Potential Effects of Electronic Cigarette Vapour on Isolated Lung Cells and in Mouse Lungs

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by

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Signature 

This dissertation is dedicated to the memory of my father, Tadele Elala.

"My Father didn't tell me how to live;
he lived,
and let me watch him do it."
Clarence Budington Kelland

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List of abbreviations

µl	Microliter
5HTT	Serotonin transporter
AECII	Alveolar epithelial type II cells
B2M	Beta2-microglobulin
Bcl2	B-cell lymphoma 2
bp	Base pair
BrdU	5-Bromo-2´-Deoxyuridine
BSA	Bovine serum albumin
Ccna1	Cyclin-A1
CD8	Cluster of differentiation 8
cDNA	Complemetary deoxyribonucleic acid
cm	Centimetre
CSE	Cigarette smoke extract
Csf2	Colony-stimulating factor 2
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
E-liquids	Electronic cigarette liquids
et al	et alia
FACS	Fluorescence-activated cell sorting
FDA	Food and Drug Administra
FEMA	Flavour and Extract Manufacturers Association
FEV_1	Forced expiratory volume in 1 second
g	Gram
GRAS	Generally recognize as safe
GSH	Glutathione
HCL	Hydro chloric acid
Hepes	2-(-4-2-hydroxyethyl)-piperazinyl-1- ethansulfonate
hESC	Human embryonic stem cells
hPF	Human pulmonary fibroblasts
hr	Hour
IARC	International Agency for Research on Cancer
IL-8	interleukin-8
iNOS	Inducible nitric oxide synthase
mg	Milligram
mL	Milliliter
mM	Milli mole
MMP9	Matrix metallopeptidase 9
mNSC	Mouse neural stem cells
mRNA	Messenger ribonucleic acid
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
NaCl	Sodium chloride
NaOH	Sodium Hydroxide
NIOSH	National Institute for Occupational Safety and Health
NNK	Nicotine-derived nitrosamine ketone
Nrf2	Nuclear factor erythroid 2–related factor 2
OD	Optical density

PASMC	Pulmonary arterial smooth muscle cells
PBS	Phosphate-buffered saline
PG	Propylene glycol
PMSF	Phenylmethane sulfonyl fluoride
ppm	Parts per million
qRT-PCR	Quantitative real time- polymerase chain reaction
RCF	Relative centrifugal force
RMPI	Roswell Park Memorial Institute medium
RNA	Ribonucleic acid
RNS	Reactive nitrogen spices
ROS	Reactive oxygen spices
RVSP	Right ventricular systolic pressure
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
TAE	Tris-acetate-EDTA
TBS	Tris-buffered saline
TEMED	Tetramethylethylenediamine
Timp3	Metalloproteinase inhibitor 3
TNFα	tumour necrosis factor-alpha
Traf1	TNF Receptor Associated Factor 1
Tris	tris(hydroxymethyl)aminomethane
TSNAs	Tobacco-specific nitrosamines
U.K	United Kingdom
UV	Ultraviolet
β	Beta

1. Introduction

1.1. Cigarette smoking

1.1.1. History of tobacco use

Tobacco use has been part of human history for a long time. Mayan Indians depicted the tobacco in stone carvings dated between 600 and 900 anno Domini (AD). Christopher Columbus and his crew were first Europeans to be introduced to tobacco use. He brought a tobacco with him back to Europe, but most Europeans didn't appreciate the taste of tobacco leaves. Since 1561 when Jean Nicot, French ambassador in Lisbon, Portugal sent tobacco to the French royal court as a potential medicinal treatment (1), tobacco use slowly has gain popularity. The tobacco plant, Nicotiana was named after Jean Nicot by Carl Linnaeus, and therefore, the addictive substance was called nicotine. By the 1800's, the number of tobacco users dramatically increased. The people use the tobacco by smoking a pipe, hand rolled cigarettes or cigars and simply by chewing (2). Especially, smoking became popular after the Industrial Revolution (3) and two significant time points when tobacco was widely popularized: 1) in Western countries during World War I and 2) in Asian countries around the 1940s (3).

Gradually, accumulating data warning about the harmful effect of tobacco use started to emerge. In 1964 Surgeon General of the United State (U.S) reported that cigarette smoking (CS) is the most important cause of lung cancer and chronic bronchitis (4). Therefore, the Congress of the U.S passed the Cigarette Labelling and Advertising Act in 1965 and the Public Health Cigarette Smoking Act in 1969. These laws required a health warning on cigarette packages, banned cigarette advertising in the broadcasting media and called for an annual report of the health consequences of smoking (4,5). Furthermore, driven by an increasingly negative public perception, CS began to be restricted in public places by U.S Federal, State and Local law (2).

1.1.2. Epidemiology of tobacco use

The implementation of tobacco control has been resulted in decrease of prevalence of smoking only in developed countries, mostly in English speaking countries (6). Still, these regulations did not stop the increase of prevalence of CS worldwide. The prevalence of smokers increased globally from 721 million in 1980 to 967 million in 2012 and the daily consumption of cigarettes increased from 4.96 trillion to 6.25 trillion (6). In 2015, there were over 1 billion smokers worldwide and more than 80% of smokers are living in low and middle income countries (7).

Before the world wars, men were main consumers of cigarettes as smoking by female were considered as bad manners. This situation was changed by the liberalization of women's roles and behaviour (2,8), consequently, the number of female smokers rose from World War I to World War II and onwards (2,9). However, the percent of male smokers is still 5 times higher, though this gap drops in younger populations (10,11). Furthermore, concern about CS among young adult is growing and young adults have the highest smoking rate of any age group (12). In 2002, the World Health Organization (WHO) estimated that approximately 20% of 13-15 years old teenagers were smokers and between 80,000 to 100,000 young teens started smoking everyday (13). Long-term predictions estimate that ~90% of smoking adolescents would be still smoking 15 to 20 years later (13).

1.1.3. Cigarette smoke and health

1.1.3.1. History: the investigation of effect of smoke on the health

Scientific and medical journals addressing the health consequences of smoking begun to surface by the early 20th century. One of the pioneering studies that statistically correlated CS with cancer was published in 1930 by researchers from Cologne, Germany (14). In 1938 Dr. Raymond Pearl from the John Hopkins University reported that smokers died prematurely compared to non-smokers (15). Since the publication of "Smoking and Health: Report of the Advisory Committee of the Surgeon General of the Public Health Service" in 1964, CS is considered as major hazard factor triggering (CS is a cause of diseases) or contributing (together with another factors such as genetic or epigenetic inheritance) to the various diseases (Figure 1 and 2). Accordingly, Framingham study of 34 years follow-up of 5209 individuals revealed the significant relationship between active smoking and the incidence of cancer of the lung, stroke and transient ischemic attacks, cardiovascular diseases (CVD), and most especially the average annual death rate (16). Currently, the research focus has shifted from the active smoking-induced health problems to the study of effects of second-hand smoking (passive smoking – exposure to the side-stream smoke) on the health (Figure 2). However, despite the intensive studies, the outcome of this research regarding the effect of passive smoking (environmental tobacco smoke) is controversial (17).

Nowadays, it is widely accepted that active and passive smoking affect nearly all physiological reactions in almost every organ of the body (15), contributing to the development of osteoporosis, psoriasis, cataracts, type 2 diabetes mellitus, rheumatoid arthritis (15) and is leading cause of chronic obstructive pulmonary disease (COPD) (Figure 1). Furthermore, CS

impairs the fertility of men and women and raises the risk of pre and postpartum complications in both the mother and child (8,15,18) (Figure 1). In addition, habitual CS is not only associated with the development of numerous pathologies, but could also contribute to a number of neurocognitive deficits, such as deficits in memory and attention (19).

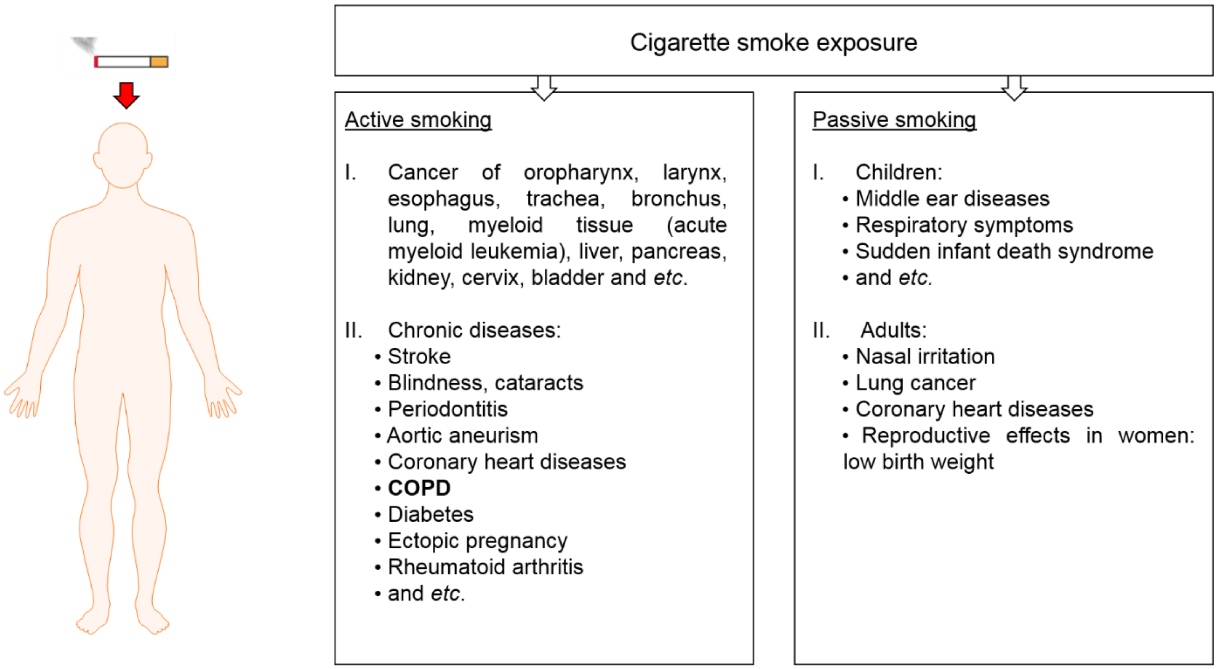


Figure 1. Health consequences of cigarette smoking (CS)

Active smoking and exposure to second-hand smoke has a multitude of pathophysiological consequences in various organs evoking numerous pathologies. CS is a main risk factor of chronic obstructive pulmonary disease (COPD).

1.1.3.2. The effect of CS on the health

Burning the modern cigarette, which on average has around 600 ingredients, releases over 7,000 chemicals, of which 250 have been identified as harmful substancs and 69 of those identified as carcinogenic (15,18,20,21). These cigarette combustion products are classified into either gaseous phase or solid particulate (tar) phase products, with most of the toxic smoking components present in the tar phase (22-24). Inhalation of mainstream (smoke drawn from the cigarette rod) and side-stream (smoke released from the lit end of the cigarette) CS results in the fast absorption and consequent systemic availability of toxins (e.g. reactive oxygen species [ROS] and reactive nitrogen species [RNS]) by the oral and airway epithelium (22,25). ROS are not present in the tobacco leaves, but are produced as a result of cigarette combustion (22).

The inhalation and absorption of cigarette combustion products (including ROS per se) initiate numerous signalling pathways including the activation of endogenous sources of ROS (iNOS, NADPH oxidases) and inflammation triggering countless cellular consequences (apoptosis,

proliferation, senescence and etc.) that can cause or contribute to development of various pathologies such as CVD, COPD and lung cancer in genetically compromised individuals (Figure 2) (26).

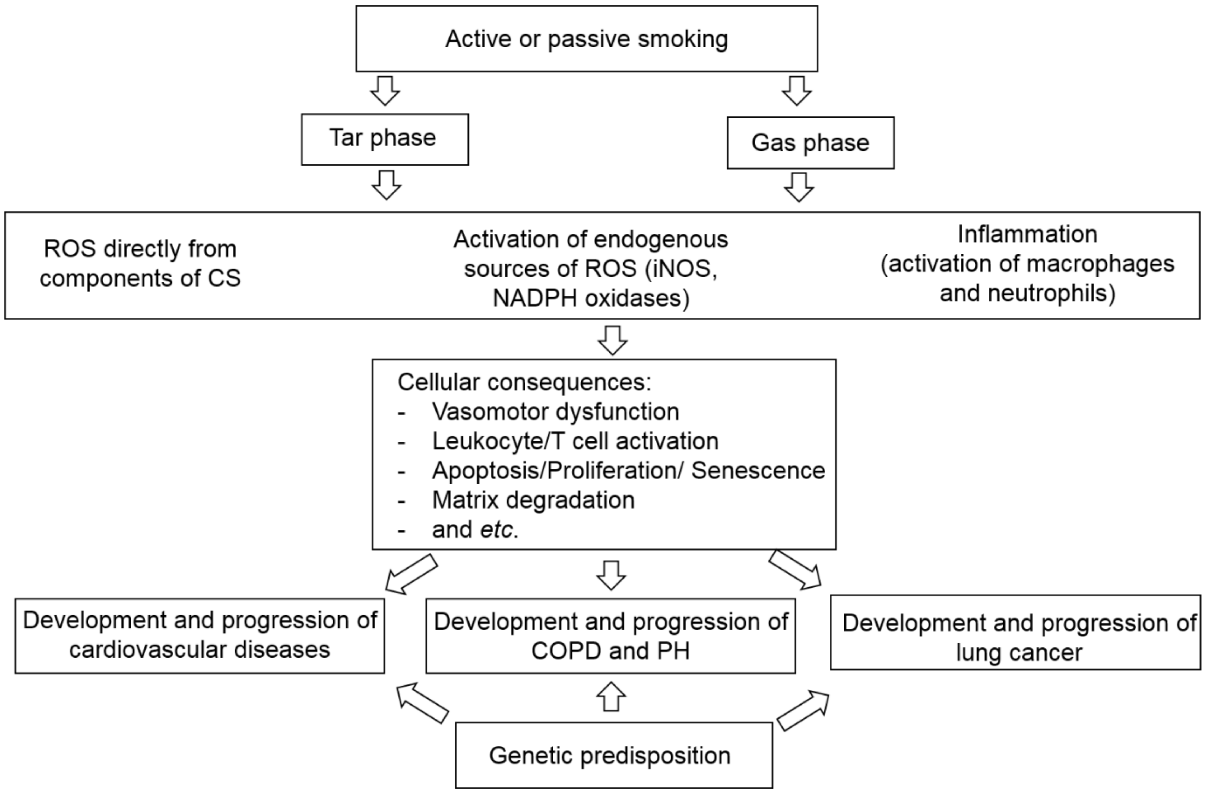


Figure 2. Putative mechanisms of pathogenesis and pathophysiology of cigarette smoke (CS) related diseases

Active and passive smoking (tar and gas phases) via ROS (component of CS), the activation of endogenous ROS and altered inflammation trigger various cellular consequences that initiate or contribute to the development of numerous pathologies in genetically compromised individuals. Cardiovascular diseases, COPD and lung cancer are major outcomes of CS. PH: Pulmonary hypertension.

CS is a major risk factor in cancers of the lung, bladder, colorectal, oropharynx, kidney, cervix, oesophagus, pancreas, stomach, nose, trachea, and bronchus, as well as haematological malignancy such as acute myeloid leukaemia (15). Approximately 50% of all cancer related deaths attributed to the CS (15,18). CS is by far the leading cause of lung cancer (27) and lung cancer is the leading cause of deaths across all cancer subtypes (15,18). Despite modern treatment (surgery + anticancer drugs), the five-year survival rate for non-small cell lung cancer (NSCLC) which accounts for ~84% of lung cancer, is roughly 56% (28). The exact pathogenesis of CS-induced growth advantage for the tumour cells at the expense of the host is still not completely understood (29). The cigarette combustion products including various

carcinogens have been shown directly or indirectly (e.g. ROS or RNS) to damage DNA (30). Chronic tobacco carcinogens-induced injury of airways and lung parenchyma triggers a hyperplasia as well as dysplasia of normal lung cells resulting in malignant tumours (30). Considering the facts that ~10-15% of all lung cancers arise in never smokers (31) and non-smokers develop cancer at some point during their lifetime (31) suggest that the contribution of other factors in the development of cancer. In this regard, numerous cancer susceptibility genes including BRCA1/2, APC, TP53 and *etc*. have been identified.

Moreover, lung cancer and COPD often co-exist. Depending on the criteria used for diagnosis, 40-70% of lung cancer patients are also affected by COPD (32,33). Within 10 years, compared to smokers with normal lung function, the probability of developing lung cancer in mild to moderate COPD patients is 3 fold higher and the probability of developing lung cancer further increases to 10 fold in severe COPD patients (34).

Furthermore, active and passive smoking play an important role not only in the initiation but also in the progression and development of fatal cardiovascular complications of CVD (35). In this regard, WHO reported that smoking is responsible for 10% of all CVD cases (36). However, how CS trigger or contribute to CVD development is still not clear. It was suggested that CS-induced endothelial dysfunction and damage could: 1) increase in the oxidation of proatherogenic lipids, as well as decrease of high-density lipoprotein, 2) initiate inflammation within vessels and 3) promote the increase of procoagulant state in the circulation (35). In conclusion, CS is the highest preventable cause of morbidity and mortality in developed countries (15,18). Globally, 10% of adult deaths are associated with the use of tobacco (13). This corresponds to about 6 million deaths per year of which 600,000 are attributable to second-hand smoke in 2002 (13). Therefore, the strategies to eliminate active as well as passive CS are absolutely necessary to prevent the development of debilitating diseases.

1.1.4. COPD

CS is a major risk factor for the development and progression of COPD (37,38). COPD, which includes both chronic bronchitis and emphysema, is severe irreversible and progressive disease and is characterized by progressive and persistent airflow limitation. The pathogenesis of COPD is based on the complex pathophysiological reactions to the inhalation of toxic particles/ gases and is still not completely understood. Although tobacco smoking is the major cause of the initial inhalation injury at COPD, many other environmental and occupational exposures partake in the COPD development (37).

The pathology of COPD goes beyond the lung, giving rise to systemic inflammation and the development of a number of comorbidities including skeletal muscle wasting, diaphragmatic dysfunction, CVD, and osteoporosis (42,77). Therefore, COPD is more and more viewed as a systemic disease (37). Furthermore, 30%–70% of patients with COPD also have pulmonary hypertension (PH), which nowadays classified as third group of modern PH classification (39). Post mortem examinations of moderate and severe COPD patients have shown drastic changes in pulmonary muscular arteries and precapillary vessels and might explain the irreversible increase of pulmonary vascular resistance in these patients (40,41). Nevertheless, these changes are not limited to patients with advanced disease only; they were also observed in patients with mild COPD (43-45) as well as in smokers without any airflow obstruction (44,45). Studies from tobacco smoke animal models (guinea pigs and mice) revealed CS causes vascular remodelling with thickening of the pulmonary artery vessel wall. These studies further indicated that pulmonary vascular remodelling might precede lung emphysema development (46-49).

Various theories to explain the COPD pathogenesis have been suggested. Laurell and Eriksson in 1963 first described an association between COPD and a genetically determined deficiency of alpha 1 antitrypsin (A1AT), which is a protease inhibitor encoded in chromosome 14 as a part of a gene cluster called the SERPIN supergene (50). To date, more than 100 genetic variants of the *SERPINA1* have been described (51). However, only 5% of people with COPD have alpha-1 antitrypsin deficiency (51). It has been reported that CS accelerate the development of emphysema in patients with alpha-1 antitrypsin deficiency compared to non-smokers with the deficiency. Lately, other theories of COPD pathogenesis have been proposed including accelerated aging, aberrant inflammatory response and increased oxidative stress (52,53)

The initial inhalation of toxic gases and particles of CS initiates the lung response including the activation of the immune system, which could later lead to pathological changes resulting in airway remodelling, increased mucus section and alveolar wall destruction in the lung manifesting to the development of COPD (Figure 3).

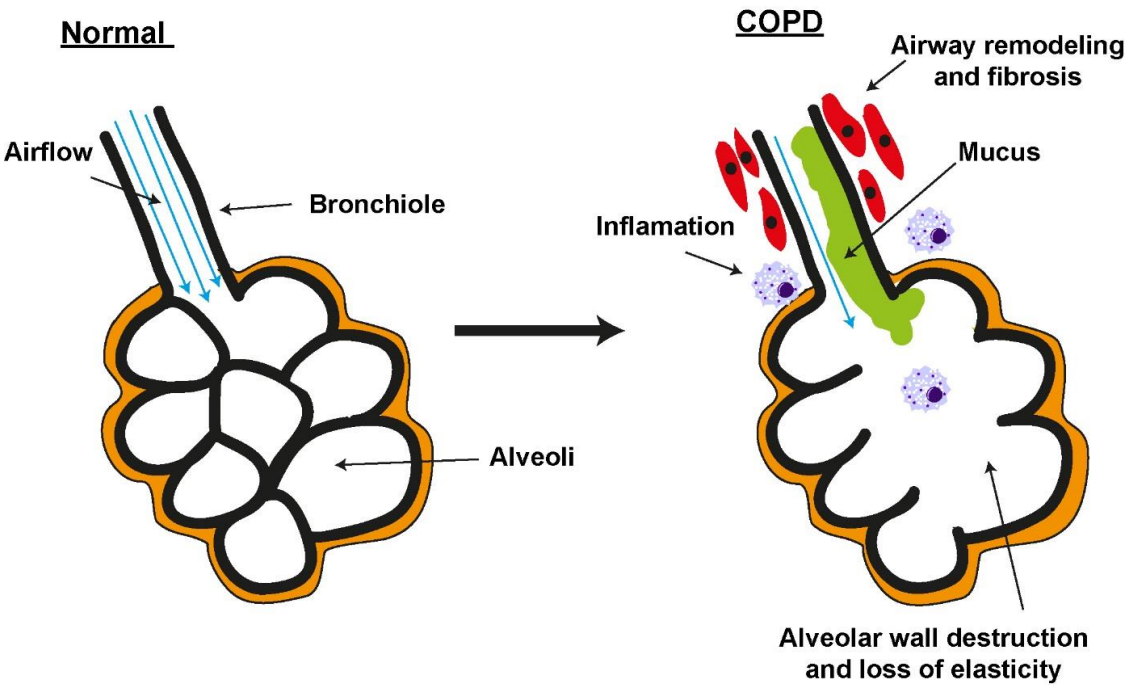


Figure 3. Mechanisms of airflow limitation in COPD

Compared to normal lungs, lungs of patients with COPD show airflow limitations due to enlargement of alveoli and structural loss, as well as obstruction of the bronchioles with mucus and airway remodelling

1.1.4.1. COPD and ROS

Oxidative stress and oxidative damage are the hallmarks of CS (54,55) and is a prognostic marker in COPD (53). The increased oxidative stress in COPD can be an outcome of: 1) direct or in indirect ROS/RNS components of CS, 2) increased activity of endogenous ROS/RNS sources and 3) inhibition of ROS defence mechanism (antioxidant systems).

Both gas and tar phase of CS contains extremely high concentrations of numerous ROS/RNS including superoxide anion, H_2O_2, reactive hydroxyl radical and peroxynitrite that can directly trigger the oxidative damage of cellular membrane lipids, proteins, enzymes and DNA (56). Moreover, CS constituents can produce ROS after metabolic conversion (e.g. benzo[α] pyrene). Benzo[α] pyrene is metabolized to quinone (57,58) that can undergo redox cycling by entering into the NADPH reductase pathway, which gives rise to labile semiquinones that are readily autooxidized (59-61). NADPH oxidase generates high levels of ROS in neutrophils as a central mechanism of host defence response. In smokers NADPH oxidases generates ROS due to an increased migration of neutrophils and macrophages to the lung (62,63).

Finally, the aldehydes in CS react through a non-radical mechanism with proteins, which results in the formation of Michael addition products with protein SH- and NH_2-groups. These types of modifications may result in altered activity of crucial enzymes, membrane receptors and transport proteins and interfere in cell signalling pathways (64).

1.1.4.2. COPD and inflammation

The altered immune response is important component of COPD development (22). However, the exact cellular signalling mechanisms underlying the CS-induced inflammation in COPD are still unclear. CS constituents (specifically ROS) can damage epithelial cells through a number of different mechanisms, including peroxidation of lipids and other essential cell membrane components and DNA damage (56, 65,66). The damage of epithelial cells can initiate a local and systemic inflammation by release of pro-inflammatory mediators [for instance, interleukin-8 (IL-8) and tumour necrosis factor-alpha (TNFα)] and by recruitment of inflammatory cells that can secret various cytokines and chemokines (56,67-68). Macrophages, neutrophils and lymphocytes are the primary immune cells that have been seen in the lungs of COPD patients and smokers (69-73).

In addition to its role in maintaining the conduit for air to and from the alveoli, the airway epithelium is central to the defence of the lung against pathogens, through the combined function of ciliated epithelial and secretory cells maintaining efficient mucociliary clearance and through a variety of other host defence mechanisms (74). Some insults like CS are able to activate epithelial cells directly and this further induces the release of chemokines and inflammatory mediators (74-76).

Particles from CS induce release of chemotactic factors from epithelial cells and macrophages, resulting in recruitment of lymphocytes and neutrophils from the circulation. These in turn activate fibroblasts then undergo abnormal tissue repair and cause ultimately bronchiolar fibrosis (77). Macrophages and neutrophils release proteases that lead to alveolar wall destruction and release of mucous (77).

1.2. Cigarette smoke cessation methods

After the discovery of the link between CS and cancer in the early 1950's and the demonstration of the health-benefits of smoking cessation, the tobacco industry, governments and public sectors were aiming to reduce the harm from cigarettes in a concerted effort (78). Therefore, strategies to eliminate CS have led to the emergence of new smoking cessation tools as

alternatives for CS including nicotine patch, gum, inhaler, lozenges, bupropion, varenicline, nasal spray and most recently electronic cigarettes (e-cigarettes) (79). The Nationally representative longitudinal online survey of US adult cigarette smokers demonstrated that e-cigarettes became most popular approach for smoking cessation in USA (82).There are a few studies dedicated to the investigation of efficacy of e-cigarettes as smoking cessation tool. In this regard, an online survey of 222 smokers who had tried e-cigarettes as a smoking cessation tool showed that 31% of participating smokers did not smoke cigarettes for 6 months after starting e-cigarettes and 66.8% of participants reduced their cigarette consumption (80). In another prospective study of 40 smokers who were not thinking of quitting, 55% of participant demonstrated 50% reduction in the number of cigarettes smoked per day (81). However, most of these studies are short-term and the effectiveness of e-cigarettes in long-term is still to be addressed. Moreover, using of e-cigarettes as smoking cessation tool, in most cases, preserve the addiction to nicotine.

1.2.1. History of e-cigarette

E-cigarettes have been introduced to the market in recent years as an alternative to smoking conventional cigarettes. E-cigarettes are nicotine delivery devices by heating and converting to aerosol a liquid mixture composed of propylene glycol (PG), vegetable glycerine (VG), flavouring chemicals, and nicotine (79). Common components of e-cigarettes include a battery, heating coil, atomizer that transforms the e-cigarette liquid (e-liquid) to an aerosol, cartridge that contains the e-liquid, and mouthpiece (Figure 4). When a user takes a puff on the end of the e-cigarette tube a battery heats up the e-liquid, which creates a vapour that is then inhaled into the lungs. In the e-cigarettes, the nicotine solution is vaporized electro-thermally, so that no active combustion takes place. In contrast to tobacco smoke, inhaled vapour does not contain either tar or carbon monoxide (83).

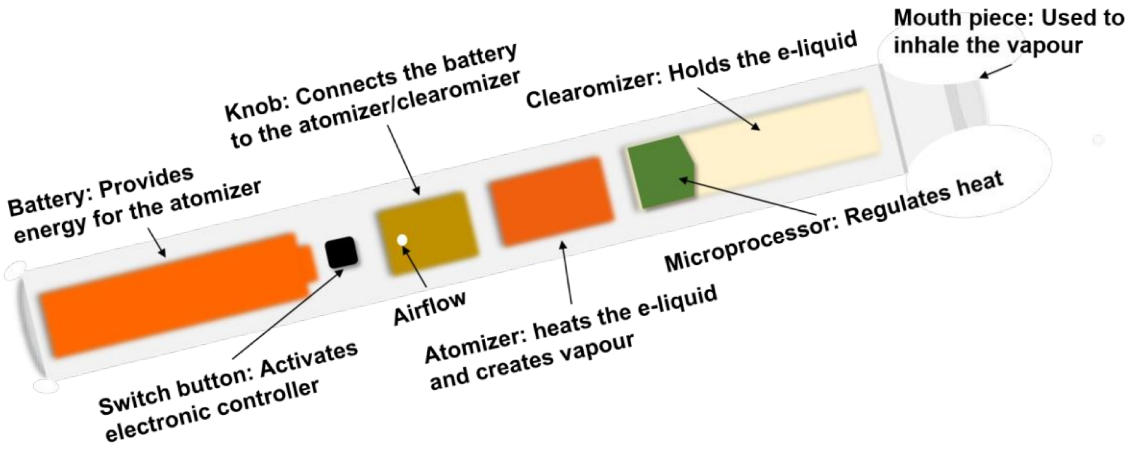


Figure 4. Diagram of electronic cigarette configuration

E-cigarettes are usually composed of a nicotine cartridge (e-liquid container), a vaporizing chamber, a heating coil (heats e-liquid) followed by an atomizer (e-vapour generator), a rechargeable battery, a voltage controller (which will adjust the amount of nicotine delivered during vaping), a microcompressor, and an LED indicator (not present in all types) to activate the battery and visually mimic the conventional cigarette, respectively.

The American Herbert A. Gilbert patented the first e-cigarette in 1963 as "smokeless non-tobacco cigarette". The device was designed to replace the burning tobacco and paper with heated, moist, flavoured air without nicotine. These smokeless non-tobacco cigarettes were never commercialized because smokers at that time did not feel pressured to replace smoking as it was considered trendy, there were no prohibition of public smoking and the report about the link between CS and increased mortality of smokers by the U.S. Surgeon General was not yet released (84,85). Contrary, Chinese pharmacist Hon Lik is often associated with the invention of e-cigarettes. Hon Lik invented e-cigarettes in its current form in 2003 (86,87).

There are generally three types of e-cigarette. First generation e-cigarettes that were designed to look and feel like conventional cigarettes. These devices consists out of a battery, a heating element (atomizer) and a reservoir for e-liquids with or without nicotine. The product was neither rechargeable nor refillable. Second generation e-cigarettes are pen-shaped and contained a cartomizer, which combined the reservoir, the wick/ fibre with the heating element in one single unit. These models are rechargeable, usually refillable and allow the user to regulate the length and duration of the puff they take by a manual switch. Third generation also known as Mods have a higher capacity and a technologically sophisticated tank system. The devices allow the user to adjust the voltage or wattage applied to the atomizer, have automatic temperature control and dual airflow slots (88).

1.2.2. Prevalence of e-cigarette

In countries where e-cigarettes are available, awareness and usage is high among current and former smokers (89). A survey conducted by Adkison and colleagues in the United Kingdom (UK), United States America (USA), Canada and Australia estimated the rates of e-cigarette usage and perception among current smokers and ex-smokers (89). Nearly half (46.6%) of respondents heard about e-cigarettes across the countries. Awareness of e-cigarettes were highest in USA (73%), followed by UK (54%) and Australia (40%). The lowest rate of awareness was observed in Canada (20%). Among those who are aware of e-cigarettes, the prevalence of usage was 20.4%, 17.7%, 11% and 10% in USA, UK, Australia and Canada, respectively. This study also analysed correlation of awareness with sociodemographic data. E-cigarette use and awareness were higher among younger, higher-income and well-educated respondents. The vast majority of respondents (70.3%) reported that e-cigarettes are less harmful than conventional cigarettes. Another similar study (the Eurobarometer survey) conducted in 27 European Union member countries assessed awareness, attitude and prevalence of ever-using e-cigarettes (90). The highest awareness was in Finland (92%) and Greece (90%), while the lowest was in Sweden (34%). 7% of European Union respondents tried e-cigarettes at least once and 2% of respondents regular use them with higher abundance in Greece, Denmark and Romania. As in Adkison's study, the highest awareness of e-cigarettes was among male and younger respondents. 38% of European Union respondents were unsure about harmful effects on e-cigarettes on health, 35% perceive them as not harmful and 27% as harmful (90).

Thus, e-cigarette use among youth and young adults has become a public health concern as flavours of e-cigarette are highly appealing to try or even regularly use (91-93). This reflects in a spike during the past 5-6 years of adolescents that have used e-cigarettes. Nowadays, e-cigarettes are the most commonly used tobacco products among adolescents and are strongly associated with the use of other tobacco products, alcohol, and other substance use as for example marijuana (94-100). In addition, the technical design of e-cigarettes can facilitate the delivery of cannabinoids and other illicit drugs (101,102). A study by Morean and colleagues showed a high rate of e-cigarettes used for vaporizing cannabis among high school students (e-cigarette 18.0%, cannabis 18.4%, and dual users 26.5%) (101).

1.2.3. E-cigarette puffing topography

Puffing topography such as puff duration, number of puffs, inter-puff interval, flow rate, and volume is highly variable among e-cigarette users. Puffing topography is an important

determinant of nicotine intake and exposure to potentially toxic substances in e-cigarette aerosol (103). There are different determinant factors that affects e-cigarette puffing topography. One of this factor is experience of e-cigarette usage. Puffing topography changes as e-cigarette–naïve users become more experienced. The puff duration and puff volume increase with experience (104-106). Nicotine concentration has been shown to play critical role in determining the e-cigarette puffing topography. Number of puffs and puff duration tend to decrease as nicotine strength of the e-liquid increases (107-109). Furthermore, e-cigarette puffing topography is different among types of e-cigarettes (first generation versus advanced models) and voltage or power (107). Because the variability of puff topography, it is very difficult to mimic (study) the effects of e-cigarette in animal models and in vitro studies. Furthermore, effect of passive e-cigarette exposure on health is completely not investigated.

1.2.4. Health effects of e-cigarette

With the increase of popularity of e-cigarettes comes an increase of unknown health risks. Despite a growing body of research investigating the impact of e-cigarettes on health, there are very limited studies available concerning their long-term effects, mainly because e-cigarettes are a more recent development. The potential harmful effects of e-cigarettes can be attributed to: 1) the chemical composition of e-liquids, 2) the liquid components (ingredients) and 3) the created aerosols (Table 1) (110). E-cigarettes deliver a mixture of chemical substances, including carcinogens such as formaldehyde, acetaldehyde, acroleine, propanal, nicotine, acetone, o-methyl-benzaldehyde (111,112). Moreover, studies by Pellegrino et al. and Schober et al. show that use of e-cigarettes impairs indoor air quality by increasing the concentration of particulate matter in ambient air (113,114).

Table 1. List of components found in e-liquid and aerosol of e-cigarettes.

Constituents of E-liquid and Aerosol in E-cigarette	
E-Liquids	Aerosols
Glycerol	Glycerol
Propylene glycol	Propylene glycol
Nicotine	Nicotine
Acetone	Acetone
Acrolein	Acrolein
1,3-Butadiene	Acetaldehyde
Formaldehyde	Formaldehyde
Cyclohexane	N'nitrosonornicotine (NNN)
Diethylene glycol	4(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)
Ethanol	Toluene
Tobacco alkaloids:	Metals:
• Nornicotine	• Cadmium
• Myosmine	• Lead
• Anabasine	• Nickel
	• Tin
	• Copper

Adapted from Dinakar et al., 2016 (110).

Currently, complete understanding of e-cigarette effects on the human body is incomplete and there are debates if e-cigarettes can generally be seen as harmless (115). Pitfalls of published studies are proper study designs, small sample size, study duration and many *in vitro* studies report results of treatment with e-liquids instead of the inhaled product of vaping.

Several *in vitro* studies on mouse lung cells, human embryonic stem cells, mouse neural stem cells, human pulmonary fibroblasts and cardiomyoblast cells have been conducted in order to investigate the effects of e-cigarette exposure (116,117). However, there are no studies that assess the effects of e-cigarette vapour extract on primarily isolated lung cells. Farsalinos et al. evaluated the potential effects of e-cigarette vapour on cardiomyoblast cell viability (116). Vapour from 20 different e-cigarette liquids with different flavouring substances was prepared and when cells were exposed over 24 hours to different concentrations, 4 out of 20 e-cigarette, showed cytotoxic effects on cardiomyoblasts. When comparing e-cigarette vapour extracts (ECVE) with cigarette smoke extracts (CSE) on cardiomyoblasts, a reduced cytotoxicity indicate that e-cigarettes might be less harmful then cigarettes (116). A study by Bahl et al.

evaluated the cytotoxic trait of 35 different flavoured e-cigarette liquids on human embryonic stem cells (hESC), mouse neural stem cells (mNSC), and human pulmonary fibroblasts (hPF) (117). Cells treated with different concentrations of refill liquids, except the flavour bubble-gum, showed cell cytotoxic to various extents. The cytotoxicity mostly depends on the dose of refill solution and flavours as well as cell type, where hESC and mNSC were more sensitive than hPF (117).

Until now, not so much data regarding the effect of e-cigarette use in animal model are available. Animal exposure to e-cigarette vapour for 30 minutes (118), 24hours (118), 72hours (119), 2weeks (120), 1month (121) and 4months (122) using different delivery approaches such as nebulizer (118,122), whole body exposure (119-121,) and intratracheal installation of the e-liquid (123) led to a significant increase in inflammation. E-cigarette aerosol exposure has also led to developmental defects (124,125), impaired lung growth in neonatal mouse (124) and heart malformation in zebrafish (125).

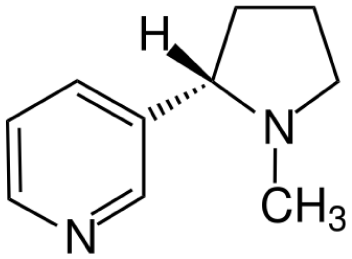
Even though inhaled compounds associated with e-cigarettes may be fewer in number and less toxic compared to conventional cigarettes, it is not possible to reach a definite conclusion based on current data to the question of whether e-cigarettes are less harmful to the user than cigarettes (111-114). Furthermore, long-term health effects of e-cigarettes have not yet been documented in humans, but experimental studies of acute effects in humans show that e-cigarette can cause physiological effects comparable to cigarettes (113,126,127). Similar to smoking, immediate reductions in exhaled nitric oxide (FeNO) (126,127) were observed, while FeNO was increased in another study (113). Other studies in naïve e-cigarette users found increased airway resistance (126,127) with simultaneous decreased specific airway conductance (127) and an increase in impedance and overall peripheral airway resistance (126). Even if these studies did not find clear detrimental effects of human use of e-cigarettes, they do raise questions regarding the health consequences that may arise from long-term use of e-cigarettes.

1.2.5. E-cigarette liquids

The PG and VG in e-liquid are used to produce the vapour while the flavouring provides the taste and aroma. In the European Union, the nicotine concentration in e-liquids is limited to a maximum of 20mg/ml (128) and surveys of e-cigarette users reveal 18mg/ml as the most popular concentration (129-131). In addition, the most preferred flavours were tobacco, mint and fruit (131). Typical e-liquid contains 95% PG and VG and the remaining being nicotine

and flavouring substances (132). Nevertheless, e-liquids are available without PG, nicotine or flavours (133-135).

1.2.5.1. Nicotine



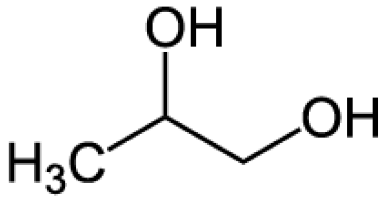
Nicotine is absorbed through the skin and the mucosal lining in nose, mouth and lungs. Smoking is a highly effective form of nicotine administration, causing a rapid rise in blood nicotine concentration (136,137). In the lungs, nicotine is first absorbed into the pulmonary venous circulation, followed by the arterial circulation, and moves from the lungs to the brain (138). As an alkaloid, basic conditions favour nicotine absorption. The slightly basic conditions in the lung combined with the vast surface area of alveoli identifies aerosol inhalation as the most efficient way to get nicotine in to the bloodstream (138). Furthermore, nicotine can also be absorbed through the gastrointestinal tract and skin and therefore it is possible to deliver nicotine to the bloodstream by chewing tobacco, gum and skin patches (smokeless tobacco products). Once absorbed, around 80% of nicotine is metabolized to cotinine by enzymes (mostly by CYP2A6) in the liver. Lungs also metabolize nicotine into cotinine and nicotine oxide. Nicotine's metabolic half life is 2 hours while cotinine has about a 20 hours half-life, making cotinine a reliable metabolite biomarker for person's nicotine intake (138,139).

Nicotine stimulates the adrenal glands to produce the hormone and neurotransmitter epinephrine (adrenaline) through its agonistic action on the nicotinic acetylcholine receptors (nAChRs) (138). Production of adrenaline stimulates the body and causes sudden release of glucose, increases heart rate and blood pressure, constricts blood vessels and ultimately leads to rapid and shallow breathing. Indirectly nicotine stimulates the production of dopamine, a neurotransmitter that plays an important role in the human body and brain. nAChRs are mainly found in neurons and neuromuscular junctions (140,141). Recent reports revealed that other cells such as human bronchial epithelial cells, human endothelial cells, and astrocytes also have nAChRs (140,141).

Generally, nicotine is not considered carcinogenic; but some of its metabolites are. For example the nicotine-derived nitrosamine ketone (NNK; tobacco specific lung carcinogen) which can be formed from aminoketone by nitrosation (142,143). Nevertheless, nicotine can also induce tumours under special conditions such as hyperoxia (144). Furthermore, nicotine acts as chemotactic agent for neutrophil migration and may contribute to inflammation (145). In mice,

nicotine has been reported to enhance leukocyte-endothelial interaction, which resulted in leukocyte adhesion in the cerebral microcirculation (146). In human monocyte derived dendritic cells, nicotine strongly stimulates an inflammatory response (147). Furthermore, nicotine may play a crucial role in producing endothelial dysfunction, lipid abnormalities, and insulin resistance (148).

1.2.5.2. Propylene glycol (PG)



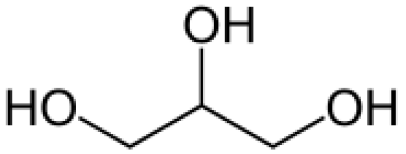
PG (propane-1,2-diol) is a viscous, colourless, odourless and completely water soluble substance derived from petroleum. PG has been classified by the Food and Drug Administration (FDA) as an additive that is "generally recognized as safe (GRAS)" for use in food. It is used in pharmaceutical industries as a solvent for drugs, a stabilizer for vitamins, in ointments for medicinal applications, cosmetics or food products as well as creating artificial smoke or fog used in firefighting training and in theatrical productions (149). However, vaporisation of PG through heating can form propylene oxide, which is classed as carcinogen by the International Agency for Research on Cancer (IARC) (150). The major route of mammalian PG metabolism is via alcohol dehydrogenase to lactaldehyde, then through aldehyde dehydrogenase, and on to glucose via gluconeogenic pathways. An alternative route is conversion to methylglyoxal via alcohol dehydrogenase, through glyoxalase metabolize to D-lactate (151-153).

There are limited human studies available regarding the health effects of PG following inhalation exposure. Wieslander et al. (149) evaluated the effects of exposure to a PG mist from artificial smoke generator in non-asthmatic human volunteers. This experimental study showed acute effects on ocular and upper airway irritation as well as decreased tear film stability in subjects after 1 minute exposure to an aircraft simulated PG mist. Four weeks exposure of patients suffering from allergic rhinitis to PG as part of pharmaceutical inhalations led to nasal burning, stinging and throat irritation. However, these effects decreased considerably with reduction of PG concentration from 20% (0.16g/ day) to 5% (0.04g/ day) (154). According to the safety report on the Ruyan® e-cigarette cartridge, the doses used in the above study are lower than the inhaled dose of PG from normal e-cigarette, which is 0.3 to 0.45g/day and during intensive use up to 0.9g/day (155).

Animal studies assessing the adverse respiratory effects after acute or intermediate PG inhalation exposure are inconclusive. A study by Konradova et al. reported acute inhalation

exposure to a concentration of 10% PG for 20 and 120 minutes increased the number of degenerated goblet cells in tracheal lining of rabbits (156). On the contrary, a study done in rats after inhaling 321 ppm PG for over 90 days showed thickened respiratory epithelium with enlarged goblet cells (157). The author also reported nasal haemorrhage in rats exposed to lower concentration of 51 ppm PG. On the other hand, continuous inhalation exposure of up to 112 ppm for 13 to 18 months in rhesus monkeys and rats failed to show any adverse effects on the respiratory system (158).

1.2.5.3. Vegitable glycerine (VG)



VG (also known as Glycerine or glycerol, propan-1,2,3-triol) is a viscous, hydroscopic liquid at room temperature and occurs naturally (as glycerides, an ester containing fatty acids and glycerol) in animals and vegetable fats and oils. Due to the presence of three hydroxyl groups, glycerine is completely soluble in water and alcohols (159). Glycerine has several commercial applications as component of drugs, cosmetics, toothpastes and processed foods (159). Since the end of 1950s, glycerine has been recognized as GRAS and is used as a general purpose food additive and as a food packaging material (Code of Federal Regulations, 1990). Nonetheless, after vaporisation through heating, glycerine forms acrolein, which can cause irritation of the upper respiratory tract and damages the lining of the lungs (160,161). Moreover, chronic exposure to even low levels of acrolein have been associated with increased CVD risk (162-166), while acute exposure can induce vascular injury (167), endothelial dysfunction (168), dyslipidemia (169) and platelet activation (170).

In biological systems, glycerine is seen as an intermediate in carbohydrate and lipid metabolism and is readily absorbed in the intestine and the stomach and incorporated in the standard metabolic pathways to form glucose and glycogen (171,172).

No relevant human glycerine inhalation studies exist. However, two key studies in Sprague-Dawley rats exposed to glycerine aerosol were found (159,173). Histopathological examination of rats exposed to nose-only inhalation of glycerine for 2 weeks (6 hours/day, 5 days/week) revealed an increase incidence of minimal to mild squamous metaplasia of the upper respiratory tract compared to controls. Further studies by the same authors described also development of minimal to mild squamous metaplasia of the upper respiratory tract of nose-only exposed rats for 13 weeks (6 hours/day, 5 days/week) (159,173).

1.2.5.4. Flavouring chemicals

Flavours are complex mixtures of substances who may be natural or manmade. As of 2014, about 8000 of commercially available e-liquids contained unique flavours (174,175). While FDA recognize most of the flavours as GRAS for ingestion, their effect on inhalation has not been evaluated. Studies have shown that some GRAS flavours can cause irreversible lung diseases upon prolonged inhalation (79,176,177). Most chemicals used in flavours have not been tested for respiratory toxicity via the inhalation route. The Flavour and Extract Manufacturers Association (FEMA) estimates that over 1000 GRAS flavour ingredients may have the potential to pose respiratory hazard due to possible volatility and irritant properties (alpha, beta-unsaturated aldehydes and ketones, aliphatic aldehydes, aliphatic carboxylic acids, aliphatic amines, and aliphatic aromatic thiols and sulphides) (178). Moreover, the flavours are highly appealing and could attract teenagers and young adults. This raised concerns among public health authorities that long-term usage of e-cigarettes may ultimately be a gateway to CS (179,180). More recently, US Centers for Disease Control revealed that there are 1080 confirmed deadly lung disease associated with using e-cigarette, or vaping, 18 of whom have died. Most of the patients were adolescents. Majority of patients report a history of using flavours and tetrahydrocannabinol (THC)-containing products (the main psychoactive compound in marijuana) (205).

Diacetyl (alpha-diketone, 2,3-butanedione) is used to give foods a buttery or creamy flavour (176). Diacetyl or acetyl propinyl are often found in sweet flavoured e-liquids (181-183). When inhaled, diacetyl-containing flavours have been shown to cause acute onset bronchiolitis obliterans, a severe and irreversible obstructive lung disease (177). The National Institute for Occupational Safety and Health (NIOSH) performed a series of studies at six microwave popcorn plants where they investigated the relationship between exposure to diacetyl vapour over time and abnormal lung function using spirometry (177,184-187). Workers exposed to higher cumulative diacetyl develop illnesses characterized by having lower level of forced expiratory volume in 1 second (FEV_1) on lung function test (177,184-187). Lung biopsies from some of the affected workers showed inflammation in and scaring of the smallest airways, which can lead to severe and disabling shortness of breath (186). In animal studies, inhaling of buttery flavours containing diacetyl caused severe airway injury (185,188). Rats exposed to diacetyl for six hours by inhalation developed multifocal necrotizing bronchitis, which was most consistently present in the main stem bronchi (185). Subchronic and subacute diacetyl

exposure causes significant epithelial injury, peribronchial lymphocytic inflammation, or fibrohistiocytic lesions in the terminal bronchioles of mice (188).

Structurally very similar to diacetyl, 2,3-pentanedione (alpha-diketone, acetyl propionyl) has received attention as a flavour substitute for diacetyl (189). Published data on the toxicity of 2,3-pentanedione after inhalation showed effects that are similar to diacetyl in laboratory animals (189,190). An inhalation study in rats with 2,3-pentanedione caused proliferation of fibrous connective tissue in the airways walls, projections of fibrous connective tissue, which sometimes extended into the air passageways (189), olfactory neuroepithelial injury and changes in gene expression in the brain (190).

Some e-liquids are flavoured with tobacco extracts (one of the most preferred flavours) and it has been shown these may contain tobacco-specific nitrosamines (TSNAs), nitrates, aldehydes and phenol (191). TSNAs, which are present in the tobacco plant, are probably the most important compounds in tobacco cigarettes that poses negative health effects due to their strong carcinogenicity. Nitrate and aldehydes, which may be both present in the tobacco plant and derived from heating, are compounds with significant toxic and carcinogenic potential (191). In saliva, nitrate is converted to nitrite (192) that can participate in the endogenous production of TSNAs (193). Phenols, which may be derived from heating cured tobacco leaves during flavour extraction, are compounds with significant genotoxic, cardiotoxic and carcinogenic properties (191).

In summary, the complexity of flavouring mixtures, flavouring exposure and the lack of documented health effects for many of the flavouring components makes the study of the health effects of individual flavouring substances challenging. In addition, the long-term effects of the ingredients in relation of e-cigarette is unknown. The current study presents an establishment of representative vaping machine specifically designed for e-cigarettes that enables the control and monitoring of relevant physical parameters during e-cigarette vapour generation. The exposure platform enables the systematic standardization of e-cigarette puffing profiles in a laboratory setting for successive studies.

1.3. Aims of the study

With a worldwide rapidly increasing prevalence of use, e-cigarettes are gaining popularity as a safe alternative to CS. Despite advertised as "safer", very little is actually known about the health risks of e-cigarettes and the long-term effects are still elusive. The hypothesis tested here is if acute and chronic exposure to e-cigarettes can induce alterations in various signalling pathways and may therefore play a role in the impairment of lung and/or pulmonary circulation functions. Against this background, the aims of this study were

1. to investigate the effects of e-cigarette vapour extract (ECVE) on cytotoxicity, cellular functions and gene expression in different primary mouse lung cells such as pulmonary arterial smooth muscle cells (mPASMCs) and alveolar epithelial type II cells (mAECII),
2. to establish an animal model for long-term exposure to e-cigarettes and
3. to investigate the long-term effects of e-cigarettes vapour on lungs and on the pulmonary circulation structure and function of mice.

In order to answer these questions, the following investigations were performed:

- Cytotoxicity, functional and transcriptome analysis following ECVE were investigated in isolated mPASMC and mAECII.
- The long-term effects of e-cigarettes were assessed by exposure of mice to e-cigarette vapour for 6 hours per day, 5 days per week for 8 months.

2. Materials and Methods

2.1.Materials

2.1.1. Reagents

Reagent	Company	Address
4x Laemmli sample buffer	Bio-Rad	Hercules, CA, USA
6X DNA loading buffer	Fermentas	Waltham, MA, USA
Agarose	Sigma-Aldrich	Steinheim, Germany
Antimycin A	Sigma-Aldrich	St. Louis, MO, USA
Bovine serum albumin (BSA)	PAA Laboratories GmbH	Pasching, Germany
Carbonyl cyanide-*4*-(trifluoromethoxy) phenylhydrazone (FCCP)	Sigma-Aldrich	St. Louis, MO, USA
DNA standard (GeneRulerTM 100 bp) ladder	Fermentas	Waltham, MA, USA
DNase/RNase-free water	Gibco	
Ethanol (70%, 95%, 99.6%)	SAV LP GmbH	Flintsbach, Germany
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich	St. Louis, MO, USA
Glyceine	Reidel-deHaen	Hanover, Germany
Hamilton syringes	OROBORS	Innsbruck, Austria
Heparin	Ratiopharm GmbH	Ulm, Germany
Hepes (2-(-4-2-hydroxyethyl)-piperazinyl-1- ethansulfonate)	Sigma-Aldrich	St. Louis, Mo, USA
Hydro chloric acid (HCL)	Sigma-Aldrich	St. Louis, MO, USA
Isoprophanol (99.8%)	Fluka Chemie	Buchs, Switzerland
iTaq SYBR Green supermix with ROX	Bio-Rad	Hercules, CA, USA
Methanol	Fluka Chemie	Buchs, Switzerland
Non-fat milk powder	Carl ROTH	Karlsruhe, Germany
Oligomycin	Sigma-Aldrich	St. Louis, Mo, USA
PBS	Sigma-Aldrich	St. Louis, Mo, USA
PMSF	Sigma-Aldrich	St. Louis, Mo, USA
RIPA Buffer	Sigma-Aldrich	St. Louis, Mo, USA

Sodium chloride (NaCl)	Sigma-Aldrich	St. Louis, Mo, USA
Sodium dodecyl sulphate (SDS)	Sigma-Aldrich	St. Louis, Mo, USA
Sodium hydroxide (NaOH)	Sigma-Aldrich	St. Louis, Mo, USA
SYBR® Safe DNA gel stain	Invitrogen	Carlsbad, CA, USA
TEMED	Sigma-Aldrich	St. Louis, Mo, USA
Tris	Bio-Rad	Hercules, CA, USA
Tween-20	Sigma-Aldrich	St. Louis, Mo, USA
β-Mercaptoethanol	Sigma-Aldrich	St. Louis, Mo, USA

2.1.2. Equipment

Equipment	Company	Address
1.4 Micromanometer Catheter	Miller Instruments	Houston, TX, USA
3RF4 Research Cigarettes	Kentucky Tobacco Research & Development Center	Kentucky, KY, USA
8x60K 60 microarray slides (design ID 028005)	Agilent Technologies	Santa Clara, CA, USA
Cell culture incubator, HeraCell Heraeus	Thermo Fisher Scientific	Waltham, MA, USA
Centrifuge	Rotina Hettich	Tuttlingen, Germany
ChemiDoc.XRS$^+$ (Bio Imaging System)	BioRad	Hercules, CA, USA
FlexiVent	SCIREQ	Montreal, Canada
Hemocytometer	LO–Laboroptik GmbH	BadHomburg, Germany
Homoeothermic plate and control unit	AD Instruments	Spechbach, Germany
IncuCyte™ Live-Cell Imaging System	Essen Bioscience	Ann Arbor, MI, USA
inExpose	SCIREQ	Montreal, Canada
Light microscope	Leica	Wetzlar, Germany
Microplate Reader (Sunrise Remote)	Tecan	Männedorf, Switzerland
Nanodrop (ND-1000)	Kisker-Biotech	Steinfurt, Germany
Oxygraph-2k	OROBOROS	Innsbruck, Austria

Pump (For smoke and vapour extract)	Knf Neuberger	Freiburg, Germany
PVDF membrane	Pall Corporation	Dreieich, Germany
The CFX Connect Real-Time PCR Detection System	BioRad	Hercules, CA, USA
Surgical instruments	Martin Medizintechnik	Tuttlingen, Germany
Thermal Block	VWR	Bruchsal, Germany
Thermal Cycler	Effendorf	Hauppauge, NY, USA
Transblot SD Semi-Dry Transfer Cell	BioRad	Hercules, CA, USA

2.1.3. Ragents and equipment for cell isolation and culture

Reagents and Equipment	Company	Address
15G Cannula	Dispomed Witt	Gelnhausen, Germany
18G Cannula	HMD Healthcare LTD	Horsham, UK
Agarose Type VII (low melting)	Sigma-Aldrich	St. Louis, Mo, USA
Airway Epithelial Cell Growth medium	Promocell	Heidelberg, Germany
CD16/32	BD Pharmingen	Heidelberg, Germany
Cell Strainer (10, 40, 70 µm)	Amersham Hybond^{TM}-N + GE Healthcare	Munich, Germany
Collagenase Type IV, 80U/ml	Sigma-Aldrich	St. Louis, Mo, USA
DNase	Roche	Basel, Switzerland
D-PBS	PAN Biotech	Aidenbach, Germany
Dulbecco's Modified Eagel Media (DMEM)	GIBCO Invitrogen	Karlsruhe, Germany
Dynabeads M-450	GIBCO Invitrogen	Karlsruhe, Germany
Dynal MPC-1 Magnetic Particle Concentrator	Thermo Fisher Scientific	Waltham, MA, USA
Dyspase	GIBCO Invitrogen	Karlsruhe, Germany
Fetal Calf Serum (FCS)	Invitrogen	Carlsbad, CA, USA
Fibronectin	Sigma-Aldrich	Munich, Germany
Iron Particles (Fe_3O_4, <5mU 98%)	Sigma-Aldrich	St. Louis, Mo, USA
Ketamine 10%	Bela-Pharm	Vechta, Germany

L-gluthamate	GIBCO Invitrogen	Karlsruhe, Germany
Medium 199	Invitrogen	Carlsbad, CA, USA
Medium for human SMC (smooth muscle cells 2)	Promocell	Heidelberg, Germany
Nile Red		
Normocine	Invivogene	San Diego, CA, USA
Nylon Mesh	BD Falcon™	Heidelberg, Germany
Penicillin/Streptomycine (100ml 10,000U/ml/10mg/ml)	PAN Biotech	Aidenbach, Germany
RMPI 1640 Medium (Without Phenol Red)	GIBCO Invitrogen	Karlsruhe, Germany
Smooth Muscle Cell Medium Supplement Mix	PromoCell	Heidelberg, Germany
Tripsin-EDTA	PAN Biotech	Aidenbach, Germany
Xylazin 2%	Bayer Vital	Leverkusen, Germany

2.1.4. Assays and kits

Assays and Kits	Company	Address
Abcam's Kinetic Apoptosis kit	Abcam	Cambridge, UK
Bradford Assay	Bio-Rad	Hercules, CA, USA
BrdU (5-Bromo-2´-Deoxyuridine) Cell Proliferation Assay	Roche	Basel, Switzerland
ECL kit	GE Healthcare	Little Chalfont, UK
GSH/GSSG-Glo™ Assay	Promega	Madison, WI, USA
iScript cDNA Synthesis Kit	Bio-Rad	Hercules, CA, USA
MTT Assay	Sigma-Aldrich	St. Louis, Mo, USA
RNeasy Mini kits	Qiagen	Hilden, German
TGX Stain-Free FastCast Acrylamide Kit	Bio-Rad	Hercules, CA, USA

2.1.5. Buffer

Buffer	Substance	Amount
FACS Buffer	PBS (1X)	920 ml
	FCS	5 ml
	Na-Azide	0.05%
	EDTA (1 v/%)	74 ml
Red Blood Cell Lysis Buffer, pH 7.4, (AECII isolation)	Ammonium chloride	155mM
	Potassium bicarbonate	10mM
	Disodium-EDTA*2H_2O	0.1 mM
Running Buffer, 10X	Tris	30.28 g
	Glycine	144 g
	SDS	10 g
	dH_2O	1000 ml
Stripping Buffer	Glycine, 1 M	10 ml
	HCL, 37 %	1 ml
	dH_2O	90 ml
TAE Buffer, 50X	Tris	242 g
	Acetic acid	27 ml
	0.5 M EDTA solution, pH 8.0	100 ml
	dH2O	1000 ml
TBS Buffer, 10X, pH 7.5	Tris	24.23 g
	NaCl	87.66 g
	dH_2O	1000 ml
TBST Buffer	TBS buffer	999 ml
	Tween-20	1 ml
Transfer Buffer	Tris	11.6 g
	Glycine	5.8 g
	SDS (20 %)	3.7 ml
	Methanol	400 ml
	dH_2O	1596.3 ml

2.1.6. Consumables

Consumable	Company	Address
6, 12, 24 and 96-well culture plate	Greiner bio-one	Frickenhausen, Germany
Blot absorbent filter paper	Bio-Rad	Munich, Germany
Falcon tubes 15 and 50 ml	Greiner bio-one	Frickenhausen, Germany
Feather disposal scalpel	Pfmmediacl	Köln, Germany
Medical adhesive bands 3M	Durapore®	St. Paul, MN, USA
Gauze 5 x 4 cm	Purzellin® Lohmann und Rauscher	Rengsdorf, Germany
Gauze balls size 6	Fuhrmann Verrbandstoffe GmbH	Much, Germany
Needle (20G, 11/2",0.9x40mm) and 26G (0.9mm x 25mm)	BD Biosciences	Heidelberg, Germany
Petri dishes	Greiner bio-one	Frickenhausen, Germany
Pipette filter tips, 10, 100 and 1000µl	Eppendorf	Hamburg, Germany
Plastic Syringe (1,3,5, 10ml and 20ml)	Braun	Melsungen, Germany
Serological pipette (5, 10, 25, 50 ml)	Greiner bio-one	Frickenhausen, Germany
Single use syringes Inject Luer®, (1, 2, 5, 10ml)	Braun	Melsungen, Germany
Tissue Culture Dish 60mm and 100mm	Greiner bio-one	Frickenhausen, Germany
Tissue Culture Flask T75	Greiner bio-one	Frickenhausen, Germany

2.1.7. Antibodies

Name of antibody	Species	Company	Address
β-actin	Mouse	Sigma-Aldrich	St. Louis, Mo, USA
IκBα	Rabbit	Cell Signalling Technology	Danvers, MA, USA
LAMP1	Rabbit	Abcam	Cambridge, UK
LAMP2	Mouse	Abcam	Cambridge, UK
NfκB (p65)	Rabbit	Cell Signalling Technology	Danvers, MA, USA
PCNA	Mouse	Cell Signaling Technology	Danvers, MA, USA
p62 and LC3-II (Autophagy Flux detection Kit)	Rabbit	StressMarq Biosciences	Victoria, Canada
Anti-rabbit		Promega	Mannheim, Germany
Anti-mouse		Promega	Mannheim, Germany

2.1.8. Primers for real time PCR

Primer	Species	Orientation	Sequences (5'->3')
5HTT (Serotonin transporter)	Mouse	Forward	AGCGACGTGAAGGAAATGCT
		Reverse	GGAGTTGGGGTGGACTCATC
B2M (Beta2-microglobulin)	Mouse	Forward	AGCCCAAGACCGTCTACTGG
		Reverse	TTCTTTCTGCGTGCATAAATTG
Bcl2 (B cell leukemia/lymphoma 2)	Mouse	Forward	TGGGATGCCTTTGTGGAACT
		Reverse	TTGGCAATTCCTGGTTCGGT
Ccl6 (Chemokine (C-C motif) ligand 6)	Mouse	Forward	GGCCCAAGATCTGGGAACAA
		Reverse	CTCTATTGTGGCAGGGCGAA
Ccna1 (Cyclin A1)	Mouse	Forward	AAGAACCTGAGAAGCAGGGC
		Reverse	CAGGGTCTCTGTGCGAAGTT
Csf2 (Colony Stimulating Factor 2)	Mouse	Forward	ACAACCTGGGGGAAGGCTC
		Reverse	AGTTCCTGGCTCATTACGCA
eNOS (Endothelial nitric oxide synthase)	Mouse	Forward	ACACAAGGCTGGAGGAGCTG
		Reverse	TGGCATCTTCTCCCACACAG
Fgf10 (Fibroblast growth factor 10)	Mouse	Forward	CCGACACCACCAGTTCCTAC
		Reverse	CTTTGACGGCAACAACTCCG
Gucy1a3 (Guanylate cyclase 1, soluble, alpha 3)	Mouse	Forward	AAAGACACCTTTGGCCCGAT
		Reverse	ACAGTCACTTCGGAAGCAGG
Gucy1b3 (Guanylate cyclase 1, soluble, beta 3)	Mouse	Forward	CTCGGATCCACTGTTCCATT
		Reverse	GAACCCAACCGACGTTCTCT
Il1b (Interleukin 1 beta)	Mouse	Forward	TGCCACCTTTTGACAGTGATG
		Reverse	TTCTTGTGACCCTGAGCGAC
iNOS (Inducable nitric oxide synthase	Mouse	Forward	TGATGTGCTGCCTCTGGCT
		Reverse	AATCTCGGTGCCCATGTACC
Mmp9 (Matrix metallopeptidase 9)	Mouse	Forward	CAGCCGACTTTTGTGGTCTTC
		Reverse	GTCGAAATGGGCATCTCCCT
Nrf2 (Nuclear factor, erythroid derived 2, like 2)	Mouse	Forward	TCTCCTAGTTCTCCGCTGCT
		Reverse	GTTTGGGAATGTGGGCAACC
Timp3 (Tissue inhibitor of metalloproteinase 3)	Mouse	Forward	TCCAAACACTACGCCTGCAT
		Reverse	CTGCTTGCTGCCTTTGACTG
Traf1 (TNF receptor-associated factor 1)	Mouse	Forward	GCGCACAGTGTGAGAAGAGA
		Reverse	AGAGAACTCTGGGCTCCGAT

2.2.Methods

2.2.1. Cell culture

Human adenocarcinoma cell line, A549, was obtained from German Collection of Microorganisms and Cell Cultures GmbH (DSMZ, Braunschweig, Germany) cultured in Dulbecco's modified Eagle's media (DMEM) containing 2mM Glutamine, 10% FCS and 50UI/ml penicillin and 50µg/ml streptomycin. Human bronchial epithelial cells (HBEpCs) were obtained from PromoCell (Heidelberg, Germany). HBEpCs were cultured in Airway Epithelial Cell Growth medium.

All cells were cultured in tissue culture flasks T 75 and maintained in a humidified atmosphere condition (5% CO_2, 21% O_2 and 74 % N_2) at 37°C. Upon reaching confluence (80-90 %), cells were routinely subcultured. Cell culture medium was removed from the flasks and cells were carefully rinsed with pre-heated PBS at 37°C. Tripsin-EDTA was added to the cells and the flask was returned to the incubator for 3 to 5 minutes or until all cells detached. After the cells were detached, PBS were added to neutralize the Tripsin-EDTA solution and the cell suspension was centrifuged at 338 x g for 5 minutes. The supernatant was discarded and the pellet was resuspended in appropriate cell culture media. Cells were counted using haemocytometer and a required number of cells were reseed in in the culture flask or for experimental procedures. All culture and subculturing experiments were performed aseptically.

2.2.2. Ethical consideration

All human cells were provided by the UGMLC Giessen Biobank, member of the DZL Platform Biobanking (*https://www.dzl.de/index.php/de/forschung/plattformen/biobank*), approved by Ethik-Kommission des Fachbereichs Medizin der Justus-Liebig-Universität Gießen, AZ 10/06. The animal studies were approval by the university Ethik-Kommission (*https://www.uni-giessen.de/fbz/fb11/dekanat/ethikkommission*) and by the governmental authorities (Regierungspraesidium Giessen, Germany) and were carried out according to the EU Directive 86/609/EEC. The corresponding approved proposal can be found under the reference: (GI 20/10, Nr. 74/2016, GI 20/10, Nr. 115/2014).

2.2.3. Primary cell isolation

2.2.3.1. Isolation of primary human pulmonary arterial smooth muscle cells (hPASMC)

The biomaterials were provided by the UGMLC Giessen Biobank, member of the DZL Platform Biobanking. hPASMC were derived from healthy donor lung tissue. Briefly, the

pieces of pulmonary artery (2-3 cm long and 0.5-1.0 cm diameters) were carefully prepared from the lung tissue. Under stereoscopic guidance, the attached parenchymal tissue was removed, and then the vessels were opened and fixed with needles. The intima was gently scraped off. The cautious incision was made to peel of the medial layer by the forceps. Afterwards, medial layer was cut into the 1 mm pieces and transferred into humidified chamber (37°C, 5% O_2) in a T 75 with 4 ml SMC-Media 2. At 90–95% confluence (about 4 weeks), cells were sub-cultured. The hPASMC were characterized morphologically and immunohistochemically to confirm purity. Cells between passages 3 and 4 were utilized for the experiments.

2.2.3.2. Isolation and culture of primary mouse pulmonary arterial smooth muscle cells (mPASMC)

mPASMC were isolated from male C57BL/6J mice (male, 20-22g body weight, Jackson Laboratory) using a modification of the method of Marshall et al and Waypa et al (194,195). Mice were anesthetized with 0.2 ml Ketamin/Xylazin by intraperitoneal injection. The abdominal cavity was opened, and mice were exsanguinated by severing the inferior vena cava and the left renal artery. After removing the salivary gland and the musculatures around trachea ligature was installed and trachea was cannulated with a 20-gauge luer stub adapter. The ribcage was opened and the thymus was removed carefully. Lung was perfused via pulmonary artery with 3 ml saline. By use of the PA cannula, growth medium 199 (M199, 3 ml) containing penicillin and streptomycin (1%) plus low-melting-point agarose (0.5%) and iron particles (0.5%) was flushed through the pulmonary vasculature. The airways were filled intratracheally with M199 (3 ml) containing low-melting-point agarose (1 %) without iron. The lungs were placed in cold PBS to cause the agarose to gel. The lungs were removed from the animal and placed in 50 ml falcon tube containing around 30 ml ice cold PBS and were incubated for 10 minutes on ice. Lungs were subsequently transferred to a 50 ml falcon tube containing 2 ml PBS and the lobes were dissected free and finely minced using 3 scissors technique. Lung tissue was washed (3 times) with PBS by use of a magnet to retain the iron-containing fragments. The iron-containing tissues were re-suspended in M199 (10 ml) containing collagenase (80 U/ml) and transferred to 100mm petri dish then incubated at 37°C for 1 hour. After incubation the tissue collagenase solution were first drawn through a 15-gauge needle and subsequently through an 18-gauge needle, to homogenize. The lung tissue were then washed 3 times with M199 containing FBS (10%) using magnetic holder. The resulting fragments were re-suspended in smooth muscle cell growth medium containing smooth muscle cell growth

medium supplement mix along with FCS (10%) and Normocin (1%) and subsequently transferred to 60mm cell culture dish. The cell culture dishes were cultured at 37ºC in a humidified atmosphere conditions (5% CO_2, 21% O_2 and 74 % N_2) for 5 to 6 days, during this time the mPASMC were observed to grow around the iron particles. After 5 or 6 days, the iron-containing particles were transferred to a new dish containing fresh media. The adherent mPASMC continued to propagate until the cells were 50% confluent.

2.2.3.3. Isolation and culture of primary murine alveolar epithelial type II cells (mAECII)

mAECII were isolated from male C57BL/6J mice (male, 20-22g body weight, Jackson Laboratory) as described previously (196) with a slight modification. Mice were anesthetized with 0.2 ml Ketamin/Xylazin by intraperitoneal injection. The abdominal cavity was opened, and mice were exsanguinated by severing the inferior vena cava and the left renal artery. After removing the salivary gland and the musculatures around trachea ligature was installed and trachea was cannulated with a 20-gauge luer stub adapter. The ribcage was opened and the thymus was removed carefully. Lung was perfused via pulmonary artery with 3 ml saline. 2 ml Dispase was intratracheally installed followed by low melting agarose (45ºC) then the tracheal ligature was closed immediately. The lungs were removed from the animal and placed in 50 ml falcon tube containing 2 ml Dispase and were incubated for 30 minutes in 37ºC water bath. Lungs were subsequently transferred to a 50 ml falcon tube containing 2 ml DMEM and 0.04 mg/ml DNase with 1 ml FCS. After mixing shortly, the lungs were then transferred to a 100-mm culture dish. The heart, esophagus and the trachea were removed gently and the lung tissue was minced using 2 tweezers. Tissue was transferred back to 50 ml falcon tube containing 2 ml DMEM and 0.04 mg/ml DNase with 1 ml FCS. The cell suspension was filtered through 70 µm, 40 µm and 10 µm nylon mesh cell strainer. Cells were then centrifugation at 200 x g for 10 minutes (4°C). After centrifugation, the supernatant was discarded and the cell palate was suspended with 1 ml DMEM. Crude cell suspensions were purified using negative selection system by placing the suspension on prewashed 100-mm tissue culture plates coated with 15 µl CD16/32 for 24 hours at 4°C. After incubation for 45 minutes in 37ºC incubator, the cell suspension was placed in uncoated 100-mm tissue culture plates and incubated for 45 minutes at 37ºC incubator. Cell suspension was collected in 50 ml falcon tube and centrifuged at 200 x g for 10 minutes (4°C). Afterwards, cell suspension was treated with 1-2 ml Erylase (red blood cell lysing buffer) for 5 minutes, after incubation the reaction was stopped by adding 2-4 ml DMEM. The suspension was then centrifuge at 200 x g for 10 minutes (4°C). mAECII were

resuspended in DMEM medium plus 2% L-glutamate, 1% Pen/Strep and 10% fetal bovine serum and cultured on fibronectin-coated plated.

2.2.4. Cigarette smoke extract (CSE) preparation

Reference research cigarettes 3RF4 were used to create the cigarette CSE. Mainstream smoke was bubbled through culture medium without FCS. One cigarette was used per 10 ml of the medium. The resulting suspension was adjusted to pH 7.4 with concentrated NaOH then filtered through a 0.20-µm pore filter and was used within 30 minutes of preparation. CSE was standardized by measuring the absorbance at weave length 290 nm (0.7±0.05 OD) and was considered as 100% CSE. Control medium was prepared by bubbling air through 10 ml of medium, and the sterile filter through a 0.20-µm pore filter.

2.2.5. Electronic cigarettes vapour extract (ECVE)

ECVE was produced by simulating of use of e-cigarette (Joyetech eGo-C, Riccardo Retail GmbH, Neubrandenburg, Germany). Commercially available e-liquid (VG: PG based) (Riccardo Retail GmbH, Neubrandenburg, Germany) with different nicotine concentrations (0 mg/ml and 18mg/ml) and without any flavouring were used to fill the cartridge. The vapour was bubbled through a cell culture medium by activating a vacuum pump. Puffing condition were following: 15 puffs with duration of 4 seconds were altered with 20 seconds of interval, the resulting suspension was filtered through a 0.20-µm pore filter and was considered as 100%.

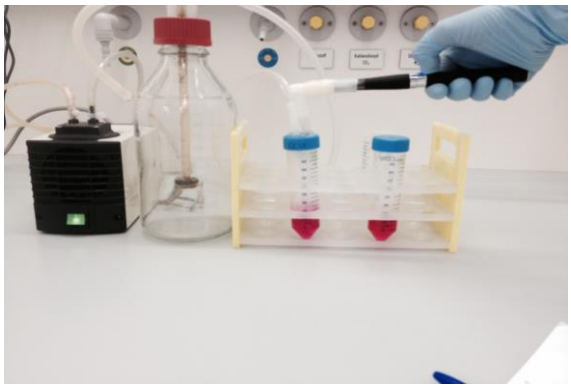


Figure 5. Set up to produce e-cigarette vapour extract

Experimental setup for a realistic simulation of e-cigarette. The suction pump on the left generates an adjustable pressure, which aspirates the vapour and bubbles it into the culture medium in the left falcon tube. The middle gas bottle is for gas cleaning to avoid pump damage.

2.2.6. Cell viability assays

A MTT [4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) Cell viability Assay was used to evaluate the cytotoxicity effects of ECVE and CSE in mPASMC, mAECII, hPASMC HBEpC and A549 cells following the manufacture's protocol. MTT is cleaved by the mitochondrial dehydrogenases of viable cells leading to the formation of insoluble in aqueous solutions purple crystals, representing formazan metabolism. Briefly, after incubation cells with ECVE or CSE for 24 hours, the culture medium was replaced by RMPI 14 medium without phenol red and 10 µl of 5 mg/ml MTT. Afterwards, the cells were further incubated for 4 hours. After incubation, the medium was replaced with 100 µl/well of acid-isopropanol (0.04 N HCL in isopropanol) to dissolve the purple crystals and plate were shake for 30 minutes at room temperature. The absorbance at 570 nm was measured with a microplate reader and absorbance at 690 nm was used as reference. The viability of the treated cells was expressed as a percent of untreated cells.

2.2.7. Proliferation assay

Cell proliferation was assessed using BrdU (5-Bromo-2´-Deoxyuridine) cell proliferation assay according to manufacturer's protocol. The assay is based on immunodetection of BrdU incorporated into replicating DNA in place of thymidine using specific anti-BrdU antibody. Cells cultured in 24-well tissue culture plate were labelled by BrdU for about 24 hours as a result this pyrimidine analog is incorporated in place of thymidine into the newly synthesized DNA of cell cycle. After removing the labelling medium, the cells are fixed, and the DNA is denatured (the denaturing of DNA is necessary to improve the accessibility of the incorporated BrdU later to the detection anti-body) in one step by adding FixDenat (500 µl/well in 24-well plate) and incubated for 30 minutes at room temperature. During this incubation time anti-BrdU-POD antibody solution was prepared by diluting anti-BrdU-POD with antibody diluting solution (1:100). Then FixDenat was removed and the anti-BrdU-POD antibody (250 µl/well) was added and incubated for 90 minutes at room temperature. The antibody will bind to the BrdU incorporated into the newly synthesized cellular DNA. The plate was 3 times with 1X washing solution and subsequently substrate (500 µl/well) was added to develop colour. The reaction product is quantified by measuring every 5 minutes the absorbance at 370 nm with a microplate reader and background subtraction was adjusted with absorbance readings at 492 nm. For all experiments the following negative controls were used: only culture medium without cells, only anti-BrdU-POD antibody solution without cells, cells plus anti-BrdU-POD antibody solution plus substrate (without BrdU labelling solution), cells plus substrate (without

BrdU labelling solution and anti-BrdU-POD antibody solution) and cells plus BrdU labelling solution plus substrate (without anti-BrdU-POD antibody solution). The average value of the negative controls was subtracted from all values. The absorbance values were normalized by setting the untreated cells in each row to 100%. Subsequently, the viability of the treated cells was expressed as a percent of untreated cells.

2.2.8. Migration assay

The culture-insert (Ibidi culture-inserts 2 well (Ibidi, Munich, Germany)) was inserted to the bottom of 24 well plate, which provided two cell culture reservoirs. 2500 hPSAMC and 5000 mPASMC cells were seeded per each reservoir. The wound healing assay was initiated by removing the culture-insert 16 hours after seeding. The wound closure (cell migration) was recorded using the IncuCyte ZOOM™ live cell imaging system and pictures were acquired every 1 hour for 24 hours. This system measured scratch closure in real time and automatically calculated the relative wound density within the initially vacant area at each time point.

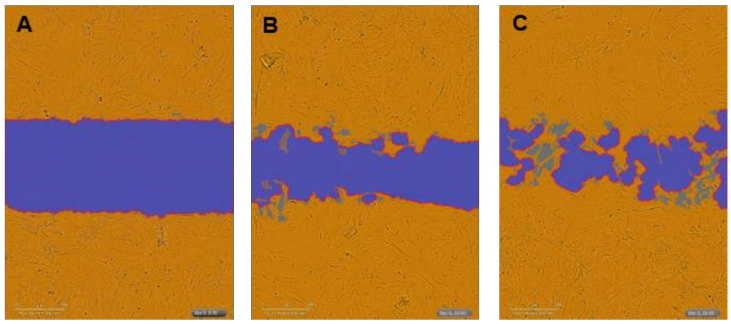


Figure 6. Representative pictures from IncuCyte ZOOM™ live cell imaging system

Red colour indicates the position of detected edge, purple colour indicates the area of the scratch and the yellow colour indicates the position of cells. The three pictures (A, B and C) indicate the area of remaining vacant space at 0, 10 and 16 hours for the control assay, respectively.

2.2.9. Cellular respiration

Cellular respiration was performed at 37°C using Oxygraph-2k (O2k). Cells (300,000 cells/dish) were seeded in 100 mm culture dish at passage 2. After exposed cells to ECVE with or without nicotine for 24 hours, they were trypcinised. After centrifugation, the supernatant was discarded and the cell pellet was resuspended with 2.5 ml SMC growth medium containing 10% 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES). 2.2 ml cell suspension (0.6-0.8 million cells/ml) was added to the chamber of O2K. After the equilibration to room air for 10 minutes, the chamber was closed. A typical recording is illustrated in Figure 7. A standard

protocol using oligomycin (2.0 µg/ml), FCCP (0.45 µM), and antimycin A (2.5 µM) was used for measurement of cellular respiration.

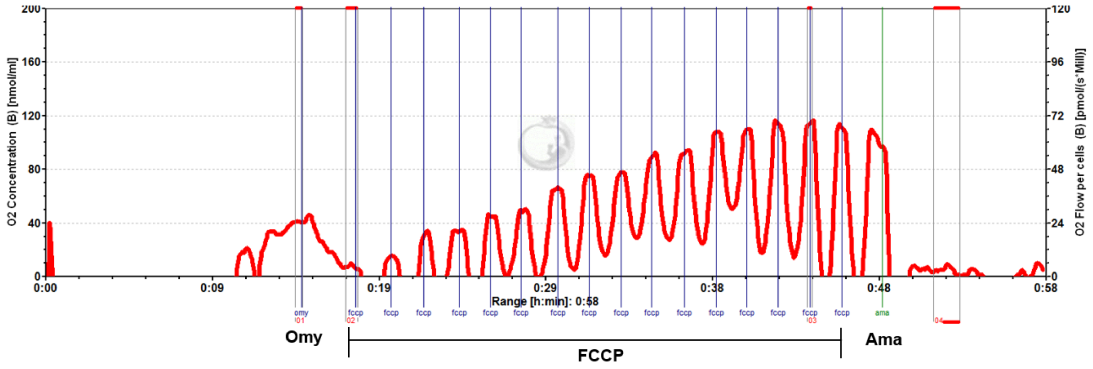


Figure 7. Representative respiratory traces of mPASMC from DataLab software

The red curve shows the oxygen consumption of the cells. Titration protocol: addition of ~2 million cells in their conditioned medium (Routine respiration), 2µg/ml oligomycin (Leak respiration), titration of FCCP to a final concentration of ~5µM (mitochondrial electron transfer system capacity), and 2.5µM antimycin A (residual oxygen consumption). Omy: oligomycin; FCCP: Carbonyl cyanide-*4*-(trifluoromethoxy) phenylhydrazone; Ama: antimycin A.

2.2.10. RNA isolation, cDNA synthesis and quantitative RT-PCR

2.2.10.1. RNA isolation

RNA (ribonucleic acid) was isolated by using RNeasy Mini kits (Qiagen, Hilden, Germany) according to manufacturer's instructions. Briefly, after aspirating culture medium from wells containing cells, cell culture plate was washed twice with ice cold PBS then 350µl RLT buffer was added to each well and incubated for 5 minutes on ice. The cells were scraped using RNase and DNase free 100 µl pipet tips, and then transferred to 1.5 µl RNase and DNase free tubes. Add 350 µl ethanol (70%) to the diluted RNA and mix well. Then transfer the sample to RNeasy mini spin column placed in 2 ml collection tube and centrifuge for 15 seconds at 10000 x g. Discard the flow through and add 700 µl wash buffer 1 on the column to wash the membrane then centrifuge for 15 seconds at 10,000 x g. Subsequently discard the flow through and add 500 µl RPE buffer on the column centrifuge for 1 minute at 10,000 x g to wash the membrane. Discard the flow through. Place the RNeasy mini spin column in a new 2 ml collecting tube and centrifuge at 14,000 x g for 1 minute to dry the membrane. Place the RNeasy mini spin column in 1.5 ml collection tube (RNase and DNase free) and add directly to the spin column membrane 30-70 µl DNase/RNase-free water, and centrifuge for 1 minute at 10,000 x g to elute the RNA.

2.2.10.2. RNA Quantification

Quantification of RNA was performed at 260 nm using a Nanodrop (ND-1000), and then stored at -80ºC for further use. To estimate RNA purity, the ratio of the absorbance contributed by the nucleic acid to the absorbance of the contaminants is calculated. For RNA a ratio of A_{260}/A_{280} 1.8 to 2.2 are acceptable, while requirements for A_{260}/A_{230} ratios are generally >1.7. In either case, a substantially lower ratio may indicate the presence of protein, phenol or other contaminants.

2.2.10.3. cDNA synthesis

cDNA (complementary deoxyribonucleic acid) was synthesis by using iScript cDNA Synthesis Kit (BIO-RAD, Munich, Germany). The cDNA synthesis reaction consisted of 1µg of RNA, 4 µl of 5X iScript reaction mix, and 1 µl of iScript Reverse Transcriptase. The samples were bought up to a final volume of 10 µl with the addition of DNase/RNase-free water. cDNA synthesis was carried out in a thermal cycler. The cycle consisted of 25º C for 5 minutes, 42ºC for 30 minutes, and 85ºC for 5 minutes, and 4ºC hold. After cDNA synthesis, each sample of the cDNA was diluted (1:5) with DNase/RNase-free water and stored at -20ºC for further experiments.

2.2.10.4. Primer Design

The sequences of the genes were attained from the NCBI *Entrez Nucleotide* database (*https://www.ncbi.nlm.nih.gov/nucleotide/*). Then the primers were designed using primer blast tool from NCBI (*https://www.ncbi.nlm.nih.gov/tools/primer-blast/*).

2.2.10.5. Real time PCR

All primers used are listed in section 2.1.8. Expression levels of target genes were normalized by concurrent measurement of *B2M* mRNA levels. Quantitative real-time polymerase chain reaction (RT-PCR) was performed with the iQ SYBR Green Supermix according to the manufacturer's instructions. In brief, a 10 µl mixture was used containing 5 µl iQ SYBR Green Supermix, 0.5 µl forward and reverse primer (10 mMol), 3.5 µl sterile RNase free water and 1 µl complementary DNA template. A negative control (non-template control) was performed in each run. Each sample was analyzed in duplicate with a Biorad (BIO-RAD, Munich, Germany) under the following conditions; initial denaturation, 1X, 95ºC for 3 minutes, followed by 40 cycles of denaturation for 1 second, 95ºC, annealing for 1 second, 59ºC, elongation for 5 seconds, 72ºC, followed by 10 seconds 95ºC, 10 seconds final extension at 72ºC, and a melt curve of 0.5ºC increments starting at 60ºC to 95ºC.

2.2.10.6. Agarose gel electrophoresis

Purity and specificity of PCR products was evaluated by electrophoresis on a 1.2% agarose gel *(Sigma-Aldrich, Steinheim)* that was prepared in 1x Tris-acetate-EDTA buffer. The agarose was boiled in a microwave for about 1 min and waited until it comes to a temperature about 50-60 °C. The gel was then stained with 2 µl *SYBR safe DNAgel stain* to visualize the DNA under ultraviolet (UV) light. A DNA standard (GeneRulerTM 100 bp Ladder, 3 µl) was applied. 10 µl of the sample and 2 µl *6X DNA loading buffer* were mixed and loaded on to the gel. The DNA loading buffer serves two purposes: first, it provides a visible dye that helps with gel loading and will allow gauging how far the gel has run. Second, as it contains high percent of glycerol, it makes the sample heavier than water and will settle to the bottom of the gel well, instead of diffusing the buffer. After separation of the products at 120 volts, 120 mA and 100W for 45 minutes the band pattern were visualized under UV light at 320 nm with Bio Imaging System (ChemiDoc.XRS$^+$ BioRad).

2.2.11. Microarray experiments

Purified total RNA was amplified and Cy3-labeled using the LIRAK kit following the kit instructions. 200ng of total RNA was used per reaction. The Cy-labeled aRNA was hybridized overnight to 8x60K 60mer oligonucleotide spotted microarray slides (design ID 028005). Hybridization and subsequent washing and drying of the slides were performed following the Agilent hybridization protocol.

The dried slides were scanned at 2 µm/pixel resolution using the InnoScan 900 (Innopsys, Carbonne, France). Image analysis was performed with Mapix 6.5.0 software, and calculated values for all spots were saved as GenePix results files. Stored data were evaluated using the R software (197) and the limma package (198) from BioConductor (199). Log mean spot signals were taken for further analysis. Data was background corrected using the NormExp procedure on the negative control spots and quantile-normalized (198,200) before averaging. Genes were ranked for differential expression using a moderated t-statistic (189). Pathway analyses were done using gene set tests on the ranks of the t-values (198). The hybridization of the samples and subsequent biostatistical analysis was performed by Dr. Jochen Wilhelm.

2.2.12. SDS-PAGE gel electrophoresis and western blotting

2.2.12.1. Protein isolation

After removing the culture medium, the wells were washed twice with ice cold PBS, then lysis buffer (RIPA buffer containing 1 mM sodium vanadate, 0.1 mM phenylmethylsulphonyl

fluoride (PMSF), 40 µl/ml protease-inhibitor mix complete (Roche, Mannheim, Germany) RIPA buffer plus protease inhibitor complex and Phosphatase inhibitor) was added (100-150µl/well) and kept on ice for 10 minutes. Then cells were scraped using scraper or 100µl pipet tips, all procedures were done on ice. The cell suspension was centrifuge at 18,620 x g at 4°C for 15 minutes, finally, the supernatant was carefully transferred (not to transfer the cell pellet) to a new eppendorf tubes and were stored at -80°C.

2.2.12.2. Protein quantification

Protein was quantified using Bradford Assay. For Bradford Assay, we used Bio-Rad dye reagent. A standard curve with known amounts of bovine serum albumin (BSA) was generated in each assay. The linear range of the assay for BSA is 0.128-2.056 µg/µl (w/v). Standards and samples were prepared at the same time. 5 µl of both standards and sample, 25µl substanceA and 200µl SubstanceB were carefully added to 96 well plate. Before measuring the absorbance, the plates were incubated at room temperature for about 5 minutes. Absorbance was measured in a plate reader at 630nm and the concentration of protein was calculated by fitting the samples to the standard curve.

2.2.12.3. SDS-PAGE

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the Bio-Rad's TGX Stain-Free FastCast Acrylamide Kit instructions.

2.2.12.4. Western blot

After quantifying the samples, they were mixed with loading buffer containing β-Mercaptoethanol (30 µl/ml 4x Laemmli sample buffer) and heated at 95°C for 5 minutes in thermal cycler block. After heating the samples were cooled on ice then centrifuged at 140000 x g at 4°C for 30 seconds. Equal amount of protein from each samples and molecular weight protein marker-Precision Plus Protein Standards (3µl) were loaded per gel. The gel was then electrophoresed in 1X running buffer at 120V, 400mAmp and 150W for 90-120 minutes. After the gel had finished separating the protein according to molecular weight, proteins were subsequently transferred to a polyvinylidene fluoride (PVDF) membrane by the semi dry-blotting method as follows. The gel was removed from the gel rig apparatus and transferred a small container containing transfer buffer to equilibrate. The PVDF was activated in methanol for 1 second then equilibrated in transfer buffer. Whatmann filter paper were cut and equilibrated as well in transfer buffer. After equilibration step, the gel was sandwiched: three Whatmann filter paper, PVDF membrane, gel, three Whatmann filter paper. The proteins were

then transferred using the Transblot SD Semi-Dry Transfer Cell by electrophoresis at 100V, 115mA, 150W for 75 minutes on to PVDF membrane. The membranes were then incubated with blocking buffer (6% (w/v) non-fat dry milk powder with TBST) for 1 hour at room temperature. Incubation with diluted primary antibodies were performed at 4°C overnight. After washing the blots 3 times for 10 minutes with TBST, appropriate secondary antibodies were applied for 1 hour at room temperature. The blots were then washed 5 times with TBST and visualization was carried out using the enhanced chemiluminescence kit (ECL). Image of the membrane was taken using ChemiDoc.XRS^{+} BioRad. Densitometry was performed using Image Lab 4.1. Briefly, the image was taken and opened in operation window. The bands present in the image were centred and the number of lanes present was entered manually. Bands were selected by selecting the tool "Bands" then "Detect Bands", after adjusting the band width to avoid overlapping, analyse table was provided on screen and a detailed report (.pdf format) of each band was obtained by selecting the "Report" button. Finally, the membranes were washed with washing buffer for 5 min and incubated for 1hr in stripping buffer to re-blot proteins of the loading control.

2.2.13. Glutathione assay

The total glutathione (reduced form [GSH] + oxidized form [GSSG]) and GSH/GSSG ratio were measured using GSH/GSSG-Glo™ Assay following manufacturer's instructions. In detail, cells were seeded in 96-Well clear bottom white Polystyrene Microplates. After the cells attached, cells were exposed to ECVE or CSE. The cells were divided in to two sets: one set of cells for the measurements of total glutathione and the other set for oxidized glutathione (GSSG) measurement. After 24 hours exposure, the medium with or without vehicle was removed and discarded then the cells were washed once with warm PBS. Prepare glutathione standards, 0-16 µM (16 µM, 8 µM, 4 µM, 2 µM, 1 µM, 0.5 µM, 0.25 µM, 0 µM) by diluting the 5mM glutathione in water and 5 µl of the standards were added to the plate. Prepare total glutathione lysis reagent and oxidized glutathione lysis reagent then add 50µl per well total glutathione lysis reagent to the total glutathione measurement set and to the wells containing glutathione standards and oxidized glutathione lysis reagent to wells GSSG measurement set. The plate was incubated on a plate shaker for 5 minutes at room temperature. Then 50µl of Luciferin Generation Reagent was added to all wells. Plate was shook briefly and incubated for 30 minutes at room temperature. After adding 100µl Luciferin Detecting Reagent to all wells and incubating for 15 minutes, luminescence was measured. All samples and standards were performed in triplicate. The standards and all reagents were prepared fresh. The value of blank

was subtracted from all values. To calculate the GSH/GSSG ratio we need the values of both total glutathione and GSSG measurements. The following formula was used:

$$\text{Ratio GSH/GSSG control} = \frac{\mu\text{M total glutathione control} - (\mu\text{M GSSG control} \times 2)}{\mu\text{M GSSG control}}$$

$$\text{Ratio GSH/GSSG treated} = \frac{\mu\text{M total glutathione treated} - (\mu\text{M GSSG treated} \times 2)}{\mu\text{M GSSG treated}}$$

2.2.14. Determination of aerosol particle size by laser diffraction

The mass median aerodynamic diameters (MMAD) and fine particle fraction (FPF) of the generated aerosols/smoke were determined by laser diffraction (HELOS, Sympatec, Clausthal-Zellerfeld, Germany). Simultaneous measurement of the particle size of e-cigarette aerosol, generated directly from e-cigarette with a 2 L/minute sampling flow rate and particle size of smoke from tobacco, were collected into syringe. The aerosol/smoke was then passed directly through the laser beam. The cloud was measured under low angles in forward direction following the premise of Fraunhofer scattered light of absorbing particles. Particle size measurements were conducted in a laboratory with a controlled temperature range of 21–22 °C and relative humidity of 55%–60%. The particle size measurements were performed in collaboration with Alexandra Dalla-Bona (Dr. Gessler team).

2.3. Animal experiments

2.3.1. C57BL/6J Mice

Adult male (6-8 weeks old) C57BL/6J mice were obtained from Charles River Laboratories (Sulzfeld, Germany). Animals were housed in the animal house of the department of Internal Medicine Justus Liebig University Giessen under controlled conditions (20-23ºC and 40-70% humidity) with a 12 hours light/dark cycle and food (Altromin®Standard Diet Food) and water supply ad libitum. Mice were randomly allocated to the exposure groups and were kept in polycarbonate type II long cages with litter, nesting material and red shelters.

2.3.2. Establishment of mouse e-cigarette vapour exposure system

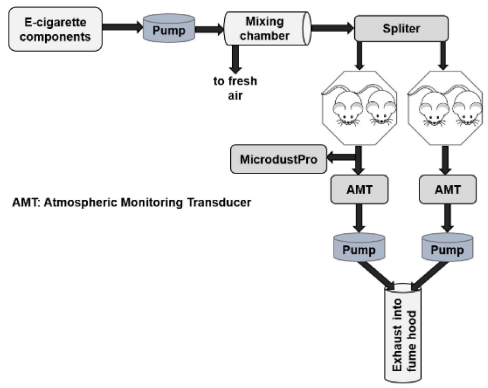
Joyetech eVic-VTC Mini e-cigarette integrated with custom-made automated inExpose inhalation exposure system (SCIREQ Scientific Respiratory Equipment Inc, Montréal, PQ, Canada) was used to generate e-cigarette vapour. The exposure devices allowed for precise control over the e-cigarette puffing parameters such as, puff duration, volume and frequency as to simulate the actual human e-cigarette use. The e-cigarette aerosol exposure system automatically activates and puffs the e-cigarette. The overall e-cigarette vapour generation system is presented in Figure 8. The e-cigarette was mounted on a power supply and was connected to enlarged and refillable reservoir for prolonged vapour generation.

A cylindrical shaped chamber (mixing chamber) was used to mix the e-cigarette aerosol to fresh air before delivered to the whole-body chamber where the mice were. The mixing chamber had three tube connectors, which were connected to the pump, to the whole-body chamber and one was open to the atmospheric air. The airflow was adjustable to achieve the desired dilution ratio in the mixing chamber. In addition, the whole-body chamber were featured with atmospheric monitoring transducer for simultaneous measurement of temperature, humidity and gases (O_2 and CO_2). The system is also integrated with the MicroDust Pro (UT-CEL712, CASELLA Measurements, Bedford, UK), which is a real-time dust monitoring instrument ideally suited for the measurement of particulate concentration such as from dust, smoke, and aerosols. To make accurate and repeatable measurements, Microdust Pro uses a proven forward light scattering principle. This can allow a quasi-quantitatively measurement of the vapour delivered to the mice.

Before each experiment, the whole system was tested for air leakages and rotameter was used to monitor the pressure changes. The inExpose system is fully managed using a software,

flexiWare 6.1, which permits real-time monitoring, visualization and recording of data as well as the exposure time and duration can be modified practically by the software. All data is recorded with a time stamped log, and can be exported to a variety of formats including Microsoft Excel.

A



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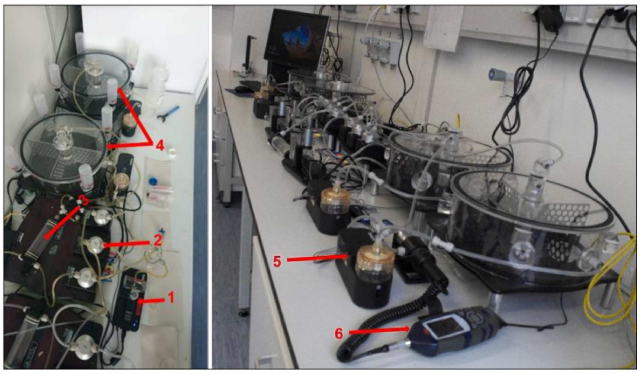
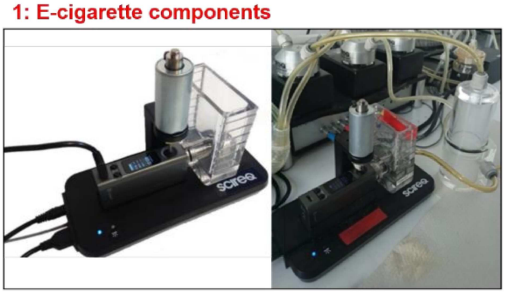
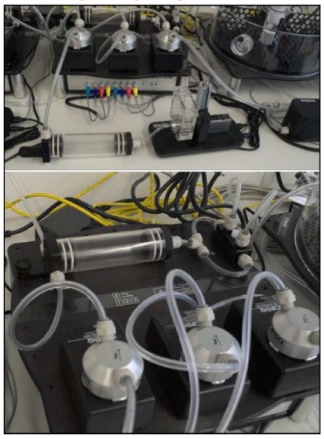


1: E-cigarette components



2: inExpose pump module



3: Mixing chamber

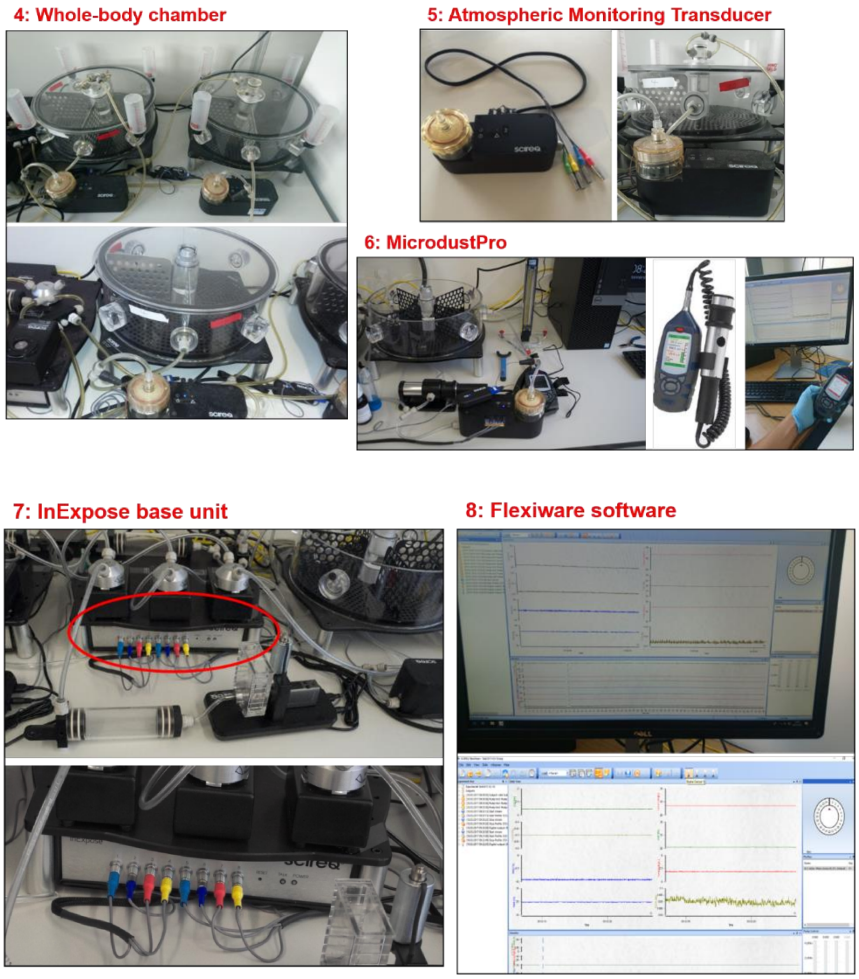


Figure 8. Electronic cigarette (E-cigarette) vapour generation and inhalation exposure system

(A) Schematic overview of the whole system. (B) Components of the e-cigarette inhalation exposure system. (1) E-cigarette aerosol generator. (2) InExpose pumps. (3) Mixing chamber. (4) Whole body chamber: the animals can be placed in chambers to expose the whole body to e-cigarette aerosol. (5) Atmospheric monitoring transducer: for simultaneous measurement of temperature, humidity, O_2 and CO_2. (6) MicroDust Pro: real time measurement of particulate concentration. (7) InExpose base unit: the base unit is connected to the software to control the exposure parameters. (8) Flexiware software: temperature, humidity O_2 and CO_2 as well as the exposure time and duration can be seen on screen.

2.3.3. E-cigarette vapour exposure

To generate e-cigarette vapour, inExpose inhalation exposure system were used. This automated system was set to take one 60 ml puff/minute, 2 second puff duration, mix the puff with room air with a flow of 3L/min, and then transfer it into the whole-body exposure chamber. Mice were exposed for 6 hours/day, 5 days/week for up to 8 months. Commercially available e-liquids (55% propylene glycol (PG), 35% glycerol (VG) and 10% water) (Avoria GmbH, Nuremberg, Germany) with (18mg/ml) or without (0mg/ml) nicotine and without flavouring

were used. Mice were randomly allocated to e-cigarette with nicotine exposed, e-cigarette without nicotine exposed and unexposed control groups. Age matched control groups were kept under similar conditions as e-cigarette exposed mice.

2.3.4. Anaesthesia

Prior to in vivo measurements, animals were anesthetized with a mixture of 3-5% v/v isoflurane (Forene® Abbott, Wiesbach, Germany) in O_2 in an anaesthetic chamber. The onset of anaesthesia was controlled by using the loss of the pedal reflex as an index.

2.3.5. Echocardiography

Anaesthesia was maintained on isoflurane (1.0 to 1.5%). Body temperature was monitored using a rectal thermometer (Indus Instruments). Mice were laid in a supine position on a heating platform with all legs taped to electrocardiogram electrodes for heart rate monitoring. Body temperature was monitored using a rectal thermometer (Indus Instruments, Houston, TX). To enhance the ultrasound quality, the chest area of each mouse was shaved. A prewarmed ultrasound gel was applied over the chest wall to provide a coupling medium for the transducer. Transthoracic echocardiography was performed with VEVO770 high resolution imaging system equipped by 30 MHz transducer (VisualSonics, Toronto) to evaluate right ventricular wall thickness (RVWT), right ventricular internal diameter (RVID), tricuspid annular plane systolic excursion (TAPSE), cardiac output (CO), cardiac index (CI), ejection fraction (EF) and ratio of pulmonary artery acceleration time (PAT) to pulmonary artery ejection time (PET) as described before (201). The echocardiograph was performed by Dr. med. vet. Simone Kraut.

2.3.6. Lung function measurements

Lung function experiments were performed on the homeothermic plate (AD Instruments, Spechbach, Germany) at 37°C. The body temperature of mice was controlled throughout the whole experiments by the rectal probe connected to the control unit (AD Instruments, Spechbach, Germany). Anaesthesia was maintained by continuous inhalation of 1.5% isoflurane mixed with O_2 (Air Liquid, Siegen, Germany). The animals were intubated to the FlexiVent (SCIREQ Inc., Canada) system via tracheostomy. The ventilation of mice was performed with tidal volume of 10 ml/kg, 150 breaths/min and a positive end-expiratory pressure was 2 cm H_2O. The pulmonary resistance and dynamic compliance were evaluated using a ‘snapshot perturbation’ manoeuvre. Pressure-volume (PV) loops were generated to measure the static compliance. Flexiware software (SCIREQ Inc., Canada) calculates and displays parameters associated with the perturbation.

2.3.7. Haemodynamic measurements

2.3.7.1. Right heart catheterization

1.4F micronanometer catheter (Miller Instruments, Houston, USA) was placed in 1ml syringe filled with air bubble free physiological saline approximately for 30 minutes. The right external jugular vein was carefully exposed using two curved forceps under it, and carefully pulling (removing) the connective tissue surrounding the jugular vein. Two surgical sutures were laid beneath the jugular vein. A knot was made to the upper suture and jugular vein was ligated and stretched by attaching the thread to the left of the homeothermic plate and a simple loose know was made in the lower suture. A small incision was made on the vein and the tip of the catheter was inserted and then the suture was tightened with enough tension to stop the bleeding but still permit the catheter advance in the caudal direction into the vein, and then the catheter was steadily moved into the right atrium and right ventricle. The pressure was recorded after stabilizing the signal. All data were collected and analyzed using PowerLab data acquisition systems and LabChart 7 for Windows software (MPVS-Ultra Single Segment Foundation System, AD Instruments, Spechbach, Germany). The parameters measured were heart rate and right ventricular systolic pressure (RVSP).

2.3.7.2. Left heart catheterization

The right carotid artery was carefully exposed by moving the connective tissue and vagus nerve aside. Similar to the jugular vein, two surgical suture were laid beneath the carotid artery. A knot was made to the upper suture and the artery was ligated and stretched by attaching the thread to the left of the homoeothermic plate and a simple loose know was made in the lower suture. The carotid artery was occulated with a Biemer microvessel clip (Aesculap, Tuttlingen, Germany) at the proximal part of the artery. A small incision was made and the tip of the 1.4F micronanometer catheter was introduced into the vessel and fixed with the suture. Simultaneously, the clip was removed and the catheter was moved into the left ventricle until the typical pressure signal was displayed on the monitor. The pressure was recorded after stabilizing the signal.

2.3.8. Blood oxygen saturation, haematocrit and plasma collection

After hemodynamic measurements, heparin was administered intraperitoneally (1000 U/kg) and blood (1 - 1.5 ml) was drawn from right ventricle. The blood was transferred to an Eppendorf tube and blood oxygen saturation was measured by blood gas analyser 348 (Siemens Healthcare Diagnostics GmbH, Eschborn, Germany). A small drop of blood was collected with

a capillary for haematocrit measurement. For haematocrit, the capillary was sealed a haematocrit sealing kit and centrifuged at 13,709 x g for 5 minutes at room temperature using haematocrit 210 (Hettich centrifuges, Tuttlingen, Germany). The value of haematocrit was read from the haematocrit scale of the centrifuge lid. The blood in the Eppendorf tube was centrifuge at 4ºC, 3024 x g for 5 min using Hettich Mikro 200R (Hettich centrifuges, Tuttlingen, Germany). The supernatant plasma was transferred into cryotubes and snap froze before storing in -80ºC for further experiment.

2.3.9. Plasma nicotine and cotinine measurement

Plasma nicotine and cotinine concentration were measure from mice exposed for 6 hours to e-cigarette with (18mg/mL) or without (0mg/mL) nicotine or room air controls. Blood was drawn from right ventricle and centrifuged at 4ºC, 3024 x g for 5 min using Hettich Mikro 200R (Hettich centrifuges, Tuttlingen, Germany). The supernatant plasma was transferred into cryotubes and was immediately snap froze before storing in -80oC. The samples were send on dry ice to ABS Laboratories (ABS Laboratories Ltd, BioPark, Welwyn Garden City, Hertfordshire, AL7 3AX, U.K.) for analysis.

2.3.10. Bronchoalveolar lavage (BAL)

After the in vivo hemodynamic and respiratory function assessments, mice were sacrificed by bleeding. The lungs were lavage three times with 600 µl of saline via a 20 G peripheral venous catheter (Braunüle) without stylet inserted into the trachea. The three fractions were pooled and centrifuged at 121 x g for 5 minutes at 4ºC. The cell pellet was resuspended in Fluorescence-activated cell sorting (FACS) buffer and fixed with ice cooled 4% paraformaldehyde for 20 minutes at room temperature. The total cell number in BAL fluid (BALF) was counted using neubauer chamber (haemocytometer).

2.3.11. Removal and fixation of the lungs

After the BAL collection, the thorax and the abdomen were disinfected with Braunoderm® and the skin was removed by cutting laterally along the lower costal arch. With a pair of fine tweezers the sternum was exposed and the diaphragm was cut with a spring scissors. The rib cage was then open and was fixed laterally with a curved 24 G cannula. A surgical thread was passed beneath the pulmonary artery and the aorta and a loose knot was made. Subsequently, a small incision was made in the right ventricle, through with rinsing cannula was inserted into the pulmonary artery then the cannula was fixed by tightening the knot. Another incision was made in the left ventricle for drainage. The lung was flushed free from blood with an isotonic

saline solution at a pressure of 22 cm H_2O. Afterwards, a 20 G peripheral venous catheter (Braunüle) without stylet was introduce into the trachea. The right lung was ligated with a suture and separated from the left lung and were immediately place in cryotubes and frozen in liquid nitrogen and then stored at -80°C until further molecular biology studies and/or for ROS measurement. The left lung lobe, depending on the purpose of the use, was handled as follows: For laser microdissection, then molecular biology studies, approximately 1 mL of Tissue Tek® (Sakura Finetek, Staufen, Germany) was installed to the lung via trachea and directly frozen in liquid nitrogen. For alveolar and vascular morphometry, the left lung was perfused through the pulmonary artery and trachea with 3.5 % formaldehyde (Otto Fischar GmbH&Co, Germany) with a constant pressure of 22 cm H_2O and 12 cm H_2O, respectively for 15-20 minutes. After removing the cannula from the pulmonary artery and the catheter from trachea were removed and the lungs were carefully removed from the animal. The lungs were afterwards, placed in in embedding cassettes and incubated overnight with 3.5 % formaldehyde at room temperature. The next day, the lungs were transferred into 0.1 M phosphate buffered saline (PBS) and incubated at 4°C. On the third day, the PBS was changed to 50% ethanol and on the fourth day in 70% ethanol. Thereafter, the lungs underwent a routine overnight dehydration program in a closed vacuum tissue infiltration machine (Leica TP1050). The lungs were embedded in paraffin using a paraffin pouring station and they were cooled down on a cooling plate. Finally, the lungs were stored at 4°C until further histological studies.

2.3.12. Assessment of right heart hypertrophy

The right heart hypertrophy was estimated by measurement of the weight ratio of RV to LV plus septum (LV+S). The data was normalized to tibia length.

2.3.13. Alveoli count via design-based stereology

Lungs were flushed blood free via the pulmonary artery and fixed by 3.5% formaldehyde infusion via both a) intratracheal (with pressure column at 15 cm H_2O) and b) intra pulmonary artery infusion (with pressure column at 20 cm H_2O) for 20 minutes. Lung volume of the left lobe was determined by water-displacement method. Consequently, the left lobe was cut into 3 mm proportionate parallel slices, dehydrated and the slices were paraffin embedded next to each other. Sections (3 mm) were stained with resorcin/fuchsin and nuclear fast red (Weigert's elastin staining). The number of alveoli was counted using a light microscope (Leica, Wetzlar, Germany) equipped with newCast software for stereology (Visiopharm, Aarhus, Denmark). The physical dissector method was used for counting of alveoli, for more detail refer (202).

Respective counts were related to the left-lung volume. The staining of the lungs and counting of the alveoli was carried out with the support of technical assistant Miriam Wessendorf.

2.3.14. Immunohistochemistry: Muscularization

The paraffin embedded lungs were cut into 3µm sections and double staining with antibodies to smooth muscle α-actin (α-SMA) and von Willebrand Factor (vWF) to assess the degree of muscularization of small peripheral pulmonary vessels using a light microscope (Leica, Wetzlar, Germany) equipped with QwinV3 software. The degree of muscularization was determined on small (20 to 70 µm, outer diameter), average (70 to 150 µm, outer diameter) and large (>150 µm, outer diameter) vessels. The respected vessels were distinguished as follows: non-mascularized (<5% muscularization), partial muscularized (5 to 70% muscularization) and fully muscularized (>70% muscularization).

2.3.15. Flow cytometry

Staining procedures, data acquisition and analysis were performed with an LSRII flow cytometer using DIVA software (BD Biosciences) as previously described (203,204). Cells were stained with antibodies to detect CD45 (clone 30-F11; BioLegend, Fell, Germany), Gr-1 (clone RB6-8C5; BioLegend), CD11c (clone N418; BioLegend), CD11b (clone M1/70; BioLegend), Ly6G (clone 1A8, BioLegend) and SiglecF (clone E50/2440; BD Pharmingen, San José, CA, USA) in the dark for 15 minutes at 4°C and then washed with FACS buffer prior to assessment. Corresponding isotype antibodies were used as negative controls. The flow cytometry analysis was performed in collaboration with Balachandar Selvakumar, PhD (Prof. Herold group).

2.4.Statistical analysis

All data are presented as mean ± standard error of the mean (SEM). Comparison of multiple groups was performed by analysis of variance (ANOVA). When comparing the variance in the group means within a sample while considering only one independent factor, a one-way ANOVA was used. When all samples were compared to each other, the Tukey post-hoc test was used and when all samples were compared to a reference (control), the Dunnett's post-hoc test was used. Two-way ANOVA with Dunnett's post-test was used when analysing the effect of two factors on a dependent variable. It was assumed that the data were relatively normally distributed and a parametric test was performed. *P* value below 0.05 was considered as statistical significant for all analysis. All statistical analysis were carried out using GraphPad Prism® software.

3. Results

3.1. Cell viability from exposure to ECVE and CSE

The effect of different concentrations of ECVE or CSE on the cell viability was evaluated in A549, hPASMC, mPASMC, mAECII and HBEpC cells (Figure 9). CSE evoked the decrease of cell viability at concentrations of 15-100% in all cell types. In contrast, exposure to higher concentrations of 50-100% ECVE with or without nicotine resulted in a slight decrease of cell viability in A549, hPASMC and mAECII or did not affect mPASMC and HBEpC cell viability. These results suggested that ECVE has the considerably lower acute toxicity in comparison with CSE. 15% ECVE, which was the maximum concentration that did not cause any change in the cell viability in all cell types, was chosen for further experiments. In case of CSE, the maximum concentration that did not cause a significant change was depended on the cell type. Therefore, we selected CSE concentration of 5% for hPASMC and mPASMC and 2.5% for mAECII in the following experiments.

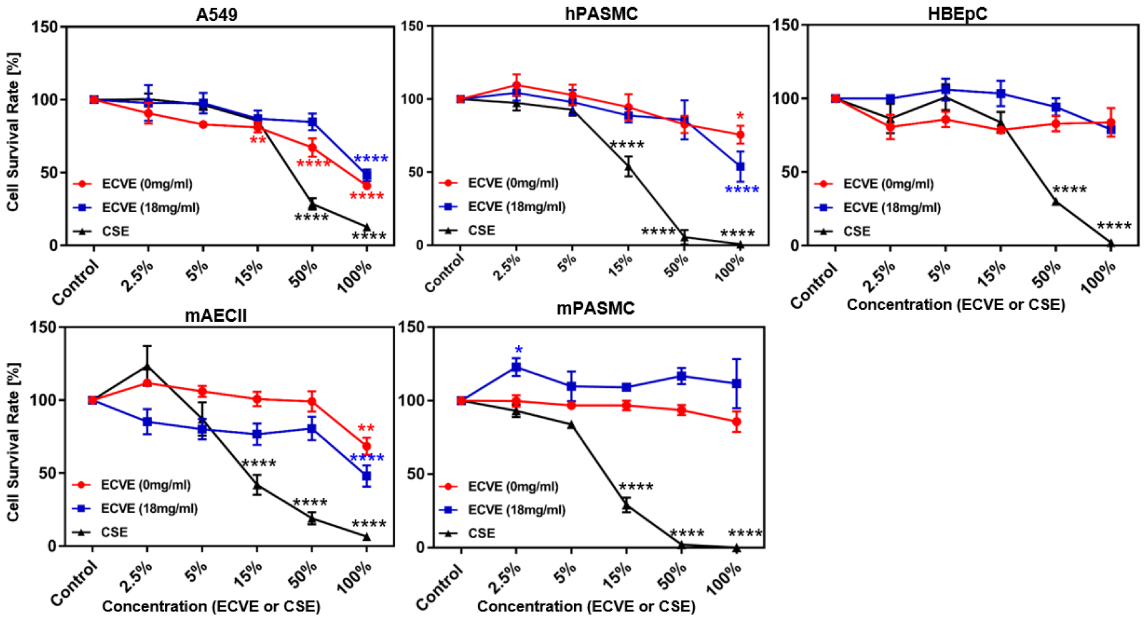


Figure 9. Dose-dependent cell viability (MTT assay) of human and mouse lung cells treated with e-cigarette vapour extract (ECVE) and cigarette smoke extract (CSE)

The cytotoxicity of ECVE with (18mg/ml) or without (0mg/ml) nicotine and CSE on A549 cells, human pulmonary arterial smooth muscle cells (hPASMC), mouse pulmonary arterial smooth muscle cells (mPASMC), mouse alveolar epithelial type two cells (mAECII), human bronchial epithelial cells (HBEpC). Values represent the mean ± SEM, n=3. *p < 0.05, **p < 0.001, ***p < 0.0001, ****p < 0.00001 using one-way ANOVA followed by Dunnett's multiple comparison test.

ECVE (0mg/ml): E-cigarette vapour extract without nicotine; ECVE (18mg/ml): E-cigarette vapour extract with 18mg/ml nicotine

3.2.Effects of ECVE/CSE on the proliferation of PASMC *in vitro*

To evaluate the effects of ECVE or CSE on the hPASMC and mPASMC proliferation, BrdU cell proliferation assay was performed (Figure 10A and 10B). Exposure to ECVE with 18mg/ml nicotine for 24 hours significantly reduced the cellular proliferation of hPASMC and mPASMC, while exposure to ECVE without nicotine did not affect cellular proliferation. Exposure of mPASMC to ECVE with 18mg/ml nicotine for 24 hours significantly decreased the protein level of PCNA compared to non-exposed cells and cells exposed to ECVE without nicotine (Figure 10C). Similar to ECVE with 18mg/ml nicotine, exposure of mPASMC to CSE resulted in a significant reduction of cellular proliferation (Figure 10A and 10B).

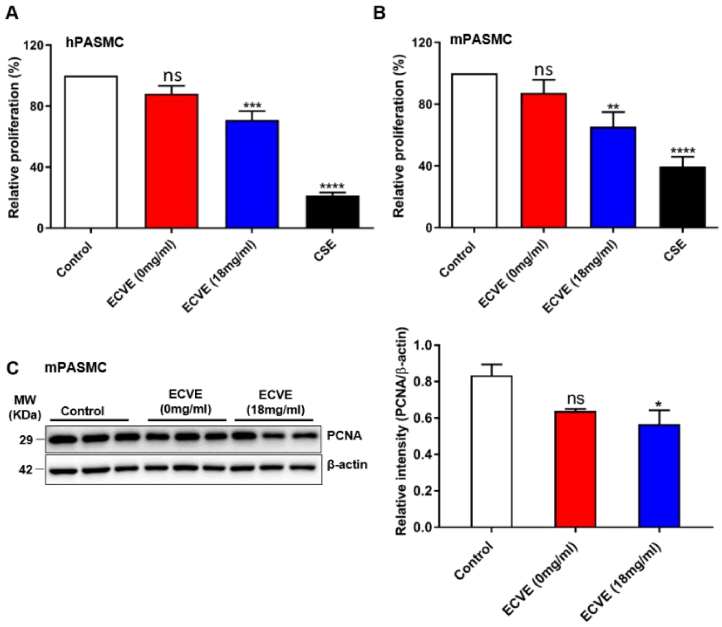


Figure 10. BrdU cell proliferation assay of pulmonary arterial smooth muscle cells exposed to e-cigarette vapour extract (ECVE) or cigarette smoke extract (CSE)

Proliferation of human pulmonary arterial smooth muscle cells (hPASMC) (A) and mouse pulmonary arterial smooth muscle cells (mPASMC) (B) after exposure to ECVE with (18mg/ml) or without (0mg/ml) nicotine or CSE at 24 hours compared to unexposed control group. (C) Western blot analysis of PCNA (proliferating cell nuclear antigen) from mPASMC normalized to β-actin. A representative blot is shown on the left and densitometry is given on the right. Values are from measurements of n=6 for 2A and 2B and n=3 for 2C. Each bar represents a mean ± SEM, *p < 0.05, **p < 0.001, ***p < 0.0001, ****p < 0.00001, ns indicates no statistical difference using one-way ANOVA followed by Dunnett's multiple comparison test.

ECVE (0mg/ml): E-cigarette vapour extract without nicotine; ECVE (18mg/ml): E-cigarette vapour extract with 18mg/ml nicotine**.**

3.3.Effects of ECVE/CSE on cell migration of PASMC *in vitro*

The wound healing assay (Ibidi cell culture insert) was used to evaluate the effect of ECVE or CSE on the migration ability of hPASMC and mPASMC. After the removal of the Ibidi insert, hPASMC and mPASMC were exposed to ECVE or CSE and their migration to the denuded area was monitored by the IncuCyte ZOOM™ live cell imaging system (Figure 11). CSE decreased migration of both hPASMC and mPASMC, while ECVE with 18mg/ml nicotine or without 0mg/ml nicotine did not altered neither hPASMC nor mPASMC migration.

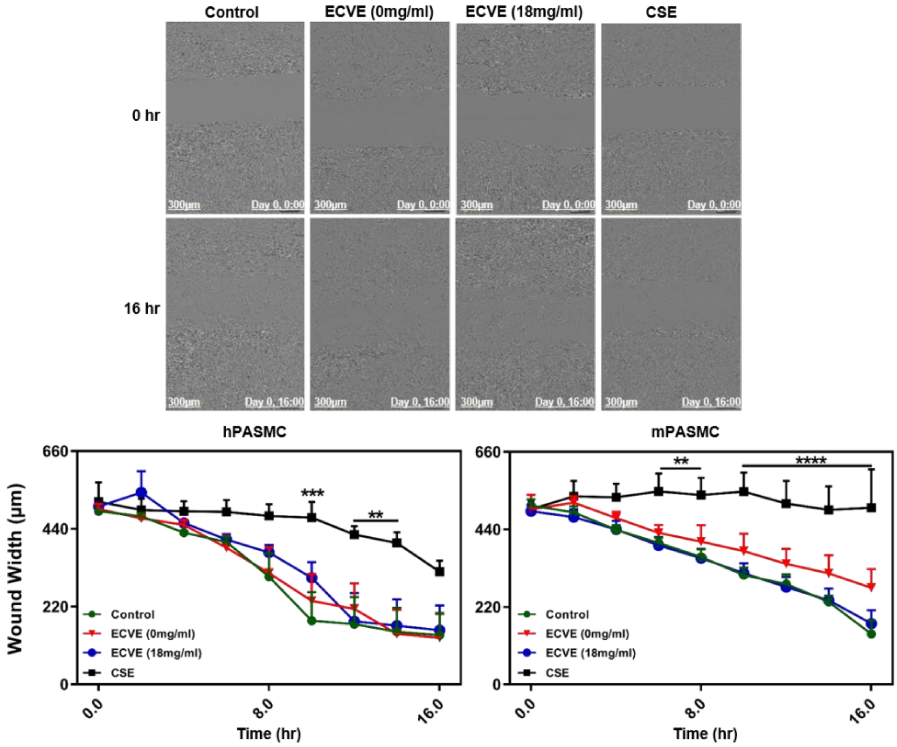


Figure 11. Cigarette smoke extract (CSE) but not e-cigarette vapour extract (ECVE) visually decrease pulmonary arterial smooth muscle (PASMCs) cells migration

Migration of the cells was evaluated using live cell imaging from the time the cells were exposed to ECVE or CSE. Representative wound closure images (top) and calculated cell migration expressed as wound width to the initial cell free zone (bottom) are shown. Values are represented as mean ± SEM, n=4-6, *p < 0.05, **p < 0.001, ***p < 0.0001, ****p < 0.00001, using one-way ANOVA followed by Dunnett's multiple comparison test.
ECVE (0mg/ml): E-cigarette vapour extract without nicotine; ECVE (18mg/ml): E-cigarette vapour extract with 18mg/ml nicotine; Day 0, 0:00 indicates the start of the migration; Day 0, 16:00 indicates the end of the migration.

3.4.Effect of ECVE on cellular respiration

To evaluate whether e-cigarettes have any effects on mitochondrial respiration, cellular oxygen consumption after exposure to ECVE for 24 hours was measured in mPASMC by a high-

resolution respirometry (oxygraph-2K). The exposure of mPASMC to ECVE did not altered the mitochondrial respiration (Figure 12).

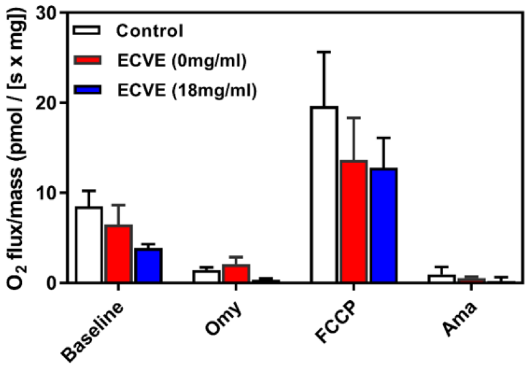


Figure 12. Mitochondrial respiration in mouse pulmonary arterial smooth muscle cells

Mitochondrial respiration of mPASMC after exposure to ECVE compared to the unexposed control group. Data are given as respiration in pmpl of O_2 consumed per minute per mg of protein. Baseline is unstimulated respiration; Omy= oligomycine-inhibited respiration; FCCP= respiration in the presence of carbonyl cyanide-4-phenylhydrazone; Ama= antimycine A-inhibited respiration. Data are from independent cell isolation, n=4. Two-way ANOVA followed by Dunnett's multiple comparison test.

ECVE (0mg/ml): E-cigarette vapour extract without nicotine; ECVE (18mg/ml): E-cigarette vapour extract with 18mg/ml nicotine

3.5. Comparison of gene expression pattern of CS and ECVE exposed cells

The expression levels of 15 genes that were known to be affected by the conventional CS were screened to evaluate whether e-cigarettes induced effects are similar to the ones of a conventional cigarette. These genes were selected based on previous results from our lab and from literature (49, 288-290). Primary mPASMC and mAECII were exposed to ECVE with (18mg/ml) or without nicotine for 24 hours, and gene expression levels were assessed via qRT-PCR. In contrast to previous study with conventional CS exposure, the mRNA levels of *Bcl2, MMP9, Nrf2 , Timp3 and Traf1* were not changed (Table 2). mRNA levels of *iNOS (Nos2), Csf2, 5HTT and* Ccna1 (Figure 13A) were significantly increased in mPASMC after exposure to nicotine containing ECVE, but no change was observed in ECVE without nicotine. The exposure of mAECII to ECVE with or without nicotine did not alter mRNA expression of those genes (Figure 13A and 13B). A comparison of our findings to published results in the literature is summarised in Table 2. Since mAECII did not show any alterations on the investigated genes, only mPASMC is displayed in the table.

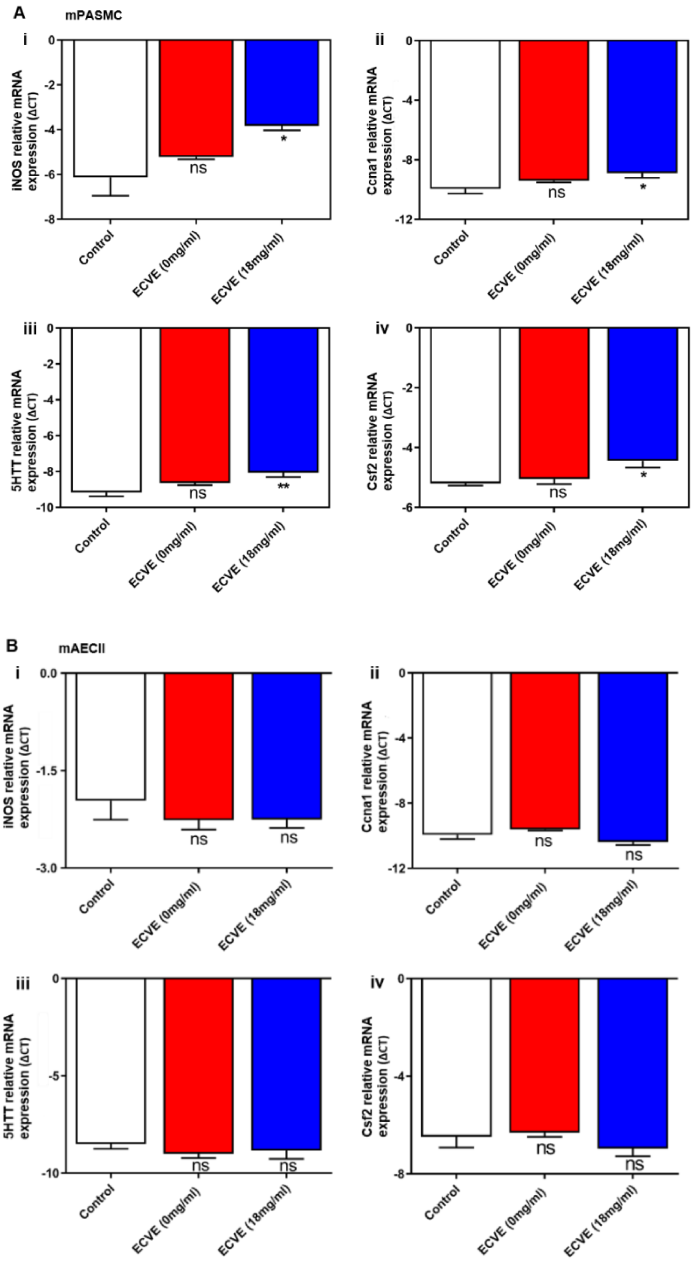


Figure 13. mRNA expression levels of selected genes in mouse pulmonary arterial smooth muscle cells (mPASMC) and alveolar epithelial type II cells (mAECII) exposed to e-cigarette vapour extract (ECVE)

mRNA-expression of (i) iNOS, (ii) Csf2, (iii) 5HTT and (iv) Ccna1 in murine (A) pulmonary arterial smooth muscle cells (mPASMC) and (B) alveolar epithelial type two cell after exposure to ECVE with (18mg/ml) or without (0mg/ml) nicotine. Data are from independent cell isolation, n=5.*$p < 0.05$, **$p < 0.001$, ns indicates no statistical difference using one-way ANOVA followed by Dunnett's multiple comparison test.

ECVE (0mg/ml): E-cigarette vapour extract without nicotine; ECVE (18mg/ml): E-cigarette vapour extract with 18mg/ml nicotine.

Table 2. Comparison of gene expression pattern of CS and ECVE exposed mPASMC.

Function	Gene Symbol	Gene name	Cigarette smoke effects (from litratures)	Electronic cigarette vapour extract effects (PASMC)
Proliferation	*Ccna1*	cyclin A1	Down (Seimetz et al., Cell 2011)	Up (p=0.05)
	Fgf10	fibroblast growth factor 10	Down (Seimetz et al., Cell 2011)	Down# (p= 0.9)
	Csf2	Colony Stimulating Factor 2	Up (Cabanski et al., Inflamm. Res. 2015)	Up (p=0.016)
Apoptosis	*Bcl2*	B cell leukemia/lymphoma 2	Up (Seimetz et al., Cell 2011)	No change
	Traf1	TNF receptor-associated factor 1	Up (Seimetz et al., Cell 2011)	Up# (p=0.7)
Extracellular Matrix	*Mmp9*	matrix metallopeptidase 9	Up (Seimetz et al., Cell 2011)	Up# (p=0.8)
	Timp3	tissue inhibitor of metalloproteinase 3	Down (Seimetz et al., Cell 2011)	No change
Inflammation	*Il1b*	interleukin 1 beta	Up (Seimetz et al., Cell 2011)	Up# (p=0.3)
	Ccl6	chemokine (C-C motif) ligand 6	Up (Seimetz et al., Cell 2011)	Up# (p=0.2)
Oxidant-responsive	*Nfe2l2*	nuclear factor, erythroid derived 2, like 2	Up (Hübner et al., Mol Med 2009)	Down# (p=0.8)
Nitric oxide regulation	Nos3	nitric oxide synthase 3, endothelial cell	No change (Seimetz et al., Cell 2011)	No change
	Nos2	nitric oxide synthase 2, inducible	Up (Seimetz et al., Cell 2011)	Up (p=0.03)
Guanylate cyclase	*Gucy1a3*	guanylate cyclase 1, soluble, alpha 3	Down (Weissmann et al.,Am J Respir Crit Care Med. 2014)	No change
	Gucy1b3	guanylate cyclase 1, soluble, beta 3	Down (Weissmann et al.,Am J Respir Crit Care Med. 2014)	No change
Serotonin	*5HTT*	Serotonin transporter	Up (Weissmann et al.,Am J Respir Crit Care Med. 2014)	Up (=0.012)

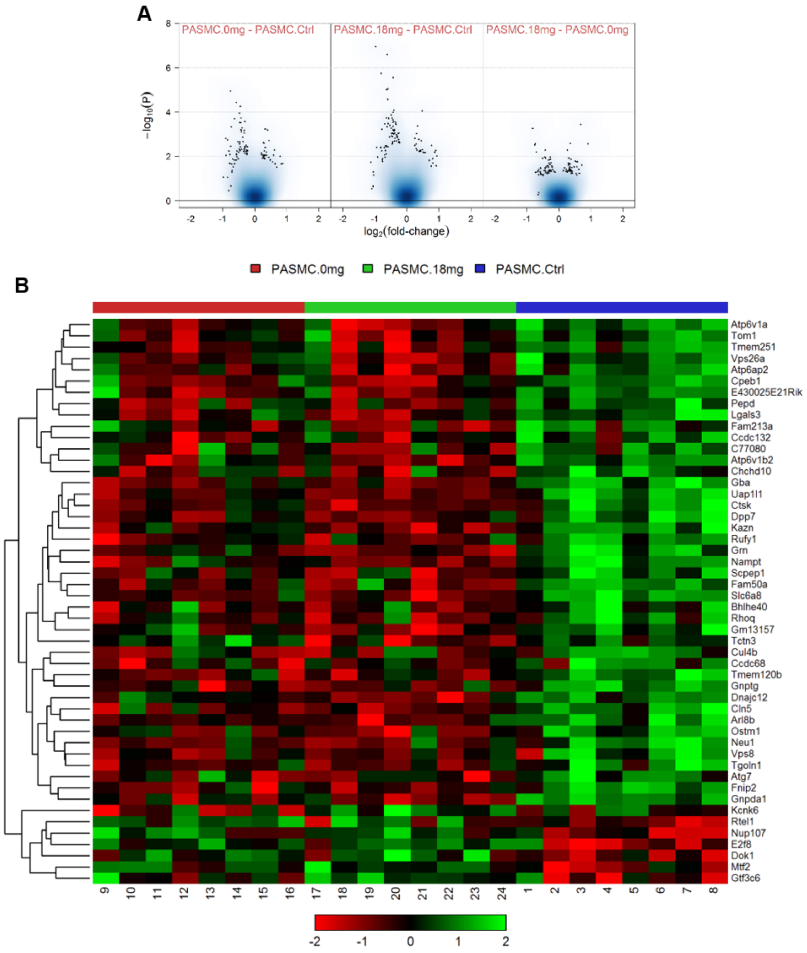
#:Tendency

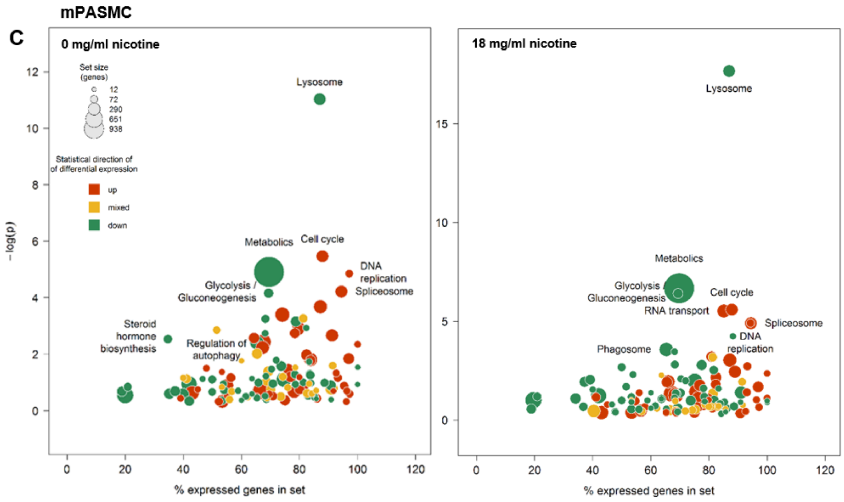
3.6.Effects of ECVE on the transcriptome of mPASMC and mAECII cells

To investigate the effects of ECVE exposure on the alterations of various signalling pathways, a whole transcriptome analysis was performed in primary mPASMC and mAECII isolated from wild type mice after exposure to ECVE with nicotine (18mg/ml) or without nicotine (0mg/ml) for 24 hours. Exposure to ECVE significantly modulated the expression of various genes in these cells (see Figure 14A/B and Figure 15A/B).

Gene ontology (GO) analysis identified significant upregulation of genes involved in cell cycle, DNA replication and spliceosome and downregulation of genes involved in lysosome and metabolic pathways in mPASMC (Figure 14C). To validate the results from the microarray, expression levels of selected proteins, such as the autophagy markers microtubule-associated protein 1A/1B-light chain 3 (LC3)-phosphatidylethanolamine conjugate (LC3-II) and the ubiquitin-binding protein p62 or the lysosomal protein lysosomal-associated membrane protein 1 and 2 (LAMP1/2), were analysed by western blot analysis. LC3-II and p62 showed a significantly decrease of protein levels, while LAMP1/2 showed a tendency for downregulation (Figure 14D). The direction of fold change of LAMP1/2, LC3-II and p62 determined by the microarray were similar to those changes in protein levels by western blot.

GO analysis of mAECII revealed an increased expression of genes involved in metabolic pathways such as cytochrome P450 (CYP 450) and glutathione (GSH) metabolism whereas genes involved in steroid biosynthesis and endocytosis were significantly inhibited (Figure 15C). An analysis of cellular GSH levels showed a depletion of intracellular GSH only in mAECII exposed to ECVE with nicotine, but the ratio of GSH to GSSG was significantly decreased in all exposed groups ECVE with or without nicotine and CSE to a comparable level (Figure 15D). Moreover, western blot analysis of proteins which can be affected by alteration in GSH level showed that exposure of mAECII to both ECVE with or without nicotine downregulated the inhibitory κB alpha (IκB-α), while did not alter the protein level of the nuclear factor-κB (NF-κB) (Figure 15E). The direction of fold change of NF-κB and IκB-α determined by the microarray were similar to those changes in protein levels by western blot.





Pathways	0 mg/ml	P.value	18 mg/ml	P.value
Cell cycle	Up	3.35E-06	Up	2.67E-06
DNA replication	Up	1.41E-05	Up	1.27E-05
Spliceosome	Up	5.93E-05	Up	1.33E-05
Protein processing in endoplasmic reticulum	Up	0.000205	Up	0.000959
RNA transport	Up	0.015289	Up	3.16E-06
Lysosome	Down	9.85E-12	Down	2.12E-18
Metabolic pathways	Down	1.31E-05	Down	1.92E-07
Glycolysis / Gluconeogenesis	Down	7.05E-05	Down	3.84E-07
Other glycan degradation	Down	0.000376	Down	5.68E-05
Steroid hormone biosynthesis	Down	0.001984	ns	0.210334
Phagosome	Down	0.003949	Down	0.000261
Glycosaminoglycan degradation	Down	0.001861	Down	0.000347

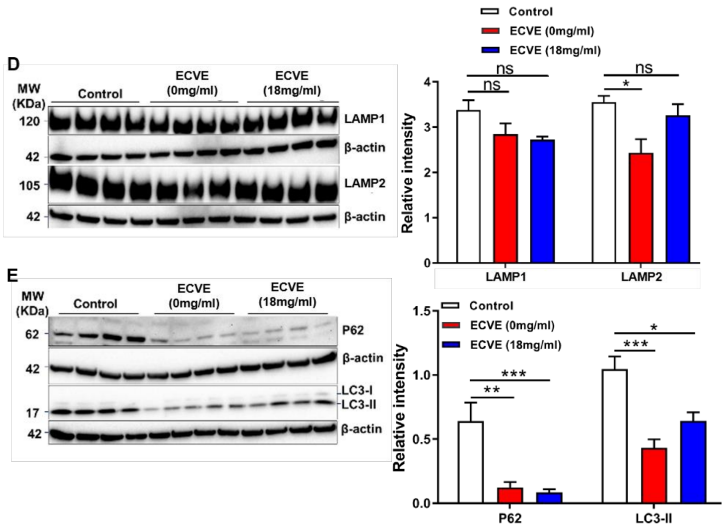
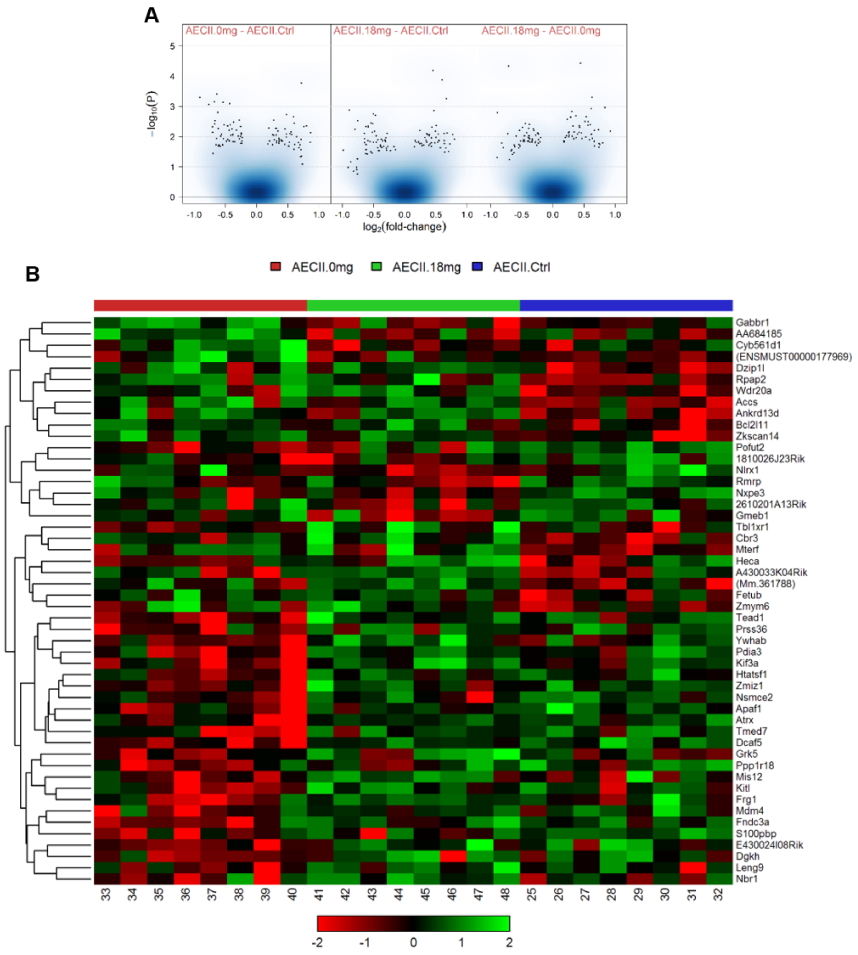
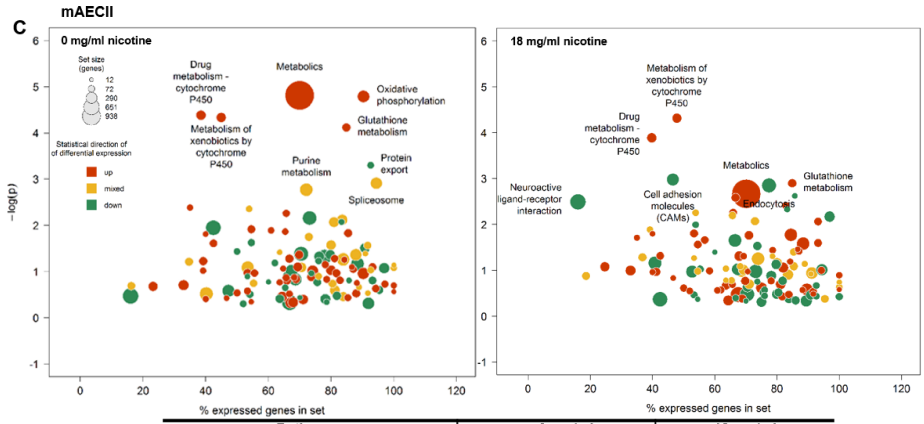


Figure 14. Differentially expressed transcripts and gene ontology (GO) enrichment analysis of mPASMC responsive to the ECVE exposure

(A) Volcano plots for the differentially expressed genes. The X-axis shows the Log2 of the fold change (FC) and the Y-axis shows the −Log10 of the adjusted p-values. (B) Heatmap showing unsupervised hierarchical clustering for mouse pulmonary arterial smooth muscle cells (mPASMC) following the e-cigarette vapour extract (ECVE) exposure compared to control. (C) GO enrichment for differentially expressed transcripts in mouse pulmonary arterial smooth muscle cells (mPASMC) exposed to e-cigarette vapour extract (ECVE) with (18mg/ml) or without

(0mg/ml) nicotine vs control. Bubble plot for the gene ontology (GO) for the top enriched GO terms. The percent of expressed genes in set is assigned to the x-axis and the negative logarithm of the adjusted p-value to the y-axis (the higher the more significant). The area of the displayed circles is proportional to the number transcripts in specified pathway, and the colour of the node corresponds to up/down enriched GO terms (red-, green- and yellow - represents up, down and mixed regulated genes respectively), n=8. The associated tables present the top 12 GO term function and p-value for the enriched term. Protein expression of LAMP 1/2 (D), and p62 and LC3-II (E) normalised to β-actin expression in mPASMC exposed to ECVE with (18 mg/ml) or without (0 mg/ml) nicotine for 24 hours. n=4, ns - not statistically difference, *p < 0.05, **p < 0.001, ***p < 0.0001by one-way ANOVA with Dunnett's multiple comparison test. ECVE (0mg/ml): E-cigarette vapour extract without nicotine; ECVE (18mg/ml): E-cigarette vapour extract with 18mg/ml nicotine





Pathways	0 mg/ml	P.value	18 mg/ml	P.value
Metabolic pathways	Up	1.42E-05	Up	0.00203
Oxidative phosphorylation	Up	1.74206E-05	ns	0.258696
Drug metabolism - cytochrome P450	Up	4.22541E-05	Up	0.000127
Metabolism of xenobiotics by cytochrome P450	Up	4.7259E-05	Up	4.74E-05
Glutathione metabolism	Up	7.88927E-05	Up	0.001254
Protein export	Down	0.000484	ns	0.367434
Focal adhesion	Down	0.0066	ns	0.105782
Steroid biosynthesis	Down	0.00830627	Down	0.004701
Endocytosis	Down	0.045818541	Down	0.001437
Phagosome	ns	0.131564471	Down	0.022463
Cell adhesion molecules (CAMs)	ns	0.257275719	Down	0.001067
Neuroactive ligand-receptor interaction	ns	0.333317891	Down	0.004938

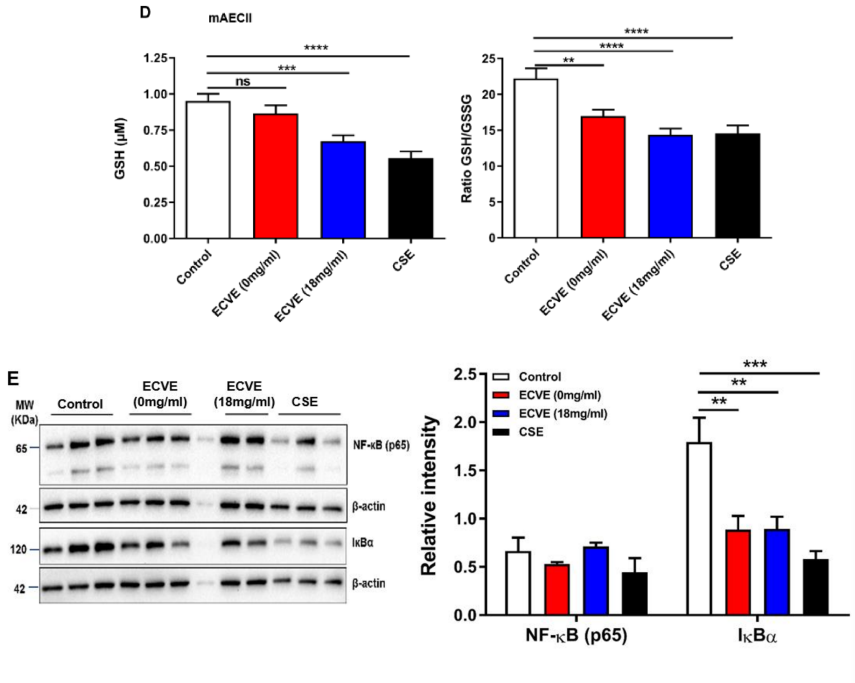


Figure 15. Differentially expressed transcripts and gene ontology (GO) enrichment analysis of mAECII responsive to the ECVE exposure

(A) Volcano plots for the differentially expressed genes. The X-axis shows the Log2 of the fold change (FC) and the Y-axis shows the −Log10 of the adjusted p-values. (B) Heatmap showing unsupervised hierarchical clustering for mouse alveolar epithelial type two cells (mAECII) following the e-cigarette vapour extract (ECVE) exposure compared to control. (C) GO enrichment for differentially expressed transcripts in mouse alveolar epithelial type two cells (mAECII) exposed to e-cigarette vapour extract (ECVE) with (18mg/ml) or without (0mg/ml) nicotine

vs. control. Bubble plot for the gene ontology (GO) for the top enriched GO terms. The percent of expressed genes in set is assigned to the x-axis and the negative logarithm of the adjusted p-value to the y-axis (the higher the more significant). The area of the displayed circles is proportional to the number transcripts in specified pathway, and the colour of the node corresponds to up/down enriched GO terms (red-, green- and yellow - represents up, down and mixed regulated genes respectively), n=8. The associated tables present the top 12 GO term function and p-value for the enriched term. GSH/GSSG-Glo™ Assay measuring cellular glutathione level in mAECII exposed to ECVE or cigarette smoke extract (CSE), n=6 (D). (E) Protein expression of NF-κB (P65) and IκBα normalised to β-actin expression in mAECII, n=3. ns - not statistically difference, *p < 0.05, **p < 0.001, ***p < 0.0001, ****p < 0.00001 by one-way ANOVA with Dunnett's multiple comparison test.

ECVE (0mg/ml): E-cigarette vapour extract without nicotine; ECVE (18mg/ml): E-cigarette vapour extract with 18mg/ml nicotine

3.7.Particle size of e-cigarette vapour and tobacco smoke

The aerosol characterization (mass median aerodynamic diameters [MMAD] and fine particle fraction [FPF]) of the e-cigarette vapour and CS was determined by laser diffraction. The average MMAD for e-cigarette vapour was 1.7 µm and 2.3 µm for tobacco smoke with geometric standard deviation (GSD) values of ≤ 1.5. While there was no difference in MMAD of the vapour generated from e-cigarette regardless of nicotine, the MMAD of cigarette smoke was significantly higher compared to e-cigarette vapour (Figure 16A). The FPF values for CS were significant lower compared to e-cigarette vapour (Figure 16B).

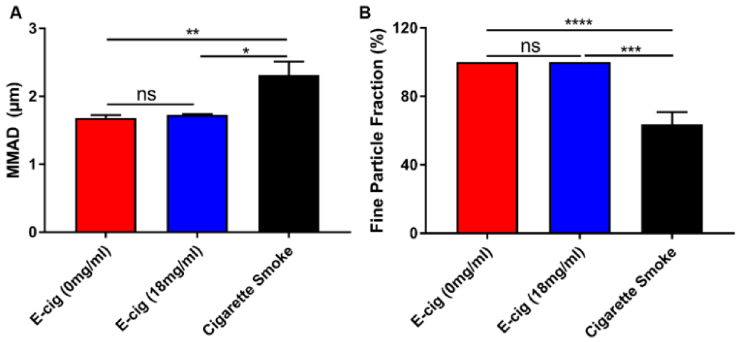


Figure 16. Particle size of e-cigarette vapour and cigarette smoke

The mass median aerodynamic diameters (MMAD) (A) and fine particle fraction (B) of the generated vapour/smoke as determined by laser diffraction. Each bar represents a mean ± SEM. N=4-6, *p < 0.05, **p < 0.001, ***p < 0.0001, ****p < 0.00001, ns indicates no statistical difference using one-way ANOVA followed by Dunnett's multiple comparison test.

E-cig: Electronic cigarette (e-cigarette); E-cig (0mg/ml): E-cigarette without nicotine; E-cig (18mg/ml): E-cigarette with 18mg/ml nicotine.

3.8.Effects of *in vivo* long-term e-cigarettes exposure

3.8.1. Blood oxygen saturation and haematocrit

To characterize the physiological response to e-cigarette vapour exposure, blood oxygen saturation (O_2 saturation) and haematocrit was assessed at the end of experiments. There were no changes in O_2 saturation between groups were observed (Figure 17A). On the other hand, the haematocrit values shows a modest increase when exposed to e-cigarette vapour without nicotine and a significant increase when exposed to e-cigarette vapour with nicotine (18mg/ml) compared to room air exposed groups (Figure 17B).

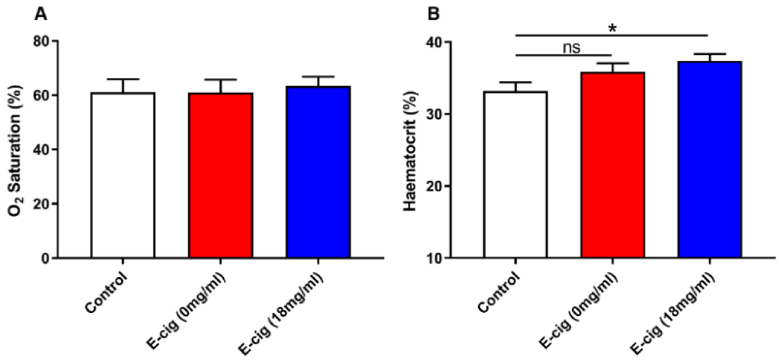


Figure 17. The level of blood oxygen saturation and haematocrit values of mice exposed to e-cigarette vapour for 8 months

8 months after e-cigarette vapour exposure blood oxygen saturation and haematocrit values were analysed. (A) Blood oxygen saturation, (B) haematocrit. Each bar represents a mean ± SEM. n=10-11. *p < 0.05, ns indicates no statistical difference using one-way ANOVA followed by Dunnett's multiple comparison test.

E-cig: Electronic cigarette (e-cigarette); E-cig (0mg/ml): E-cigarette without nicotine; E-cig (18mg/ml): E-cigarette with 18mg/ml nicotine.

3.8.2. Mouse exposed to nicotine containing e-cigarette vapour showed elevated plasma nicotine and cotinine levels

Acute exposure (6 hours) of mice to e-cigarette vapour with nicotine (18mg/ml) showed measureable plasma nicotine and cotinine (the predominant metabolite of nicotine) levels (Figure 18A/B). The cotinine level indicates that systematic absorption of nicotine by the mice.

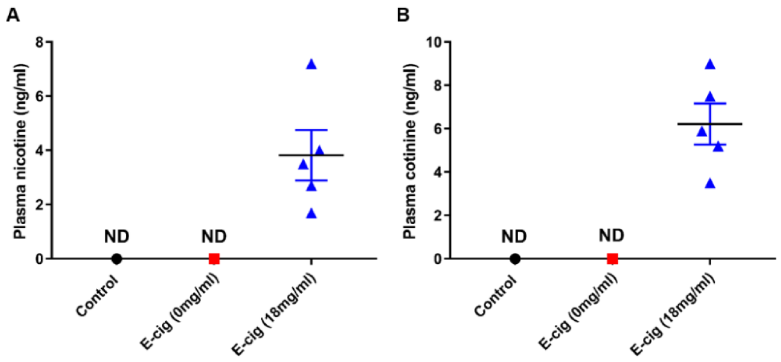


Figure 18. Plasma nicotine and cotinine levels of mice exposed to e-cigarette vapour for 6 hours

Six hours after e-cigarette vapour exposure plasma nicotine and cotinine levels were measured. (A) Plasma nicotine levels, (B) Plasma cotinine levels. Graphs are represented as mean ± SEM, n=5.

ND: Not detected; E-cig: Electronic cigarette (e-cigarette); E-cig (0mg/ml): E-cigarette without nicotine; E-cig (18mg/ml): E-cigarette with 18mg/ml nicotine.

3.8.3. Influence of e-cigarette vapour exposure on the development of pulmonary emphysema in mice

After a period of 8 months exposure to e-cigarette vapour, lung function measurements were carried out including, lung static and dynamic compliance, lung hysteresis and pulmonary resistance. Pulmonary resistance, lung compliance and lung hysteresis were not altered in mice exposed to e-cigarette vapour with (18mg/ml) or without nicotine compared to room air control groups (Figure 19A-C). To support these findings, quantification of alveolar numbers via design-based stereology analysis of lung sections was performed and a trend to increased alveolar numbers for e-cigarette vapour exposed mice was observed, but did not reach significance (Figure 19D).

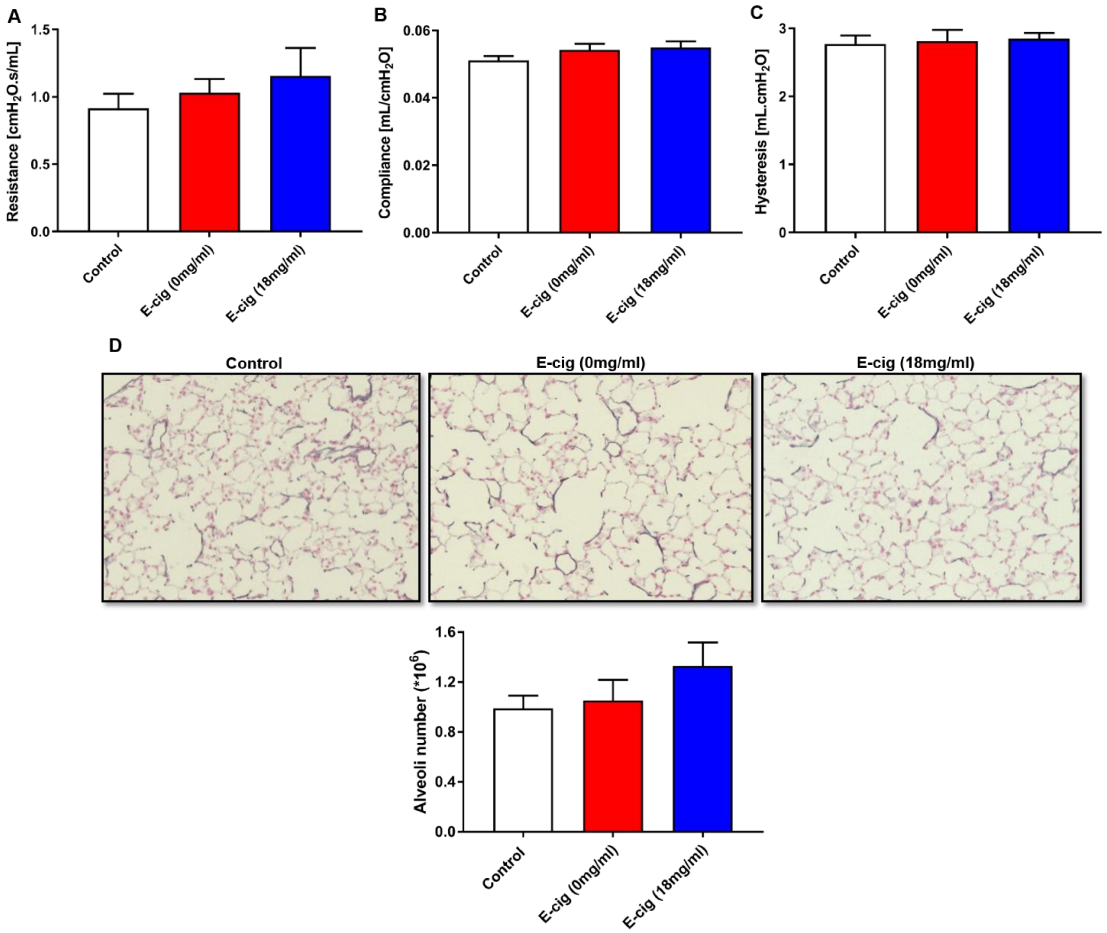


Figure 19. Long-term effects of e-cigarette vapour exposure on the development of emphysema in mice assessed by structural and functional parameters

Mice were exposed to e-cigarette vapour for 8 months. In vivo lung function tests; pulmonary resistance (A) (n=10-12), dynamic compliance (B) (n=10-12), and hysteresis (C) (n=10-12). Representative histology from lung sections stained with Weigert's elastin staining (D, up), total number of alveoli assessed by quantitative stereology (D, down) (scale bar, 50 μm, data are given from n = 6-7). Each bar represents a mean ± SEM.

E-cig: Electronic cigarette (e-cigarette); E-cig (0mg/ml): E-cigarette without nicotine; E-cig (18mg/ml): E-cigarette with 18mg/ml nicotine.

3.8.4. Effect of e-cigarette exposure in the development of PH and right heart hypertrophy in mice

Whether e-cigarette exposure leads to the development of PH and/or right heart hypertrophy was further deciphered. To characterize PH and cardiac function, hemodynamic and echocardiographic measurements were carried out. Systemic arterial pressure (SAP) and right ventricular (RV) systolic pressure (RVSP) values did not differ between groups exposed to e-cigarette or room air (Figure 20A/B). Moreover, e-cigarette exposure did not result in pulmonary vascular remodelling as shown by unchanged degree of muscularization in small vessel (Figure 20C). Furthermore, there were no deterioration of RV function detected (Figure

20D-I). To determine the presence of right ventricular hypertrophy, the heart ratio (ratio of RV mass to left ventricle (LV) mass plus septum) was calculated. Heart ratio, ratio of RV mass to tibia length and ratio of LV mass to tibia length were similar in all experimental groups (Figure 20J-L).

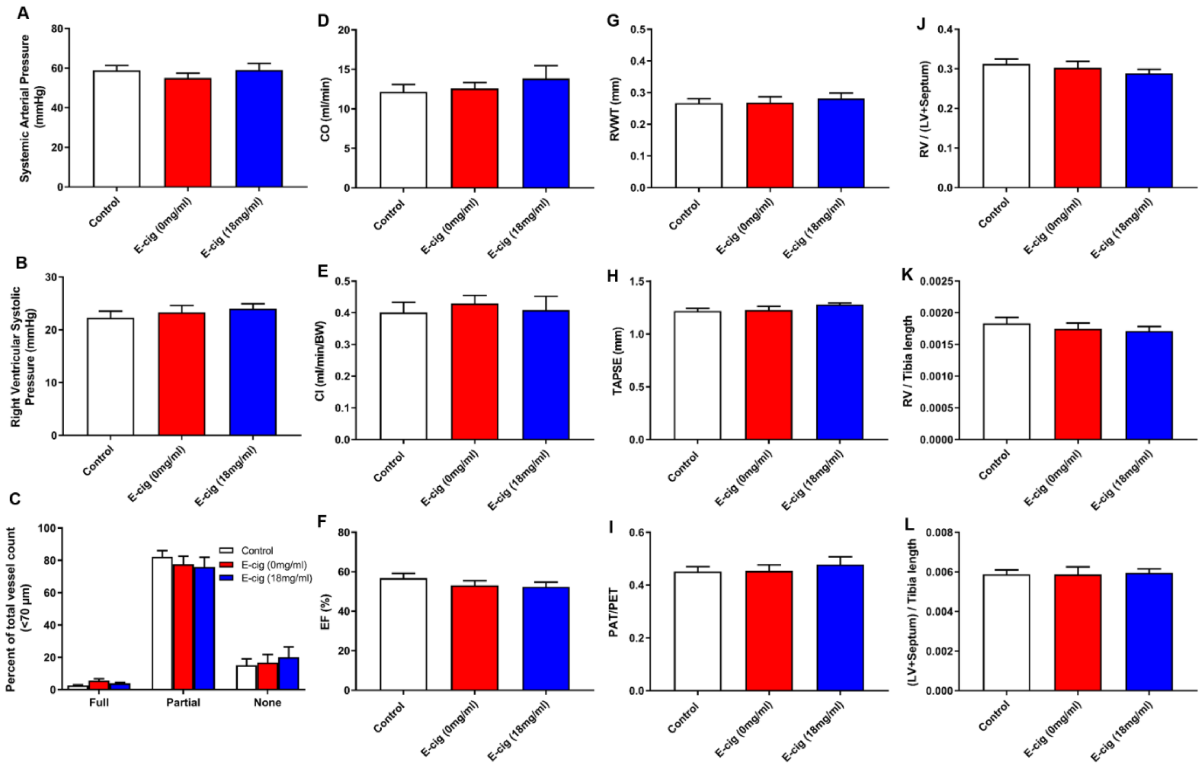
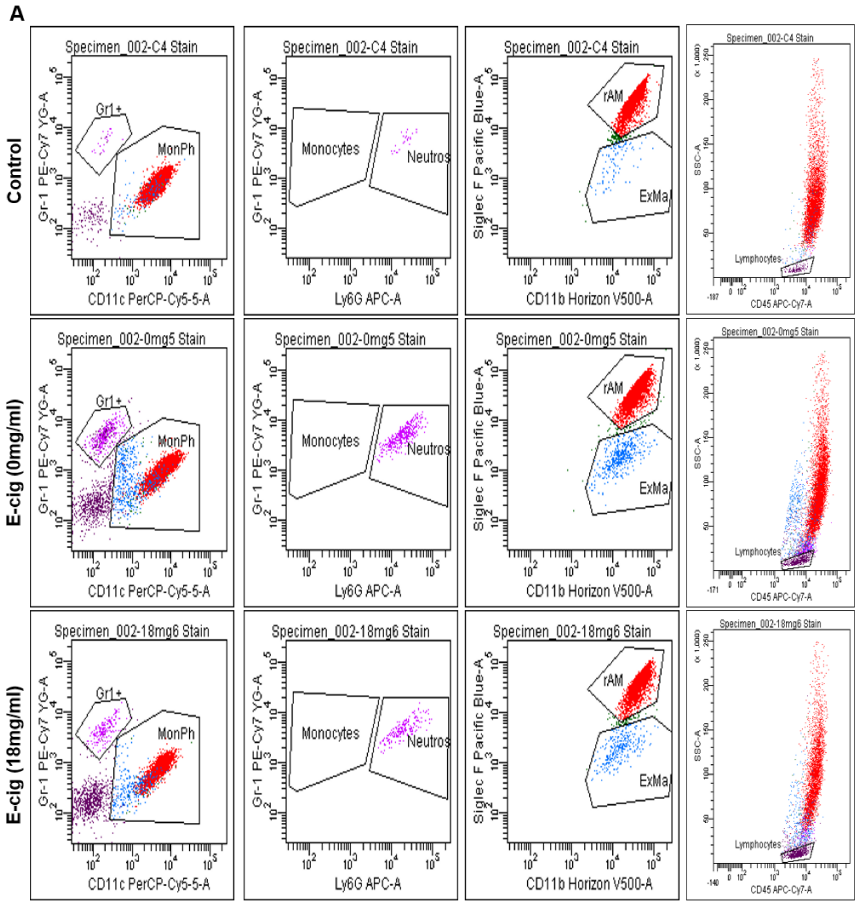


Figure 20. Long-term effect of e-cigarette exposure on the development pulmonary hypertension, cardiac function and right heart hypertrophy in mice

After 8 months of e-cigarette vapour exposure, pressure changes for (A) systemic arterial pressure, (B) right ventricular systolic pressure were determined using hemodynamic. (C) Degree of muscularization of small pulmonary arteries (outer diameter <70 µm) percentage of total vessel count for fully muscularized (full), partially muscularized (partial), and nonmuscularized (none) vessels. Cardiac function in mice after 8 months of e-cigarette vapour exposure, (D) cardia output (CO), (E) cardiac index (CI), (F) ejection fraction, (G) right ventricular wall thickness (RVWT), (H) tricuspid annular plane systolic excursion (TAPSE) and (I) ratio of pulmonary artery acceleration time (PAT) to pulmonary artery ejection time (PET). Ratio of right ventricle (RV) to left ventricle (LV) + septum (J), RV mass to tibia length (K) and LV+S mass to tibia length (L) in e-cigarette aerosol exposed and room air exposed control mice. Each bar represents a mean ± SEM, A, B and I-K, n=11-12; C-H, n=6.
E-cig: Electronic cigarette (e-cigarette); E-cig (0mg/ml): E-cigarette without nicotine; E-cig (18mg/ml): E-cigarette with 18mg/ml nicotine.

3.8.5. Mouse exposed to e-cigarette vapour leads to altered immune cell dynamics in the lungs

To evaluate the immune response of e-cigarette vapour exposure in the lung, BALF was collected from control mice and mice exposed to e-cigarette vapour with (18mg/ml) or without nicotine. The overview of inflammatory cells in BALF from mice in each group is shown in Figure 21. Exposure of mice with e-cigarette vapour containing nicotine or without nicotine for 8 months increased neutrophils population. The lymphocyte population in mice exposed to e-cigarette vapour containing nicotine were significantly higher when compared to mice exposed to e-cigarette vapour without nicotine or to room air. However, there were no significant differences between the latter two. The population of resident alveolar macrophages (rAMs) in the BALF of e-cigarette vapour exposed mice was decreased compared to the control mice. Additionally, a trend to increased exudate macrophages (ExAM; a population that may include lung interstitial macrophages (287)) in e-cigarette exposed groups was observed.



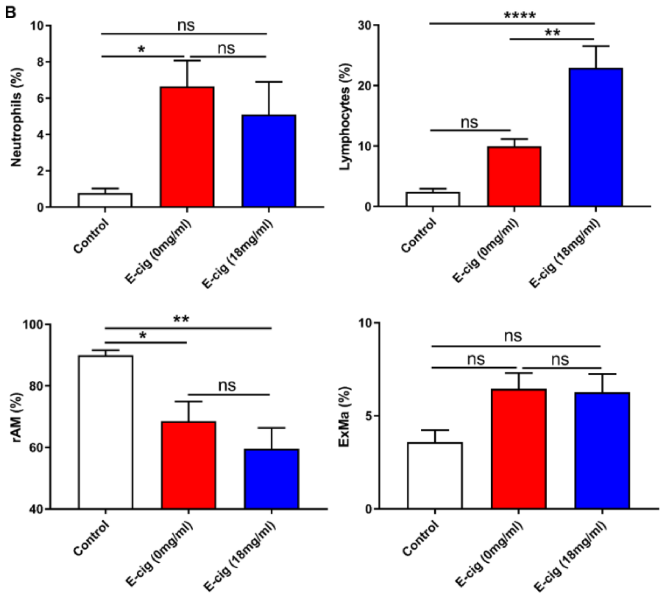


Figure 21. The number of immune cells in bronchoalveolar lavage fluid (BALF) after e-cigarette exposure

Flow cytometry of BALF from control and from mice exposed to e-cigarette vapour with (18mg/ml) or without (0mg/ml) nicotine for 8 months. (A) Representative flow cytometry plots. After the exclusion of doublets and debris, immune cells were identified by CD45 staining. A sequential gating strategy was used to identify populations expressing specific markers: mononuclear phagocyte (Gr-1^{-} CD11c^{+}), within mononuclear phagocyte gate distinguishes residence alveolar microphages (rAM) (SiglecF^{+} CD11b^{-}) and exudate macrophages (ExMa) (SiglecF^{-} CD11b^{+}), neutrophils (Gr-1^{+} Ly6G^{+}). Lymphocytes were gated on SSC-A vs CD45. (B) The amount of neutrophils, lymphocytes, residence microphages and exudate microphages in BALF from control and mice exposed to e-cigarette vapour with (18mg/ml) or without (0mg/ml) nicotine for 8 months. Values represent the percentages from the parent gate. Data from independent mouse, n=5-6. ns - not statistically difference, *p < 0.05, **p < 0.001, ****p < 0.00001 by one-way ANOVA by Tukey multiple comparison test.

E-cig: Electronic cigarette (e-cigarette); E-cig (0mg/ml): E-cigarette without nicotine; E-cig (18mg/ml): E-cigarette with 18mg/ml nicotine; CD45: Leukocyte common antigen; Gr-1: Granulocyte-differentiation antigen-1; Ly6G: Lymphocyte antigen 6 complex, locus G; Siglec F: Sialic acid–binding, Ig-like lectin F; SSC-A: Side scatter area. CD11b is a pan-macrophage marker and CD11c is mouse dendritic cells and other immune cells marker.

4. Discussion

Despite many unsolved questions regarding their safety and efficacy, e-cigarettes are gaining popularity as an alternative to smoking among smokers who want to quite conventional cigarette (79). Additionally, e-cigarettes are used by 1) smokers with no plan to quit as addition to CS and 2) by people who never smoke before. Moreover, the use of products of e-cigarette company JUUL has increased rapidly among the youth population in USA because their trendy design (called the 'iPhone of e-cigarettes'), youth-friendly flavours like Fruit Medley and Crème Brulee and the various social media platforms where there are numerous colourful pictures of "Juuling" including those which are posted by celebrities (295). It was published that 8% of American in aged 15–24 years used Juul in the 30 days prior to the survey conducted in 2017 (217). Furthermore, there is a rising concern that e-cigarette may also serve as a gateway drug for conventional CS.

The health effects of short-term as well as long-term use of e-cigarettes are poorly understood. Therefore, this study focused on (i) assessing of the effects of e-cigarette on the lung cells, and (ii) examining of the potential long-term effects of e-cigarette vapour exposure of mice. The *in vitro* results are based on different lung-derived cells exposed to ECVE, while the *in vivo* results are based on long term exposure of C57BL/6J mice to e-cigarette vapour for 8 months.

4.1.*In vitro* experiments: exposure of cells with ECVE

4.1.1. E-cigarette vapour on cell

In current study, ECVE showed less profound (cell viability and proliferation) or no effect (migration assay and mitochondrial respiration) on cellular functions of PASMC (isolated from human and mouse), mAECII, A549 and HBEpC cells. Our data are in line with previous reports, which showed little to no effect of ECVE on cytotoxicity (206-211) and migration (212).

The presence of nicotine in ECVE (18mg) resulted in more profound difference compared to ECVE without nicotine only in proliferation assay. In line with this finding, the expression of cell proliferation-related protein, PCNA was also reduced in mPASMC cells exposed to the ECVE with nicotine. These results are in agreement with previous reports demonstrating that e-cigarettes significantly reduced proliferation in the airway epithelial cells (213), human gingival fibroblasts (214), human periodontal ligament fibroblasts (215) and human vascular endothelial cells (216). The possible cellular pathways underling the ECVE nicotine-induced impaired proliferation are discussed later.

In conclusion, most of the cytotoxic effects of ECVE that are described in existing scientific literature were observed after exposure various cells to ECVE containing different flavours (116,210,213,219,220). Contrary to these studies, the current study did not use any flavours, this allows to study solely the effects of the base chemicals of e-cigarettes.

4.1.2. Differential effects of e-cigarette vapour extract on differential gene expression

The determinant effects of conventional CS are well studied and documented as contributing factors to the development of COPD, lung cancers, PH and CVS (18,37,45,221). In hypothesis-driven approach, 15 candidate genes that regulated by conventional CS were investigated in primary isolated mPASMC and mAECII after exposure to ECVE. Only mPASMC showed features of differential gene expression. Only four of the 15 candidate genes (*iNOS [Nos2], Csf2, 5HTT* and *Ccna1)* were significantly upregulated when cells where exposed to ECVE containing nicotine (Tabel.1). No significant change was found in cells exposed to ECVE without nicotine. These findings are in agreement with previous reports showing significant increase of expression of *iNOS* (49,222)*, Csf2* (223), *5HTT* (224) and *Ccna1* (49) in smokers without COPD and/or animals exposed to conventional tobacco smoke (49,222). Some of this effects can be attributed to nicotine per se, since it has been reported that nicotine can induce mRNA expression in *iNOS* (225) and *5HTT* (226) genes. Moreover, the regulation of the expression of all these genes can be a cause of impaired proliferation which was discovered in mPASMC exposed to ECVE with nicotine (218,245,246). In this regard, it has been shown that *iNOS, Csf, 5HTT* can affect the cell proliferation via mitogen-activated protein kinase signal transduction cascades (257,293,294).

Furthermore, a non-hypothesis driven transcriptome approach was performed in mPASMC and mAECII by microarray and affected genes were determined and assigned to corresponding molecular mechanisms via pathway analysis. Significantly upregulated and downregulated genes were found in both cells exposed either to ECVE with (18mg/ml) or without nicotine.

Thus, the work presented here clearly shows that the observed effects of ECVE on mAECII and mPASMC transcriptome level occur regardless of the presence of nicotine in ECVE. However, not all genes were regulated by ECVE with or without nicotine in the same manner, suggesting additive effect of nicotine. Genes associated with the cell cycle, DNA replication and spliceosome were upregulated, while genes involved in metabolic and glycolysis/gluconeogenesis pathways were downregulated in mPASMC. Alterations in the

lysosome, phagosome and regulation of autophagy pathways were also observed. The genes involved in these pathways were downregulated. In mAECII exposed to ECVE with nicotine or without nicotine, upregulation of genes involved in the metabolism of xenobiotics by CYP 450 were observed. It is well documented that CYP 450 induction results in overproduction of ROS, which is associated with occurrence of new cancers through a mechanism called co-carcinogenesis (227,228). Sprague-Dawley rats exposed to e-cigarette liquid containing nicotine evoked a similar upregulation of xenobiotic metabolism in the lung (229).

The lysosome pathway is of interest since the autophagy-lysosome pathway has been suggested as potentially important for the pathogenesis of multiple pulmonary diseases, such as acute lung injury (230), pulmonary vascular diseases (231), cystic fibrosis (232,233), COPD (234) and lung cancer (235). LAMP1 and LAMP2 are major protein components of the lysosomal membrane and they are routinely used as a markers for degradative autophagy-lysosomal organelles (236,237). In the current study, exposure of mPASMC to ECVE resulted in a moderate decrease of LAMP1/2 proteins levels. The autophagy pathway was also altered in the mPASMC exposed to ECVE as well as a significant decrease in protein levels of LC3-II and p62 were found after exposure. The protein levels of LC3-II and p62 mirror the regulation at mRNA level that is observed from microarray data. LC3-II is the first mammalian protein discovered to be specifically associated with autophagosomal membranes and the assessment of a cytosolic form of LC3 (LC3B-I) to LC3B-II conversion is a reliable method to evaluate whether autophagy is activated *in vitro* or *in vivo* (238,239). The selective autophagy receptor p62 sequesters ubiquitinated proteins into autophagosomes by interacting with LC3. Moreover, p62 is a substrate for autophagic degradation, hence its degradation can be used as a marker of autophagic clearance (240). In contrast to current study, human gingival fibroblasts exposed to e-liquids showed time dependent activation of lysosomal compartment and no changes in LC3 expression (241). The possible explanation of this contradiction is that in contrast to the current study, which used vapour extracted from e-liquids, the mentioned study used diluted e-liquids.

GSH, a small thiol, which plays crucial role in antioxidant defence, nutrient metabolism, and the regulation of pathways essential for whole body homeostasis, such as gene expression, DNA and protein synthesis, cell proliferation and apoptosis, signal transduction, cytokine production, and immune response (242,243). The results from the present study showed upregulation of gene sets that are associated with metabolism of GSH. The measurement of the ratio of GSH to GSSG is used as a marker for redox status and oxidative stress (250-252). In normal cells most GSH exists in its reduced form, while a rapid dropping of the ratio occurs in case of disruption

of cellular redox homeostasis (252). Measurements of mAECII exposed to ECVE or CSE revealed decreased level of cellular GSH and GSH/GSSG ratio. This might explain the increased transcription of genes involved in GSH metabolism, as it has been described previously that cellular GSH depletion led to an increased GSH metabolism (244,253). The decreased levels of GSH in ECVE exposed cells might be due to the consumption of GSH during the detoxification of oxidants of ECVE. In this regard, it has been reported that an increased amount of oxidants consumes the antioxidants during chemical conjugation of oxidants (291). In line with current study, reduced level of GSH were previously observed in lungs of C57BL/6J mice to acute exposure of e-cigarette aerosols (119) and in the lungs of different mice strains exposed to acute tobacco smoke (254).

Among others, NF-κB regulates the transcription of important enzymes in GSH metabolism and it is part of a family of transcriptional factors that mediate immune responses to viral and bacterial infections, inflammation and cell proliferation (255). GSH effects NF-κB activity in multiple ways (255). In the cytoplasm, the inactive NF-κB forms a complex with the IκB that cannot enter the nucleus. Here GSH depletion increases NF-κB activity by IκB glutathionylation. When NF-κB relocates to the nucleus, GSH increases the binding of NF-κB to DNA through the feedback loop (255). Exposure of mAECII cells to ECVE or CSE showed decreased levels of IκBα protein, but no difference in NF-κB protein levels were observed compared to the control group. One potential explanation might be that in the current study whole cell lysate was analysed. Therefore, experiments with the sample fractionated into cytoplasmic and nucleus phase should be performed in the future. Although, analysing signalling mechanisms underlying effect of ECVE is outside of the scope of this thesis, the identification of molecular targets directly affected by depletion of intracellular GSH levels will be a vital goal of future studies.

In summary, the results of current study indicate that ECVE induced alterations of cellular redox homeostasis and various signalling pathways including NF-κB and iNOS which might further lead to lung inflammatory responses as discussed below.

4.2.E-cigarette vapour particle size

It was suggested that *in vitro* measurements of particle size distribution could be a predictive factor for *in vivo* lung deposition patterns (257). Laser diffraction is a rapid and efficient light scattering method used to assess particle sizes in the range of submicron to millimetre (258). Regardless of nicotine content, the MMAD, GSD and FPF of e-cigarette vapour tested were

found to be of similar size. This aligns with the results of studies assessing aerosols generated from e-cigarettes and conventional cigarettes using fast mobility particle size spectrometry (259), spectral transmission methods with the electrical mobility procedure (260) and electrostatic low-pressure impactor (261) that described comparable particle sizes. Contrary, a study by Bertholon and colleagues (262) used an electrostatic low-pressure impactor and found bigger particle size for aerosols from e-cigarettes compared to cigarettes. The difference in particle size might be due to the techniques used to measure the particle size. It has been demonstrated that airborne particles with smaller aerodynamic diameters are likely to be deposited deep in the lung (256). Taking this into consideration, the result from this study suggested vapour produced from e-cigarettes is capable of being deposited in the smaller airways.

4.3.*In vivo* e-cigarette exposure experiments

In order to address the effects of e-cigarette vapour, an integrated e-cigarette vapour generation platform was developed. The current study presents an establishment of representative vaping machine specifically designed for e-cigarettes that enables the control and monitoring of relevant physical parameters (O_2, CO_2, temperature and humidity) during e-cigarette vapour generation. The computer-controlled platform enables the systematic standardization of e-cigarette puffing profiles in a laboratory setting for continuous studies. The standard automated puffing profiles was used: 60 mL puff volume, 2 seconds puff duration, 60 seconds puff interval. Even though, this standard condition for e-cigarette puffing is not in accordance with the standard conditions for CS machines (35 mL puff volume, 2 seconds puff duration, 60 seconds puff interval) ISO3308 (263), it fulfils the "Vapour products–Routine analytical vaping machine–Definitions and standard conditions", ISO 20768, puffing requirements (264).

4.3.1. Plasma nicotine and cotinine levels, blood O_2 saturation and haematocrit

To study the systematic absorption of nicotine by the mice, the plasma cotinine levels were measured after mice were exposed to e-cigarette vapour for 6 hours (acute exposure). Similarly, to exclude hypoxic changes in the e-cigarette vapour exposed mice, the blood O_2 saturation and haematocrit were measure at the end of the *in vivo* measurements.

Nicotine is one of the major constituents of CS and e-cigarettes. In human, 70% to 80% of absorbed nicotine is metabolized to cotinine (265). Plasma cotinine has been widely used as an exposure marker of CS because of its longer half-life and the smaller diurnal variation in

smokers than that of nicotine (136,265). In the presented work, plasma nicotine and cotinine levels were measured after mice were exposed to e-cigarette vapour for 6 hours (acute exposure) and the result indicated measurable plasma nicotine (1.7 to 7.2 ng/ml) and cotinine (3.5 to 9 ng/ml) levels in mice exposed to e-cigarette containing nicotine (18mg/ml) group. The cotinine level indicates systematic absorption of nicotine by the mice. Previous studies have demonstrated elevated plasma nicotine and/or cotinine concentrations in both mice and humans exposed to aerosolized nicotine-containing e-cigarettes (122,266-269). In the presented work nicotine concentrations in plasma after e-cigarette vapour exposure were lower but comparable to human cigarette smokers (10–50 ng/ml) (269,270).

O_2 saturation, the amount of oxygen in the bloodstream transported by haemoglobin, is used as early diagnosis of hypoxia as well as an indicator of disease severity in asthma attacks, chronic lung disease, acute bronchiolitis, and pneumonia (271). The O_2 saturation observed in this study did not vary between experimental groups. Mice exposed to nicotine-containing e-cigarette vapour showed an increase in haematocrit compared to room air exposed control mice. Since the blood O_2 saturation was not significantly different between any of the experimental groups this difference could not be explained by tissue hypoxia, however, this might be a consequence of nicotine. Nicotine has been shown to disrupt oxygen delivery to tissue and stimulates the bone marrow to produce more RBCs and thereby increase haematocrit (272).

4.3.2. E-cigarette aerosol and pulmonary emphysema and PH

CS is main risk factor of development of COPD. COPD is characterized by a slow progressive development of airflow limitation due to chronic obstructive bronchiolitis and destruction of lung parenchyma (emphysema) (37). Moreover, PH is an underdiagnosed condition in COPD patients and up to 70% of COPD patients also suffer from PH (39). PH characterized by increased mean pulmonary arterial pressure greater than 25 mm Hg at rest measured by right heart catheterization. Severe PH increases right ventricular afterload and leads to RV hypertrophy, which is initially compensatory but often leads to RV failure (284). The pathogenesis of COPD is complex and multifactorial (38). The inflammatory response of the lungs to noxious particles and gases, predominantly characterized by increased neutrophil, macrophage and $CD8^+$ T-lymphocyte numbers, contributes to the development of progressive airflow limitation (277). The analysis of conventional smoke-induced alterations of structure and function of airways/alveoli in emphysema and vascular structure/function in PH have been reported previously using animal models of COPD (46,49,273-276). The work presented here was based on a similar experimental design. Mice were exposed to e-cigarette vapour for 8

months and structural and functional lung parameters were assessed. However, whereas some changes were observed in *in vitro* experiments as discussed above, 8 months e-cigarette vapour exposure did not show any major alterations in the lung as well as in pulmonary circulation function/structure in mice. The findings of the current study are consistent with the study that demonstrated no significant changes in the lung function/structure in mice exposed to e-cigarette for 8 months (279). Conversely, a study by Garcia-Arcos et al., (122) showed significantly reduced lung function and airspace enlargement in A/J mice exposed to the inhalation of nicotine (18mg/ml) containing e-cigarette liquids, thus indicating emphysema development. Another study by Larcombe and colleagues (280) using female BALB/c mice exposed for 8 weeks to e-cigarette showed significant impairments in lung function at both functional residual capacity and high transrespiratory pressure.

To the best of my knowledge, at the time of thesis submission there are no studies indicating effects of short- or long-term e-cigarette vapour exposure on the pulmonary circulation and the development of PH. There are a few studies that investigated cardiac function in general in mice exposed to e-cigarette. The result of these studies is also contradictory. Similar to the findings of current work, a short term (3 hours per day for 14 consecutive days) exposure to e-cigarettes resulted in no significant changes in cardiac function (285). In contrast, another study showed only a small changes in cardiac function in mice exposed to chronic e-cigarette for 8 months (279). A potential explanation for the heterogeneity of reported results and the result of this thesis regarding the effects of e-cigarette on function/structure of lung and pulmonary circulation might be due to: i) difference in duration, ii) mode of exposure (nose only verses whole body), iii) difference in puffing topography (this thesis uses a more realistic e-cigarette use), iv) types of e-liquid (the presence of flavour) and v) strains differences. For example A/J mice have been shown to be extremely susceptible to CS-induced emphysema (281).

Moreover, in line with experiments conducted in animals, there is discrepancy in studies of the effect of e-cigarette on humans. 3.5 years cohort study of nine e-cigarette users who have never smoked showed no significant changes in lung function using spirometry tests and no structural abnormalities assessed by high-resolution computed tomography of the lungs from baseline in the e-cigarette users or between e-cigarette users and the control groups (282). In contrast, other studies reported an increased airway resistance and/or decreased specific airway conductance after acute exposure of e-cigarettes with or without nicotine (127,283). There is no available literature regarding the effect of e-cigarette on the development of PH in human.

4.3.3. E-cigarette and cell composition in BALF

Inflammation is a major feature in the pathogenesis of many pulmonary diseases such as pneumonia, acute respiratory distress syndrome, asthma, and COPD (286). Mice exposed to e-cigarette vapour showed altered inflammatory cell composition of BALF. The number of neutrophils, lymphocytes and exudate macrophages were increased, whereas resident alveolar macrophages were decreased in BALF after e-cigarette exposure. Impairments in cellular composition of BALF occurred in the absence of alteration in pulmonary function and in the absence of significant morphological differences of lung structure as discussed above. Moreover, the inflammatory changes were independent of the presence of nicotine. These findings might indicate that the observed changes in BALF composition are due the process of e-liquid vaporisation but not due to the inhalation of nicotine. Previous studies have shown that e-cigarette vapour exposure altered lung inflammatory responses in mice (119,120,122,282). Sussan et al. (120) demonstrated that nicotine containing e-liquid exposure resulted in macrophage-mediated inflammation (58% increase BALF macrophages). However, there was no impact on infiltration of neutrophils, eosinophils, or lymphocytes. In a study of allergic airway disease in mice (123), intratracheal installation of e-liquid containing nicotine led to elevated airway inflammation. Even though e-cigarette exposure did not increase BALF inflammatory cells in studies that employed inhalation exposure (119,121), the levels of pro-inflammatory cytokines in BALF were significantly elevated. Increased inflammation in response to e-cigarette exposure have the potential to elicit pulmonary defences against bacterial or viral infection. Impaired anti-bacterial and anti-viral responses have been shown in mice after e-cigarette exposure (120,121).

The alteration of various signalling pathways which we found by hypothesis and non-hypothesis driven approaches including iNOS and LAMP can be cause of e-cigarette induced modification in immune cell dynamics in the lungs via direct effect on the function of immune cells (248,249, 292) or via altered release of inflammatory cytokines (249, 278).

4.4.Limitations

While interpreting the presented date, the following limitations need to be considered. No standardized methods for preparing and testing e-cigarette vapour extract or even for the e-liquid constituent and e-cigarette atomizers are currently available. There is a need for the scientific community to generate a standard for comparison of research results, as was done for research regarding conventional cigarettes by the University of Kentucky. Furthermore, it should be noted that the presented results cannot be generalized to flavour containing e-liquids

or other e-liquids differing in composition. It is possible that the experiments with e-liquid containing flavouring agents may result in different outcomes. Additionally, for *in vitro* studies the levels of nicotine in the extract were not measured, nonetheless, the extract were prepared by simulating realistic conditions of use. Moreover, the *in vitro* scenario may not entirely reflect the *in vivo* events. Besides, due to the differences in respiratory physiology between humans and mice, and exposure method (active versus passive exposure), direct comparison of results is complicated. Furthermore, accurate measurements of consumption of e-cigarette vapour in the whole body chamber is not possible since skin exposure and ingestion of aerosol residues may occur during grooming. Finally, the result of this study can only be extrapolated to understand the potential effects of e-cigarettes users with no history of smoking. In contrast, most e-cigarette users were conventional cigarette smokers before. This increases the complexity of outcome interpretation of current study.

5. Summary

Cigarette smoking (CS) is the main preventable cause of chronic obstructive pulmonary disease (COPD) and lung cancer. Research of potentially less harmful nicotine delivery devices and tools for smoking cessation resulted in the development of electronic cigarettes (e-cigarette). Despite the lack of appropriate studies regarding their safety, efficacy and health effects, e-cigarettes are gaining popularity as an approach of CS cessation and the number of e-cigarettes users is rapidly growing worldwide, especially among younger generations.

Against this background, the aim of the current study was to investigate the effects of e-cigarette vapour extract (ECVE) on a cytotoxicity, cellular functions and gene expressions in different human cell lines (adenocarcinoma cell line [A549] and human bronchial epithelial cells [HBEpCs]), primary mouse cells (murine alveolar epithelial typeII cells [mAECII] and pulmonary arterial smooth muscle cells [mPASMCs]) and human lung cells (human pulmonary arterial smooth muscle cells [hPASMCs]) as well as to study the long-term effects of e-cigarette vapour on the function and structure of mouse lung and pulmonary circulation in vivo.

Regardless of nicotine, ECVE showed a little (A549, mAECII, and hPASMCs) or no effect (HBEpCs and mPASMCs) on the cell viability compared to the cigarette smoke extract (CSE). Furthermore, ECVE with (18mg/ml) or without nicotine did not alter the cellular respiration of mPASMC as well as the migration of hPASMC and mPASMC. However, only nicotine containing ECVE decreased the proliferation of hPASMC and mPASMC compared to ECVE without nicotine. The hypothesis driven approach showed increased mRNA expression of *iNOS*, *Csf2*, *5HTT* and *Ccna1* in mPASMC after exposure to ECVE with nicotine indicating their possible roles in ECVE nicotine-induced decrease of mPASMC proliferation. Non-hypothesis driven transcriptomic analysis by microarray revealed that genes related to cell cycle, DNA replication and spliceosome were upregulated, while genes of lysosome and metabolic pathways were downregulated in mPASMCs exposed to ECVE regardless of nicotine. Moreover, various metabolic pathways, particularly genes involved in cytochrome P450 and glutathione metabolism were upregulated in mAECII.

Exposure of wild type mice (C57BL/6J) to e-cigarette vapour with (18mg/ml) or without nicotine for 8 months (6 hours/day, 5 days/week) did not result in development of neither lung emphysema nor pulmonary hypertension assessed by lung function, hemodynamic and morphological analysis, respectively. However, long-term exposure to e-cigarette vapour evoked changes in the neutrophils, lymphocytes and macrophages amounts in bronchoalveolar lavage fluid indicating that e-cigarette vapour could induce the alteration of immune cell dynamics in the mouse lungs.

SUMMARY

In conclusion, despite that ECVE is less cytotoxic compared to CSE, exposure to ECVE alters nicotine-independently various cellular signalling pathways in lung cells *in vitro*. Moreover, even though long-term exposure of mice to e-cigarette vapour does not show any functional and structural alterations in the lungs, similar to conventional CS, exposure to e-cigarette prompts the distinct inflammatory responses in the mouse lung. Further experiments are necessary to elucidate the effects of various puffing regimen parameters (e-cigarette topography) and especially the presence of numerous flavours in e-cigarette liquids on the human health.

6. Zusammenfassung

Zigarettenrauchen (CS) ist die vermeidbare Hauptursache für chronisch obstruktive Lungenerkrankung (COPD) und Lungenkrebs. Die Erforschung potenziell weniger schädlicher Nikotinverabreichungsgeräte und Werkzeuge zur Raucherentwöhnung führte zur Entwicklung von elektronischen Zigaretten (E-Zigarette). Trotz des Fehlens geeigneter Studien über ihre Sicherheits-, Wirksamkeits- und Gesundheitsauswirkungen gewinnen E-Zigaretten als Ansatz zur Raucherentwöhnung an Popularität und die Zahl der E-Zigarettennutzer wächst schnell, vor allem bei jüngeren Generationen.

Vor diesem Hintergrund wurden in dieser Arbeit die Auswirkungen von Dampfextrakten von E-Zigaretten (ECVE) auf die Zytotoxizität, Zellfunktion und Genexpression in unterschiedlichen Zelllinien (humane Lungenadenokarzinomzelllinie [A549]; humane Bronchialepithelzellen[HBEpCs]), primären Mauszellen (alveolären TYP-II Epithelzellen der Maus [mAECII]; glatte Muskelzellen der Lungenarterie der Maus [mPASMC]) und humanen Lungenzellen (glatte Muskelzellen der Lungenarterie des Menschen [hPASMC]) untersucht. Weiterhin wurden Langzeiteffekte von ECVE auf die Lungenfunktion, Lungenstruktur und den Lungenkreislauf im Mausmodell analysiert.

Im Vergleich zu Dampfextrakten von herkömmlichen Zigaretten (CSE) hatte ECVE, mit oder ohne Nikotin, einen geringen (A549, mAECII und hPASMC) bzw. gar keinen (HBEpCs und mPASMCs) Effekt auf die Zelllebensfähigkeit. Weiterhin beeinflusste ECVE mit oder ohne Nikotin nicht die zelluläre Atmung von mPASMC und die Migration von hPASMC und mPASMC. Allerdings verringerte im Vergleich von ECVE mit oder ohne Nikotin nur nikotinhaltiger ECVE die Proliferation von hPASMC und mPASMC.

Der hypothesengetriebene Ansatz zeigte die erhöhte mRNA-Expression von *iNOS*, *Csf2*, *5HTT* und *Ccna1* in mPASMC nach Exposition gegenüber ECVE mit Nikotin und deutet auf eine mögliche Rolle in der, durch nikotinhaltigen ECVE induzierten, Abnahme der mPASMC-Proliferation an. Die nicht hypothesengesteuerte transkriptomische Analyse von ECVE exponierten mPASMC-Zellen durch Microarray ergab, dass mit Zellzyklus, DNA-Replikation und Spleißosom assoziierte Gene hochreguliert, während lysosomale und Gene involviert in Stoffwechselwegen nikotinunabhängig herunterreguliert wurden. Darüber hinaus wurden in mAECII verschiedene Stoffwechselwege hochreguliert, insbesondere Gene, welche am Zytochrom P450- und Glutathionstoffwechsel beteiligt sind.

Die Exposition von Wildtyp-Mäusen (C57BL/6J) gegenüber E-Zigarettendampf mit (18mg/ml) oder ohne Nikotin für 8 Monate (6 Stunden/Tag, 5 Tage/Woche) führte weder zur Entwicklung

eines Lungenemphysems noch zu pulmonaler Hypertonie, welches anhand von Lungenfunktionstest und hämodynamischer bzw. morphologischer Analyse beurteilt wurde. Die langfristige Exposition gegenüber E-Zigarettendampf veränderte jedoch die Anzahl der Neutrophilen Granulozyten, Lymphozyten und Makrophagen in der bronchoalveolären Lavageflüssigkeit. Dies deutet darauf hin, dass E-Zigarettendampf die Dynamik der Immunzellen in der Lunge von Mäusen zu beeinflussen bzw. zu verändern vermag.

Zusammenfassend lässt sich sagen, dass durch die Exposition zu ECVE nikotinunabhängig verschiedene zelluläre Signalwege in Lungenzellen gestört werden, aber ECVE im Vergleich zu CSE dennoch geringere zytotoxische Wirkung *in vitro* aufweist.

Auch wenn die langfristige Exposition von Mäusen gegenüber E-Zigarettendampf keine funktionellen und strukturellen Veränderungen in der Lunge aufzeigte, ähnlich wie bei herkömmlichen Zigarettenrauch, lösen E-Zigaretten eindeutige Entzündungsreaktionen in der Lunge von Mäusen aus. Weitere Experimente sind absolut notwendig, um die Auswirkungen von unterschiedlichen Parametern des Puffingschemas und insbesondere das Vorhandensein zahlreicher Aromen in E-Zigarettenflüssigkeiten auf die menschliche Gesundheit zu ermitteln.

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