NO-mediated vasodilation through upregulated Ca^{2+} channels in vascular smooth muscle cells and an altered PKG-pathway. This suggests that nicotine in snus may be the key component causing endothelial dysfunction (Fig. 16).

Combustion derived products, including CS, are well known for their pro-inflammatory effects which alter the endothelium and lead to endothelial dysfunction [189-191]. Lind et al.

demonstrated impaired EDV and a trend towards attenuated EIDV in daily cigarette smokers [192]. Also, exposure to diesel exhaust, which contains a large amount of combustion derived particles and associated products, decreases both EDV and EIDV [91]. Due to these findings, it has been suggested that endothelial dysfunction in cigarette smokers is largely attributed to combustion derived compounds. Our results imply that nicotine also alters endothelial function, possibly through the GC/PKG pathway.

The findings of Paper IV may explain the higher mortality rates in survivors of myocardial infarction that continue using snus [34]. Glyceryl tri-nitrate (GTN) is commonly used in patients with myocardial infarction and chronic ischemic heart disease. Repeated use of GTN causes nitrate tolerance, possibly through the GC/PKG pathway, resulting in an attenuated EIDV [193, 194]. As chronic snus users also display attenuated EIDV, we speculate that chronic nicotine exposure may have similar effects on the PKG pathway as chronic GTN exposure resulting in an attenuated EIDV.

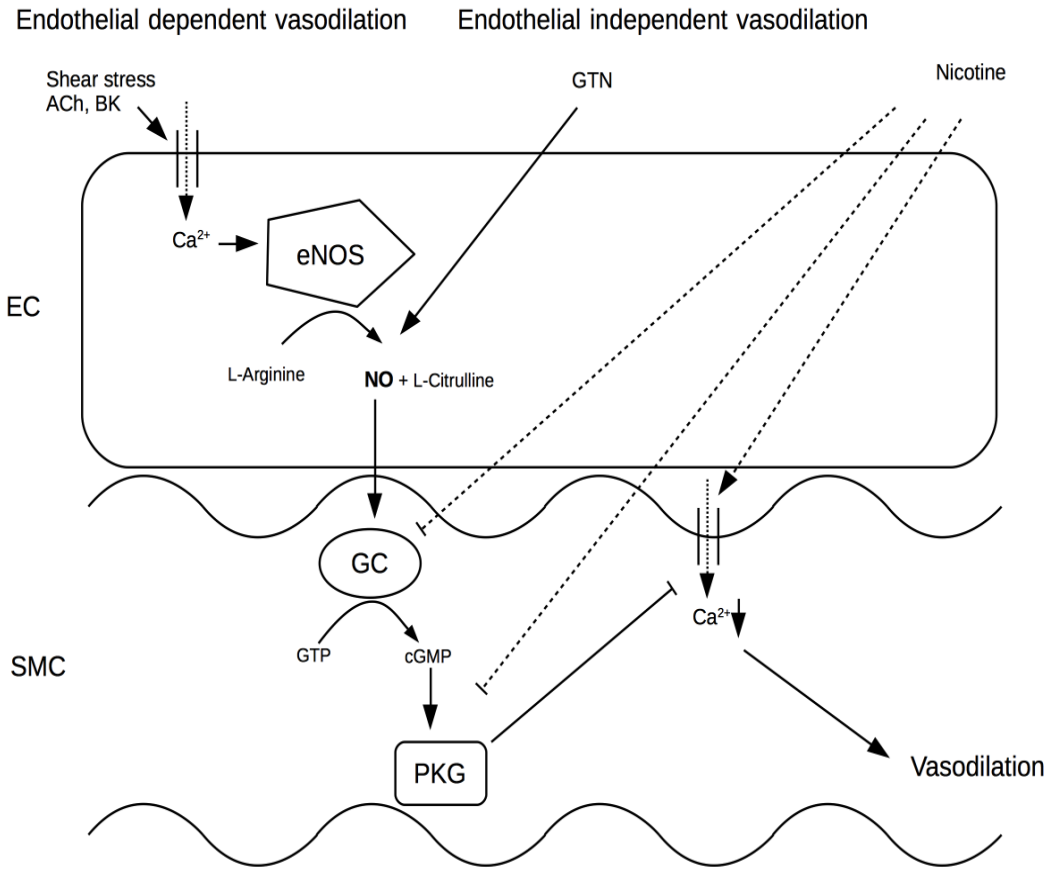

Fig. 16. Schematic over endothelial-dependent and independent vasodilation and potential acting points for nicotine. Shear stress, acetylcholine (ACh) or bradykinin (BK) cause Ca^{2+}-influx into the endothelial cell (EC), which activates endothelial nitric oxide synthase (eNOS). eNOS catalyzes NO-formation. NO diffuses into the surrounding smooth muscle cell (SMC) and activates guanylate cyclase (GC), which catalyzes the formation of cyclic guanosine-monophosphate (cGMP) from guanosine tri-phosphate (GTP). cGMP activates protein kinase G (PKG) which regulates the vascular tone through a reduction of Ca^{2+}-influx into the SMC. Glyceryl tri-nitrate is a direct NO-donor.

5.3.3 Fibrinolytic function

Pellegrini et al. demonstrated that NRT use for seven days increased fibrinolytic function in healthy volunteers as measured by endothelial tPA/PAI release [195]. They speculated that chronic exposure to NRT may lead to a depletion of tPA reserves and thus to a decreased fibrinolytic function, which has been observed in daily smokers [89, 90]. Results from Paper IV do not support this hypothesis, as participants in our study have been exposed to high and continuous nicotine levels for many years, and still did not display a reduced fibrinolytic function. However, it is well established that CS and other air pollutants do decrease fibrinolytic function in humans, hence this process may be instigated by combustion derived products [90, 91, 196].

5.3.4 Endothelial progenitor cells

There was no significant difference in EPC counts between chronic snus users and controls. We previously speculated that the inhalation of nicotine is per se the main trigger for EPC mobilization. It is possible that the manner of nicotine delivery and absorption (alveoli vs oral mucosa) plays an important role in how nicotine impacts the endothelium. Currently, there are no studies investigating the acute effects of snus or pure nicotine on EPC levels. An animal hind-limb ischemia model demonstrated increased EPC counts following systemic nicotine exposure, yet only in states of ischemia [197]. Consecutively, it is possible that inhalation of nicotine together with other compounds which trigger ischemia may mobilize EPCs.

6 GENERAL DISCUSSION

Combustion derived compounds in CS are well known for their negative impact on vascular health [6, 189, 191]. This thesis shows that combustion-free products like e-cigarettes or snus also have an impact on vascular integrity, mainly driven by nicotine (Fig. 17).

Nicotine acts primarily on nicotinic acetyl-cholinergic receptors (nAChr) commonly found in the brain, autonomic nervous system and skeletal muscle cells, but also at non-neuronal sites like on endothelial cells or lymphocytes [6, 198]. It exerts its sympathomimetic effects primarily through the presynaptic release of noradrenaline and through stimulation of the adrenal gland, thus increasing blood pressure and heart rate [199]. Newer studies have focused on possible long-term nicotine effects and the association to atherosclerosis and cancer [200, 201].

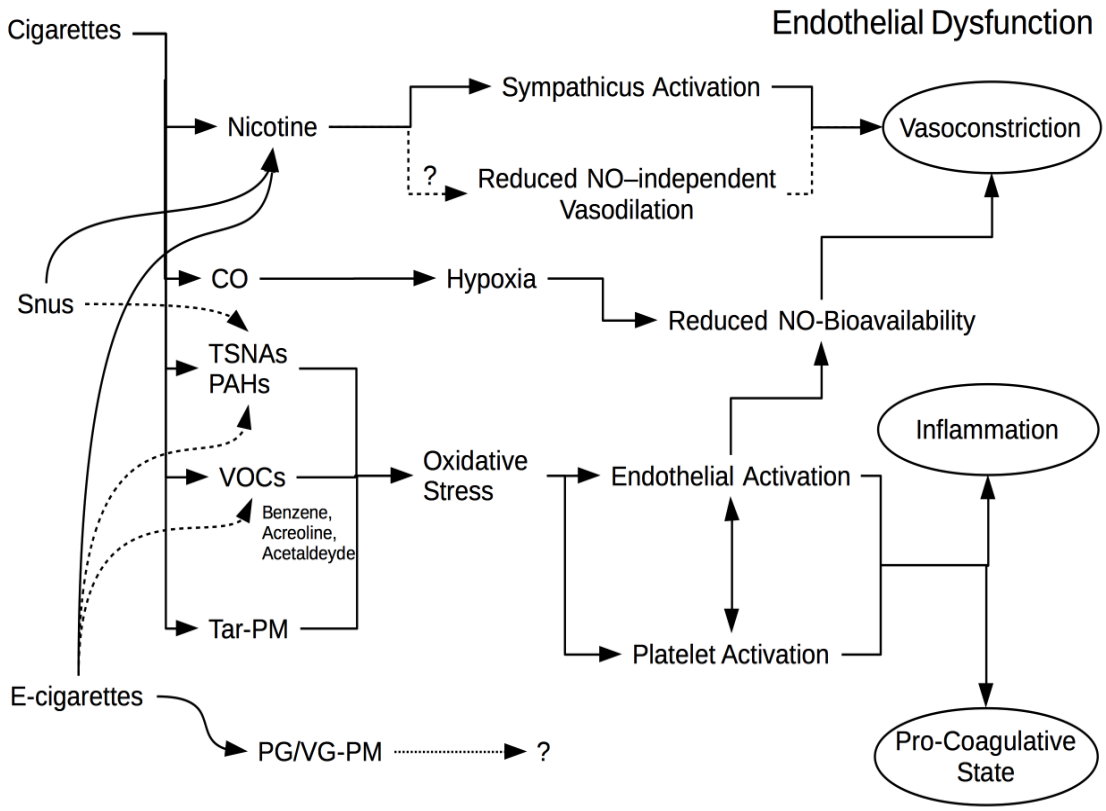

Fig. 17. Schematic over compounds of cigarettes, e-cigarettes and snus and established and potential (dotted lines) pathways that lead to endothelial dysfunction. The effect of particulate matter (PM) from propylene glycol (PG) and vegetable glycerin (VG) on the endothelium is not known. CO: carbon monoxide, TSNAs: tobacco-specific nitrosamines, PAHs: polycyclic aromatic hydrocarbons, VOCs: volatile organic compounds, NO: nitric oxide.

We demonstrate that inhaled nicotine may be an important trigger for acute mobilization of EPCs. Acute mobilization of EPCs is often triggered by hypoxia in the vascular wall and/or endothelial activation [114]. Repeated mobilization may lead to a depletion of circulating EPCs which is observed in daily cigarette smokers [122]. Diminished levels of EPCs is a known risk factor for CVD [113, 114, 202]. Chronic snus use however seems not to be associated with a decline in EPC counts. This indicates that inhaled nicotine, which is absorbed via the lung, may have a divergent impact on EPCs than more slowly absorbed nicotine via the oral mucosa (Fig. 18) [203]. Nicotine in CS and ECV is quickly absorbed through the large surface area of the alveoli, resulting in a quick increase in serum nicotine levels. Therefore, varying nicotine pharmacokinetics may exert different impacts on vascular health. It was also suggested that the lungs are an important reservoir for EPCs and therefore inhaled nicotine may cause an EPC mobilization but not snus [204, 205]. This has to be investigated in future studies.

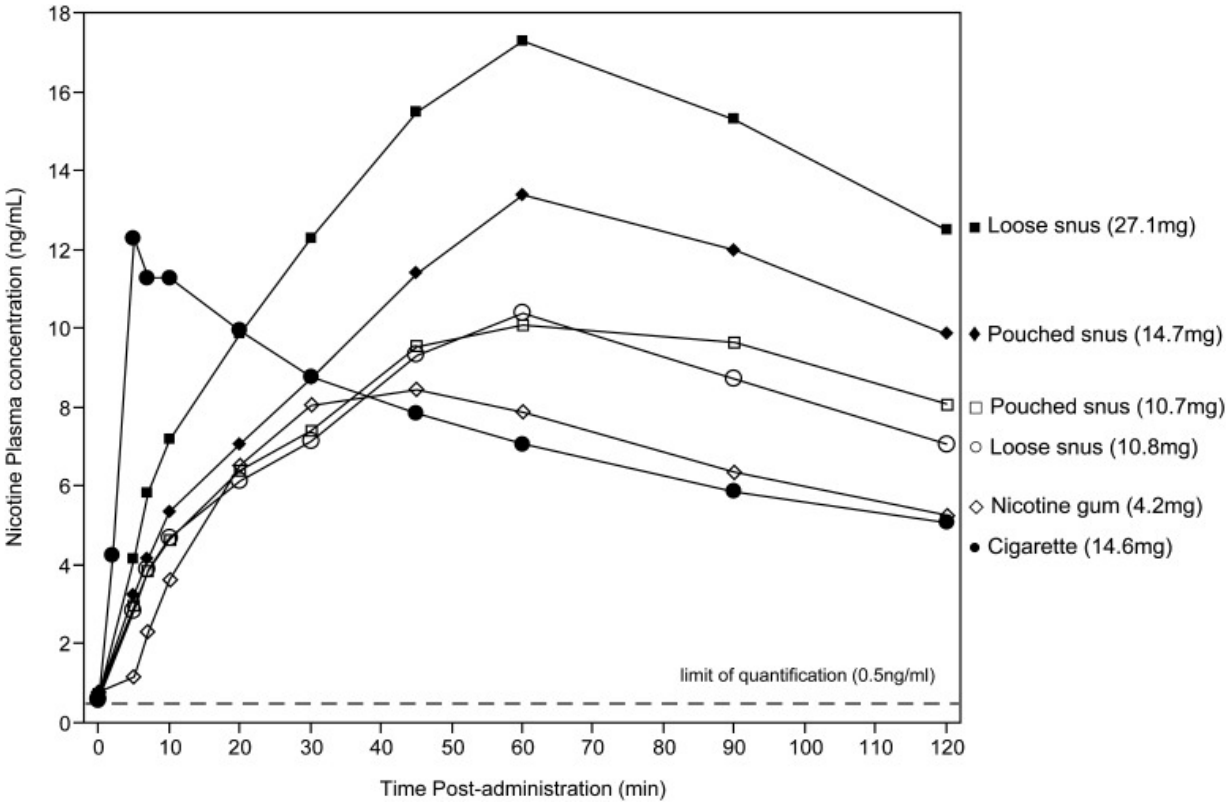

Fig. 18. Nicotine plasma concentrations following exposure to cigarette inhalation, snus and nicotine gum. Adapted from: Digard, H., et al., *Determination of nicotine absorption from multiple tobacco products and nicotine gum.* Nicotine Tob Res, 2013. **15**(1): p. 255-61.

Paper III shows that inhalation of ECV containing nicotine causes an acute increase in arterial stiffness as also observed immediately following conventional cigarette smoking [178, 179]. In Paper IV, we demonstrate increased arterial stiffness and altered vasodilatory function in chronic snus users. Daily cigarette smokers display increased arterial stiffness and endothelial dysfunction as well, both of which are independent risk factors for the future onset of CVD [178, 192]. This illustrates that both acute and chronic exposure to nicotine may alter vascular function (Fig. 17). Taken together, caution is warranted when new nicotine delivery devices like e-cigarettes are introduced to the market, as nicotine itself may alter endothelial function. Furthermore, Swedish snus should not be regarded as a harmless recreational tobacco product.

Paper I shows an acute increase in MVs following a brief CS exposure. Budding of MVs is mainly triggered by receptor activation in pro-inflammatory conditions, but also due to hypoxia [142, 206, 207]. CS contains oxidative gases, PAHs and particulate matter, which are known to lead to endothelial inflammation through oxidative stress and carbon monoxide, which strongly binds to hemoglobin and therefore causes hypoxia (Fig. 17) [208, 209]. However, nicotine alone has the ability to stimulate dendritic cells and monocytes, with the concomitant release of pro-inflammatory cytokines [210]. This indicates that combustion derived products in CS may, together with nicotine, exert endothelial activating and disruptive properties, primarily through oxidative stress. This may be true, as acute exposure

to CS and simultaneous red-wine consumption attenuates the release of MVs [149]. The phenols in red-wine have anti-oxidative properties and therefore may, to a certain degree, attenuate the effect of CS on the vascular wall.

We demonstrated that acute exposure to CS increases EPCs. Likewise, acute exposure to particulate matter (PM) and secondhand CS increase the levels of EPCs and induce EPC dysfunction, which may be elicited by oxidative stress in the lungs [150, 205, 211]. As discussed before, nicotine may also induce EPC mobilization. Hence, the acute mobilization of EPCs following CS may therefore be triggered by combustion derived constituents and possibly potentiated by inhaled nicotine.

7 FUTURE PERSPECTIVES

New biomarkers for endothelial function like MVs and EPCs have been associated with the development and progression of cardiovascular disease [142]. Even though these biomarkers are often discussed as risk factors for CVD, it is important to keep in mind that an additional reason for their release may be initially as vascular protection [93-96, 109-112, 212, 213]. The role of MVs and EPCs in the complex system of vascular hemostasis needs to be further investigated and understood. Nevertheless, these new markers may serve as future biomarkers to detect states of endothelial dysfunction in a clinical setting, predicting the onset of atherosclerosis.

Electronic cigarettes have quickly risen in popularity and are often promoted as a healthier alternative to conventional cigarette smoking. As these products are still relatively new and continually developing, it will take some time for long-term health effects to be investigated in epidemiological studies. Meanwhile, it is important to conduct studies, in human subjects, animals and in vitro, in order to analyze any possible adverse health effects from acute and long-term use. The scientific community has a responsibility to objectively investigate this novel product and to clearly communicate results to the general public, medical community as well as regulatory organizations.

Reviews and studies often discuss e-cigarettes in comparison to conventional cigarettes. As e-cigarettes are conspicuously advertised towards a younger, smoking-naïve population, it is a more realistic approach in future studies to compare e-cigarettes to not consuming them at all [214].

The largest distributor of snus in Sweden - Swedish Match™ – regularly promotes that the use of snus in Sweden is associated with the low smoking rates, although this correlation is merely inferred rather than scientifically proven. “Harm Reduction” instead of “smoking cessation” has recently emerged as a new focus for the tobacco industry. Nicotine is the mutual substance in all harm-reduction products and often considered as a relatively harmless substance. With accumulating evidence that this is not the case, it is important to focus on nicotine cessation and not on shifting the source of nicotine delivery.

7.1 THE IMPORTANCE OF INDEPENDENT RESEARCH

The economic profit from these addictive, nicotine-containing products is enormous. International tobacco companies have consistently proven that their economic interests overshadow any concern about the negative health effects of their products [9, 215]. Through advertisement, donations, lobbying-efforts and lawsuits they seek to influence individuals and governments to keep public opinion, media coverage and legislation in their favor [216-219].

During the last couple of years new opinions have emerged in scientific journals as well as on several blogging and social media sites questioning the work of the WHO-Framework Convention for Tobacco Control (WHO-FCTC) [220-225]. This is a worrisome development as the main goal of the WHO-FCTC is the prevention of tobacco associated diseases mainly through guidance in implementing health policies.

In the scientific field, it has long been acknowledged that conflicts of interest (COIs) interfere with objective conclusions and relevant study designs. This is now considered incontrovertible for many of the studies regarding the negative health effects of cigarette smoking and second-hand CS exposure [226, 227]. Nevertheless, studies with clear COIs are steadily published that dismiss or attenuate negative health effects of Swedish snus and electronic cigarettes [21, 26, 52, 180, 228]. There are journals that predominantly publish data from scientists with COIs [229]. Furthermore, some researchers try to dismiss the fact that COIs may influence conclusions drawn from data and even acknowledge tobacco industry funded work [230, 231]. As our study results indicate possible negative health effects of e-cigarettes they have been questioned through letters to the editor and on social blogs often by individuals with openly stated COIs [232-235]. In light of these recent developments, independent and impartial research funded by neutral financial sources remains of outmost importance.

8 CONCLUSIONS

Short-term inhalation of cigarette smoke mobilizes EPCs and MVs of endothelial, platelet and leukocyte origin, which may be interpreted as an acute endothelial activation/injury as well as a pro-inflammatory and pro-coagulative response.

Short-term inhalation of ECV with nicotine mobilizes EPCs but not MVs, which may be due to endothelial activation mainly triggered by nicotine.

Short-term inhalation of ECV containing nicotine increases arterial stiffness as compared to vapor without nicotine. Hence, chronic exposure to nicotine may alter aortic distensibility and increase the risk for CVD.

Long-term snus use is associated with increased arterial stiffness and endothelial dysfunction. This effect may be primarily nicotine driven and strengthens the argument that snus use is associated with CVD.

9 POPULAR SCIENCE SUMMARY IN ENGLISH

Background

Cigarette smoking is considered to be one of the major causes of premature death worldwide and is associated with an increased risk for heart attack and stroke. Smoking affects the blood vessels by damaging the innermost cell-lining (called endothelial cells). As a consequence, inflammation, blood coagulation and blood vessel stiffness increases. This condition is called endothelial dysfunction, which precedes the development of atherosclerosis. With continually increased smoking restrictions and negative public perceptions, the tobacco industry has started to shift its focus to alternative products such as Swedish snus and electronic cigarettes (e-cigarettes).

Snus is common in Sweden where approximately 22% of men and 4% of women use it on a daily basis. The use in other European countries is restricted due to sales bans by the European Union. During the last few years, snus has been introduced to the US market primarily by Altria (Marlboro) and R.J. Reynolds (Camel). Snus is often marketed as an alternative to cigarettes as well as towards smokers for locations where smoking bans are in place. Those who argue that snus is an acceptable replacement for smokers refer to studies that show no negative health effects. In recent years, more studies have been published that were able to detect a statistical relationship between snus and increased mortality in heart attacks and stroke. This has led to considerable attention by the media where it has been speculated whether snus really should be considered a healthier alternative to smoking.

E-cigarettes have also fairly recently been launched as an alternative to traditional cigarette smoking. The e-cigarette consists of a battery driven vaporizer which heats a liquid. The resulting vapor is inhaled in the same way as cigarette smoke. The liquid (e-liquid) is based on vegetable glycerin and propylene glycol. E-liquid is available in various nicotine strengths (also nicotine-free) as well as numerous flavors, such as tobacco, spices, candy, desserts and fruits. The e-cigarette is currently being marketed as an alternative to cigarettes, as a smoking cessation aid as well as a novel product with an adventurous, youthful appeal. In addition to nicotine, several toxins have been found in the vapor and the e-liquid, but the levels of these compounds were lower compared to cigarette smoke. So far, there is limited information about the possible health risks of e-cigarette use.

The aim of this thesis was to investigate vascular effects of cigarettes, e-cigarettes and snus using various methods.

Methods and Results

Vessel function can be examined in several ways. One method is analyzing markers called microvesicles (MVs) and endothelial progenitor cells (EPCs) in blood samples. MVs are released by all types of cells involved in vessel function such as endothelial cells, white blood cells and platelets. A rapid increase in MVs in the blood is usually associated with an

inflammation and vessel injury. EPCs are involved in vessel repair and are also released when damage to the blood vessel wall occurs.

Another method is the measurement of arterial stiffness which is easily evaluated with a sensor around the neck and thigh as well as with a probe at the wrist, which all sense and record pulse waves.

A more time-consuming method is the measurement of vessel function in the forearm, while simultaneously examining whether the vessels respond to specific substances which relax the smooth muscles in the vessel walls. This method is called venous occlusion plethysmography (VOP).

In Paper I, we investigated acute effects of cigarette smoking in 12 young healthy volunteers who were subjected to cigarette smoking on one occasion and to non-smoking on another. Blood samples were drawn prior to exposure and 1h, 4h and 24h after exposure. Samples were analyzed for MVs and EPCs. Smoking was shown to cause a rapid increase in MVs and EPCs.

In Paper II, acute effects of e-cigarette vapor inhalation (with nicotine) were investigated. Sixteen young healthy volunteers were subjected to either e-cigarette use or non-use. Blood samples were taken prior to exposure and 1h, 4h and 24h after exposure and evaluated for MVs and EPCs. E-cigarette use with nicotine caused a rapid increase in EPCs but not in MVs.

In Paper III, 17 young healthy volunteers were subjected to e-cigarette vapor with nicotine and without nicotine. Arterial stiffness was measured before and after exposure during 4 hours. Inhalation of e-cigarettes containing nicotine caused a temporary increase in heart rate and vessel wall stiffness, but this was not seen after the use of e-cigarettes without nicotine.

Paper IV examined 24 healthy daily snus users (average age 44.8 years) and 26 non-users of similar age. Arterial stiffness and VOP were measured. Snus users had elevated arterial stiffness and a decreased response to the vessel-relaxing substance known as nitroglycerin.

Discussion

Smoking one cigarette causes a strong response in the blood vessels with signs of vessel wall damage, increased inflammation and activation of platelets. E-cigarettes do not appear to have an equally strong negative effect on the blood vessels. However, the nicotine content in both cigarettes and e-cigarettes, seems to increase EPCs. Repeated use of e-cigarettes may lead to lower levels of EPCs, which is associated with an increased risk for cardiovascular disease.

Inhalation of e-cigarette vapor with nicotine increases arterial stiffness and daily snus users have chronically elevated arterial stiffness, which is also a known risk factor for the development of cardiovascular disease. In addition, chronic snus users have a decreased susceptibility to nitroglycerin, which is commonly used in patients with heart disease to

improve blood flow to the heart. This may worsen the outcome for snus users who suffer a heart attack.

In summary, we demonstrate that primarily the nicotine component in snus and e-cigarettes has a negative impact on vascular health. E-cigarettes and snus should therefore not be considered as harmless products.

10 POPULAR SCIENCE SUMMARY IN SWEDISH

Bakgrund

Fem miljoner människor beräknas dö årligen på grund av cigarettrökning enligt World Health Organisation (WHO). Rökning räknas som en av de största orsakerna till död och sjuklighet, bland annat i hjärtinfarkt och stroke. Rökning påverkar blodkärlen genom att skada det innersta cell-lagret (endotelcellerna). Detta leder till inflammation, ökad koagulation och styvhet av artärerna och detta tillstånd kallas för endoteldysfunktion som är ett förstadium till åderförkalkning. Runt om i världen börjar dock restriktionerna kring rökning skärpas och med detta ökar drivkraften för tobaksindustrin att hitta alternativa vägar för att upprätthålla sina intäkter. Förbränningsfria produkter som snus och e-cigaretter har därför blivit alltmer uppmärksammade som alternativa nikotinprodukter.

Snus är välkänt i Sverige och cirka 22 % av män och 4 % av kvinnor använder produkten dagligen. Användningen i övriga Europa är begränsad då EU införde ett snusförbud 1992. Snusprodukter har dock tagits fram av både Altria (Marlboro) och RJ Reynolds (Camel) och dessa företag (tillsammans med Swedish Match) lanserar nu intensivt snus i USA. Snuset marknadsförs som ett hälsosammare alternativ till rökning. De som förespråkar snus som en ersättning till rökning hänvisar till de stora svårigheterna att koppla snusning till ökad sjuklighet. På senare år har allt fler studier publicerats som kunnat påvisa ett statistiskt samband mellan snusning och ökad dödlighet i hjärtinfarkt och stroke. Å andra sidan finns publicerade studier med motsägelsefulla resultat avseende hjärt- och kärlrisk. Detta har lett till medial uppmärksamhet och det har spekulerats huruvida snus bör anses som ett hälsosammare alternativ till rökning.

Utöver snusning har på senare år e-cigaretten lanserats som ett alternativ till cigarettrökning. E-cigaretten består av en elektronisk förångare som hettar upp en vätska. Denna ånga andas in på samma vis som vid vanlig cigarettrökning. Vätskan (e-vätska) är baserad på glycerol och propylenglykol. Den finns tillgänglig i olika nikotinstyrkor (det finns även nikotinfria alternativ) och ett flertal olika smaker, såsom tobak, mentol och olika fruktsmaker. E-cigaretten lanseras just nu som ett alternativ till cigarettrökning och även i en del fall som rökavvänjning. Utöver nikotin har flera gifter hittats i e-cigarettånga och e-vätskan, men nivåerna av dessa föreningar är i mycket lägre koncentration än det som kan detekteras i cigarettrök. Det finns än så länge mycket begränsade uppgifter om eventuella hälsorisker med e-cigarrettanvändning.

Målet med avhandlingen var att undersöka kärleffekter av cigaretter, e-cigaretter och snus med olika metoder.

Metoder och Resultat

Kärlfunktion kan undersökas på flera olika sätt: I blodprover kan man analysera mikrovesiklar (MVs) som utsöndras av celler som är involverad i kärlens funktion som endotelceller, vita blodkroppar och blodplättar. En akut ökning av MVs i blodet brukar vara

förknippat med inflammation och kärlskada. Man kan även analysera antalet endoteliala progenitorceller (EPCs). Dessa celler är involverade vid kärlreparation och utsöndras också vid skada på endotelceller.

En annan metod är mätning av kärlstyvhet som enkelt analyseras med en detektor runt halsen och låret samt med en speciell detektor vid handleden.

En mer tidskrävande metod är mätning av kärlfunktion i underarmen där man samtidigt undersöker huruvida kärlen svarar på kärlvidgande substanser. Denna metod kallas för venös-ocklusionspletysmografi (VOP).

I delarbete I studerade vi akuta effekter av rökning bland 12 unga friska frivilliga försökspersoner. De fick röka en cigarett vid ett tillfälle, jämfört med att inte röka vid ett annat tillfälle. Blodprover togs innan rökning/inte-rökning och 1h, 4h och 24h efter. Blodprover analyserades för MVs och EPCs. Rökning orsakade en snabb ökning av MVs och EPCs.

I delarbete II undersöktes akuta effekter av e-cigarett-inandning (med nikotin). 16 unga friska försökspersoner utsattes för antingen e-cigarettanvändning eller icke-användning. Blodprover togs innan exponeringen och 1h, 4h och 24h efter. Blodprover analyserades för MVs och EPCs. Inandning av e-cigarett ånga orsakade en snabb ökning av EPCs men inte av MVs.

Sjutton unga friska försökspersoner använde i delarbete III e-cigarett med och utan nikotin. Kärlstyvhet mättes innan och efter användning under 4 timmar. E-cigarett med nikotin orsakade en övergående ökning av puls och kärlstyvhet men inte e-cigarett utan nikotin.

I delarbete IV undersöktes 24 friska snusare (medelålder 44,8 år) och 26 icke-snusare i samma ålder. Kärlstyvhet och VOP mättes. Jämförd med icke-snusare så hade snusande individer förhöjd kärlstyvhet och ett försämrat svar på den kärlvidgande substansen nitroglycerin.

Diskussion

Rökning orsakar ett kraftigt svar från blodkärlen som uppvisar tecken till skada, inflammation och aktivering av blodkoagulering. E-cigaretter verka inte ha en lika kraftigt negativ effekt på kärlen. Däremot observerade vi en ökning av EPCs efter både cigarett och e-cigarettanvändning som talar för en aktivering av kärlen och som kan vara orsakad av nikotin. E-cigarett med nikotin ökar dessutom kärlstyvhet tillfälligt och daglig användning av snus ger ökad kärlstyvhet. Ökad kärlstyvhet är en riskfaktor för utveckling av hjärt- och kärlsjukdom. Dessutom uppvisar kroniska snusare ett försämrat svar på en kärlvidgande substans (nitroglycerin) som ofta används i samband med akut hjärtinfarkt för att förbättra blodflödet till hjärtat. Detta kan försämra prognosen för snusande personer som drabbats av hjärtinfarkt.

Våra fynd tyder på att nikotinkomponenten i snus och e-cigaretter påverkar kärlen på ett negativt sätt. E-cigaretter och snus bör därför inte anses som ofarliga produkter.

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