Universidade do Minho
Escola de Medicina

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UMinho | 2018

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The impact of E-cigarette aerosols in lung development

Estudo do impacto dos aerossóis derivados de cigarros eletrónicos no desenvolvimento pulmonar

Dissertação de Mestrado

Mestrado em Ciências da Saúde

Trabalho efetuado sob a orientação da

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e da

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Outubro de 2018

“Science means constantly walking a tightrope between blind faith and curiosity; between expertise and creativity; between bias and openness; between experience and epiphany; between ambition and passion; and between arrogance and conviction - in short, between an old today and a new tomorrow.”

Heinrich Rohrer (1933 - 2013)

ACKNOWLEDGMENTS

Quero exprimir o meu o meu profundo agradecimento a todas as pessoas que de alguma forma contribuíram para esta dissertação. Agradeço à Universidade do Minho, especialmente à Escola de Medicina e ao Instituto de Ciências da Vida e Saúde (ICVS) e ao Centro de Biologia Molecular e Ambiental (CBMA) que foram o meu lar durante o desenvolvimento deste trabalho.

Primeiramente gostaria de expressar o meu agradecimento às minhas orientadoras. Agradeço à Doutora Rute Moura por ter aceitado entrar neste desafio, não só novo para mim, mas também para ela. Por me ter motivado e desafiado sempre, o que me permitiu crescer não só como pessoa, mas também como futuro investigador, pelos conselhos, motivação e pela simpatia. Sem a sua ajuda este trabalho não se teria desenvolvido. À Doutora Paula Sampaio, quero agradecer a forma calorosa como me recebeu e aceitou também este desafio, por me mostrar que devemos ter confiança e certeza no trabalho que fazemos, e me aconselhar que mesmo o caminho seja laborioso, há sempre uma forma de atingir os nossos objetivos.

Aos colaboradores do Centro de Engenharia Biológica (CEB), isto é, à Zlatina Genisheva, ao Eduardo Coelho e ao Professor Doutor José Maria Oliveira, que se disponibilizaram para ajudar na parte crucial deste trabalho e permitiram que o mesmo passasse ao nível seguinte. Transmitindo-me novos conhecimentos e outras perspetivas científicas no processo. Quero também deixar um agradecimento ao Doutor Pedro Carvalho, que contribui neste trabalho com o seu conhecimento em cigarros eletrónicos, permitindo que este trabalho se tornasse mais equilibrado, e ao Professor Doutor Jorge Correia Pinto pelo apoio.

De seguida quero agradecer às pessoas com que me acompanharam todos os dias ao longo deste trabalho, a todo pessoal do laboratório I1.03, do domínio das Ciências Cirúrgicas e também laboratório Micro II. No entanto quero deixar o meu agradecimento às pessoas que se destacaram, começando pela Ana Gonçalves e o Hugo Silva, pelos concelhos e motivação que transmitiram, à Violina Barbosa que me acompanhou neste trabalho sempre com uma palavra amiga, ao Pedro Silva pela motivação e energia e ao Henrique Silva pela sua animação e, mais importante, pelas suas capacidades enquanto operador de câmara. Quero também agradecer à Joana Pereira, à Patrícia Silva e à Marisol Dias que muito bem me receberam no seu grupo e me acompanharam neste trabalho e deixar um agradecimento especial ao Augusto Barbosa que me ensinou e supervisionou neste trabalho.

Quero também agradecer às minhas colegas de mestrado, principalmente à Ana Catarina Matos, à Sónia Celeiro, à Laura Silva e à Marta Lopes pela sua amizade.

Quero também agradecer aos meus pais pelo apoio. À minha irmã e também à Ana Delgado que foram as minhas companheiras de tese fora do laboratório, e aos meus amigos, especialmente à Andreia Brandão, Joana Ferreira, Helena Costa e Ana Mendes, que sempre se preocuparam com o estado do meu trabalho e me transmitiram forças, um muito obrigado.

The work presented in this dissertation was performed at the Surgical Sciences Research Domain of the Life and Health Sciences Research Institute (ICVS), School of Medicine, University of Minho, Braga, Portugal (ICVS/3B's – PT Government Associate Laboratory, Braga/Guimarães, Portugal). The work was supported by FEDER funds, through the Competitiveness Factors Operational Programme (COMPETE), and by National funds, through the Foundation for Science and Technology (FCT), under the scope of the Project POCI-01-0145-FEDER-007038; and by the Project NORTE-01-0145-FEDER-000013, supported by the Northern Portugal Regional Operational Programme (NORTE 2020), under the Portugal 2020 Partnership Agreement, through the European Regional Development Fund (FEDER).

ABSTRACT

Smoking is a major public health issue associated with high mortality rates. Currently, new nicotine delivery systems have been developed aiming to overcome the well-known toxic effects of conventional cigarettes (c-cig). Electronic cigarettes (e-cig) are advertised as a healthier way to smoke and are becoming popular worldwide.

E-cigs vaporize a liquid creating an aerosol that reaches the lung and, subsequently, its components move to the bloodstream thus influencing other tissues. Recent studies have shown that c-cig smoke and nicotine exposure affects lung development, however, the effect of e-cig aerosol in lung development is less known so far.

Macrophages are the innate immune cells that serve as the first line of defense against toxic substances, *e.g.* smoking and vaping particles. Several studies revealed that smoking alters macrophage function due to its toxicity. In fact, smoking-related conditions, such as chronic obstructive pulmonary disease, present an altered innate immunity profile.

In this sense, the aim of this work was to compare the potential effect of e-cig aerosol and c-cig smoke in chick lung development and in macrophage survival. The chick model is a good option to study lung development since it presents both morphological and molecular similarities with the mammalian embryo, in early developmental stages.

For this purpose, a smoke/aerosol system was optimized to obtain a saturated culture medium. Then, the exposed medium was used to perform *in vitro* chick lung explants and to grow a macrophage cell line. The impact of aerosol/smoke in lung development was evaluated by morphometric analysis and toxicity assays (extracellular LDH activity and TNF-α release). The impact of aerosol/smoke in macrophages was assessed by cell viability assays (MTT and LDH).

Comparing to control, e-cig aerosol leads to a decrease in lung size of approximately 10% and increases pro-inflammatory cytokine release about 7 times. Regarding c-cig smoke, e-cig aerosol has a less harmful impact in lung growth with an increase between 12 and 30% in morphometric parameters. The viability of the macrophages exposed to e-cig aerosol suggests that metabolic viability is altered, however, it seems that aerosol does not promote cell damage.

This work shows, for the first time, that e-cigs affects lung development, however, the effect is less severe than c-cig. E-cig and c-cig have an impact in macrophages viability, although e-cig aerosol effect is not as exacerbated as c-cig smoke.

RESUMO

O tabagismo é um problema de saúde pública associado a elevadas taxas de mortalidade. Recentemente, foram desenvolvidos novos sistemas de libertação de nicotina para substituir os cigarros convencionais (c-cig) e assim evitar os seus efeitos nocivos. Os cigarros eletrónicos (e-cig) são publicitados como uma alternativa menos nociva que o tabaco convencional e, por esse motivo, a sua utilização é cada vez mais frequente.

O e-cig vaporiza um líquido criando um aerossol que é inalado, atinge os pulmões e, posteriormente, passa para a corrente sanguínea alcançando outros tecidos. Estudos recentes mostraram que a exposição à nicotina e ao fumo do cigarro prejudicam o desenvolvimento pulmonar. No entanto, o efeito do aerossol do e-cig no desenvolvimento pulmonar ainda não é conhecido.

Os macrófagos são um tipo celular, pertencente à imunidade inata, que atua como a primeira linha de defesa contra substâncias tóxicas tais como, por exemplo, o fumo do tabaco ou o aerossol do e-cig. Estudos anteriores mostraram que o tabaco altera a função dos macrófagos devido à sua toxicidade. Por outro lado, há estudos que sugerem que o aerossol do e-cig provoca o mesmo efeito. Na verdade, o tabagismo está associado a doenças pulmonares, tais como a doença pulmonar obstrutiva crónica, que apresentam um perfil alterado da imunidade inata.

Assim sendo, o objetivo deste trabalho foi comparar o potencial efeito do aerossol do e-cig com o fumo do tabaco no desenvolvimento pulmonar de galinha, e na sobrevivência dos macrófagos. O modelo de galinha surge como uma alternativa válida para avaliar o pulmão em desenvolvimento, visto que apresenta semelhanças morfológicas e moleculares com o embrião de mamífero, nos primeiros estadios do desenvolvimento.

Para este propósito, foi necessário otimizar um sistema de aquisição de fumo/aerossol de forma a obter meio de cultura saturado. Posteriormente, este meio foi utilizado para realizar culturas de explantes de pulmão de galinha *in vitro*, e culturas de uma linha celular de macrófagos. Os efeitos no desenvolvimento pulmonar foram avaliados pela análise morfométrica e também por ensaios de toxicidade (atividade da LDH extracelular e libertação de TNF-α). Por outro lado, ensaios de viabilidade celular (MTT e atividade da LDH extracelular) foram realizados na linha celular de macrófagos.

Comparando com o controlo, o aerossol diminui o tamanho do pulmão em aproximadamente 10%, e aumenta em 7 vezes a libertação de citocinas pro-inflamatórias. Em relação ao fumo do c-

cig, o aerossol do e-cig tem um efeito menos prejudicial no crescimento pulmonar, com um aumento dos parâmetros morfométricos entre 12 a 30%. A viabilidade dos macrófagos expostos aos aerossóis do e-cig sugere que a viabilidade metabólica pode estar alterada, no entanto, o aerossol parece não provocar danos celulares.

Este trabalho mostrou, pela primeira vez, que o aerossol do e-cig afeta o pulmão em desenvolvimento, no entanto, o seu efeito é menos agressivo que o fumo do c-cig. O e-cig e o c-cig têm impacto na viabilidade dos macrófagos, no entanto, o efeito do e-cig não é tão exacerbado como o do c-cig.

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Abbreviation List

ANOVA	Analysis of variance
C-cig	Conventional cigarette
DAD	Diode array detector
E-cig	Electronic cigarette
e-juice	Electronic juice
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
g	Gravitational force
GC-MS	Gas chromatography-Mass spectrometry
GRAS	Generally recognized as safe
h	Hour
HNB	Heat-not-burn tobacco
HPLC	High-performance liquid chromatography
IL	Interleukin
ISO	International Organization for Standardization
LSD	Least Significant Difference
LDH	Lactate dehydrogenase
LPS	Lipopolysaccharide
min	Minute
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
Mφ	Macrophages
nAChRs	Nicotinic acetylcholine receptors
NDS	Nicotine delivery systems
NK	Natural killer
PB	Phosphate buffer

PBS	Phosphate buffered saline
PG	Propylene glycol
rpm	Rotations per minute
TNF-α	Tumor necrosis factor α
TPM	Total particle mass
UPLC	Ultra performance liquid chromatography

FIGURE LIST

TABLE LIST

1. Introduction

1.1. General considerations

Tobacco is a major public health issue associated with high mortality rates [1] mostly related to pulmonary diseases [2]. Only in 2016, 7.1 million worldwide deaths were caused by tobacco use, of which approximately 884000 deaths were caused by passive smoking. Smoking impacts not only the respiratory system (e.g., cancer, chronic obstructive pulmonary disease) but increases the risk of dying due to heart diseases or stroke. Moreover, tobacco use during pregnancy has serious consequences, and infants are more susceptible to develop congenital disorders, cancer, lung diseases, and sudden death [3].

Economic costs of smoking represent a huge economic burden for society. Expenses are related to smoking-related illness and death, second-hand smokers, loss of income, disability, among other things. In 2009, in Europe, the impact of smoking was 544 million euros, and it is expected to be higher in 2030 [4]. Accordingly, it is crucial to develop effective prevention programs and to encourage tobacco cessation. In this sense, alternatives are being offered by the industry as, for instance, electronic nicotine delivering systems. Nonetheless, the impact of these new systems in health is yet to be determined.

1.2. Smoking

The use of tobacco, also called conventional cigarette (c-cig) is maintained due to the addictive effect of the nicotine present in dry leaves [5]. C-cigs are a blend of dry tobacco leaves processed with tar, humectants, and other countless compounds. More than 7000 compounds are detected [6] and the great majority identified as toxic (tar, nitrosamines, etc.), carcinogenic (*e.g.* mycotoxin, cadmium) or teratogenic [6-8].

The deleterious effects of tobacco smoking have been already extensively documented. At the molecular level, smoking can cause several consequences, such as oxidative stress [9] and an increase of the inflammatory response [10]; these features are the basis for several lung problems as, for example, chronic obstructive pulmonary disease, asthma and lung cancer [11,12]. The lung is not the only organ affected by tobacco smoking and there are multiple tobacco-induced diseases, as for instance rheumatoid arthritis [13] and ulcerative colitis [14]. Moreover, it has been shown that maternal smoking promotes placental development deficits [15]; impairs embryo development causing low birth weight, premature birth, spontaneous abortion [16,17], congenital heart malformations [18], musculoskeletal [19] and brain defects [6]; may be genotoxic [20]; and

alters the innate and acquired immune of newborns [21]. Overall, smoking is noxious to human health triggering several adult diseases and negatively affecting embryo development.

Toxic effects of smoking during embryo development have been reported by several studies. Kim *et al.* (2017) showed a dysregulation in the cell cycle and an increase in cell cytotoxicity and migration in placental cells due to smoking [22]. Massarky *et al.* (2015) assessed the total particle mass (TPM) generated by smoking in the zebrafish embryo. Zebrafish embryos exposed to TPM displayed a decrease in body size, impairment in vascular development, high mortality rate as well as hyperactivity. Altogether, these results point to the teratogenic effect of smoke [23]. Kemény *et al.* (2017) exposed mice to c-cig smoke over 6 months and evaluated pulmonary emphysema progression; the authors observed that chronic smoke exposure causes an increase in airway spaces, thus increasing pulmonary emphysema, and triggers inflammation and atelectasis [24].

1.3. Nicotine delivery systems

Nicotine Delivery Systems (NDS) were developed more recently in the attempt of finding alternatives to conventional cigarettes thus reducing the noxious effects associated with smoking. In this context, the electronic cigarette (e-cig) and the heat-not-burn tobacco (HNB) emerged (Figure 1).

Figure 1. **Alternatives to conventional cigarettes.** Representative examples of an electronic cigarette (A) and a heat-not-burn tobacco system (B) [25,26].

HNB is a device that instead of burning, heats around 260 °C, a disposable tobacco stick soaked in propylene glycol. This process produces smoke that is composed of nicotine and other chemicals. HNB is commercially available since 1988 and it has been advertised as a healthier option than tobacco burning. However, this heating device did not lure consumers to replace

tobacco burning. Moreover, the recent development and popularity of the e-cig caused a decrease in the sales of the HNB device. For this reason, the tobacco companies tried to overcome this issue by developing an improved HNB device [27]. These new products emerged recently and for this reason, there is not much information [28].

Regarding the e-cig, one of the first systems was created in 2003, by Hon Lik, as an electronic device that generated a vapor/aerosol containing nicotine that was then inhaled by the user. This type of devices [29] are commercialized around the world and advertised as a less harmless option than c-cig smoking [30]. The e-cig was created not only with the purpose of replacing burning c-cig, thus reducing the toxic and carcinogenic substances generated by smoking, but also to help quit smoking [31]. However, there is some controversy on this issue. Part of the public health community considers that e-cig is a helpful tool to quit smoking hence resulting in public health benefits. On the other hand, the opposing part considers e-cig as a threat since it reverts all the improvements made over the past decades in tobacco control policies. These new habits converted *vaping* in the new type of socially accepted smoking [32]. The e-cigs subject is, indeed, a hot topic. These devices have become quite popular over the last years, however, their safety has not been fully proven. Thus far, there are no epidemiological studies to determine the effects of vaping.

1.4. Electronic cigarette

1.4.1. Composition and mechanism of action

Since the development of the first device, e-cigs have been constantly improved, however, the mechanism of action is still the same. E-cig devices (Figure 2) are composed by a lithium battery, a heating element (normally a coil or resistance) that, when activated, can reach temperatures around 200-300 °C [33,34]. The heating element is near or inside a cartridge (or reservoir) where the electronic juice (e-juice) or electronic fluid (e-liquid) is stored [23,35].

E-cig can be used either by manual action, *i.e.*, activating the device by pressing a button or automatically, by aspirating the mouthpiece which then activates an airflow sensor [37]. Both events lead to the activation of the battery, which provides power to the coil or resistance to increase the temperature of the cartridge. When exposed to high temperatures, the e-juice vaporizes creating

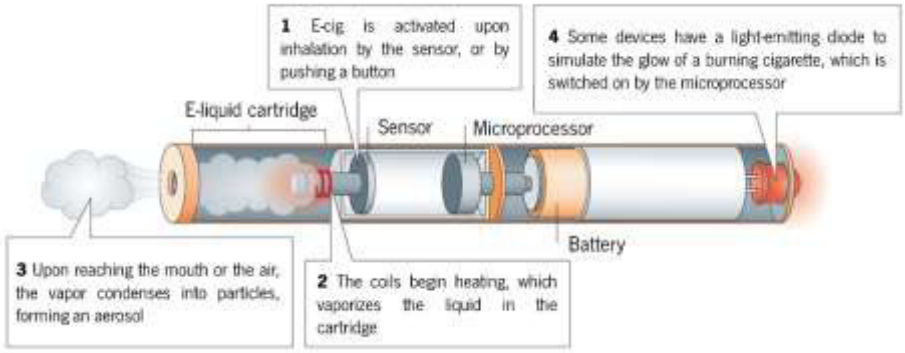

Figure 2. **Electronic cigarette components and mechanism of action.** Electronic nicotine delivery device composed by a lithium battery which provides energy to a heating element (coil). The coil temperature rises up to 300 °C and vaporizes the nicotine solution in the cartridge. Adapted from Benowitz *et al.* (2017) [36].

a vapor (aerosol) that is inhaled by the user, known as vaper [38,39]. For this reason, the usage of e-cig is also designated as *vaping*. Unlike smoking, vaping does not burn tobacco, and this feature contributed to the popularity of this type of devices [1,37].

Since 2003, e-cigs were progressively updated to improve the vaper experience and can be classified into 4 groups (Figure 3): first, second, third and fourth e-cig generation [23,34]. The first-generation e-cigs had the same appearance as a c-cig and were meant to be used only once (Figure 3 A). The second-generation e-cig presented the first rechargeable lithium battery, they were refillable and known as *vape pen* because they looked similar to a pen or a laser pointer (Figure 3 B). The third-generation e-cig, called *mod*, had powerful batteries (with higher voltages) and a larger tank allowing to store higher volumes of e-juice (Figure 3 C). Finally, the last generation e-cig named *regulated mod* has improved batteries with a higher range voltage allowing the vaper to regulate the desired nicotine dose (Figure 3 D) [23,29,40].

These new features allow tailoring of the e-cigs by the vaper by changing the cartridge to bigger tanks or by using more potent batteries. However these "homemade" changes create huge variability in the aerosol that is produced; for instance, high voltage batteries may overheat the coil thus increasing the chance of explosion [45] and the probability of releasing toxic compounds from the e-juice [46,47]. These "domestic" device modifications make it very difficult, virtually impossible, for a health organization to evaluate the effects of e-cigs aerosol, since vapers do not use e-cig correctly after purchasing it. Another important source of variability is the e-juice; this solution has a standard composition, however, the amount of nicotine and the presence of flavors may have a different impact on human health.

Figure 3. **Electronic-cigarette types.** A) first-generation, B) second-generation, C) third-generation and D) fourth-generation [41-44].

1.4.2. E-juice

E-juice, i.e. the solution loaded in the e-cigs cartridge, is composed of propylene glycol and vegetable glycerol [8]; these two compounds serve as carriers for other substances and are known as humectants. The e-juice may contain different concentrations of nicotine ranging from 0-24 mg/L [48]. Furthermore, e-liquids may contain different flavors [49-51].

1.4.2.1. Glycerol

Glycerol or glycerin is present in many products, such as detergents or soaps, in cosmetics, medicines, paper, and dyes or even in foods and drinks; it has been generally recognized as safe (GRAS) since 1959 as a general-purpose food additive and is permitted in food-packaging materials [11,52]. Glycerol is considered a non-toxic substance and widely used in everyday products [12].

However, in these products, glycerol is maintained in its natural form, while in the e-juice it is vaporized. When heated, glycerol can generate acrolein, glycidol, and formaldehyde that are considered toxic substances [53]. In fact, Shen *et al.* (2016), reported that exposure to aerosol from e-juice carrier fluid, *i.e.*, without nicotine, does not induce cell toxicity responses in human bronchial epithelial cells; however, the aerosol exposure is not risk free, since it induces discrete transcriptomic alterations associated with metabolism, immunity and signal transduction pathways, independently of the nicotine content [33]. Additionally, it has been shown in rat that short-term and long-term exposure to aerosolized glycerol (3.91, 1.93, or 1.0 mg/L; 0.662 mg/L, respectively), induced minimal to mild squamous metaplasia of the epithelium lining the base of the epiglottis, without any changes in the lung [54].

1.4.2.2. Propylene glycol

Propylene glycol (PG), in the same way as glycerol, is a GRAS substance (Regulation 21 CFR 184, GRAS) [55], *i.e.* it is considered non-toxic in its natural form. This substance is present in pharmaceutics, cosmetics, and food. PG is used to produce the artificial smoke or mist by "fog" machines used, for example, on television [56]. When heated, PG suffers a thermal breakdown and generates propylene oxide, acetaldehyde, acetol, formaldehyde, and other toxic compounds. Moreover, it has been shown that depending on PG concentration in the e-juice, free radicals may appear and set cells under oxidative stress, therefore promoting apoptosis [53,57].

1.4.2.3. Nicotine

Nicotine is the main alkaloid present in tobacco leaves and it is the addictive substance present in c-cig and in the e-juice. Nicotine can reach the organism through different NDSs, *e.g.*, nicotine gum or transdermal patch. Nonetheless, nicotine is often obtained by inhalation of c-cig smoke or e-cig aerosol. Nicotine is easily absorbed through the skin and lungs [36,58,59]. After being absorbed nicotine enters the bloodstream and reaches the liver, kidney, and lung where it is metabolized, and the brain where it exerts its main action [60]. However, there is evidence that indicates that the impact of nicotine in the lung is different from the impact of circulating nicotine in the whole organism [61]. In fact, nicotine is highly poisonous and the average lethal dose to humans is about 1-13 mg per kg of body weight [62]. Therefore, it is crucial to eliminate nicotine from the human body. Approximately 80% of nicotine is converted to cotinine by the liver and excreted through urine [60].

Nicotine binds to nicotinic acetylcholine receptors (nAChRs) acting as an agonist of acetylcholine [63]. This means that nicotine activates these ion gate channels, promoting cation influx and activating signal transduction cascades that regulate cellular processes such as apoptosis, proliferation, and differentiation. nAChRs are present in neuronal and in non-neuronal cells, *e.g.*, nAChRs can be found in lung, immune and endothelial cells [35,58,64]. There are numerous studies about the impact of nicotine in the nervous system. For instance, it has been reported that, in rats, nicotine at 5.01, 5.72, 6.44, 7.13, 20.41 and 43.1 mg/kg/day causes degeneration in specific brain areas (medial habenula and fasciculus retroflexus) in a dose-dependent manner [65]. On the other hand, it has been shown that 0.4 mg/kg/day improves memory and information processing (learning) in adult rats [66]. Regarding the lung, it has been

reported that nicotine administration in mice (2 mg/kg/day, 100 μg/mL) increases air spaces, and in rats (6 mg/kg/day or 40 μg/μL) promotes fibrosis [67,68]. Concerning the immune system, it has been shown that nicotine suppresses inflammation in mouse placenta (10 μmol/L) and induces oxidative stress, in a dose-dependent manner (1, 5, 10, 25 and 50 mmol/L), in mice peritoneal macrophages [69,70].

Nicotine is classified as a teratogenic molecule, *i.e.*, it induces malformations during embryogenesis and fetus development [71,72]. For instance, Bao *et al.* (2016) have shown that 10 μmol/L of nicotine inhibits LPS (lipopolysaccharide present in gram-negative bacteria wall)-induced cytokine production and leukocyte infiltration in rat placenta explants [69]. El-Betagy *el al.* (2015) have reported that nicotine injection in fertilized eggs (1.6 mg/kg/egg) induced histological and structural changes in cerebellar cortex of the chicken embryos [73]. Additionally, chick embryos exposed to a 0.0001% nicotine solution, *in ovo,* exhibited a decrease in femur size and collagen production, thus affecting bone formation [74]. Moreover, zebrafish embryos exposed to nicotine (30, 60, 120 or 240 μmol/L) displayed a dose-dependent increase in locomotor activity due to the stimulation of neurons by nicotine [75].

1.4.2.4. Flavors

There is a myriad of flavoring options for the e-juice [23]. The addition of flavoring was a strategy that convinced many smokers to become vapers and attracted non-smoker adolescents [8]. Flavors, in its natural form, are considered GRAS and are widely used in the food industry [76].

There are few studies reporting the effect of different flavors in e-juices [49,77], however, the products that are formed after heating are still unknown [76]. Bitzer *et al.* (2018) reported that flavorings may influence reactive free radical production. For instance, cinnamon flavor is one of the most harmful, because it produces free radical species that promote lung cell inflammation [76]. Also, mice exposed to flavored tobacco e-juice displayed signs of lung inflammation and a decrease in cell viability when compared to fluids without flavoring.

Menthol is one of the preferred flavors and can be present in both e-cig and c-cig. Therefore, e-fluid aromatized with this flavor was already studied. Neilson *et al.* (2015) performed an air-liquid interface culture exposing tracheobronchial epithelial cells directly to c-cig smoke and e-cig aerosol aromatized with menthol and tobacco flavor, via a smoking machine. These authors showed that

the aerosol obtained from menthol e-fluid did not have a significant impact in the metabolic viability and transepithelial electrical resistance when compared with controls; additionally, when compared to c-cig smoke exposure, viability was significantly higher [78]. Moreover, Sundar *et al.* (2016) exposed human periodontal ligament fibroblasts directly to a set of e-cig aerosols, with different flavors (tobacco and menthol) and nicotine levels. This study revealed that menthol flavor increases the release of pro-inflammatory cytokines and other inflammation mediators, similarly to the other flavor used in the study [79]. Regarding burned tobacco products, there is no evidence that menthol exacerbates the toxic effects of tobacco, the only known effect is that menthol reduces nicotine metabolism rate, thus prolonging nicotine effects [80].

Overall, these studies have shown that flavorings can affect lung cells and interfere with the immune system, however, the toxicity of these substances in the e-cig aerosol is not fully recognized and, for this reason, additional studies are required [63].

1.4.3. E-cig vs. c-cig & NDS

There are studies reporting noxious e-cig health effects, mostly in relation to the toxic substances present in the aerosol (please see previous sections). Nevertheless, there are scientists defending these devices and arguing that e-cig studies should be carefully reviewed, mainly because some researchers tend to exaggerate the obtained results [81]. Conversely, the deleterious effects of conventional smoking are widely accepted. In table 1 the main features of both c-cig and e-cig are summarized.

E-cig use is not completely risk-free since the aerosol contains some toxic compounds; however, it has fewer toxic substances than c-cig smoke and the detected levels fit the safety range [71]. In table 2, the main substances detected in smoke or aerosol are displayed. Additionally, some studies have quantified the nicotine present in smoke/aerosol, or in their extracts, and in general, smoke or smoke-saturated solutions exhibits more nicotine ($1x10^{-3}$ - 45.6 mg/L) than aerosol or aerosol-saturated solutions ($1x10^{-3}$-2.54 mg/L) [20,83,84].

Table 1. **Comparison between conventional and electronic cigarette.**

	Conventional	**Electronic**
Basic composition	>7000 constituents	Propylene glycol and glycerol
Nicotine	Present	Optional, different concentrations
Flavorings	Limited	Countless options
Combustion	Present	Not present
Generates	Smoke	Vapor (aerosol)
Odor generation	Present	Not Present
Consumption time	Approximately 2 min	Vaper option, maximum until e-juice fully consumed
Cost	More expensive (disposable)	Cheaper (reusable)
Danger	Toxic and carcinogenic compounds detected	Toxic compounds detected (more studies required)
Cytokine release	Present	Present
ROS production	Present	Present
DNA damage	Present	Present

Table 2. **Comparison of the amount of the most common substances detected in smoke/aerosol in μg/L and the detection methods.**

Compounds	Smoke (μg/L)	Aerosol (μg/L)	Detection method
Acetaldehyde	$1.2x10^{-1}$-2919	$2.0x10^{-3}$-18	HPLC[a] [84,82], HPLC-DAD[b] [85]
Acetone	$6.4x10^{-2}$-1222	$2.0x10^{-2}$-11	GC-MS[c] [84], HPLC [82]
Acrolein	289	4-11	HPLC [82], HPLC-DAD [85]
Carbon monoxide	$1.2x10^{-5}$	ND[d]	QC[e] measurements [82]
Carbonyls	5404	64	HPLC [82]
Cotinine	3130	ND	HPLC [82]
Formaldehyde	$8.6x10^{-2}$-176	$1.2x10^{-2}$-22	GC-MS [84], HPLC [82], HPLC-DAD [85]
N-nitrosonornicotine	5.4-79	$4.1x10^{-4}$-1.9	UPLC-MS[f] [85], GC-MS [51]
4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone	3.1-21.7	$2.7x10^{-3}$-5.2	UPLC-MS [85], GC-MS [51]
Toluene	$4.4x10^{-2}$	$<1.0x10^{-3}$	GC-MS [84,85]

[a]HPLC (High-performance liquid chromatography)

[b]HPLC-DAD (HPLC coupled to a diode array detector)

[c]GC-MS (Gas chromatography-mass spectrometry)

[d]ND (not detected)

[e]QC (quality control)

[f]UPLC-MS (Ultra performance liquid chromatography-MS)

This year, the UK government reviewed e-cig devices and revealed that they are at least 95% less harmful than tobacco [81,86]. There are some studies that corroborate this conclusion. For instance, Palpant *et al.* (2015) compared the effects of e-cig aerosol and c-cig smoke in heart development, and in *in vitro* human embryonic stem cells stimulated to differentiate in cardiac progenitor cells. C-cig had a more aggressive impact than e-cig; furthermore, cells displayed a decrease in the expression levels of cardiac transcription factors after e-cig and c-cig exposure. These results suggest that smoking and vaping delay early heart development and cardiac cell differentiation [15] but demonstrate that c-cig is more damaging that e-cig. However, further studies are needed for both e-cig and c-cig to complement 2D-cell studies.

Another important aspect of the e-cigs is the quitting effect when compared with other NDS, such as the nicotine gum and patches; e-cig seems to be more efficient to help smokers to quit [31]. Moreover, e-cigs display a quicker nicotine delivery when compared to other types of NDS, *i.e.* nicotine provided by the e-cig reaches the brain faster. However, the most efficient way to quit smoking is abstinence [31].

To conclude, e-cig is becoming progressively more important at the global scale so new approaches/studies to gain knowledge about its impact/risks are mandatory.

1.5. Lung development

The lung is a crucial organ for life since it is responsible for performing gas exchange and uptaking oxygen and eliminating CO_2 [16]. Simultaneously, this organ also uptakes other molecules/compounds present in the inhaled smoke/aerosol from c-cig and e-cig, respectively.

Lung development is a complex process characterized by multilevel anatomical, mechanical and biochemical events [87]. It depends on an intricate crosstalk between mesenchymal and epithelial cells mediated by different signal pathways, *e.g.*, wingless-related integration site (WNT) or retinoic acid, that ultimately regulate proliferation, differentiation, and apoptosis [88].

1.5.1. Lung embryonic stages

Mammalian lung development is characterized by five stages: embryonic, pseudoglandular, canalicular, saccular and alveolar (Figure 4) [89,90].

In the embryonic phase, the anlage of lung and trachea is formed from the primitive foregut, giving rise to the two primary buds (main bronchi) that later will become the lung lobes [16]. Next, in the pseudoglandular phase, branching morphogenesis occurs, *i.e.*, the primary buds repeatedly bifurcate and elongate distally to originate the new buds (secondary buds): this process is responsible for the formation of the tree-like lung structure [91]. Additionally, later in this stage, mesenchymal cells start to differentiate to cartilage and smooth muscle cells, while epithelial cell differentiates to goblet, neuroendocrine and ciliary cells [92].

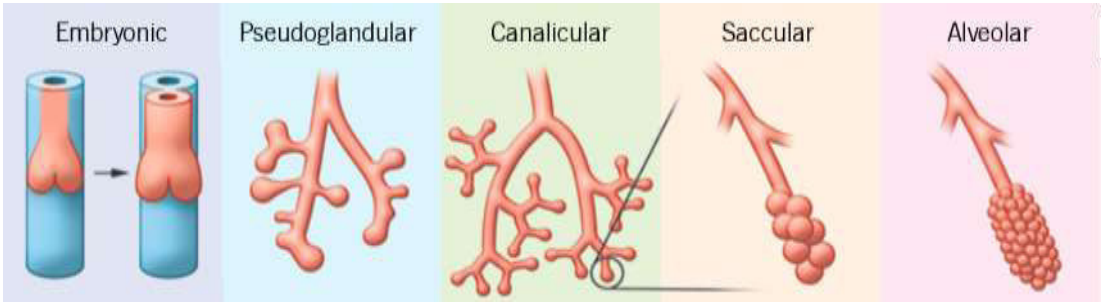

Figure 4. **Lung developmental stages.** Schematic representation of the five stages. Adapted from Rackley *et al.* (2012) [90].

In the canalicular phase, bronchioles develop, the vascular network responsible for gas exchange is completed [76] and the surfactant starts to be produced [16]. Regarding the saccular phase, the air spaces expand, type I and II epithelial cells are well defined, cartilage formation is complete and smooth muscle emerges around the bronchioles [16,89]. Finally, the alveolar phase is characterized by alveoli formation and secondary septation of the bronchioles gives rise to the alveolar ducts and terminal alveoli; angiogenesis is extended thus maximizing the gas exchange surface, preparing the fetus to breathe after birth [89,92].

1.5.2. Chick model

In the early developmental stages, embryos are very similar amongst different species since, in fact, they undergo comparable molecular and anatomic processes. The chick embryo (*Gallus gallus*) is no exception to this rule. Indeed, the chick embryo is widely used for embryological studies [93] and presents some advantages and disadvantages over rodent models, which are summarized in table 3.

Table 3. **Chick model: advantages vs. disadvantages.**

	Advantages
Chick model	Inexpensive [94]
	Does not require animal facilities and maintenance [93]
	Develops *ex utero*, no need for progenitor sacrifice [93]
	Easy to manipulate [94]
	Development precisely staged [95]
	Genetic and molecular similarities in the first stages of life [93]
	Disadvantages
	Not appropriate for genetic manipulation
	Nonmammalian
	Not suitable for late development studies
	Long breading cycle (over six months)

Regarding lung development, the chick embryonic lung shares molecular and morphological similarities with the mammalian lung despite the obvious differences in the adult organ. Chick lung branching occurs laterally from the primary bronchus, shaping the secondary buds, while the main bronchus elongates distally [96]. The lateral branching is very similar to one of the branching routines of the mammalian lung [89]. Moreover, the molecular mechanisms underlying the basic events of branching morphogenesis are conserved between the two species [97-101]. For these reasons, the chick embryo is a good option to study lung development at early stages.

1.5.3. Impact of vaping in the lung

C-cig is composed of toxic and carcinogenic compounds that elicit the noxious effects of smoking in the adult and developing lung, as previously referred (please see section 1.2.). On the other hand, there are some sociological studies [102-106] and case reports [107,108] describing the impact of e-cig in general, and in the adult lung, in particular. Finally, some studies have shown the impact of aerosols in cell lines, e.g. fibroblast, skin, lung (cancer, endothelial), macrophages [109-112]; however, only a few studies have addressed the impact of aerosols in

embryo development as mentioned previously [15,113]. Furthermore, the effect of vaping in lung development is still quite unknown.

McGrath-Morrow *et al.* (2015) exposed neonatal mice to e-cig aerosol and control to room air. Nicotine and cotinine levels in plasma and urine were quantified, and lung inflation and cell proliferation evaluated in lung tissue. The authors found that aerosol exposure during the first 10 days of life modestly impairs alveolar growth and decreases proliferation [113]. However, there is no data regarding the effect of aerosol exposure during lung development. E-cigs are becoming increasingly attractive to pregnant women. Taking into consideration that it has been shown that aerosols may contain toxic substances, it is crucial to understand whether aerosols impact in lung development and properly advise the population about their potential effect [58].

1.6. Innate immunity

The immune system is responsible for protecting the human body against harmful influences/events. This system can be divided into three levels (Figure 5): the anatomic and physiologic barriers, the innate immunity and the acquired immunity [114]. The anatomic and physiologic barriers includes, for example, the skin; however, if the barriers are damaged, and consequently, their protective effect fails, the innate immunity compensates this failure. Cells from this immunity level are the organism's first line of defense against threats [115,116], such as microorganisms or compounds/particles found in smoke and aerosols. The innate immunity is composed by neutrophils, basophils, eosinophils, dendritic cells, natural killer (NK) cells, monocytes and macrophages (Mφ) [116]. These cells are responsible for recognizing threats and interact with T and B lymphocytes (acquired immunity cells) to eliminate them and restore homeostasis [117].

1.6.1. Macrophages

Macrophages are mononuclear phagocytes that have an important role in development and homeostasis. The majority of Mφ present in tissues are derived from embryonic progenitors, such as fetal monocytes and yolk sac-derived Mφ [119], that are called tissue-resident Mφ. However, when inflammation occurs, these cells can derive from bone marrow monocytes that are recruited from the blood into the inflammation site; when monocytes leave the blood vessel and enter in the tissue (extravasation and diapedesis) they give rise to monocyte-derived macrophages [120].

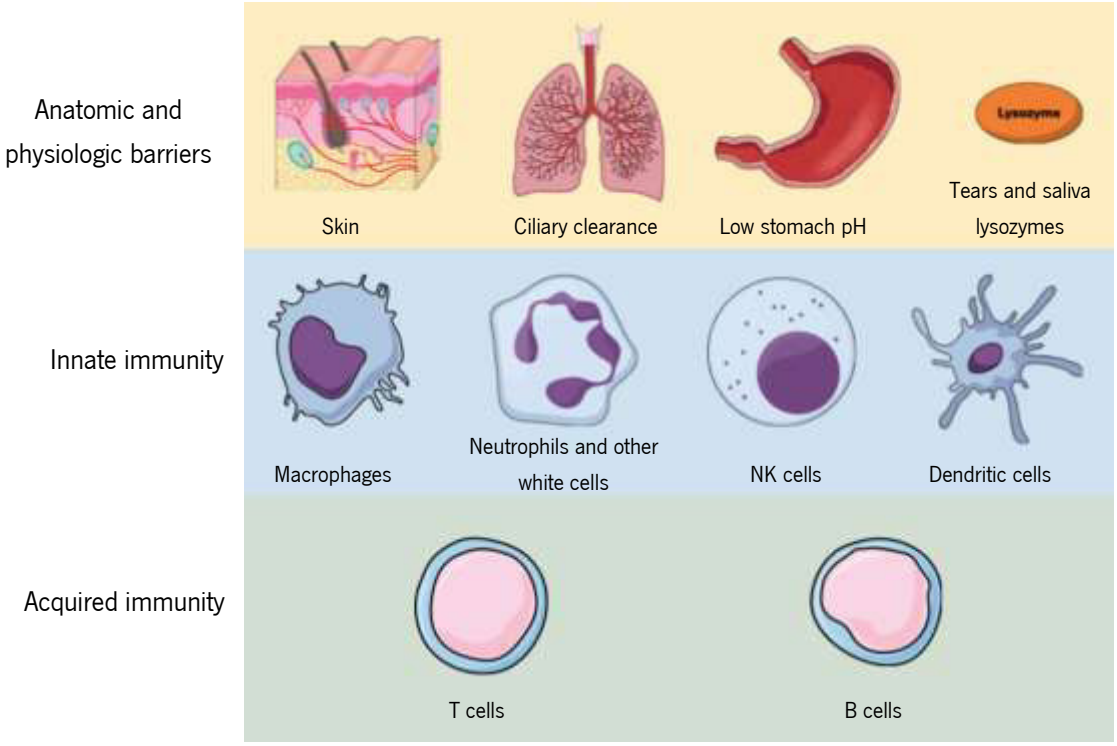

Figure 5. **Immune system barriers.** The immune system is divided in 3 levels: the anatomic and physiologic barriers, the innate immunity and the acquired immunity. NK – natural killer. Adapted from Turvey *et al.* (2010) [118].

As mononuclear phagocytes, Mφ have high phagocytic capacity; they are secretory cells with key roles in promoting and regulating the immune response, homeostasis maintenance, tissue remodeling and wound healing. In the tissue, the stimuli of Mφ can drastically change their physiology [121], transforming them into a pro-inflammatory or an anti-inflammatory phenotype; the balance between these two phenotypes is responsible for protecting our tissues [122].

It is well accepted that c-cig smoking affects innate immunity, since it is involved in inflammatory cytokine production which leads to an abnormal innate immune response [122,123]. Studies have shown that human Mφ treated with smoke increase interleukin (IL)-8 production, thus facilitating inflammation [122]. Ko *et al.* (2015) exposed human Mφ, differentiated from THP-1 monocytes to different concentrations of smoke extract (0-20 μg/mL) and observed an increase of IL-8 in a concentration-dependent manner [124]. Murine studies have shown that smoking suppresses Mφ phagocytic ability, thus enhancing bacterial survival [125,126]. Chronic smoking can trigger changes in Mφ morphology and convert them in less mature cells; in fact, a high expression of CD14 (monocyte marker) was observed in these cells. The immune system requires innate and acquired immunity communication to protect the organism; smoking induces lung Mφ

to play an inhibitory role in the proliferation of lymphocytes and NK cells and, for this reason, makes it easier to acquire a lung infection from opportunistic pathogens [127,128]. In conclusion, smoking leads to IL-8 release and, consequently to inflammation, and reduces phagocytosis [122].

1.6.2. The effects of aerosols on macrophages

E-cig is, supposedly, less toxic than c-cig. However, Hwang *et al.* (2016) showed *in vivo* that mice exposed to e-cig vapor affects Mφ by increasing the pro-inflammatory cytokine release, reducing phagocytic function and increasing necrosis. In fact, this study suggests that smoke and aerosol can have the same effects in macrophages [129]. More recently, a study by Clapp *et al.* (2017) revealed that Mφ obtained from human bronchoalveolar fluid cultured with aerosols generated from e-juices with different flavors displayed a decrease in phagocytic activity and increase in IL-8 and IL-6 levels. In this work, cinnamon flavoring revealed higher immunosuppressive effects, corroborating previous studies (please see section 1.4.2.4) [115]. Ween *et al.* (2017) evaluated how phagocytosis is altered by e-cig aerosol and concluded that the effect on phagocytosis is not nicotine-dependent, but flavoring-dependent; in fact, cinnamon, cherry and tobacco flavors were more toxic than the others [130]. On the other hand, cell viability was similar regardless of nicotine doses or flavorings. Additionally, when compared with c-cig smoke, e-cig aerosol exposure reduced the expression of a receptor that mediates phagocytosis. This finding explains the reduction of phagocytic function but no pro-inflammatory cytokine release. In Figure 6, the smoking/vaping effects on Mφ are summarized.

In summary, smoking has deleterious effects on the immune system and increases the probability to develop an autoimmune and/or infectious disease [126]. Recent studies revealed that e-cig aerosol can affect Mφ function and that it is mostly dependent on flavoring. Nonetheless, further studies are needed to clarify the underlying molecular mechanisms.

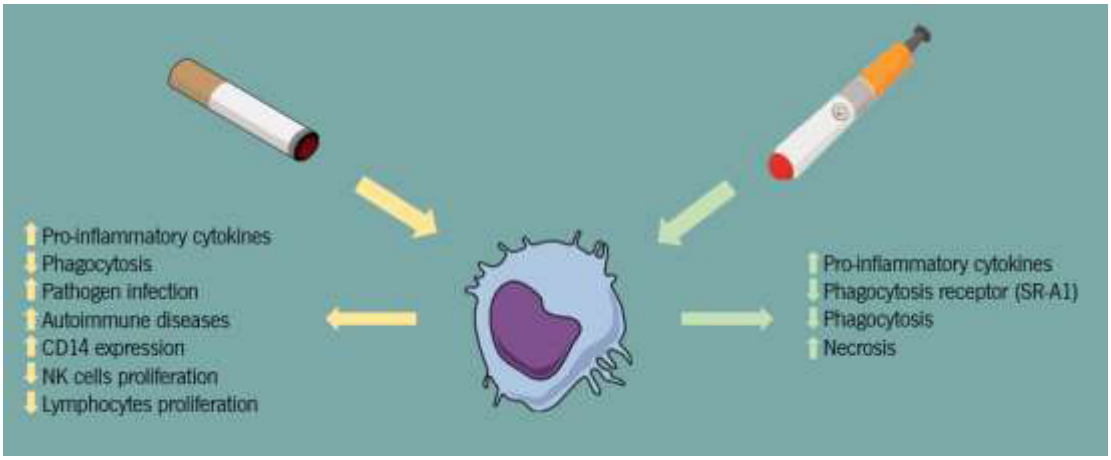

Figure 6. **Smoking/vaping effect on macrophages.** Smoking leads to pro-inflammation, alters Mφ morphology, reduces Mφ phagocytic ability thus disrupting innate immunity against pathogenic agents and, eventually, leading to autoimmune diseases, and altering other immune cells functions. Vaping shows overlapping effects: e-cig aerosol appear to decrease scavenger receptor (SR)-A1 synthesis, which decreases phagocytosis.

2. Aims

AIMS

Tobacco smoking (c-cig) is known to have a prejudicial effect on embryo development. On the other hand, data regarding the impact of aerosols originated by electronic cigarettes (e-cig) in embryo development are scarce. In addition, it is recognized that tobacco smoking induces tissue damage, particularly in the lung. Macrophages are, at the cellular level, the first line of defense of the immune system against foreign particles/molecules, such as the ones present in smoke. These cells activate an inflammatory response that contributes to maintaining homeostasis of the whole organism. However, data about how aerosols can affect immunity are inconsistent.

In this sense, the main goal of this study was to determine the impact of e-cig aerosols in lung development and compare it with the impact of c-cig smoke as well as to evaluate and compare the effect of aerosol/smoke exposure in macrophages. For this purpose, this thesis aims to accomplish the following specific objectives:

1. Optimize a manual aerosol/smoke acquisition system
2. Evaluate aerosol/smoke impact on lung morphology, by *in vitro* lung explant culture
3. Assess aerosol/smoke toxicity in the embryonic chick lung
4. Determine aerosol/smoke cytotoxicity in a mouse macrophage cell line

3. MATERIALS AND METHODS

3.1. Ethical Statement

This work was performed in the chick model, at early embryonic stages, and in a mouse macrophage cell line. According to the Directive 2010/63/EU of the European Parliament, Council of 22 September 2010 on the protection of animals used for scientific purposes, and to the Portuguese Directive 113/2013 of 7 August 2013, ethical agreement from review board institution or ethical commission was not needed.

3.2 Medium preparation

The aerosol/smoke acquisition process was adapted from Romagna *et al.* (2013) [109] and performed in a chemical fume hood. 20 mL of medium was put in a 500 mL Büchner flask. Two types of medium were used depending on the subsequent application: 199 medium (Sigma, USA) for tissue culture and DMEM (Dulbecco's Modified Eagle's Medium, 42430-025, GIBCO, USA) supplemented with 55 mg of pyruvic acid sodium salt (pyruvate) (Merck, USA) for cell culture. A cigarette (nicotine: 0.7-0.8 mg; Marlboro, Philip Morris USA Inc.) or an electronic cigarette (Green technical material, FR) (Figure 7) filled with e-liquid (Ice Bear: 16 mg/mL nicotine, <40% vegetable glycerin, <60% propylene glycol and menthol flavor; L'ABSOLU, GREEN LIQUIDES SAS, FR) were connected, through a tube, to neck of the flask. In both cases, the acquisition was performed with gentle rocking, 100 rotations per minute (rpm), to increase the liquid-aerosol/smoke contact surface. The medium was incubated with one puff every 1 minute (min), until the end of the acquisition (please see sections below: 3.2.1. and 3.2.2.). At the end of the procedure, the exposed medium was stored at 4 °C for culture (and used in the same week), or at -20 °C for liquid/liquid extraction to avoid the deterioration of the compounds that diffused into the medium.

Figure 7. **Electronic cigarette.** Green technical material, France.

3.2.1. Aerosol acquisition

To obtain aerosols from the electronic cigarette (e-cig), the side-arm of Büchner flask was connected to a vacuum pump (N035.1.2 AN.18, KNF Lab, Germany) by a hose; this connecting tube had a small hole, on the pump´s side, to decrease the vacuum pressure thus creating suction comparable to human breathing. E-cig was filled with e-liquid and weighed. Then, it was connected to the neck of the flask via a tube. By pressing the knob on the device for 3 seconds (s), and activating the pump for the same period of time, a puff was made and allowed to be in contact with the medium for 1 min. After this period, the aerosol was released and e-cig was weighed. This procedure was repeated until 200 mg of e-liquid were consumed [109].

3.2.2. Smoke acquisition

To obtain smoke from the conventional cigarette (c-cig), the side-arm of Büchner flask was connected to a 50 mL syringe, because the pressure created by the vacuum pump is too high and causes the consumption of the c-cig in a few seconds. The c-cig was connected to the neck of the flask via a tube. The c-cig was lit and, with the syringe, a 35 mL puff was made and allowed to be in contact with the medium for one minute (according to the standardized International Organization for Standardization (ISO) method 3308:2012 for obtaining c-cig extract). This procedure was repeated until the cigarette was fully consumed. In this case, only after the last puff, the smoke was released [109,131,132].

3.3. Gas chromatography-Mass spectrometry analysis

Gas chromatography-Mass spectrometry (GC-MS) is an analytical technique that allows the separation and detection of volatile compounds in a sample. Samples injected into the gas chromatograph are vaporized and transported by an inert gas, such as helium (mobile phase), through a chromatographic column. Compounds are then separated and isolated according to their ebullition temperature and affinity for the stationary phase. When compounds reach the end of the column they are ionized and fragmented using electron or chemical ionization sources, creating different charged fragments. These fragments are then detected by the mass spectrometer based on the difference between mass-to-charge ratios (m/z). The fragments and the corresponding ions provide data on the compounds, and their quantity, in the sample.

To confirm that the smoke/aerosol compounds were diffused into the medium, nicotine was quantified by GC-MS. This substance was selected since it is common to both c-cig and the e-juice

that is vaporized. Prior to gas chromatograph injection, nicotine needs to be extracted to a solvent that easily vaporizes. The extraction protocol used was performed according to Oliveira *et al.* (2006) [133]. The extraction methods may not extract 100% of the compound and, for this reason, it was necessary to evaluate its efficiency for this specific compound; therefore, a nicotine calibration curve was performed (section 3.3.3.1.).

3.3.1. Nicotine Extraction

Samples were thawed and diluted 5x (with the corresponding fresh medium). In a Pyrex tube (Pyrex, UK), 100 μL of 4-nonanol at 35.4 mg/L (818773, Merck) (that serves as an internal standard), and 400 μL of dichloromethane (SupraSolv, Merck) (extraction solvent) were added to 8 mL of diluted medium. Each medium sample was run in triplicate. Subsequently, this mixture was left stirring vigorously with a magnet, for 15 min at room temperature, over a magnetic stirrer (JENWAY 1000, UK). This step allows a better intermix between the solvent and the compounds in the media, namely nicotine, thus improving its recovery from the solution. The tubes were left at – 20 °C (10 min for 199 medium; 2 hours (h) for DMEM) so that the nicotine-enriched dichloromethane does not evaporate; moreover, it allows a better separation of the organic phase (dichloromethane) from the aqueous phase (medium).

Afterward, tubes were centrifuged for 3 min at a rotational speed of 860 g for 199 medium, and 30 min at 2634 g for DMEM, at 4 °C, to increase the retrieval of organic phase; this spin down separates and concentrates dichloromethane at the bottom of the tube. To obtain the organic phase, dichloromethane was collected with a Pasteur's pipette in the chemical fume hood. To eliminate any medium contamination, *i.e.* water, an excess of anhydrous sodium sulfate was added (≥ 99%, Fluka). Finally, the organic phase was recovered and stored in a clean vial (Sigma) at – 20 °C, to avoid compound evaporation until the samples were injected in the gas chromatograph.

3.3.2. Injection

The GC-MS (GC: Varian CP-3800, MS: Varian Saturn 2000, USA) program was set for injection. Before starting, the Hamilton injection syringe (1 μL) was washed 10x with clean dichloromethane to eliminate potential residues that may produce background peaks in the chromatogram. Then, it was washed 5x with the sample and, after that, 1 μL of the sample was injected in splitless mode (30 s) in a Sapiens-Wax MS column (30 m × 0.15 mm; 0.15 μm film thickness, Teknokroma). The carrier gas was helium 49 (Praxair) at a constant 1.3 mL/min flow.

The detector was set to electronic impact mode with an ionization energy of 70 eV, a mass acquisition range (*m/z*) from 35 to 260 and 610 ms acquisition interval. Oven temperature was initially set to 60 °C for 2 min and then raised from 60 °C to 234 °C at a rate of 3 °C/min, raised from 234 °C to 260 °C at 5 °C/min and finally maintained at 260 °C for 10 min. The injector temperature was 250 °C with 30 mL/min split flow. To avoid sample evaporation, only one tube per injection was removed from the freezer at –20 °C. Each run lasted, approximately 1 h and 20 min. During this period, the syringe was washed 10x with dichloromethane. This procedure was repeated each time a new injection was performed. The chromatogram data was recorded in MS Workstation software version 6.9.3. (Varian Inc., USA) that was connected to the GC-MS, and compounds were identified by comparing mass spectra and retention indices with those of pure standards.

3.3.3. Data analysis

Chromatograms were analyzed with MS Workstation software, version 6.9.3. (Varian Inc.). First, a baseline for the peaks was settled allowing to determine the best way to measure the peak area. Next, the peaks of interest (4-nonanol and nicotine) were identified through retention indices, the mass spectra compared with an own library, and the peak area determined. Given that the amount of 4-nonanol in the sample was known, it was possible to determine the quantity of nicotine in the sample by comparing peak areas; subsequently, nicotine concentration was calculated according to 3.3.3.1.

3.3.3.1. Nicotine Standard Curve

The extraction methods may not extract the compound of interest completely and/or equivalently to that of the internal standard. In this sense, it was necessary to evaluate the efficacy of this protocol for this specific goal. For this purpose, a nicotine calibration curve was performed. A stock solution of 240 mg/L nicotine (N3876, Sigma) was prepared in the culture medium and diluted accordingly to prepare 0 mg/L, 4 mg/L, 6 mg/L, 8 mg/L, 10 mg/L for the standard curve. These solutions were extracted and injected as previously described (section 3.3.1. and 3.3.2., respectively). The peak areas were determined and, since the nicotine amount was known beforehand, a standard curve was obtained by plotting the detected concentration (C_{d}) vs. standard concentration of nicotine (C_{n}). Detected concentration was determined by the following equation:

$$C_{\mathrm{d}} = \frac{A_{\mathrm{n}} \times C_{\mathrm{is}}}{A_{\mathrm{is}}}$$

A_{n} is the detected peak area of nicotine, C_{is} is the internal standard concentration and A_{is} the internal standard peak area. From the standard curve linear regression, it was possible to determine the Response Factor for the extraction of nicotine relatively to the internal standard (F).

$$F = \frac{C_{\mathrm{n}}}{\frac{A_{\mathrm{n}} \times C_{\mathrm{is}}}{A_{\mathrm{is}}}} = \frac{C_{\mathrm{n}}}{C_{\mathrm{d}}} = \frac{1}{a}$$

In the above formula, F is obtained by the inverse of the standard curve slope (a), which it is the division of the variable C_{d} by the variable C_{n} (section 4.1.2.). This value takes into account the yield of extraction and was applied to determine the precise concentration of nicotine in the sample.

3.3.3.2. Samples

The nicotine concentration of the different samples was used to determine the average and standard error to the mean of nicotine concentration in the c-cig or e-cig exposed medium.

3.4. *In vitro* lung explant culture

In vitro explant culture, or organotypic culture, is a technique that allows studying organogenesis since tissue structure is maintained. Tissues are cultured in an air-liquid interface on top of a semipermeable membrane. Furthermore, the culture medium can be manipulated with the addition of specific drugs, growth factors, chemical inhibitors, and their impact on morphogenesis revealed by morphological or molecular techniques [134]. In this study, chick lung explants were challenged with c-cig and e-cig exposed medium, and its effects assessed by morphometric analysis.

3.4.1. Lung dissection

Fertilized chicken eggs (*Gallus gallus*) were incubated for 5 days, at 37 °C and 48% of humidity, to obtain stage b2 lungs (two secondary buds per bronchus). To have access to the embryos, a "window" was opened in the eggshell. Next, embryos were dissected using a stereomicroscope (SZX16, Olympus, Japan) and sterile dissection tools. Lungs were collected in Phosphate buffer saline (PBS) 1x until they were transferred to the culture plate (section 3.4.3).

3.4.2. Medium supplementation

In the laminar flow hood, c-cig and e-cig exposed 199 medium was filtered through a 0.22 μm filter (Sarstedt, Germany). Control and filtered 199 medium were supplemented with: 10% of chick serum (Invitrogen, USA), 5% of inactivated fetal Bovine serum (FBS) (Invitrogen), 1% of L-glutamine (Invitrogen), 1% of penicillin/streptomycin (5000 IU/mL (Invitrogen) and 0.25 mg/mL of ascorbic acid (Sigma) [135]. Subsequently, the medium was aliquoted and kept at 4 °C, until further use.

3.4.3. *In vitro* explant culture

In the laminar flow hood, 400 μL of 199 medium/well was added to a 24 well-plate; next, a nucleopore membrane (Whatman, USA) was put floating on top of the medium, for at least 1 h before culture. Before transferring the lungs, 199 medium was replaced by 200 μL of supplemented control medium. On the bench, and near a Bunsen burner, lungs were picked by using a micropipette with a cut tip and transferred gently to the top of the membrane. Lungs were incubated for 30 min, at 37 °C and 5% CO_2. Then, lungs were photographed (D0_0 h) by a camera coupled to the stereomicroscope and cellSens software (Olympus).

Lungs were randomly assigned an experimental group (n≥16): control (non-exposed), c-cig or e-cig exposed, and the culture medium replaced accordingly. The floating culture was incubated for 24 h, at 37 °C in a 5% CO_2 atmosphere. After this period, explants were photographed (D1_24 h) and the medium replaced by the corresponding fresh medium. The floating culture was incubated for another 24 h, at 37°C in a 5% CO_2 atmosphere. After this period, lungs were photographed (D2_48 h), punctured with a 30G needle and washed 3x with PBS 1x. Finally, lung explants were kept overnight at 4 °C, with fixation solution (4% of formaldehyde in PBS 1x and 2 mmol/L ethylene glycol). The following day, lungs were dehydrated through a methanol series and kept at -20 °C with methanol.

The culture medium from D1 and D2 was collected, centrifuged at 1000 g, 20 min at 4 °C, and stored at -80 °C for further analysis (section 3.6. and 3.7.).

3.5. Morphometric analysis

AxioVision Rel. 4.9.1 software (Carl Zeiss, Germany) was used to analyze D0 and D2 images. With this software, it is possible to outline both compartments of the lung (epithelium and mesenchyme) (Figure 8) and obtain total, epithelium, and mesenchyme area, and epithelium and mesenchyme perimeter. The results of the morphometric analysis were expressed as D2/D0 ratio and reflect lung growth.

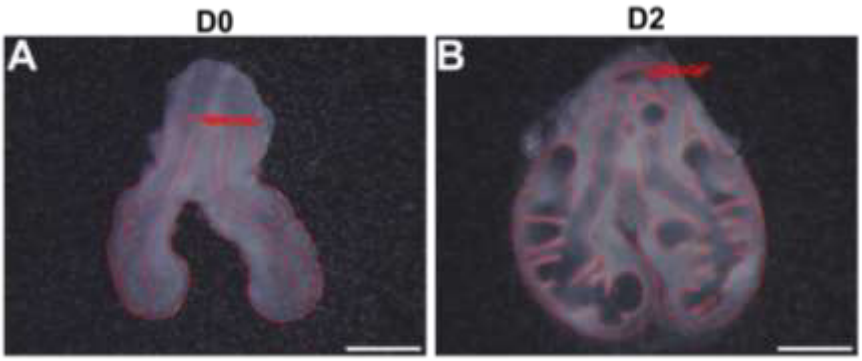

Figure 8. **Morphometric analysis**. Representative example of a b2 lung at 0 hours (A) and 48 hours (B) of culture. The red line represents the epithelial and mesenchymal outline drawn by AxioVision Rel. 4.9.1 software and the values the corresponding areas in pixels; these measurements can be used to evaluate growth. Scale bar: 500 μm.

Statistical analysis was performed with SPSS 25.0 (IBM, USA) software. Since the normality test did not fail, the results were analyzed through one-way analysis of variance (ANOVA) test, and for multiple comparisons, the post-hoc Fisher's Least Significant Difference (LSD) test was used. All quantitative morphometric data are presented as mean ± SEM, and statistical differences set at $p<0.05$.

3.6. Toxicity assessment

To evaluate cytotoxicity, extracellular lactate dehydrogenase (LDH) activity was measured. This assay is based on the principle that, when tissue/cells are exposed to a toxic substance or when an injury occurs, LDH leaks from the cytoplasm and it is released from the cell to the extracellular environment/medium. This test detects the colorimetric conversion of NAD^+ to NADH, in the presence of LDH, that is proportional to the amount of enzyme in the extracellular environment/medium.

Medium collected at 24 (D1) and 48 (D2) hours were analyzed to assess the total cytotoxicity since the medium was replaced/refreshed after 24 h.

3.6.1. LDH activity

The culture medium collected during explant culture (D1 and D2; n≥6) and the components of the LDH assay kit (K726, BioVision, USA) were thawed on ice. D1 and D2 media from different conditions were diluted 250x in assay buffer. Additionally, lung culture medium (frozen at D0, not used in culture) was processed similarly to be used as negative control.

The assay was performed according to the manufacturer's instructions, in a 96 well-plate. Briefly, the NADH standards (0, 2.5, 5, 7.5, 10, 12.5 nmol), positive control and the diluted medium were brought to a final volume of 50 μL, with assay buffer. Each sample or standard was run in duplicate. Then, an equal volume of substrate solution was added to the wells and gently mixed to homogenize. Next, in a multiplate reader (VARIOSKAN FLASH, Thermo Fisher, USA), OD was measured (A0_0 min) at 450 nm. The reaction was incubated, protected from light, at 37 °C for 30 min, then OD was measured again (A1_30 min).

3.6.2. Data analysis

To obtain the standard curve, a linear regression line through the standard points was plotted using average values. To outline the curve, only the OD_{450} values collected after 30 min were taken into consideration. Additionally, 0 nmol/well NADH background was subtracted from all readings.

For samples, ΔA (A1-A0) was calculated and, also, average values were used. To eliminate lung medium background, ΔA from lung medium (D0) was subtracted to ΔA of all samples. Then, this value was used to find the concentration (B) by interpolating on the regression line. Finally, B was used to determine LDH activity through the equation:

$$LDH\ activity = \frac{B}{\Delta T * V} * sample\ dilution$$

Where ΔT is 30 min, V is 50 μL (sample volume) and 250 the sample dilution. The results are expressed as mU/mL.

3.7. Inflammation assessment

An enzyme-linked immunosorbent assay (ELISA) was used to quantify TNF-α, a pro-inflammatory cytokine, as a complementary tool to determine toxicity. Pro-inflammatory cytokines

can lead to the activation of the immune system and, eventually, in highly toxic environments to cell death. For this reason, it is crucial to understand TNF-α profile in culture conditions and assess chick lung inflammation promoted by c-cig or e-cig exposure.

Medium collected at 24 (D1) and 48 (D2) hours (n=6) were analyzed to assess the total cytotoxicity since the medium was replaced/refreshed after 24 h.

3.7.1. TNF-α ELISA

The culture medium collected during explant culture (D1 and D2) and the components of TNF-α ELISA kit (E-EL-Ch0215, Elabscience, USA) were thawed on ice. D1 and D2 media from different conditions were diluted 10x in sample diluent. Additionally, lung culture medium (frozen at D0, not used in culture) was processed similarly to be used as negative control.

The assay was performed according to the manufacturer's instructions, in a 96 well-plate. Briefly, 100 µL of standards (0, 31.25, 62.5, 125, 250, 500 and 1000 pg/mL) and the diluted medium was added to the 96 well-plate provided in the kit, sealed and incubated 90 min at 37 °C. Each sample or standard was run in duplicate. Afterward, the solution in the wells was removed and 100 µL of Biotinylated Detection antibody working solution was added immediately to each well. The plate was sealed, gently mixed, and incubated 1 h at 37 °C. Next, the solution was decanted and 350 µL of washing buffer was added to the wells. The plate was soaked for 1 min, then the solution was emptied and the plate dried with absorbent paper. This washing step was repeated 3 times. Subsequently, 100 µL of Avidin-Horseradish Peroxidase conjugate working solution was added to each well. The plate was sealed, gently mixed, and incubated 30 min at 37 °C. After the solution was decanted, the washing step was performed 5 times, as previously described. Next, 90 µL of substracte reagent was added to the wells, the plate covered with a new sealer and incubated 25 min at 37 °C, protected from light. Finally, 50 µL of stop solution was added to each well and the OD read at 450 nm.

3.7.2. Data analysis

Average zero standard optical density was subtracted to both standard and samples average values. To obtain the calibration curve, a 4-parameter logistic curve was plotted using standard OD_{450} average values. TNF-α concentration in the samples was calculated by substituting OD_{450} average values in the 4-parameter logistic curve equation. The obtained values were multiplied by

the dilution factor (10). To determine total TNF-α, after 48 h in culture, D1 and D2 amount (from the same explant) were added up.

TNF-α data did not fail the normality test, thus the results were analyzed through one-way ANOVA test and, for multiple comparisons, post-hoc Fisher's LSD test was used. Results are represented as mean ± SEM and statistical differences were considered if $p<0.05$.

3.8. Macrophage cell line culture

To evaluate e-cig Mφ toxicity, a mouse macrophage cell line (RAW 264.7) was incubated with aerosol/smoke-exposed medium and cell viability assessed. With this approach, it was possible to compare cytotoxicity between both conditions.

3.8.1. Maintenance of cell culture

Cells were incubated with supplemented DMEM (DMEM, 10% of FBS and 55 mg of pyruvate), at 37 °C, 5% of CO_2. The cells were subcultured to new flasks every 2/3 days or when, at least, 70% of confluency was observed.

To subculture the cells, the culture medium was discarded and cells were gently washed with DMEM 3 times, to remove cells that died during culture. Afterward, supplemented DMEM was added to the flask (3 mL to T25 and 5 mL to T75). Next, cells were gently scraped off from the bottom of the flask into the medium; then the medium was transferred to a tube, and an aliquot was used to count the number of cells (section 3.8.2.). Cells were then transferred to new flasks, 1.5 mL for T25 and 2.5 mL for T75, and supplemented DMEM was added until a final volume of 10 mL or 25 mL, respectively. Cells were placed in the 37 °C, 5% of CO_2 incubator, until the next subculture was performed.

3.8.2. Cell counting

Trypan blue was used to count viable cells. If cells take up trypan blue, they are considered non-viable because their membrane is disrupted. In brief, 10 µL of cells were mixed with 90 µL Trypan blue; 10 µL were then loaded into a Neubauer chamber which was placed on the 10x objective microscope. Cells were counted and the number of cells/mL calculated. Before the assays, cells were diluted with supplemented DMEM to a final concentration of 10^5 cells/mL.

3.8.3. Incubation with exposed medium

C-cig or e-cig exposed medium (section 3.2.) was filtered with 0.22 μm filter and supplemented with 10% FBS. Afterward, the medium was diluted with supplemented DMEM (section 3.8.1.) to 1:2, 1:3, 1:4 and 1:8.

In a 96-well plate, 100 μL of cell suspension (10^5 cells/mL) were plated. After plating, cells were left to adhere for 24 h in the CO_2 incubator. Next, the medium was collected from the well and it was replaced by 100 μL medium of the following culture conditions: supplemented DMEM (controls), c-cig and e-cig-exposed DMEM with different dilutions. Next, plates were incubated for 24 and 72 hours. At these time points, the culture medium was collected and stored at -20 °C for LDH assay (section 3.9.2); the corresponding adhered cells were used for MTT viability assay (section 3.9.1).

3.9. Cytotoxicity assessment

Cell viability is used as an indicator of cytotoxicity. MTT is a metabolic assay which relies on the presence of metabolically active (viable) cells that are capable of transforming water-soluble MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) into formazan. Formazan is a purple compound that precipitates inside viable cells. The color change is directly related to metabolic activity and, therefore, with cell viability.

Additionally, extracellular LDH activity was also measured to evaluate cytotoxicity. This assay is based on the principle that cell death triggers LDH release from the cytoplasm to the medium. The activity of this enzyme can be detected by kinetic assays and it is inversely proportional to cell viability. Thus, it is a useful method to determine cell viability.

3.9.1. MTT metabolic assay

After 24 and 72 hours in culture, the medium was collected and stored at -20 °C while cells were used to perform MTT viability assay (n≥6). MTT (Thiazolyl Blue Tetrazolium Bromide, Sigma) stock solution (5 mg/ml H_2O) was diluted (1:10) in supplemented DMEM, added to adherent cells and incubated for 2 hours (37 °C, 5% CO_2). Subsequently, the solution was discarded and an EtOH:DMSO (1:1) solution was added to dissolve the formazan crystal. Finally, OD was read at 570 nm. Each condition was run in triplicate.

3.9.1.1. Data analysis

Cell viability of 100% was assumed for control samples. Data are expressed as a percentage compared to controls. Average values were used in all cases. Statistical analysis was performed with SPSS 25.0 (IBM) software. Since normality test did not fail, the results were analyzed through one-way ANOVA and post-hoc Fisher's LSD test was used for multiple comparisons. The results are presented as mean ± SEM and statistical significance was set at $p<0.05$.

3.9.2. LDH activity assay

A kinetic assay was used to detect LDH activity in Mφ cell line based on a previously described method [136,137]. In the presence of LDH, pyruvate is converted into lactate and NADH is oxidized to NAD+. In this assay, the consumption of NADH is evaluated by reading OD at 340 nm. For this protocol, it was necessary to prepare 0.36 mmol/L NADH (β-nicotinamide adenine di-nucleotide reduced disodium salt hydrate, Sigma) on pH=7.4 phosphate buffer (PB), and 4.91 mmol/L pyruvate on PB. Briefly, in a 96 well-plate, 20 µL of sample (n≥3) and 200 µL NADH solution were mixed and incubated at 30 °C for 2 min in the plate reader (VARIOSKAN FLASH). Next, 15 µL of pyruvate solution was added to each well, and OD was read every 10 seconds until 3 min. Additionally, control cells were previously treated with Tris-HCL 5 mmol/L pH 7.5 to induce cell lysis thus releasing the intracellular LDH. This suspension was used as positive control for the assay.

3.9.2.1. Data analysis

The results were plotted in a time vs. OD graph; next, a tangent line was made and the slope obtained. LDH activity per mL (U/mL) was calculated using the following formula:

$$U/mL = \frac{Slope * 0.332}{1000 * 6.22 * V_{sample}}$$

By multiplying LDH activity per mL (U/mL) with the volume of the medium in culture well (mL) the LDH activity (U) was calculated. LDH activity is inversely proportional to cell viability. Cell viability of 100% was assumed for control samples, and 0% cell viability was assumed for Tris-treated cells. In each case, cell viability (Viab%) was calculated through the following formula:

$$Viab\% = 100 - \left(\frac{U_{sample} - U_{DMEM}}{U_{Tris} - U_{DMEM}}\right) * 100$$

Statistical analysis was performed with SPSS 25.0 (IBM) software. LDH activity did not fail on the normality test, thus the results were analyzed through one-way ANOVA test, post-hoc Fisher's LSD test was used for multiple comparisons. Data are presented as mean ± SEM, and statistical differences set at $p<0.05$.

4. Results

Smoking constitutes a major public health issue associated with high mortality rates. Currently, new nicotine delivery systems, electronic cigarettes (e-cig), have been developed as an alternative to conventional cigarettes (c-cig) and them well-known toxic effects. E-cig vaporizes a liquid creating an aerosol that reaches the lungs. Previous studies have shown that c-cigs and nicotine exposure impairs embryo development [73,74], however, the effect of e-cig aerosol in lung development is not known so far.

In this sense, this work aims to determine the impact of e-cig aerosols in lung development and compare it to the effects of c-cig smoke. To achieve this goal, a reproducible and efficient system to obtain aerosol/smoke enriched medium was optimized. Subsequently, chick lung explants were exposed to aerosol/smoke enriched medium and analyzed morphologically. Additionally, and taking into consideration the recognized effect of c-cig in macrophages, the effect of e-cig aerosol in these cells was evaluated and compared to c-cig smoke.

4.1. Aerosol/smoke acquisition system

Several studies have used different methods to expose cells or tissues to smoke or vapor, as for instance: using commercially available extracts [22], designing their own manual acquisition system [109], or using a smoking machine [38,138] to obtain extracts.

In this work, we have optimized a manual acquisition system based on the one described by Romagna *et al.* (2013) [109]. Basically, every minute, a cigarette puff is generated and allowed to flow, through a hose, into a Büchner flask containing culture medium that will be then used to explant and cell culture. This system mimics human cigarette usage, as the hoses represent the airways whereas the Büchner flask represents the lung.

4.1.1. Aerosol/smoke acquisition optimization

Several optimization steps were needed to adapt the described system to the specifics of this study, such as culture medium, e-cig device and vacuum pump. Parameters such as agitation type and time, suction pressure and puff duration and number had to be adjusted. Each time the settings were changed, the medium was analyzed for nicotine content (section 4.1.2.).

Stirring the culture medium during the acquisition process allows a better aerosol/smoke diffusion. A magnetic stirrer and a horizontal rocking platform at different speeds (75-100 rpm) were tested. The magnetic stirrer dispersed the medium all over the flask and it was virtually

impossible to recover the total volume of the medium. Eventually, the best option was the horizontal rocking platform at 100 rpm, because it contributed to a higher contact surface between medium and the aerosol/smoke and, consequently, allowed a higher nicotine concentration in the culture medium.

Initially, the aerosol (e-cig) acquisition process was performed with the minimum suction pressure of the vacuum pump. However, the pressure was too high and, instead of creating an aerosol puff, the e-juice was fully aspirated from the cartridge. To solve this issue, the hose connected to the pump was perforated to decrease the suction pressure. When tested, this alteration led to the formation of a consistent puff and e-juice suctioning no longer occurred. Additionally, the minimum suction pressure of the vacuum pump was also too high to be used to obtain the c-cig smoke. In this case, c-cig smoke puff was produced with a manual syringe and by aspirating 35 mL of air to simulate inhalation (Figure 9).

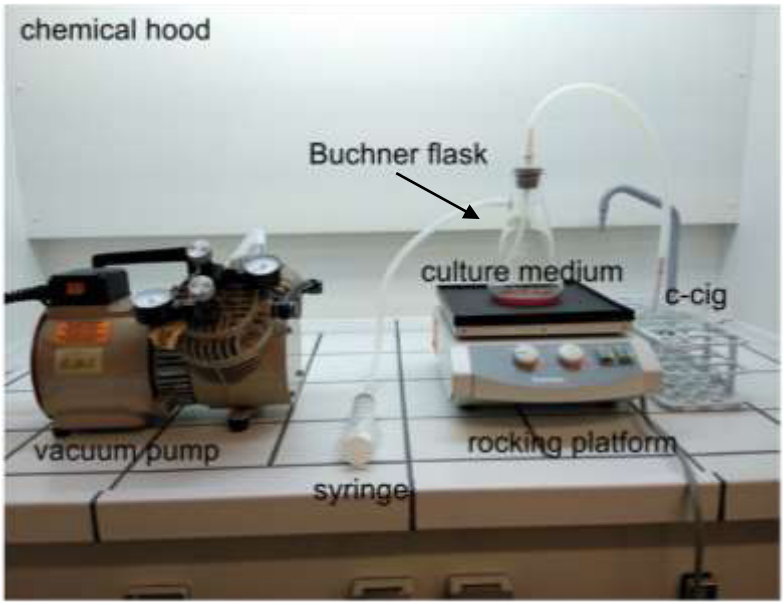

Figure 9. **Smoke acquisition system**. Experimental setting to obtain c-cig smoke: the syringe is connected to the sidearm of a 500 mL Büchner flask while a hose connects the cigarette through the neck of the flask, 20 mL of culture medium were poured to the flask.

The number of e-cig puffs was also optimized. We try to use the same number of puffs as in the c-cig (6 puffs), however, nicotine concentration in the medium oscillated significantly. Eventually, a previously described approach was selected and we produced enough number of puffs to consume 200 mg of e-liquid. This approach gave more reproducible and reliable results.

Moreover, the air flow position of the e-cig device was also taken into consideration in order to create puffs with constant e-juice consumption (approximately 10 mg of e-juice/puff).

The 35 mL of air aspirated in 2-second per puff is considered to be the standardized ISO 3308:2012 method for obtaining a c-cig extract [131, 132]. In the case of c-cig, "inhalations" were repeated, in these conditions, until one cigarette was fully consumed (6-7 puffs/cigarette). Curiously, we noticed that c-cig consumption rate fluctuated depending on whether c-cigs had been exposed to air for a long period of time or used directly after opening a new pack. In this sense, and to avoid tobacco degradation, cigarettes were kept in sealed bags (2/bag) immediately after opening the original package. Regarding e-cig, the device was filled with e-liquid and weighed with a precision scale. Then a 3-second puff was produced and repeated until approximately 200 mg of liquid were consumed according to [109]. On average, with this device, 15 to 20 puffs were needed to achieve this goal (Figure 10). It is important to refer that each optimization step only could be performed after nicotine quantification and data analysis.

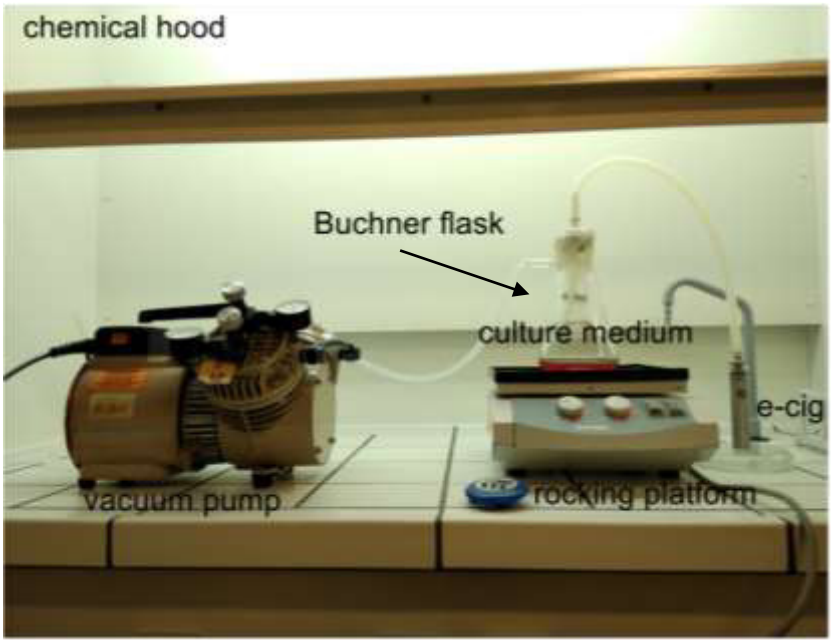

Figure 10. **Aerosol acquisition system**. Experimental setting to obtain e-cig aerosol: the vacuum pump is connected to the sidearm of a 500 mL Büchner flask, whereas the device is connected by a tube through the neck. In both case, 20 mL of culture medium were poured to the flask.

4.1.2. Process validation

To validate the acquisition process, it was necessary to confirm whether the compounds present in the aerosol/smoke were indeed present in the culture medium. Considering that nicotine is a common substance to both c-cig and e-cig, the nicotine levels in the culture medium after exposure were assessed.

To achieve this goal, we established a collaboration with the Centre of Biological Engineering of UMinho that has expertise in detecting volatile compounds by GC-MS. This technique is highly sensitive, reproducible and precise as a result of the combination of the two methods [139].

Volatile compounds need to be separated and extracted in specific organic solvents that can, then, be injected and volatilized. The extraction process was based on a liquid-liquid extraction method used to extract wine volatile compounds [140]. To determine the efficiency of this method, a nicotine standard curve was performed (Figure 11) and the response factor *(F)* determined. Subsequently, this value must be then applied to sample nicotine concentration detected thus correcting for the extraction process. From the linear regression, we obtained the *F* factor (section 3.3.3.1.), which was 2.6.

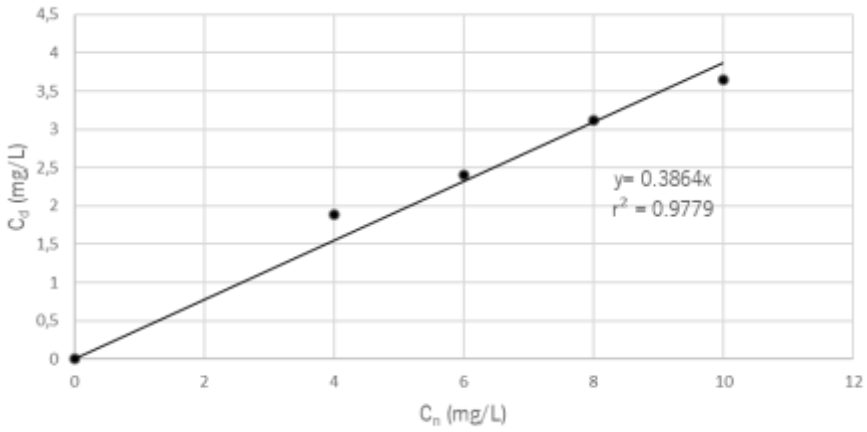

Figure 11. **Nicotine standard curve.** Linear regression curve between detected concentration of nicotine (C_d) and standard concentration (C_n) of nicotine. The response factor (F) is calculated as the inverse of the slope and used to correct nicotine concentration in the samples. *F*=2.6.

Nicotine was extracted from the culture medium and collected in the dichloromethane phase [140]. After GC-MS analysis, we obtained a chromatogram with several peaks (Figure 12) that were then compared and identified through a compound library.

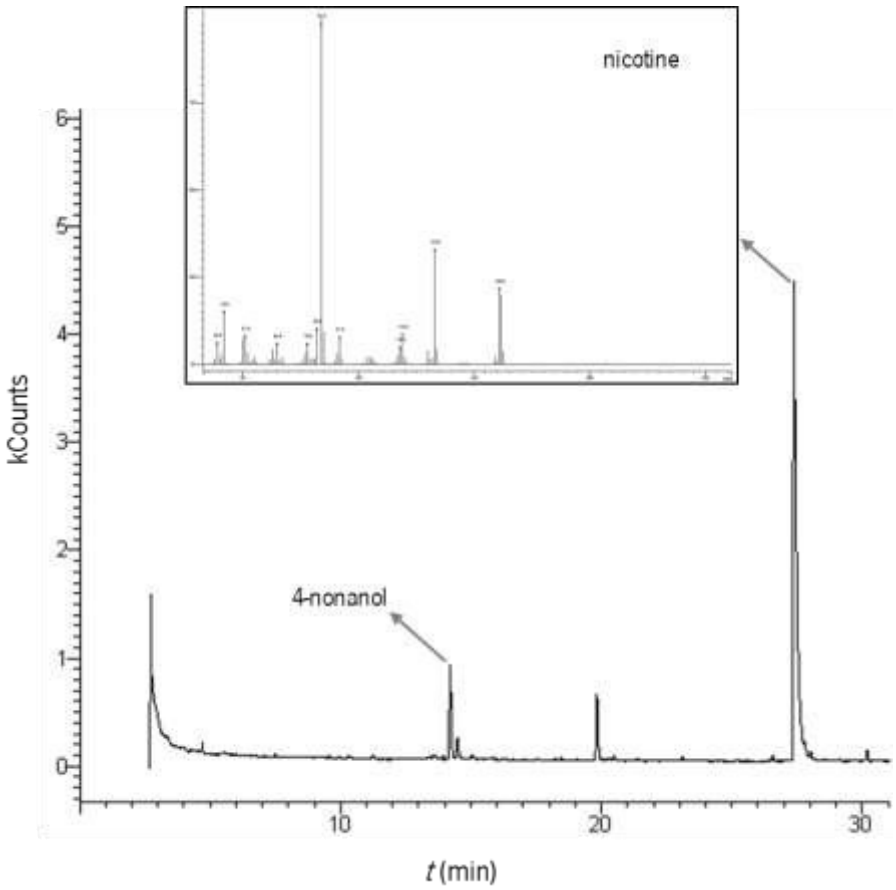

Figure 12. **GC-MS Chromatogram**. Representative example of a sample chromatogram: elution time *(t)* vs. signal (*kCounts*). The peaks are identified as: 4-nonanol, internal standard, detected by MS (± 14 min); nicotine, detected by MS (± 27 min). In the rectangle is presented the mass spectrum of nicotine.

Since samples were spiked with an internal standard of known concentration (4-nonanol), it was possible to determine the amount of nicotine present in the medium by comparing peak areas and taking into consideration the dilution of the samples. The final concentration of nicotine was adjusted according to the *F* factor. Data obtained are summarized in table 4.

Table 4. **GC-MS data**. Corrected average nicotine concentration (according to *F* factor) of all samples tested *(C_n),* standard error to the mean *(SEM)* and the sample size *(n).*

Cigarette	***Cn* (mg/L)**	***SEM* (mg/L)**	***n***
C-cig	7.15	1.02	14
E-cig	12.63	1.71	14

4.2. Impact of aerosols in lung development

To determine whether e-cig aerosol affects normal lung development, chick embryonic lungs were treated, *in vitro*, with the aerosol-exposed medium. Up to now, the impact of e-cig aerosols had been only evaluated in different cell lines and in *in vivo* mouse studies [61,130]. On the other hand, there is plenty of information regarding c-cig smoke impact in different cell lines [111,112] as well as developmental studies as, for instance, in zebrafish embryos [75]. For this reason, chick lung explants were also exposed to c-cig enriched medium for comparison.

In vitro explant culture uses the whole organ and provides more information about the impact of aerosols/smoke than a cell line. With this system, it is possible to examine not only the impact at the morphological level (morphometric analysis) but also, by analyzing the medium after 48 hours in culture, to detect the molecular response of the tissue.

Lung explant culture was performed simultaneously with the aerosol/smoke acquisition system adjustments. In fact, throughout the optimization process, explant analysis served as an additional method to help to determine whether the system was functioning properly. For each variable tested the corresponding culture was performed. However, the images are not displayed in the manuscript because it would greatly increase the number of images and could be misleading. Ultimately, only explants corresponding to the final settings are shown in the following section.

4.2.1. Morphometric analysis

Stage b2 lungs were cultured *in vitro*, for 48 h, with either non-exposed (control), e-cig or c-cig exposed medium (Figure 13). C-cig treated lungs (Figure 13 D) look smaller than control and e-cig treated explants (Figure 13 B and F, respectively); moreover, they presented a different aspect since they appeared more “whitish” and with some signs of tissue disruption (Figure 13 D). Regarding e-cig treated lungs (Figure 13 F), they seemed to be smaller than control (Figure 13 B) but with similar shape and color.

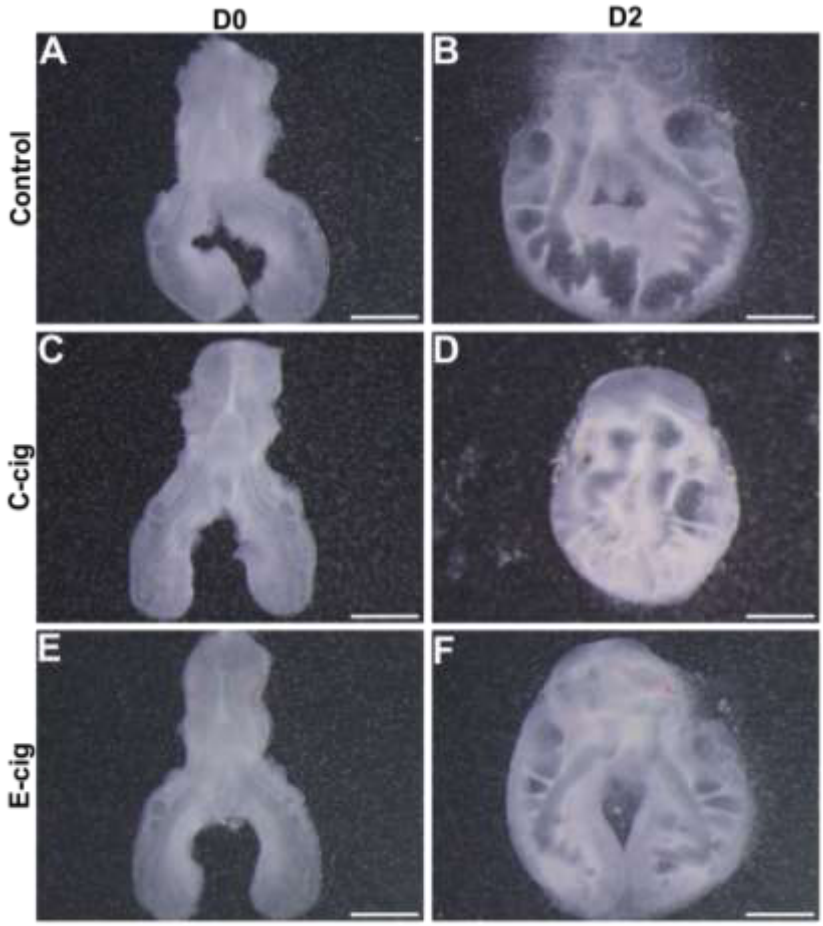

Figure 13. ***In vitro*** **chick lung explants**. Representative examples of stage b2 lung explant culture. Lungs cultured with: unexposed (n≥16) (A-B), smoke (C-D) and aerosol (E-F) exposed medium (n≥16). D0: 0 h and D2: 48 h of incubation. Scale bar: 500 μm.

The average nicotine concentration in the aerosol-exposed medium is approximately 43% higher than average concentration in smoke-exposed medium (see table 4). For this reason, an additional set of explants were cultured with different dilutions of e-cig medium to mimic nicotine concentrations similar to c-cig exposed medium, namely 1:2, 1:3, 1:4 and 1:8. In all the cases, lung explants were even more similar to controls, as expected (data not shown).

Lung explants were morphometrically analyzed at D0 (0 h) and D2 (48 h) of culture. The internal perimeter and area (epithelium) and the outer perimeter and area (mesenchyme) were assessed and results expressed as D2/D0 ratio (Figure 14). These parameters provide information about lung growth and the impact of the treatment in both compartments, independently.

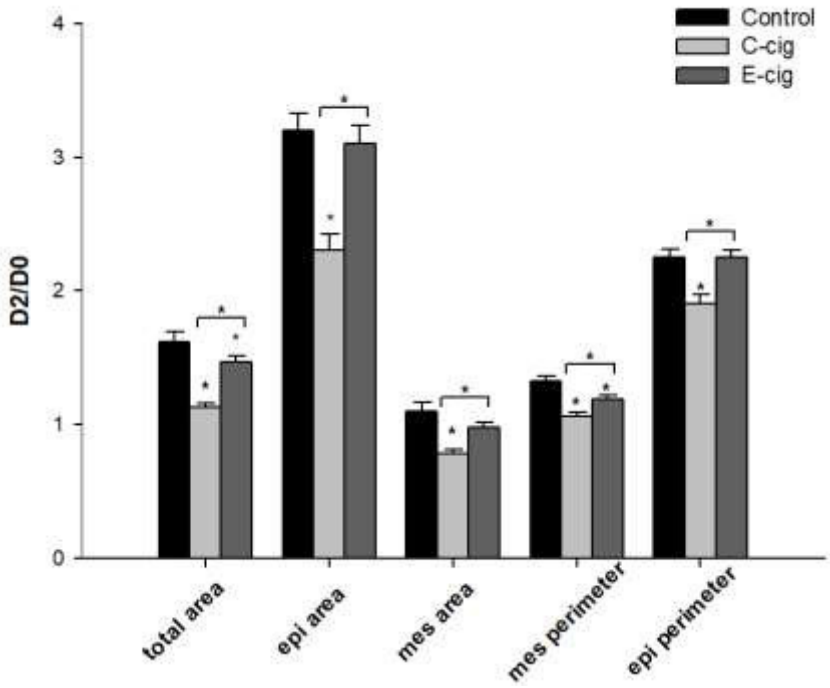

Figure 14. **Morphometric analysis of lung explants.** Control, unexposed medium; c-cig, smoke-exposed medium; e-cig aerosol-exposed medium. Results are expressed as D2/D0 ratio; n≥16. Data is represented as mean ± SEM. *p<0.05 indicate the statistical differences for each treatment. Epi: epithelial. Mes: mesenchymal.

The post-hoc analysis using Fisher's LSD test revealed a statistically significant decrease in all c-cig parameters, when comparing to controls: the total area of c-cig treated explants decreased approximately 30% (p=.000000002); epithelial and mesenchyme area, around 28% (p=.000008 and p=.000003, respectively); epithelial perimeter approximately 20% (p=.0003) and mesenchyme perimeter nearly 15% (p=.0000006).

On the other hand, e-cig parameters were very similar to controls. For instance, epithelial and mesenchyme area displayed a decrease in 3% and 11%, respectively, whereas the epithelial perimeter was equal to control; for these parameters, no statistical differences were found. Nevertheless, the total area and mesenchyme perimeter showed a 10% statistically significant decrease (p=.043, p=.012, respectively).

Conversely, all e-cig parameters showed a statistically significant increase when compared to c-cig: 30% in the total area (p=.000006), 34% in the epithelial area (p=.00003), 25% in the

mesenchyme area (p=.001), 12% in the epithelial perimeter (p=.0002) and 18% in the mesenchyme perimeter (p=.005).

4.3. Lactate dehydrogenase activity

Cytotoxicity assays are often based on assessing damage to cellular membranes. For example, enzyme leakage into the culture medium is an indicator of cell/tissue injury or death. In this case, post-incubation culture medium collected at 24 (D1) and 48 (D2) hours were analyzed to assess the presence/activity of LDH to determine the impact of smoke/aerosol-exposed medium in the embryonic lung.

For this assay, several medium dilutions were tested (1:50, 1:100, 1:200, 1:250, 1:500, 1:1000, 1:2000). In all conditions and dilutions, the supplemented control medium (i.e., D0 medium that was not aerosol/smoke-exposed) displayed a basal LDH activity. On the other hand, 199 medium (medium without any kind of supplementation) did not exhibit LDH activity. This prompt us to conclude that the observed basal LDH activity is due to the medium supplements. The LDH activity of the samples (D1 and D2 medium) was determined by eliminating this effect and, ultimately, no LDH activity was detected in the samples suggesting that lung tissue may not be significantly altered.

4.4. Tumor necrosis factor α levels

Tumor necrosis factor α (TNF-α), a pro-inflammatory cytokine, is a multipotent modulator of immune response that responds to different stimuli as, for instance, infectious agents or tissue injury. Previous studies reported that c-cig exposure induced inflammation and, consequently, pro-inflammatory cytokine release. In this work, we aimed to compare TNF-α release into the culture medium in both e-cig aerosol and c-cig smoke conditions. For this purpose, post-incubation culture medium collected at 24 (D1) and 48 (D2) hours were analyzed.

For this assay, several sample medium dilutions were tested (undiluted, 1:5, 1:10, 1:15) to determine which one fits in the standard curve. Eventually, dilution 1:10 proved to be the best option. The undiluted and 1:5 dilution TNF-α values did not fit the standard curve; on the other hand, 1:15 dilution was below the ELISA detection limit. The total amount of TNF-α (Figure 15) was calculated considering the values from D1 and D2 since the medium was replaced/refreshed after 24 hours.

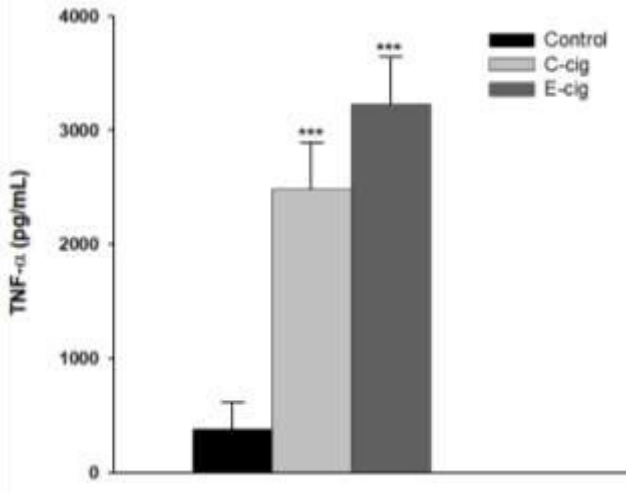

Figure 15. **TNF-α levels in the culture medium** in unexposed, smoke and aerosol-exposed medium. Control: unexposed; c-cig, smoke and e-cig, aerosol-exposed medium. Results are expressed as pg/mL; n=6. Data is represented as mean ± SEM. ***p<0.001 indicate the statistical differences for control.

The post-hoc analysis using Fisher's LSD revealed a statistically significant increase of TNF-α levels in c-cig and e-cig exposed medium (p=.0007 and p=.00004, respectively) when compared to control; the absolute values are about 7 and 9 time higher, respectively. On the other hand, e-cig TNF-α concentration is roughly 1.3 times higher than c-cig, however, this increase is not statistically significant. Overall, e-cig induces cytokine release similarly to c-cig.

4.5. Impact of aerosols in a macrophage cell line

4.5.1. Cell viability

Macrophages are the first line of defense against exogenous pathogens and toxic substances and, accordingly, protect the lung from c-cig smoke and e-cig aerosols [116]. In this sense, it is important to understand the effect of smoke/aerosols in Mφ cells. For this purpose, Mφ were cultured with aerosol or smoke-exposed medium and cytotoxicity was evaluated.

4.5.1.1. Metabolic viability assay (MTT)

Cell viability was assessed in the aerosol and smoke-exposed medium by measuring cellular metabolic activity, which reflects the number of metabolically active cells in a population, through the MTT assay. Culture medium, collected at 24 and 72 hours, was analyzed and the results are presented in Figure 16.

Overall, a particular substance is considered toxic when it induces a cell viability below 80%. In Figure 16 A we can observe that in all dilutions of c-cig smoke exposure, at both time points,

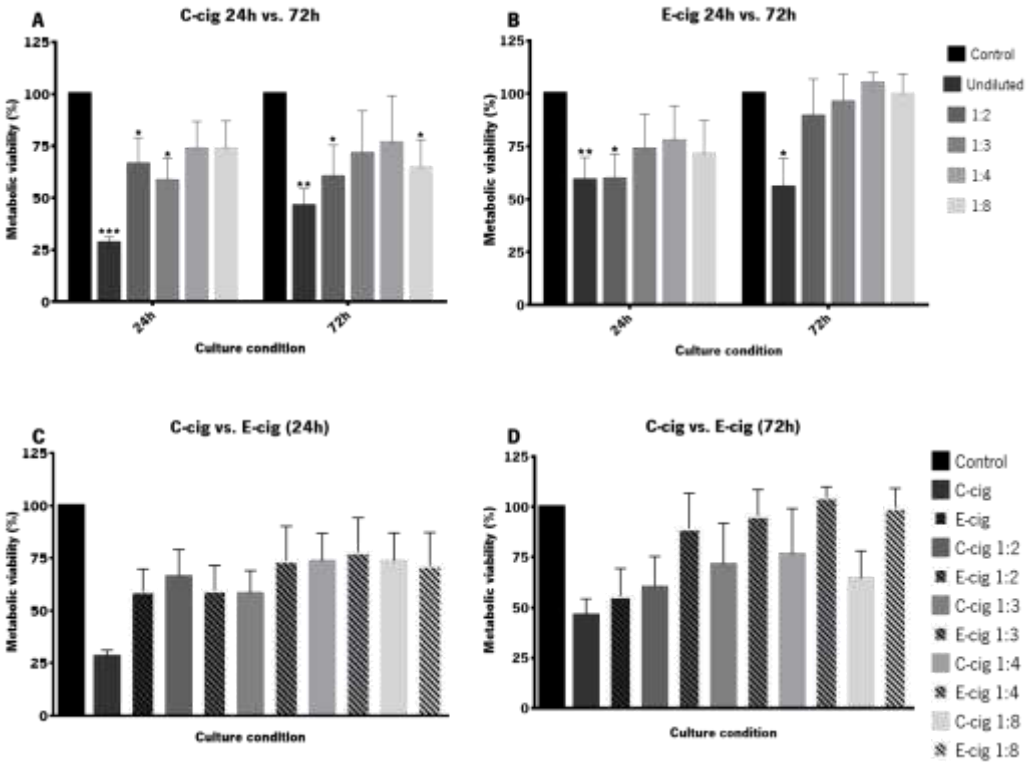

Figure 16. **Mφ cell viability evaluated by MTT assay.** Effect of c-cig smoke (A) and e-cig aerosol (B) on cell viability at 24 and 72 h of incubation. Data grouped for all conditions at 24 h (C) and 72 h (D). Control: unexposed medium. C-cig: smoke-exposed medium. E-cig: aerosol-exposed medium. Assay conditions: undiluted, 1:2, 1:3, 1:4, and 1:8. Cell viability of 100% was assumed for control medium. Data is represented as mean ± SEM (n≥6). *p<0.05, **p<0.01, ***p<0.001 indicate the statistical differences for each dilution treatment and time point.

metabolic activity was below 80%, which confirms that c-cig smoke is toxic. Regarding e-cig aerosol exposure (Figure 16 B), at 24 h and in all the dilutions, the metabolic activity was also below 80% pointing to a toxic effect at this time point; however, after 72 h of exposure, the e-cig aerosol is not considered toxic from the 1:2 dilution onwards. In all cases, as expected, metabolic viability increased with increasing medium dilutions, in a dose-dependent manner.

We can observe that Mφ, treated with the c-cig smoke medium at 24 and 72 h (Figure 16 A), display a dose-dependent metabolic viability since, as expected, metabolic viability increased when the medium was diluted. A statistically significant decrease in cell viability, at 24 h in the undiluted (p= .00006), 1:2 (p= .042) and 1:3 dilution (p= .017) was observed when compared with control; the dilution 1:4 and 1:8 also exhibited a decrease in viability, however, no statistical differences were found when compared with control. At 72 h a dose-dependent increase was also observed

and the undiluted, 1:2 and 1:8 dilution condition also displayed a statistically significant decrease in cell viability (p= .0012; p= .016; p=.032; respectively). The remaining conditions were statistically similar to control, even though the viability was decreased. In table 5, the variations in percentage (%) between groups are stated.

Table 5. **Comparison of the metabolic viability** between c-cig and control condition, at 24 and 72 h, in percentage (%). ↓, decrease.

Culture condition	C-cig vs. Control (%)	
	24 h	72 h
Undiluted	↓ 72	↓ 54
1:2 dilution	↓ 34	↓ 40
1:3 dilution	↓ 42	↓ 29
1:4 dilution	↓ 27	↓ 24
1:8 dilution	↓ 26	↓ 36

Comparing the c-cig dilutions, at 24 h and 72 h, no statistical differences were found, but an increase of metabolic viability in the undiluted, 1:3 and 1:4 treatment was observed, in contrast to a decrease in the 1:2 and 1:8 dilutions. In table 6, the variations in percentage (%) between groups are presented.

Table 6. **Comparison of the metabolic viability** between c-cig condition at 24 and 72 h, in percentage (%). ↓, decrease. ↑, increase.

Culture condition	C-cig 24 vs. 72 h (%)
Undiluted	↑ 64
1:2 dilution	↓ 10
1:3 dilution	↑ 22
1:4 dilution	↑ 4
1:8 dilution	↓ 12

Aerosol-exposed cells showed a dose-dependent increase in cell viability with increasing dilutions. A statistically significant reduction in cell viability at 24 h in the undiluted and 1:2 dilution (p= .008, p= .011, respectively) was detected when compared to controls (Figure 16 B); the other conditions did not show a significant reduction. At 72 h, only the undiluted condition presented a decrease in cell viability (p= .004). The remaining conditions were statistically similar to control; 1:2 dilution still presents a reduction in viability, overall from 1:3, the other dilutions present very similar values to control, in fact, 1:8 condition was equal to control. In table 6, the decrease in percentage (%), between groups, is stated.

Table 7. **Comparison of the metabolic viability** between e-cig and control condition, at 24 and 72 h, in percentage (%). ↓, decrease, **-** without variation.

Culture condition	E-cig vs. Control (%)	
	24 h	72 h
Undiluted	↓ 41	↓ 44
1:2 dilution	↓ 41	↓ 20
1:3 dilution	↓ 26	↓ 4
1:4 dilution	↓ 22	↓ 5
1:8 dilution	↓ 29	-

Comparing the effect of e-cig dilutions, at 24 h vs. at 72 h, no statistical differences were found, but an increase in metabolic viability in the 1:2, 1:3, 1:4 and 1:8 dilution was observed and

a decrease in the undiluted. In table 8, the variations in percentage (%) between groups are presented.

Table 8. **Comparison of the metabolic viability** between e-cig condition at 24 and 72 h, in percentage (%). ↓, decrease. ↑, increase.

Culture conditions	E-cig 24 h vs. 72 h (%)
Undiluted	↓ 6
1:2 dilution	↑ 50
1:3 dilution	↑ 30
1:4 dilution	↑ 35
1:8 dilution	↑ 40

When compared amongst each other, smoke and aerosol-exposed cells did not display statistically significant differences in cell viability, at both time points (Figure 16 C, D). Nevertheless, at 24 h (Figure 16 C) a decrease in cell viability in the undiluted, 1:3 and 1:4 dilution of c-cig treatment was detected when compared to e-cig treatment; moreover, an increase in 1:2 and 1:8 dilution when compared with e-cig was also identified. As it can be observed, when we dilute the samples the c-cig and e-cig effect in viability becomes more similar among them. At 72 h (Figure 16 D), a decrease in cell viability of all c-cig treatments when compared to e-cig. In table 9, the variations in percentage (%) between groups are presented.

Table 9. **Comparison of the metabolic viability** between c-cig and e-cig condition, at 24 and 72 h, in percentage (%). ↓, decrease; ↑, increase.

Culture conditions	C-cig vs. E-cig (%)	
	24 h	72 h
Undiluted	↓ 52	↓ 17
1:2 dilution	↑ 11	↓ 33
1:3 dilution	↓ 21	↓ 26
1:4 dilution	↓ 5	↓ 27
1:8 dilution	↑ 3	↓ 35

These results suggest that aerosol exposure affects Mφ viability similarly to smoke exposure, since no differences between treatments were found. However, when compared with control, c-cig smoke is more cytotoxic than e-cig aerosol.

4.5.1.2. Lactate dehydrogenase activity

LDH leakage into the culture medium is an indicator of cell/tissue injury or death and its activity is inversely proportional to cell viability. Hence, cell viability was assessed in the aerosol and smoke-exposed medium by measuring LDH activity. Culture medium collected at 24 and 72 hours was analyzed and the results displayed in Figure 17.

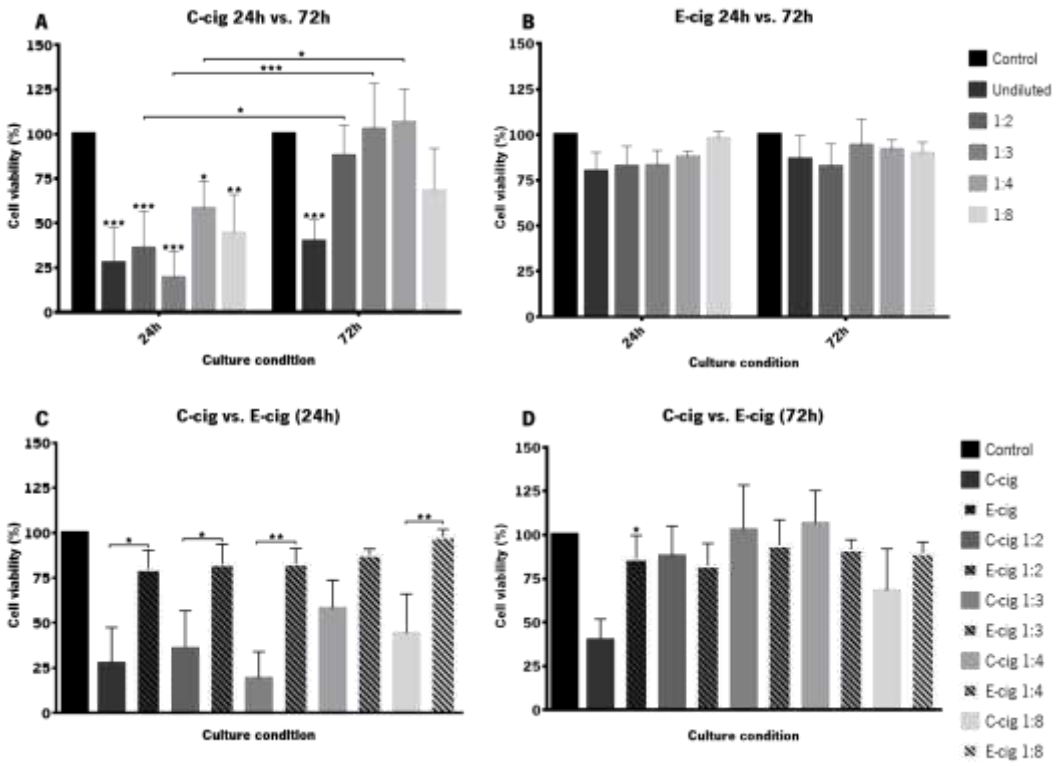

Figure 17. **Mφ cell viability assessed by LDH activity.** Effect of c-cig smoke (A) and e-cig aerosol (B) on cell viability at 24 and 72 h of incubation. Data grouped for all conditions at 24 h (C) and 72 h (D). Control: unexposed medium. C-cig: smoke-exposed medium. E-cig: aerosol-exposed medium. Assay conditions: undiluted, 1:2, 1:3, 1:4, and 1:8. Cell viability of 100% was assumed for control medium. Data is represented as mean ± SEM (n≥3). *p<0.05, **p<0.01, ***p<0.001 indicate the statistical differences for each dilution treatment and time point.

Taking into consideration that it is widely accepted that viability below 80% implies toxicity, in Figure 17 A it can be observed that all the dilutions, at 24 h, of c-cig smoke exposure, are toxic, corroborating the results obtained in the section 4.5.1.1. Nonetheless, at 72 h, only the undiluted

and the 1:8 dilution exhibited toxic effects. The result obtained with the 1:8 dilution is unexpected since the previous (and more concentrated) conditions did not display toxicity.

In the Figure 17 B it is possible to observe that, at 24 h of exposure to e-cig aerosol, only the undiluted treatment is slightly below 80% and, for this reason, may be considered as modestly toxic. This result revealed that the impact of c-cig smoke in LDH release is more severe than e-cig; since LDH release to the environment is associated with cell death, this result suggests that the c-cig exposure is more toxic, thus promoting more cell death then e-cig exposure.

Figure 17 A presents the effect of c-cig smoke on cell viability after 24 and 72 h. When compared to control, smoke-exposed cells showed a statistically significant decrease in cell viability at 24 h, in all dilutions, in a dose-dependent manner (undiluted to 1:8: p= .00009, p= .0004, p= .00002, p= .017, p= .002, respectively). However, at 72 h, only in the undiluted condition (p= .0008) presented a statistically significant decrease; nevertheless, the dose-dependent effect is still observed. The 1:2 dilution presented a decrease in viability at 72 h, comparing to control, whereas the 1:3 and 1:4 presented an increase, however, no statistical differences were found between the dilutions and the control; 1:8 dilution displayed a decrease in cell viability, still no statistical differences were found. In table 10, the variations in percentage (%) between groups are presented.

Table 10. **Comparison of the cell viability** between c-cig and control condition, at 24 and 72 h, in percentage (%). ↓, decrease; ↑, increase.

Culture condition	C-cig vs. Control (%)	
	24 h	**72 h**
Undiluted	↓ 72	↓ 60
1:2 dilution	↓ 64	↓ 12
1:3 dilution	↓ 81	↑ 3
1:4 dilution	↓ 42	↑ 6
1:8 dilution	↓ 56	↓ 32

After 72 h, cell viability increased significantly when compared to the corresponding condition at 24 h (1:2, 1:3 and 1:4 dilutions: p= .011, p= .00009, p= .017, respectively). No statistically significant differences were detected when comparing the 1:8 dilution to its matching at 72 h, even

that an increase in viability is observed. The viability in the undiluted condition increases from 24 h to 72 h, however, this increase is not statistically significant (table 11).

Table 11. **Comparison of the cell viability** between c-cig condition at 24 and 72 h, in percentage (%). ↑, increase.

Culture condition	C-cig 24 vs. 72 h (%)
Undiluted	↑ 44
1:2 dilution	↑ 145
1:3 dilution	↑ 436
1:4 dilution	↑ 83
1:8 dilution	↑ 54

When comparing e-cig aerosol-exposed cells with control no differences were detected (Figure 17 B), however a decrease in viability is observed in all treatments comparing to controls (24 h and 72 h). In table 12, the variations in percentage (%) between groups are presented.

Table 12. **Comparison of the cell viability** between e-cig and control condition, at 24 and 72 h, in percentage (%). ↓, decrease.

Culture condition	E-cig vs. Control (%)	
	24 h	72 h
Undiluted	↓ 20	↓ 14
1:2 dilution	↓ 18	↓ 18
1:3 dilution	↓ 17	↓ 6
1:4 dilution	↓ 12	↓ 8
1:8 dilution	↓ 2	↓ 10

Additionally, there were no differences between 24 h and 72 h exposure, however, in this case, an increase of viability is noted in all conditions, except in 1:2 and 1:8, which are similar (table 13).

Table 13. **Comparison of the cell viability** between e-cig condition at 24 and 72 h, in percentage (%). ↑, increase, **-** without variation.

E-cig 24 h vs. 72 h	%
Undiluted	↑ 8
1:2 dilution	-
1:3 dilution	↑ 13
1:4 dilution	↑ 83
1:8 dilution	-

On the other hand, at 24 h (Figure 17 C), aerosol-exposed cells showed a statistically significant increase in cell viability when compared to the corresponding c-cig conditions (undiluted, 1:2, 1:3 and 1:8 dilutions: p= .0102, p= .02, p= .002, p= .009, respectively) except in the 1:4 dilution condition. At 72 h (Figure 17 D), only e-cig undiluted condition is statistically different from c-cig, presenting an increase of viability (p= .021); the other conditions exhibited a decrease, except for 1:8 that increased, without statistical differences. In table 14, the variations in percentage (%) between groups are presented.

Table 14. **Comparison of the cell viability** between c-cig and e-cig condition, at 24 and 72 h, in percentage (%). ↓, decrease; ↑, increase.

Culture conditions	C-cig vs. E-cig (%)	
	24 h	72 h
Undiluted	↑ 190	↑ 118
1:2 dilution	↑ 131	↓ 6
1:3 dilution	↑ 333	↓ 8
1:4 dilution	↓ 51	↓ 14
1:8 dilution	↑ 122	↑ 32

These results suggest that c-cig smoke is more toxic than e-cig aerosol. The smoke toxicity observed in this assay is in agreement with the data obtained from MTT assay (section 4.5.1.1), while the e-cig aerosol appears less toxic.

5. Discussion

DISCUSSION

Tobacco smoking is a major public health issue associated with pulmonary diseases and high mortality rates. New nicotine delivery systems (NDS) such as electronic cigarettes (e-cig) have been developed and publicized as less noxious than conventional cigarettes (c-cig). In fact, inhaled e-cig aerosol components are assumed to be less toxic than the thousands of known toxicants in smoke [110]. Additionally, the possibility to personalize e-cigs (nicotine concentration, flavorings) has contributed to its widespread use amongst young people [35]. E-cigs device vaporizes a liquid creating an aerosol that reaches the lungs. The effect of c-cig smoke in lung development and the immune system has been already documented; however, the effect of e-cig aerosol is not known so far. Nonetheless, the assumption that e-cig is harmless does not completely discard the potentially damaging effects of inhaling e-cig aerosol [86]. Therefore, the goal of this work was to compare the impact of e-cig aerosols and c-cig smoke in lung development and in macrophages.

This work required the production of an enriched smoke/aerosol culture medium. Accordingly, a manual acquisition system was established and optimized. As a starting point, we based our method in the system described by Romagna *et al.* (2013) [109]. Alternatively, we could have used a commercial smoke/aerosol extract [22] or, eventually, employed a smoking machine [110, 141]. The smoking machine can expose cells/tissues that are already in culture directly to smoke or aerosol; this system may lead to culture contamination and more aggressive results due to particle accumulation in the culture medium. Moreover, when using these machines it is not possible to quantify compounds present in the medium. When culturing lung-specific cells, this could be an excellent option since they are one of the first cell types that are exposed to smoke/aerosol. On the other hand, other types of cells are normally exposed to c-cig and e-cig components that are transferred to the bloodstream. In section 1.4.2.3. it was referred that the inhaled nicotine affects the lungs differently than other organs that are exposed to this substance via the circulating blood [64]. Indeed, it is likely that the same may happen with e-cig aerosol. For this reason, producing an extract seemed to be the best option to perform this work since both the developing lung and Mφ are not directly exposed to smoke/vapor (except alveolar Mφ). Nonetheless, recent studies performed with c-cigs or e-cigs used an acquisition system to produce the extract [142,143].

Nicotine was the compound selected to validate the quantification method for two reasons: it is possible to compare aerosol and smoke levels, since it is common to both cigarettes, and it can be easily extracted and quantified by gas chromatography-mass spectrometry [85,144]. There are

other validation methods described in the literature, for instance, quantification of specific compounds present in smoke or aerosol, or even in the e-juice. Etter *et al.* (2013) analyzed e-juice by Ultra-high performance liquid chromatography; Trehy *et al.* (2011) evaluated nicotine levels in smoke and e-juice by HPLC; Farsalinos *et al.* (2014, 2015) estimated the aerosol compounds by HPLC. Schripp *et al.* (2013) assessed e-cig aerosol components by GC-MS, and Goniewicz *et al.* (2014) used a set of analytical methodologies (HPLC coupled to DAD, UPLC-MS, GC-MS and inductively coupled plasma associated to a mass spectrometer) to assess the e-cig vapor [23,51,83,84,85,145]. All the above mentioned analytical methods are extremely sensitive and precise, and its selection depends on the compounds that need to be detected and the extraction method performed. For example, to evaluate nicotine by HPLC a liquid-liquid extraction would not be necessary since culture media is a stable (not volatile) liquid. In our case, the selected method (GC-MS) requires nicotine extraction into a volatile solvent that can then be vaporized in the gas chromatograph. Sample components are isolated in the gas chromatograph since the different compounds have different retention times. Subsequently, the mass spectrometer only detect fragments of one compound and, therefore, this technique is highly sensitive, reproducible and robust [139]. Our study GC-MS analysis revealed that there is around 43% more nicotine in aerosol-exposed medium than in smoke-exposed medium. On average our aerosol-saturated samples present about 5 times more nicotine than the maximum detected in other studies (section 1.4.3.). These divergences may be due to the distinct experimental approaches used, namely: different acquisition process (*e.g.* smoke machine [20,82,83]) or different nicotine quantification method (*e.g.* HPLC or UPLC [20,83]). Conversely, the nicotine levels detected on smoke-saturated samples fit the concentration range of previous studies (section 1.4.3.). Nicotine was determined by applying the *F* factor to the concentrations detected by this instrument. The need to apply the *F* factor is because the extraction process is not 100% efficient. However, our objective was achieved, since we optimize a simple, reproducible system to acquire the aerosol/smoke that was validated by the GC-MS nicotine detection.

Considering that e-cig popularity is increasing and that pregnant women are a vulnerable population to the effect of nicotine [58], it is crucial to determine the impact of aerosol in embryo development, particularly in lung development, and compare it with the effect of c-cig smoke. It is widely known that smoking can provoke serious lung problems [146]. In this work, we asked whether the effect of e-cig aerosol in lung development if any, it is more or less aggressive than the

impact of c-cig smoke. For this purpose, embryonic chick lungs were cultured, *in vitro*, with smoke (c-cig) or aerosol (e-cig) exposed medium and analyzed morphometrically. Our results suggest that smoke impairs lung development. Lung explants treated with c-cig smoke were 30% smaller than controls. All the morphometric parameters examined corroborated this finding. Moreover, lung tissue displayed a "whitish" appearance, and the epithelial compartment was difficult to visualize. On the other hand, explants treated with e-cig aerosol looked similar but 10% smaller than controls. Despite the fact that nicotine levels in e-cig exposed medium (12.63 mg/L) is higher than c-cig exposed medium (7.15 mg/L), the e-cig exposed medium treatment had less impact; in fact, only the total size and mesenchyme perimeter was decreased in e-cig exposed lungs when compared to controls.

To the best of our knowledge, this is the first study describing e-cig aerosol impact in lung development, using an organotypic culture. Thus far, there was only one study employing mouse lung explants to determine the effect of nicotine in lung branching morphogenesis, made by Wongtrakool *et al.* (2007). In this study, it was reported that 162 mg/L of nicotine in the culture medium stimulated branching morphogenesis and that this effect was mediated by α7 nAChR. In our study, the observed effects are likely due to all the components of smoke/aerosol and not just nicotine. The nicotine concentration in our exposed medium is approximately 22 times less for c-cig and 13 times less for e-cig compared to this study [147]. As expected, and considering the numerous toxic substances present in c-cig smoke, explants display an unhealthy appearance. On the other hand, e-cig aerosol has allegedly fewer toxic compounds and its impact on lung morphology and growth is not as exacerbated as c-cig smoke. For example, in table 2 (section 1.4.3.) the most common toxic substances detected in the smoke/aerosol are indicated. In general, all these substances are present in lower concentrations in the aerosol when compared to smoke. Furthermore, Taylor *et al.* (2016) reported that carbon monoxide is not detected in e-cig aerosol by quality control measures, whereas smoke has an average of $1.2x10^{5}$ μg/L; additionally, the carbonyl-containing substances in e-cig aerosol are at least 86% less than in c-cig smoke [82]. Overall, c-cig smoke has more toxic components than e-cig aerosol that may justify the obtained results.

Additionally, and in order to determine the cellular impact of smoke/aerosol, toxicity and inflammatory response were assessed by evaluating LDH and TNF-α release into the culture medium, after culture. LDH is associated with cell/tissue injury and pro-inflammatory cytokines

release are associated with smoking/vaping [38,122,129]. LDH assay revealed an absence of enzymatic activity in all conditions, suggesting that, in our conditions, lung tissue was not significantly injured. This result was not expected in the c-cig condition considering the well-known toxic effect of smoking [113]. On the other hand, a possible explanation of these results is that our samples may not be compatible with the used kit. In the future, it will be interesting to analyze LDH using another activity detection kit or method. TNF-α levels were quantified by ELISA and we observed that, like c-cig smoke, e-cig aerosol significantly increases TNF-α release comparing to controls. These results are in agreement with published data reporting that e-cig aerosols can promote inflammation when compared to untreated groups [39,112]. Al-Aali *et al.* (2018) evaluated the pro-inflammatory cytokine release in the fluid surrounding dental implants and observed that e-cig users exhibit an increase in TNF-α production when compared to non-smokers [39]. Scott *et al.* (2018) exposed alveolar Mφ to e-cig aerosol condensate and observed an increase in TNF-α comparing to nicotine-free aerosol exposure and a significant increase compared to untreated groups [112]. In this work, nicotine concentration in the aerosol condensate was 132.5 mg/L, which is approximately 10 times more than the nicotine concentration obtained in our study. Our results establish a lower nicotine level in the e-cig aerosol, and eventually lower levels of other constituents, as still capable of inducing inflammation. To conclude, our results show that c-cig smoke has a more deleterious effect on lung growth than e-cig aerosol. Nonetheless, aerosol affects lung growth and promotes inflammation similarly to smoke.

Smoking can cause lung diseases and an alteration of immunity profile. It is already known that c-cigs affect Mφ function and viability [125,126]. Recent studies have shown that e-cig aerosol can also affect Mφ in the same way as c-cig smoke [115,130], however additional data is needed to fully understand how aerosols influence Mφ. To determine aerosol cytotoxicity, and compare it with smoke, we treated a Mφ cell line with culture medium exposed to smoke or aerosol, and performed viability test in cells (MTT assay) and in the medium post culture (LDH assay). We assessed the impact of smoke and aerosol at two time-points, after 24 h for acute exposure and 72 h for a longer exposure. The results of MTT metabolic assay allow inferring cell viability at the end-point of culture. Data revealed that, after 24 h of exposure, macrophage metabolic viability was significantly decreased in smoke and aerosol treatment in the undiluted samples, however when the aerosol/smoke was diluted cell viability increased, showing a dose-dependent effect. The same is observed at 72 h of incubation, however, Mφ viability was higher in both treatments when

compared to 24 h. No differences between aerosol and smoke treatment were found. The results corresponding to c-cig exposure are in accordance with previous studies [148,149]. Briefly, Park *et al.* (2018) exposed mouse alveolar Mφ, for 24 h, to increasing concentrations of a saturated smoke solution, with nicotine concentrations corresponding to 2.3, 4.7 and 9.4 ppm, which corresponds approximately to 2.3, 4.7 and 9.4 mg/L, respectively, and that match the nicotine concentration obtained in our work. In that study, a significant dose-dependent decrease in Mφ viability was observed which is in agreement to our results; moreover, an increase in apoptosis and necrosis was detected, and a cell cycle S phase arrest was also observed [148]. Cyprus *et al.* (2018) exposed Mφ differentiated from mouse bone marrow cells and human bone marrow-derived mesenchymal stem cells for 3 days (72 h) to a batch of smoke extract dilutions (1:100, 1:1000 and 1:10000). Afterward, cell viability was assessed and a dose-dependent decrease in cell viability was observed. In this study, the smoke components were not quantified, however, the nicotine composition of the c-cig used (0.73 mg) is similar to the one used in our work [149].

Kim *et al.* (2017) exposed human placental cells to a batch of smoke saturated medium with different nicotine concentrations ($4.8x10^{-2}$, $9.7x10^{-2}$, $1.5x10^{-1}$, $1.9x10^{-1}$, $2.4x10^{-1}$, $2.9x10^{-1}$, and $3.4x10^{-1}$ mg/L), for 6 days, and evaluated the metabolic viability (MTT). Increasing smoke concentrations lead to a decrease in viability, in a dose-dependent manner, similarly to our results. Nonetheless, the maximum viability decrease was of approximately 50%, whereas we observed around 70% decrease [22]. This may be explained because our maximum exposure concentration (7.15 mg/L) is almost 30 times higher than the maximum concentration used in the study. Moreover, it is likely that cell types may be differently affected by smoke, and placental cells can be more resistant to smoke solution exposure than Mφ. Campos *et al.* (2017) treated a J774A.1 macrophage cell line with a smoke extract with different concentration (0.1, 0.25, 0.5, 0.625, 1.25, 2.25, 5 and 10%) for 3, 6 and 24 h, and assessed metabolic viability by MTT. The authors observed that, after 3 h, only 10% of smoke extract exposed cultures displayed a significant decrease in Mφ viability. Moreover, after 6 and 24 h, the lower concentration revealed to be cytotoxic in a dose-dependent manner [150], similar to what is observed in our results. Given that c-cig smoke is toxic, and that smoke can activate cell death pathways (apoptosis and necrosis) in a dose-dependent manner, a decrease in cell viability is expected and it is in agreement with previous studies with similar concentrations [148].

LDH activity is also a good method to evaluate cell viability. A significant decrease in cell viability at 24 h and 72 h was observed, after smoke treatment; however, at 72 h, cell viability was higher in lower smoke concentration when comparing to 24 h incubation. At 72h cell viability was significantly higher than at 24h (in 1:2, 1:3 and 1:4 dilutions; section 4.5.1.2), suggesting that macrophages can recover from the insult of smoke over time. Again, a dose-dependent effect was observed. Conversely, aerosol treatment did not show any differences after 24 h and 72 h incubation, when compared to control. In fact, cell viability in e-cig treatment is significantly higher than c-cig. These data show that though metabolic viability is lower than control and similar to c-cig, aerosol exposure does not induce damage to Mφ. Cervellati *et al.* (2014) evaluated LDH release after exposing skin cells (keratinocytes) and lung epithelial cells to UK research cigarette smoke (12 mg tar, 1.1 mg nicotine) and different e-cig aerosols (with or without nicotine, and with or without balsamic flavors); a dose-dependent increase of LDH activity (which implies a decrease in cell viability) in smoke and aerosols containing nicotine and flavor was observed. Our findings concerning smoke-treated samples are in agreement with this study, corroborating that c-cig smoke is toxic [110]. Nevertheless, the same was not verified in the aerosol-treated samples. Aerosols have a damaging effect in skin and lung epithelial cells [110], however, it is possible that other cell populations, *e.g.* Mφ, may respond differently. For instance, Schweitzer *et al.* (2015) exposed rat lung endothelial cells to nicotine solutions (0, 162, 406, 811, 1622, 2433 and 3245 mg/L) and e-cig aerosols with different nicotine concentrations (0, 406, 811, 1622 and 2433 mg/L), but did not observe an impact in LDH release [111]. Our results are in accordance with this study since cytotoxicity is observed with increasing aerosol concentration but no changes in LDH activity are detected [111]. The nicotine concentration used in our work is not as higher as the above mentioned, and it is possible that Mφ may be more sensitive than endothelial cells. Moreover, the characteristics of the e-cig extracts used in these studies may account for these differences and may reflect differences in the method used to create the extract, the device used, the e-juice composition.

This data suggests that e-cig aerosol does not interfere with cell viability, however, it is plausible that it might be impairing cell proliferation. In fact, it has been reported that, in similar conditions to our study, c-cig smoke exposure can lead to a decrease in cell proliferation and to cell cycle S phase arrest [148,149]. Moreover, it has been demonstrated that e-cig aerosol decreases the expression of cell proliferation markers in a nicotine-dependent manner (0, 406,

811, 1622 and 2433 mg/L) [111]. This outcome may be explained by the fact that nAChR activation by nicotine can indirectly promote cation influx that regulates signal transduction cascades that, therefore, control cellular processes such proliferation [58]. Another possible explanation for the fact that aerosol-treated cells display a decrease in their metabolic activity, but no evident signs of death, may be because they are using cellular energy for survival and repair. In this hypothetical scenario, cell division is halted and, consequently, the Mφ proliferation rate decreased when compared to control because energy is being used to try to recover from the aerosol's toxic stimulus. Nonetheless, cell proliferation and apoptosis should be evaluated to confirm or discard both hypotheses.

Overall our data indicate that e-cig aerosol affects lung development by promoting inflammation and impairing lung growth. However, the e-cig effect in lung growth is less severe than the effect induced by c-cig smoke exposure. Our Mφ data showed that both c-cig and e-cig treatment affect metabolic viability at acute exposure (24 h) and chronic exposure (72 h), in a dose-dependent manner. However, our LDH data reveal that e-cig aerosol does not induce cell damage whereas c-cig smoke does, proving again that e-cig has less impact than smoking.

6. Final remarks and future perspectives

FINAL REMARKS AND FUTURE PERSPECTIVES

Smoking is a worldwide concern because its harmful effects cause several adult and developmental diseases. Electronic cigarettes emerged aiming to replace tobacco smoking. E-cig is being sold as a healthier way to smoke, which is globally attracting people (including pregnant women who are not willing/able to quit smoking) to this tobacco alternative. However, the knowledge regarding e-cig effect in development, particularly lung development, is still scarce.

The work developed during this thesis aimed to compare the effect of vaping and smoking in lung development, and provide more information regarding their effect in macrophages. We concluded that both c-cig smoke and e-cig aerosol impair lung growth and induces lung inflammation, although the e-cig effect is less severe than c-cig. Regarding macrophages, our data suggest that aerosols do not affect macrophage survival but affects metabolic viability, and confirm that c-cig smoke is toxic for these cells. In this sense, our results revealed that, in fact, e-cig seems to be a less harmful alternative to c-cig.

Nevertheless, further studies are needed to unveil the molecular mechanisms that account for the effect of e-cig in both lung development and macrophage survival. For instance, a cell death assay could be performed to complement and corroborate the toxicity studies. Regarding macrophages, it would be interesting to analyze the pro-inflammatory cytokine profile, after e-cig and c-cig exposure, by ELISA, and also to determine if cell proliferation is altered.

Our results clearly demonstrated that aerosols have a mildly negative impact on lung size, however, new questions emerged that should be answered. Specifically, if the observed effects are due to the humectants, present in the e-juice, or nicotine. To solve this questions, *in vitro* lung explants should be treated with different doses of nicotine and, on the other hand, should also be exposed to aerosol-saturated medium obtained from nicotine-free e-juice. With this approach, the individual contribution of nicotine and humectants will be disclosed.

Additionally, the expression levels of key components of different signaling pathways could be evaluated in lung explants exposed to e-cig aerosol saturated medium. This analysis would bring new insights about how the developing lung copes with this type of insult. Moreover, since HNB products are a fresh tobacco alternative product a similar approach using these devices could be tested.

7. References

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