The effects of electronic cigarettes on human gingival cells and Candida albicans

Thèse

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Résumé

Plusieurs alternatives ont été mises au point pour réduire les effets de la cigarette sur la santé buccale et générale. La plus récente de ces initiatives est la cigarette électronique. Plusieurs études montrent que la cigarette électronique contient moins de produits toxiques comparativement à la cigarette standard. Ces études concluent que la cigarette électronique est moins nocive pour la santé. Cependant, d'autres études émettent des doutes sur l'innocuité de la cigarette électronique étant donné la présence de multiples produits chimiques. Ces derniers peuvent interagir négativement avec plusieurs parties du corps, dont la cavité buccale.

Les objectifs de cette étude sont **(i)** d'évaluer les effets d'expositions répétées (1, 2 ou 3 fois) au condensé de cigarette électronique sur la morphologie, la croissance, la migration et l'apoptose des fibroblastes gingivaux humains **(ii)** d'évaluer les effets de la vapeur de la cigarette électronique sur la croissance, la production de chitine et l'expression de certains gènes codant pour des protéines de la famille des "secreted aspartyl proteinases (SAP par *C. albicans* avec des temps d'exposition de 15 min, deux fois par jour, pendant 2 et 3 jours. **(iii)** d'évaluer l'interaction des cellules épithéliales gingivales avec *C. albicans* préalablement exposé à la cigarette électronique. Nous avons utilisé différentes techniques de biologie cellulaire, de biologie moléculaire et de microbiologie.

Nos travaux montrent que les fibroblastes exposés au condensé de cigarette électronique ont une morphologie anormale (cellules plus grosses, vacuolées,.) et un taux de prolifération plus faible comparativement aux cellules non exposées. Ces observations sont consolidées par un taux plus élevé de cellules apoptotiques comparativement aux cellules non exposées. L'analyse de la migration cellulaire montre que le condensé de cigarette électronique réduit de façon significative la capacité de migration des fibroblastes. Il est à noter que les effets sont plus importants avec la cigarette, suivi de la cigarette électronique contenant la nicotine, puis celle sans nicotine. Les effets de la cigarette électronique sont moins importants que ceux du condensé de cigarette, mais plus sérieux comparativement aux cellules

non exposées. Nos études montrent que l'exposition de *C. albicans* à la cigarette électronique entraîne une augmentation de sa croissance. Cette observation est supportée par un taux plus élevé de chitine produite par *C. albicans* exposé à la cigarette électronique. L'analyse de la transformation montre des formes hyphes plus longues après l'exposition à cigarette électronique. Nous avons aussi observé que la cigarette électronique augmente l'expression des gènes *SAP2, SAP3* et *SAP9* par *C. albicans* comparativement au contrôle (non exposé). L'exposition de *C. albicans* à la cigarette électronique favorise l'adhésion de la levure aux cellules épithéliales, augmente le taux de tyransformation de la levure. L'exposition de C. albicans à la cigarette électronique, puis son contact avec les cellules épithéliales cause une libération importante de la lactate deshydrogénase (LDH), et la différenciation des cellules épithéliales, mais réduit le taux de croissance de ces cellules gingivales.

Les résultats globaux indiquent que les cigarettes électroniques peuvent interagir avec le microbiome buccal de l'utilisateur. Parce que les cigarettes électroniques réduisent la croissance des cellules gingivales et augmentent l'apoptose cellulaire, cela peut diminuer l'immunité innée dans la cavité buccale, ce qui pourrait augmenter le risque d'infections buccales, telles que la candidose.

Abstract

Electronic cigarettes (e-cigarettes) were designed to replace regular cigarette smoking and to contribute to smoking cessation. E-cigarettes require the use of vaping liquid that contains propylene glycol (PG) and vegetable glycerin (VG) as well as nicotine in various concentrations and flavours. Several studies comparing e-cigarettes to conventional cigarettes show that e-cigarettes contain lower levels of toxic compounds and for this reason are deemed safer. However, a growing body of evidence shows that e-cigarettes contain many chemicals including formaldehyde, acetaldehyde, acrolein, and toluene, which may have adverse effects on different body parts, including the oral cavity.

The first objective of this study was to investigate the impact of repeated exposures (1, 2, or 3 times) to e-cigarette condensates with or without nicotine on normal human gingival fibroblast morphology, proliferation, migration, and apoptosis. The second objective was to evaluate the effect of e-cigarettes vapors on the growth changes of *C. albicans* from blastospore to hyphal form and the expression of secreted aspartic proteinases (SAPs) SAP2, SAP3, and SAP9 genes by *C. albicans*, with exposure times of 15 min twice a day for 2 and 3 days. The third objective was to shed light on the interaction between e-cigarette-exposed *C. albicans* and gingival epithelial cells. Various cell biology, molecular biology, and microbiology protocols were deployed.

Results show that exposure of gingival fibroblasts to nicotine-rich e-cigarette condensate altered both cell morphology and proliferation rate. Exposure to the e-cigarette condensate also increased the levels of apoptotic fibroblasts. Fibroblast migration was delayed after culture scratches were exposed to e-cigarette condensate. Although e-cigarettes are considered to be less harmful than are conventional cigarettes, e-cigarettes significantly harmed the fibroblasts compared to non-exposed cells. E-cigarette exposure also increased *C. albicans* growth and hyphal length. The exposed *C. albicans* produced high levels of chitin and expressed high mRNA levels of *SAP2*, *SAP3*, and *SAP9* genes. When in contact with gingival

epithelial cells, e-cigarette-exposed *C. albicans* adhered better compared to the controls. Indirect communication between e-cigarette-exposed *C. albicans* and gingival epithelial cells led to epithelial cell differentiation, reduced cell growth, and increased lactate dehydrogenase (LDH) activity.

Overall results indicate that e-cigarettes may interact with the user's oral microbiome. Because e-cigarettes reduce gingival cell growth and increase cell apoptosis, this may decrease the innate immunity in the oral cavity, which could increase the risk of oral infections, such as candidiasis.

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Liste des abréviations

a4b2	alpha-4 beta-2
A549 cells	human alveolar cell cultures
ACT1	actin 1
AIDS	acquired immunodeficiency syndrome
APVs	advanced personal vaporizers
BOP	bleeding on probing
bpm	beats per minute
BrdU	bromodeoxyuridine
C. albicans	Candida albicans
Ca9-22	human gingival epithelial carcinoma cell line
CCS	conventional cigarette smoke
CDC	Centers for Disease Control
cDNA	complementary DNA
C°	celsius
cig-a-likes	cigarette-like
CO	carbon monoxide
Ctrl	control
COPD	chronic obstructive pulmonary disease
CS	cigarette smoke
SC	smoke cigarette
CSC	cigarette smoke condensate
CT	cycle threshold
CVDs	cardiovascular diseases
CXCL8	C-X-C motif chemokine ligand 8
DMEM	Dulbecco's Modified Eagle's medium
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
e-cigarette/e-cig	electronic cigarette
e-liquid	electronic cigarette liquid
e-vaping	electronic cigarette vaping
e-VC	electronic cigarette vapor condensate
EAP1	Enhanced Adherence to Polystyrene
EC	electronic cigarette
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent Assay
EU	European Union
ex-smokers	former smoker
FBS	fetal bovine serum
FDA	Food and Drug Administration
hBD	human β-Defensin
HSRRB	Health Science Research Resources Bank
HWP1	Hyphal Wall Protein 1

IL-1β	Interleukin 1 beta
IL-6	Interleukin 6
LDH	lactate dehydrogenase
Li-ion	lithium ion
Li-poly	lithium polymer
LiMn	lithium manganese
LL-37	cathelicidin antimicrobial peptides
MEC	mobile exam center
mRNA	messenger RNA
MTT	thiazolyl blue tetrazolium bromide
nAChR	nicotinic cetylcholine receptors
NF/NR e-vapour	nicotine free/ nicotine rich electronic cigarette vapor
NHANES	National Health and Nutrition Examination Survey
NiCad	nickel-cadmium
NiMh	nickel metal-hydride
NIOSH	National Institute for Occupational Safety and Health
NRT	nicotine replacement therapy
ns	non-significant
OSCC	oral squamous cell carcinoma
P. gingivalis	*Porphyromonas gingivalis*
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PD	periodontal disease
PD	probing depth
PG	propylene glycol
pH	power of Hydrogen
PI	plaque index
ppm	parts per million
ppn	parts per billion
qPCR	quantitative PCR
qRT-PCR	real-time quantitative reverse transcription PCR
RAGE	receptor of advanced glycation end products
RBL	radiographic bone loss
RNA	ribonucleic acid
RNase	ribonuclease
ROS	reactive oxygen species
RPMI	Roswell Park Memorial Institute
S. mutans	*Streptococcus mutans*
SA	*Staphylococcus aureus*
SAPs	secreted aspartic proteases
SD	standard deviation
sp	species
TdT	terminal deoxynucleotidyl transferase
TLR	toll like receptor
Tm-Exp	experimental melting temperature optimized

Tm-m	melting temperature from the manufacturer
TNF-α	Tumour necrosis factor alpha
TUNE	Terminal deoxynucleotidyl transferase dUTP Nick End Labeling
US	United States
USB	universal serial Bus
VG	vegetable glycerin
WHO	World Health Organization

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From 2011 to 2020, my life experience in Quebec, Canada, has strengthen me a lot in many ways - humanity, the basics -living principles, perseverance, diligence, and faith. In the beautiful location of the Faculty of Dental Medicine at Université Laval University, was a laboratory where, I really discovered myself, I loved working there a lot, and I felt at home despite the pressures of life. In this laboratory, I met Dr. Mahmoud Rouabhia as the one of the most amazing people I have met. He taught me the principles of research and helped me getting started and improved my skills, with his beautiful way. Dr. Rouabhia, words of thanks will not be sufficient to express my feeling for your kindness.

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Many thanks go to my father for his support.

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Thanks for my country, the Kingdom of Saudi Arabia and King Saud University for the great support we are receiving in both financial and moral matters. PROMISE, we will build SUCCESS in many aspects.

Avant-propos

Cette thèse présente les travaux de recherche réalisés durant mes activités de recherche doctorale en microbiologie et immunologie. Ma formation doctorale s'est déroulée de septembre 2016 à juin 2020. Les différents sujets abordés dans cette thèse sont mis en contexte dans l'introduction, suivi des objectifs. La thèse intègre deux publications (Chapitre 2et chapitre 3) relatant nos travaux qui sont publiés dans différents journaux scientifiques internationaux, avec comité de paires. Ces deux publications forment le corps scientifique de cette thèse. Ces publications sont listées ci-dessous, avec des informations relatives à leur statut de publication, leur contexte de recherche, mes contributions ainsi que celles des coauteurs dans la réalisation de la recherche et sa publication.

CHAPITRE 2 :

Titre de la publication:	Comparative study of the effects of cigarette smoke and electronic cigarettes on human gingival fibroblast proliferation, migration and apoptosis.
Auteurs :	Alanazi H, Park HJ, Chakir J, Semlali A, Rouabhia M.
Journal et date de publication :	Food Chem Toxicol, 2018 Aug;118:390-398. doi: 10.1016/j.fct.2018.05.049. Epub 2018 May 22.
Facteur d'impact du journal :	3.775 (2018)

Mon implication dans cette publication consiste en la réalisation des expériences au laboratoire, la collecte des données et leurs analyses. Mon rôle dans ces différentes étapes est estimé à 80% de la finalisation, car j'ai eu le support des autres coauteurs dans l'accomplissement, surtout des analyses des résultats et la finalisation des figures qui sont incluses dans cette publication. J'ai écrit la première version de la publication qui a été révisée par mon directeur de recherche, le Dr Rouabhia. Cette

version a été envoyée aux autres coauteurs. À la réception de leurs commentaires, j'ai finalisé le manuscrit que j'ai soumis au journal, avec l'aide de mon directeur de recherche. Les évaluateurs ont suggéré des modifications mineures. J'ai révisé et soumis le manuscrit révisé qui a été accepté pour publication dans *Food Chem Toxicol.*

CHAPITRE 3:

Titre de la publication:	E-Cigarettes Increase *Candida albicans* Growth and Modulate its Interaction with Gingival Epithelial Cells.
Auteurs :	Alanazi H, Semlali A, Chmielewski W, Rouabhia M.
Journal et date de publication :	Int J Environ Res Public Health. 2019 Jan 21;16(2). pii: E294. doi: 10.3390/ijerph16020294.
Facteur d'impact du journal :	2.468 (2018)

Mon implication dans cette publication consiste en la réalisation des expériences au laboratoire en lien avec la culture cellulaire et bactériologie, la collecte des données et leurs analyses/interprétation, leurs mises en forme en tableaux et figures. Mon rôle dans ces différentes étapes est estimé à 90%. J'ai eu une aide, apprécié, de la part des coauteurs, surtout pour les analyses des résultats et la finalisation des figures qui sont incluses dans cette publication. J'ai écrit la première version du manuscrit qui a été révisé par mon directeur de recherche, le Dr Rouabhia. Cette version a été commentée par les coauteurs. Leurs commentaires ont été intégrés dans le manuscrit pour en faire une version finale que j'ai soumise au journal (Int J Environ Res Public Health). Les évaluateurs ont suggéré des modifications mineures. J'ai modifié le manuscrit en intégrant les commentaires des évaluateurs, et je l'ai soumis. Ce manuscrit a été accepté sans modifications supplémentaires.

INTRODUCTION

 "*We @CDCgov are saddened to hear of the **first death** related to the outbreak of severe lung disease in those who use of e-cigarettes or e-vaping devices. We will continue to educate all Americans about the serious risks associated with these products*." Dr. Robert R. Redfield, Director of Centers for Disease Control and Prevention, **Atlanta**, Georgia, **USA,** tweeted on **2019-08-23** on **Twitter**.

Electronic cigarettes and their prevalence

E-cigarettes are battery-powered nicotine-delivery devices not containing tobacco but rather a liquid (e-liquid) that is vaporized to form a nicotine-containing aerosol. It uses tobacco that is directly or indirectly heated (but not burned) using a variety of heat sources to create an inhalable tobacco aerosol. The aerosol is generated by the presence of humectants, such as propylene glycol (PG) and vegetable glycerin (VG), as well as by nicotine at various concentrations, and flavours.

E-cigarettes were designed to play an active role in cigarette smoke replacement and cessation and were also proposed as a safe product. Indeed, several studies comparing e-cigarette aerosol to cigarette smoke concluded that e-cigarettes contained lower levels of potentially toxic compounds (e.g. formaldehyde, acetaldehyde, acrolein, and toluene), compared to conventional cigarettes (Bekki et al., 2014). Data from clinical trials (Caponnetto et al., 2013; Bullen et al., 2013) and meta-analyses (Hartmann-Boyce et al., 2016) indicate that e-cigarettes may help smokers to quit or reduce their tobacco consumption and that their use is well tolerated. These clinical observations, along with advertisements promoting e-cigarettes as being safe, have contributed to e-cigarette popularity with smokers, non-smokers and youth, in particular. Indeed, in the US alone, it was estimated that 20.8 % of high school students and 4.9 % of middle school students were current e-cigarette users in 2018, representing a 78 % increase in use among high school students and a 48 % increase among middle school students during the 2017–2018 school year (Cullen et al., 2018).

Researchers have suggested that the rise in e-cigarette use among youth is linked to the availability of appealing flavours and the recent popularity of discreet e-cigarette models shaped like a USB flash drive (Cullen et al., 2018; Tsai et al., 2018). In Canada, 15.4 % of Canadians aged 15 and older (4.6 million) reported having ever tried an e-cigarette, while 2.9 % (~863,000) had used an e-cigarette in the past 30 days. Prevalence of ever using e-cigarettes increased significantly between 2015 and 2017, while past 30-day use showed no significant change (https://uwaterloo.ca/tobacco-use-canada/e-cigarette-use-canada/prevalence-e-cigarette-use).

Concerns about e-cigarettes

Although e-cigarettes were developed and marketed as a healthier alternative to smoking tobacco products, a growing body of evidence shows that even if their quantity is generally lower than that found in conventional tobacco cigarettes, their aerosols in fact contain numerous toxicants, carcinogens and organic compounds produced through the thermal decomposition of the solvents (Goniewicz et al., 2014). Indeed, several reports have associated e-cigarette use with respiratory, gastrointestinal, and even cardiovascular complications including pulmonary damage (Thirion-Romero et al., 2019), relapse of ulcerative colitis (Hua et al., 2016) and disrupted endothelial functions (Skotsimara et al., 2019).

While many studies focus on the respiratory and cardiovascular systems, the impact of e-cigarette use on oral health could be one of the first red flags signaling the deleterious effects of e-cigarettes. Few studies have addressed the direct health effect of e-cigarette usage on the oral cavity. In a cross-sectional analysis, daily e-cigarette use was associated with risk factors for such poor oral health outcomes such as periodontal diseases and tooth loss (Huilgol et al., 2019). A correlation has also been found between e-cigarette use and a higher likelihood of cracked/broken teeth, pain in the tongue, and/or inside the cheek, compared to never use e-cigarette smokers, among adolescents (Cho, 2017). E-cigarette use has also been associated

with gingival mucosa lesions (Bardellini et al., 2018). Indeed, in a small sample of patients, the prevalence and characteristics of oral mucosal lesions were evaluated in former smokers (n = 45) compared to e-cigarette consumers (n = 45), with the results showing the prevalence of these mucosal lesions to be approximately 65.4 % among e-cigarette users compared to 34.6 % among former smokers. In the same study, other oral symptoms were recorded, such as nicotine stomatitis, hairy tongue, and hyperplastic candidiasis. The frequency of these symptoms was greater among e-cigarette users than among former smokers (Bardellini et al., 2018).

Context

Following a puff, the aerosol generated by an e-cigarette is delivered into the user's mouth, thus entering in direct contact with the different constituents of the mouth, before reaching the lower airways. The entry of aerosol into the mouth and its direct contact with these different oral constituents (e.g. oral mucosa, teeth, saliva, and the oral microbiome) may change the physiological equilibrium of the oral cavity and oral microbiome. We thus sought to investigate the effect of e-cigarettes on gingival cells and oral microorganisms.

Hypotheses

Previous studies including those from our research team have shown that e-cigarettes reduce the growth of gingival epithelial cells through an apoptotic-necrotic pathway (Rouabhia et al.,2017). Thus, we hypothesise that:

1) Exposure of gingival fibroblasts to e-cigarettes may therefore lead to an impairment of gingival fibroblast functions,
2) E-cigarette use may dysregulate the oral microbiome as well as oral microorganism interactions with gingival epithelial cells.

Specific Objectives

The specific objectives of this projects are:

1. To investigate the effects of e-vapour condensate with or without nicotine on normal human gingival fibroblast morphology, proliferation, migration and

apoptosis. We examined the effect of e-vapour condensate on gingival fibroblast adhesion, viability/proliferation, apoptotic process, and migration. This objective includes both nicotine-free and nicotine-rich e-cigarettes treated cells. The effects of combustible cigarette smoke condensate and e-vapour condensate on gingival fibroblasts were also compared and analyzed.

2. To investigate the effects of e-cigarettes on *C. albicans* pathogenesis. In this objective, we investigated the effect of e-cigarette aerosols on the growth and morphology changes of *C. albicans*. We also studied the effect of e-cigarette aerosols on the expression of secreted aspartic proteases (SAPs), SAP2, SAP3, and SAP9 genes by *C. albicans*. The interaction between e-cigarette-exposed *C. albicans* and gingival epithelial cells was also evaluated.

To achieve these objectives, we adopted the following specific experimental protocols:

Preparation of the smoke/e-vapour condensates

We prepared the e-cigarette vapour condensate (e-VC) by vaping 500 µL of e-liquid using an e-cigarette device in 20 ml of culture medium, as previously reported (Lerner et al., 2016). The e-VCs were prepared from both nicotine-rich and nicotine-free e-liquids. We also prepared cigarette smoke condensate solution (CSC) by burning one cigarette in 20 ml of culture medium (Semlali et al., 2014). The generated condensates were first filtered through a 0.2-µm filter to sterilize them and were then aliquoted and frozen at -80 °C until use.

Human gingival cells

- Cells were extracted from gingival tissue (gingival connective tissue) collected from healthy, never-smoked donors (18–25 years of age) following their signature of informed consent. Extracted gingival fibroblasts were cultured in Dulbecco's modified Eagle's (DME) medium containing 10 % fetal calf serum.

- We also used gingival epithelial cells in this study. These refer to a specific cell line, namely, Ca9-22 extracted from gingival squamous cell carcinoma (purchased from Health Science Research Resources Bank (HSRRB) (Osaka, Japan)). This gingival epithelial cell line was maintained in Roswell Park Memorial Institute medium (RPMI)-1640 supplemented with L-glutamine and 10 % fetal calf serum (FBS).

Candida Strain

We used *C. albicans* in this study. *C. albicans* (ATCC-SC5314) was grown in Sabouraud liquid medium supplemented with 0.1 % glucose. The culture was grown to the stationary phase for 18 h at 30 ºC in a shaking water bath. The blastoconidia were then collected, washed with phosphate-buffered saline (PBS), and counted by means of a hemacytometer. The cell suspension was adjusted to 10^8 *C. albicans* cells/ml prior to being used.

The protocol for objective 1: The different steps related to objective 1 are summarized in the figure below

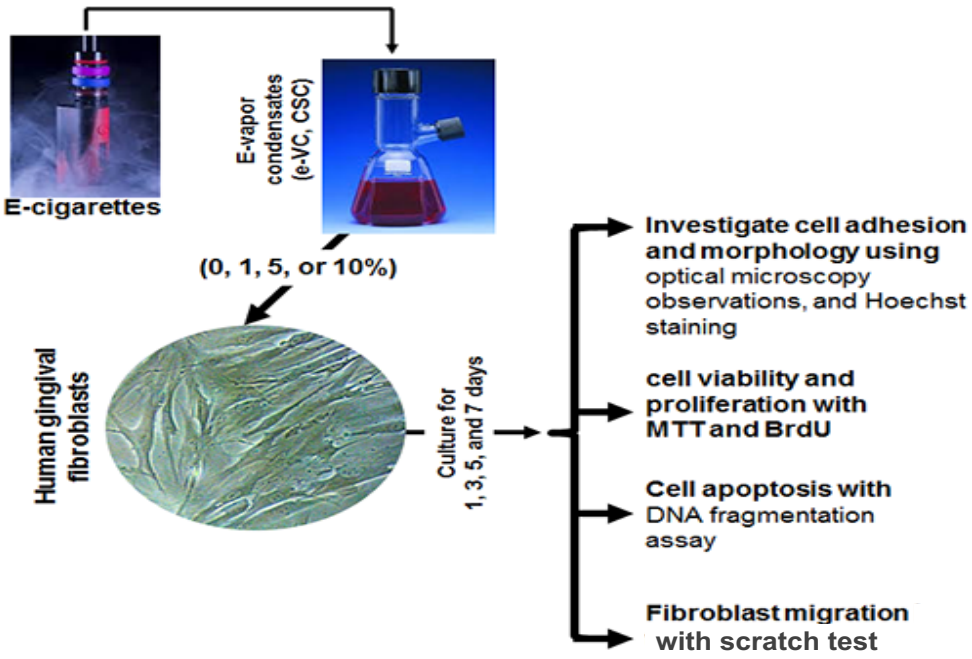

Figure 1.1: Experimental protocol for Objective 1

The protocol for objective 2: The different steps related to objective 2 are summarized in the **in the figure** below

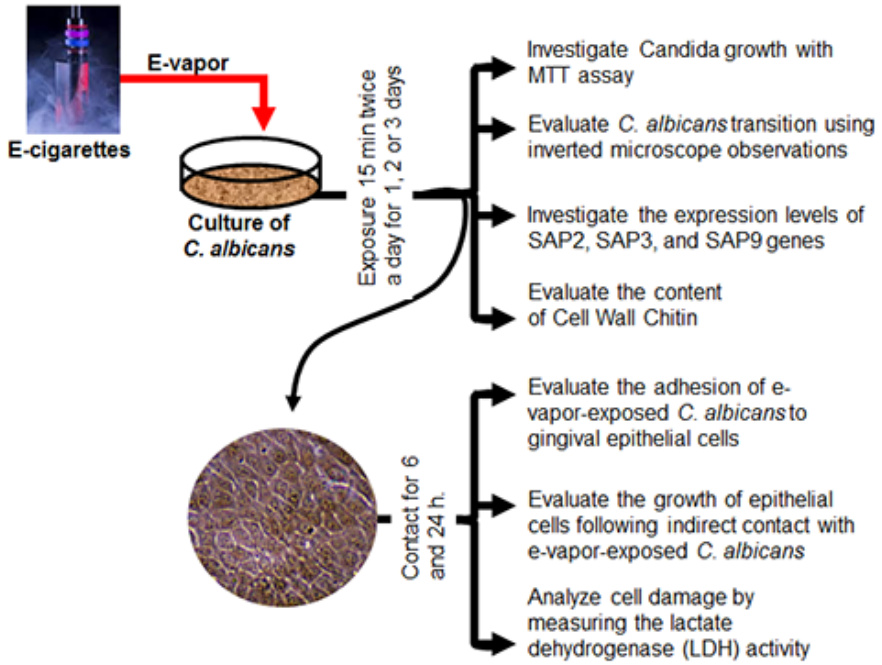

Figure 1.2: Experimental protocol for Objective 2

CHAPTER 1: LITERATURE REVIEW

1.1. **Tobacco smoke across civilisation**

Tobacco has had a longstanding role in many societies across civilisation. It is believed that the tobacco plant was the first domesticated in the Americas predating the farming of maize and other food plants (Winter, 2000). Tobacco species include *Nicotiana quadrivalvis*, *Nicotiana attenuata*, and *Nicotiana obtusifolia* (Tushingham et al., 2013). Interestingly, the process of domestication led to the development of other tobacco varieties, such as *Nicotiana rustica* and *Nicotiana tabacum* species, which have larger leaves and high nicotine content (Winter, 2000).

Historically, the domesticated species *Nicotiana rustica* was thought to reach the eastern part of the continent from South America between 2000 and 3000 years ago, while *Nicotiana tabacum* likely spread to parts of southwestern United States and the Caribbean some time thereafter (Winter, 2000; Rafferty, 2006). For a long time, various species of tobacco were used by indigenous communities throughout North and South America, then in 1492, tobacco was introduced to Columbus in the Bahama Islands during his first encounter with the Americas. European explorers to the Americas quickly recognized tobacco's unique properties and adopted it for good use. Later in the 1500s, *Nicotiana tabacum* was selected and farmed in different British and American colonies. As early as the 1600s, tobacco reached Africa, Asia, and Europe to become a global trade commodity (Tushingham et al., 2013). Following its emergence in Europe, tobacco plants were widely used throughout the Americas thanks to different processes, including plant farming, gathering, or trading. Tobacco was, and probably still is, a central product during ritual and ceremonial life of Native Americans (Winter, 2000).

African history confirms that tobacco was introduced to Africa in the late 1500s. Tobacco gained significant popularity due to various production modes and uses. In

this part of the world, but not limited to it, tobacco has contributed to economic development, particularly during the colonial and postcolonial periods (Duvall, 2017), although the commercial value was moderated by the need for fertile land and hard work involved in tobacco production.

The reputation tobacco has acquired across civilisation lies in its effect on human feelings. Although tobacco is most often smoked, it can be chewed, eaten, or snuffed. It was believed that tobacco procured sharp mental acuity, vigilance, and increased sense of calm to users. Furthermore, tobacco was frequently used as an offering in religious contexts and peace time (e.g. peace pipe). Smoking is the preferred method of tobacco use. In early times, smoking took place through dry pipes or water pipes (Figure 1.3) and was restricted to adult males. With the now-known adverse effects of tobacco smoke, this restriction was indeed an important lifesaver for teens and women. Unfortunately, almost all age ranges of people around the world currently smoke tobacco.

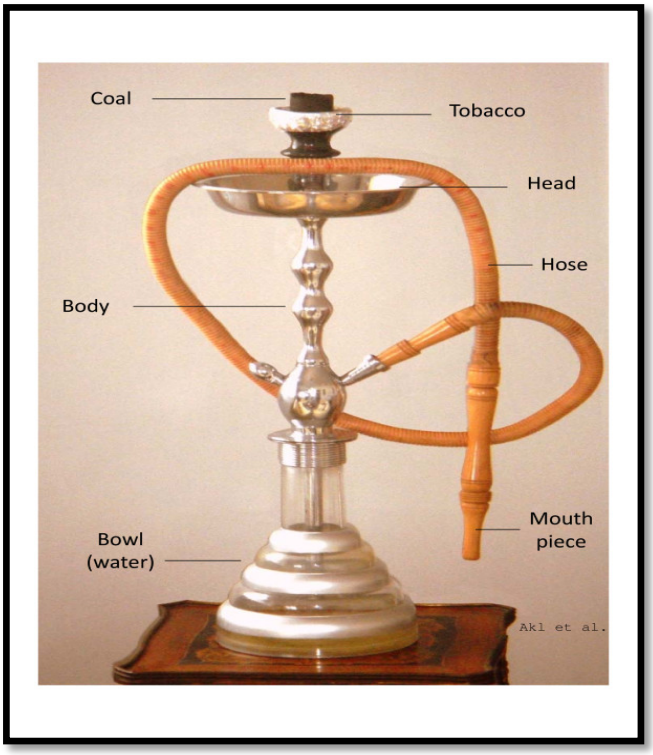

Figure 1.3: Waterpipe smoking device: An old device used for tobacco smoke. Coal heats the tobacco. The smoke is then filtered by boiled water and inhaled by the user through a rubber pipe. This figure is adapted from Akl et al., (2011).

1.2. **Smoking prevalence among youth and adult populations**

The prevalence of smoking varies from one population to another around the world (Eriksen et al., 2012; World Health Organization, 2014). Such variations are linked to multiple factors known to modulate smoking prevalence. Among the key factors are education level, national economic development, and implementation of tobacco control policies (Gilmore et al., 2000; Pomerleau et al., 2004).

In 2016 in the United States (US), more than 16 % of individuals over 18 years of age were smokers, thus an estimated 37.8 million adults smoked cigarettes. It should be noted that in 2005, the average of smokers in the US was approximately 21 % (Jamal et al., 2018). Smoke cigarette use by youth in the US is critical; indeed, tobacco use begins and is established primarily during adolescence (U.S. Department of Health and Human Services, 2012; U.S. Department of Health and Human Services, 2014). It was estimated that 9 out of 10 youth smokers have tried smoking by the age of 18[1]. Every day in the US, more than 3,200 youth 18 years or younger smoke their first cigarette, and an additional 2,100 youth and young adults become daily cigarette smokers. (U.S. Department of Health and Human Services, 2012).

Cigarette companies are continuously launching new initiatives such as adding flavour*s* to their tobacco products to attract new cigarette users (Corey et al., 2015). In 2014, 73 % of high school students and 57 % of middle school students who used tobacco products in the past 30 days reported using a flavoured tobacco product during that time (Corey et al., 2015). Adult men are more likely than women to use cigarettes in the US. Close to 18 % of men and 13 % of women were smokers at the time of the survey (Jamal et al., 2018). Ethnicity also appears to be a factor in smoking. In a 2016 survey, approximately 32 % of non-Hispanic American Indians/Alaska Natives were found to be smokers, while close to 25 % of non-Hispanic multiple-race individuals were smokers. Approximately 17 % of non-Hispanic Blacks and nearly 17 % non-Hispanic Whites were also smokers. Finally, 11 % of Hispanic and 9 % of non-Hispanic Asians were smokers (Jamal et al., 2018).

Poverty is also a possible smoke-promoting indicator, as 25 % of adults living below the poverty level were smokers compared to 14 % of adults living above the poverty level (Jamal et al., 2018)

A European survey conducted from 2013 to 2015 with participants over the age of 15 showed that the proportion of daily smokers ranged from 8.7 to 27.3 % (Eurostat, 2018) showed that the proportion of daily smokers ranged from 8.7 to 27.3 %. The lowest number of smokers was recorded in Sweden, and the highest level in Bulgaria (Figure1.4). Among the 27 EU Member States, men were more prone to smoking than were women, except for Sweden, where 7.5 % men compared to 9.8 % women were smokers during the survey (Eurostat, 2018). The highest number of smokers was in the 25–54-year range, dropping thereafter at over 65 years of age. As for smoking frequency, close to 6 % of the European population over 15 years of age smoked at least 20 cigarettes per day and about 12 % smoked less than 20 cigarettes per day. The greatest numbers of heavy smokers were recorded in Greece and Turkey (Eurostat, 2018).

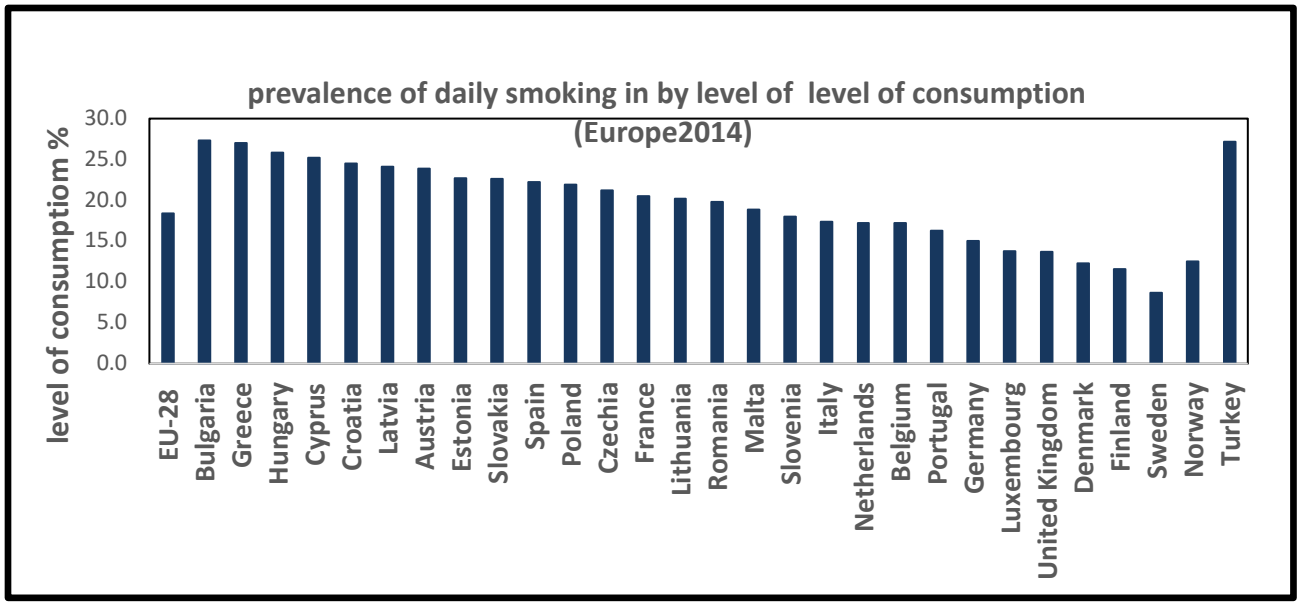

Figure 1.4: 2014 Europe smoking prevalence. Statistical results showing adults daily consumption of cigarettes in Europe in 2014. Lowest smoke level was registered in Sweden 8.7%, and the highest level was registered in Bulgaria 27.3%. This figure is adapted from Eurostat Statistics Explained: https://ec.europa.eu/eurostat/statistics-explained/index.php/Tobacco_consumption_statistics

In Canada, tobacco smoking is also a major health concern. From a 2012 Statistics Canada survey, we learned that between 2001 and 2011, the percentage of light daily male smokers increased from approximately 51 to 62 % compared to an increase of 36 to 43 % in females (Statistics Canada, Canadian Community Health Survey, (2012). Of interest, is that the number of cigarettes smoked per day by light smokers dropped from 17 in 2001 to 15 in 2011. During the same period (2001 to 2011), the percentage of heavy male smokers decreased from 31 to 23 %, while heavy female smokers dropped from 20 to 14 %. These heavy smokers consumed approximately 28 cigarettes per day during this study period (Statistics Canada, Canadian Community Health Survey 2012). Similar to other world populations, Canadians start smoking at an early age. In 2011, approximately 19 % of Canadian smokers were 15 to 17 years old and close to 12 % of young smokers were living in lower-income households (Health Canada, 2012; Reid et al., 2017).

The most recent surveys report that in 2015, nearly 13 % of Canadians were smokers. Most of these were daily smokers (9.4 %) (Figure 1.5). It should be noted that during 2015, smoking prevalence was higher in males (15.6 %) than in females (10.4 %). In terms of distribution, 9.7 % of smokers were 15–19 years old, 18.5 % were 20–24 years old, and 10.6 % were over 55 (https://uwaterloo.ca/tobacco-use-canada/highlights).

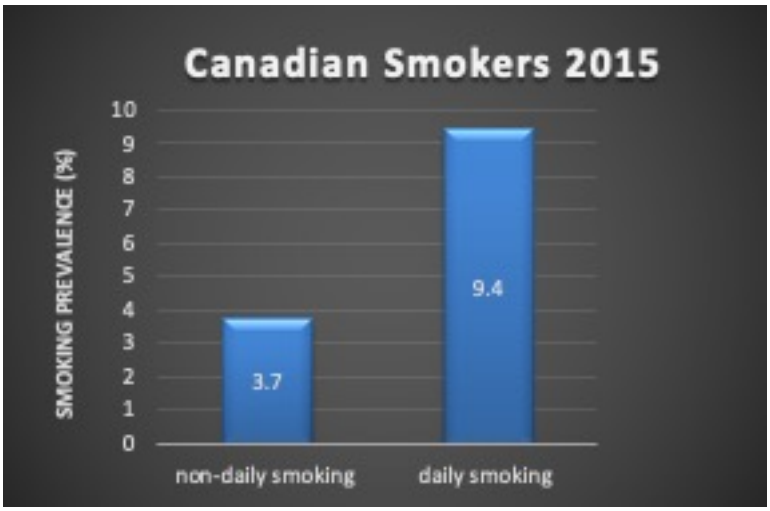

Figure1.5: 2015 Canadian adults smoking prevalence: 3.7% were non-daily smoking and 9.4% were daily smoking. This figure is adapted from Reid and Hammond (2017)

One Canadian report (Statistics Canada, Canadian Community Health Survey 2018) has shown that in 2017, daily or occasional smokers were close to 5 million (almost 16 %) in number, from the age of 12 years old. More smokers were males (19.1 %), compared to females (13.4 %) (Figure 1.6).

Globally, populations record a high number of smokers. According to the World Health Organization, tobacco is the largest public health threat worldwide, as tobacco kills over 7 million people each year, with 6 million of these deaths attributed directly to cigarette smoking (World Health Organization, 2013). There are an estimated 1 billion smokers worldwide (Eriksen et al., 2013), involving close to 30 % of men and 7 % of women (Gowing et al., 2015), with 80 % of these users in the low-to-mid income bracket. The most frustrating situation is that in several countries, children from poor families are frequently employed in tobacco farming to provide family income, which contributes to their early exposure to various tobacco chemicals (WHO, 2018). The worldwide prevalence of smokers was estimated to be 39 % in men in 2007 compared to 35 % in 2015. In women, the global prevalence estimation was approximately 6 % in 2015 and 8 % in 2017. Smoking prevalence was modulated by the countries' income (Figure 1.7, WHO report, 2017). The global concern regarding cigarette smoking is the risk of contracting a wide range of long-term morbidities, and ultimately mortality.

1.3. **Impact of smoking on health**

Tobacco use has significant direct and indirect effects on life expectancy (Manuel et al., 2016), as unhealthy behaviours tend to cluster with tobacco use (Schuit et al., 2002; Alamian et al., 2009). Indeed, the WHO estimated that smoking was responsible for over 6 million premature deaths worldwide each year (WHO, 2013). Several of these premature deaths occur in people who have stopped smoking but whose health has already been harmed by this habit (Jha & Peto, 2014).

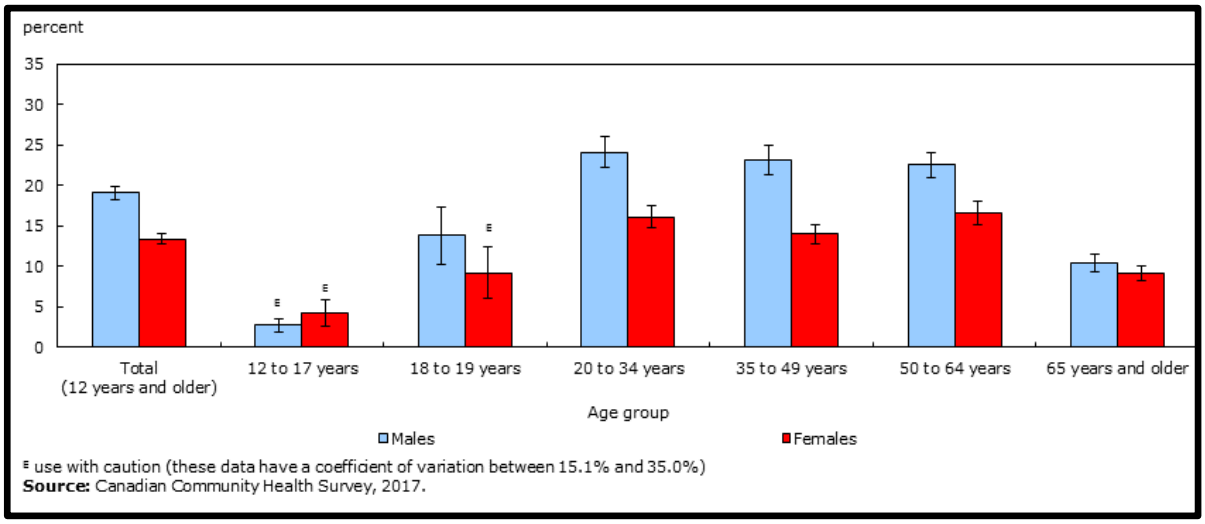

Figure 1.6 2017 : Canadian smoking prevalence proportion 2017: adult group aged 12 and older, higher smoking prevalence among males by (19.1%) compared to females (13.4%). This figure is adapted from Statistics Canada, Canadian Community Health Survey,2018 https://www150.statcan.gc.ca/n1/pub/82-625-x/2018001/article/54974-eng.htm

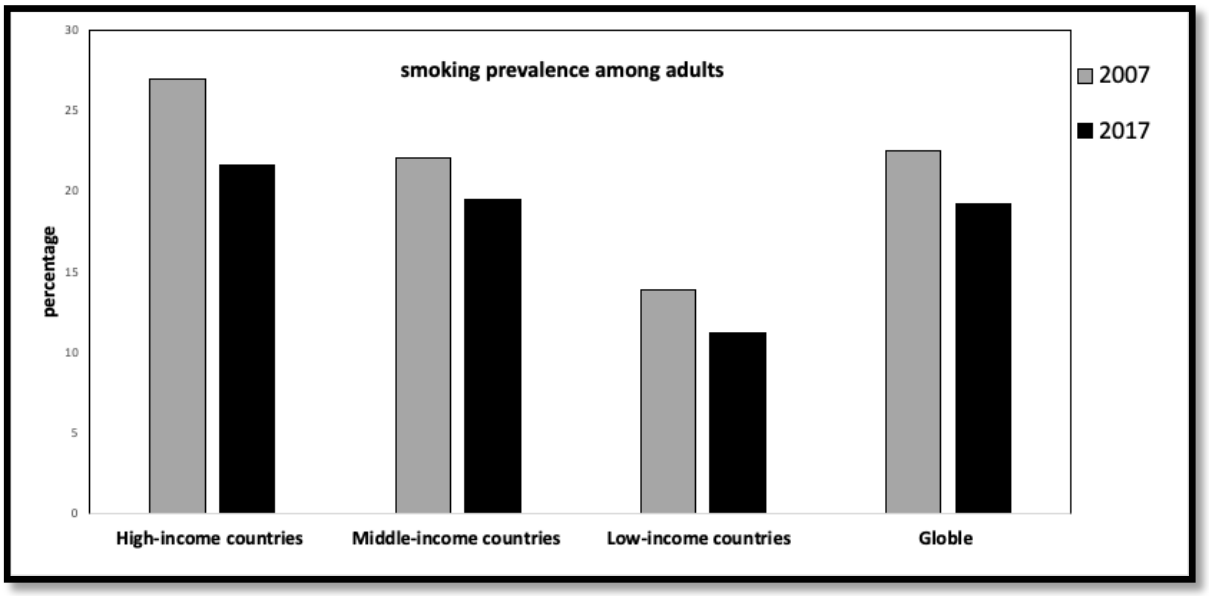

Figure 1.7: 2007-2017 Global tobacco smoking prevalence by countries' income level. : The highest adult smoking prevalence was registered in high-income countries with 27% in 2007. The lowest one was reported in low-income countries with 11.2% in 2017. This figure is adapted from *(WHO, 9 March 2018) (Tobacco Free Initiative (TFI) ; WHO report on the global tobacco epidemic 2017)*

Indeed, smokers are prone to various chronic diseases such as cardiovascular diseases (CVDs), respiratory diseases, diabetes and cancer, to name only these (Public Health Agency of Canada, 2017). In addition, smoking has created a significant economic consequences by imposing a substantial and unnecessary economic strain to healthcare systems (Krueger et al., 2016).

1.3.1. **Tobacco and cardiovascular diseases**

Multiple experimental and clinical studies have reported that tobacco remains an important risk in the development and progression of coronary and peripheral vascular diseases (Mainali et al., 2015). Cigarette smoking has long been suspected of increasing the risk of atherosclerosis and coronary diseases (Leone, 2003; Ngu et al., 2017). Indeed, cigarette smoke has been shown to increase the development and progression of atherosclerosis by increasing endothelial inflammation and vasomotor dysregulation through an oxidative stress process (Ross, 1999). Tobacco smoke can induce acute cardiovascular events by increasing heart rate, cardiac output, and blood pressure (Rahman et al., 2007). These effects have been frequently attributed to nicotine, despite the presence of other toxic and chemically active ingredients in tobacco which may be toxic to the vessel wall (Tracy et al., 1997; Kawada, 2016).

It is also important to note the link between cigarette smoke and coronary artery disease. Multiple studies have shown that over 30 % of coronary artery disease-associated mortality is due to tobacco consumption (Gepner et al., 2011). One of the mechanisms leading to coronary artery disease by tobacco is that smoking increases the oxidation of lipids which are toxic to endothelial cells, resulting in endothelium dysfunction (Wiest et al., 2017; Messner et al., 2014). In smokers, arterial plaque contains high levels of triglycerides and low-density lipoproteins. Such changes in plaque biology have been found to increase arterial thrombogenicity (Gać et al., 2017). Smokers also display low levels of secreted plasminogen activator which can lead to an altered fibrinolytic state (Newby et al., 1999).

A positive correlation between tobacco use and hypertension has also been reported. The effects of smoking on hypertension were indeed shown to be prevalent, particularly in mid and heavy smokers (Gać et al., 2014). Smokers are predisposed to increased blood pressure readings and hypertension diagnoses (Al-Safi et al., 2005; US Centers for Disease Control and Prevention, 2012). In addition, experimental research has shown that tobacco exposure decreases the synthesis of nitric oxide leading to the endothelial dysfunction of the endothelium and activation of the renin-angiotensin system (Abdelghany et al., 2018; Milara et al., 2010). Furthermore, the activation of the renin-angiotensin system by cigarette smoke is shown to lead to an increased conversion of angiotensin I to II (Stolle et al., 2010; Xiao-Ling et al., 1992), which correlates with blood pressure increases (Bennett et al., 1984).

1.3.2. **Cigarette smoke and pulmonary diseases**

It is well documented that smoking is a major lung disease-related factor responsible for morbidity and mortality. Unfortunately, the adverse effects of smoking on lung disease are not limited in time. They can begin at an early age and progress throughout the smoker's life, with increasing deterioration in terms of quality of life. Smoking is a global epidemic problem among young people, as many users begin at an early age (Ribeiro et al., 2016; Reubi et al., 2016). It is estimated that 80,000 to 100,000 children worldwide start smoking every day (Ankola et al., 2007). By their middle age and old age, smokers frequently suffer chronic lung diseases such as COPD and pulmonary fibrosis (Gometz, 2011)

Cigarette smoke can affect both the upper and lower respiratory tracts. It has been demonstrated that children exposed to parental smoking are more likely to suffer recurrent otitis media which leads to an increasing need for tympanostomy tube placement (Csakanyi et al., 2012; Yilmaz et al., 2012). Furthermore, preventing their exposure to parental smoke improves children's health quality and decreases health-care facility visits (Spangler et al., 2014).

The lower respiratory tract is also affected by smoking, as exposure to smoke can lead to a significant increase in lower respiratory tract infections, such as pneumonia (Miyahara et al., 2017; Ho et al., 2017; Jones et al., 2011). In addition, environmental tobacco smoke is an important asthma aggravator. Among children with asthma, chronic exposure to smoke has been associated with increased frequency of nighttime asthma symptoms (Morkjaroenpong et al., 2002). Smoke may also interfere with asthma medications by reducing the efficacy of inhaled corticosteroids, which may contribute to steroid resistance in asthmatics (Lazarus et al., 2007; Sheehan et al., 2015).

Deregulating lung functions could lead to the development of chronic obstructive pulmonary disease (COPD), which includes chronic obstructive bronchitis and emphysema responsible for air flow limitations (Verhamme et al., 2014). COPD, an increasing global health concern, was in fact predicted to become the third public cause of death and the fifth cause of disability in the world by 2020 (Lopez et al., 1998). Smoking was indeed found to promote COPD (Daher et al., 2019; Golpe et al., 2017) through the initiation and promotion of an abnormal inflammatory condition (Yao et al., 2009; Barnes et al., 2016). The most common pathological feature of COPD is emphysema caused by the reduction of the small airways and breakdown of lung tissue (Edwards et al., 2015). Most COPD cases can be prevented by reducing exposure to risk factors such as cigarette smoke (Rosenberg et al., 2015; Salvi, 2014). Cigarette smoke activates macrophages and epithelial cells in the airways to produce a variety of chemokines. These chemokines are involved in the recruitment of neutrophils, monocytes, and lymphocytes for damaged tissues (Costa et al., 2016; Blidberg et al., 2012). It has also been suggested that COPD is linked to an imbalance of T cells, increased inflammatory cells, and the release of inflammatory mediators (Sales et al., 2017; Kalathil et al., 2014). Thus, exposure to cigarette smoke is associated with an expanding list of serious diseases, including respiratory diseases, contributing to smokers' overall health degradation.

1.3.3. **Cigarette smoking and cancer**

It is well known that cancers are major causes of morbidity and mortality worldwide. Cancer incidence is increasing, with an estimation of over 20 million people affected by 2030. Cancers affect different organs/tissues including the lungs, breasts, stomach, mouth, and skin, to name only a few. As a pathology, cancer is found in high- and low-income societies, with two-thirds of cancer-related deaths occurring in low- and middle-income countries (WHO, 2014; International Agency for Research on Cancer, 2011).

Different stressful toxic agents including cigarette smoke are cancer promoters. Indeed, it is well documented that tobacco is an important cause of cancer all over the world (International Agency for Research on Cancer, 2004). It is estimated that smoking causes over 30 % of all cancer mortalities (International Agency for Research on Cancer, 2004). Among the organs most affected by tobacco smoke are the lungs. Before cigarettes were commercialized, the incidence of lung cancer was extremely low. Immediately after cigarettes became available to smokers, a potential causative relationship between tobacco exposure and lung cancer cases was suggested (White et al., 1990). The rapid increase in lung cancers and lung cancer-related deaths was confirmed during the 20th century and this increase was first observed in male smokers (Thun et al., 2013). Lung cancer is not the only one promoted by smoking, as tobacco use is also associated with breast cancer risks (Anderson et al., 2012; Reynolds, 2013).

Pancreatic cancer is also highly attributed to smoking (Patel et al., 2005; Fuchs et al., 1996). Cigarette smoke was estimated to be responsible for 25 % of pancreatic cancer case, with the risk increasing with smoking period and frequency using cigarette smoke (Lowenfels et al., 2006). Smoking has also been shown to increase the risk of patients with a familial predisposition to pancreatic cancer (Lowenfels et al., 2000). Bladder cancer has also been intimately linked to smoking (International Agency for Research on Cancer, 2004; US Surgeon General, 2004). One group reported an increase of bladder cancer in men (Chen et al., 2005). One

year later, the association between smoking and bladder cancer in women in workplace exposure was reported. An estimated 50 % of bladder cancers has been attributed to smoking (Jin et al., 2017). Furthermore, current or past smokers have been shown to have a threefold higher risk of developing bladder cancer (Pitard et al., 2001).

Before reaching the different organs in the body, smoke comes in contact with oral tissues, which may lead to oral cancer. Oral cancer is responsible for millions of deaths around the world, with oral squamous cell carcinoma (OSCC) representing the most common histologic type of oral cancer. However, malignant tumors may originate from any tissue in the oral cavity (Warnakulasuriya, 2009). Cigarette smoke was reported as playing a major role in the occurrence of oral cancers (Warnakulasuriya et al., 2005), and smokers were shown to have an almost 3.5 -fold increased risk of developing oral cancer compared to non-smokers (Gandini et al., 2008). Thus, almost every tissue in the body can be subjected to the adverse effects of smoking which may lead to cancer development.

1.4. **Cigarette smoke and oral health**

Tobacco smoke enters into the human body through the oral cavity. Such contact with smoke products may therefore lead to physical and physiological damage to the oral cavity constituents. The first adverse effect of smoking on the oral cavity could be the increased temperature inside the mouth which burns oral mucosa and changes tooth color (Zhao et al., 2017). Several clinical investigations have in fact reported hyper-pigmentosis, black hairy tongue, superficial glossitis, periodontitis, leucoedema, nicotinic stomatitis, leukoplakia, and neoplasm in smokers (Taybos, 2003). Tobacco has also been shown to deregulate the innate immune system in the oral cavity, which may explain the frequent oral infections observed in smokers (Semlali et al., 2012).

1.4.1. **Dental caries promotion**

Smoking has indeed been associated with caries, as it decreases the buffering effect and pH of saliva. Smokers harbour a high density of bacteria, such as *Lactobacilli sp.* and *Streptococcus mutans* that are responsible for caries (Johnson et al., 2000; Kassirer, 1994). The association of smoking with dental caries is well documented in senior groups (Locker, 1992; Jette et al., 1993). Non-smokers have been shown to demonstrate more frequent healthy oral health behaviours compared to daily smokers. Daily smoking has been also associated with an increased use of sugar in tea or coffee (Telivuo et al., 1995), thereby promoting bacterial growth and tooth damage. Furthermore, brushing habits have been found to be less effective in smokers than in non-smokers (Kelbauskas et al., 2005).

The WHO estimates that nearly 700 million children are exposed to tobacco smoke through the second-hand smoke in their living environment (parents) (World Health Organization, 1999). The most damaging cigarette is the non-filtered one, as harmful substances come in direct contact with the teeth and bacteria to promote tooth decay (Zhou et al., 2014; Vellappally et al., 2007).

1.4.2. **Periodontal disease promotion**

Periodontitis, an oral infection disease, is also promoted by smoking. One survey was conducted with participants aged 30 years and older using periodontal assessments between 2009 and 2012 (Eke et al., 2015). This survey showed that 46.3 % of participants had periodontal disease (PD): 19.1 % had severe, 67.8 % had moderate, and 13.1 % had mild PD. Of note is that the PD prevalence ranged from 32.1 % in non-smokers to 62.4 % in cigarette smokers (Vogtmann et al., 2017). Comparative results were also obtained in Swedish (Bergstrom, 2003), Brazilian (Susin et al., 2004), and Thai (Torrungruang et al., 2005) populations. Clinical studies revealed smokers to have greater probing depth and attachment loss, compared to non-smokers (Grossi et al., 1995). PD severity was also shown to be

dependent on smoking length and frequency (Bergstrom et al., 2000; Tomar et al., 2000).

Smoking may also promote PD through the prevalence of periodontal pathogens, such as *Porphyromonas gingivalis*, *Aggregatibacter actinomycetencomitans, Bacteroides forsythus, Prevotella intermedia*, and *Fusobacterium nucleatum (*Zambon et al., 1996; Gomes et al., 2006; Teixeira et al., 2009). Smoke can either promote the virulence of these bacteria or decrease the host innate immune defense (Rouabhia et al., 2011; Semlali et al., 2012). Indeed, gingival epithelial cells exposed to cigarette smoke showed a different Toll -like receptors (TLR) expression and produced various levels of pro-inflammatory cytokines, compared to that observed in non-exposed cells (Mahanonda et al., 2009; Rouabhia et al., 2011). Smoking was also found to lead to PD by up-regulating RAGE receptors on gingival cells (Katz et al., 2005), promoting a pro-inflammatory response.

1.4.3. **Oropharyngeal candidiasis promotion**

Candida is the most common yeast found in the oral cavity in approximately 70 % of healthy individuals (Arendorf et al., 1980). Multiple endogenous (deregulation of the host immune system) and exogenous (smoking) factors promote *Candida* colonization and opportunistic infection, which give rise to candidosis (McCullough et al., 2002). Oliver and Shillitoe (1984) reported that the prevalence (35 %) of *C. albicans* was comparable in smokers and non-smokers but that the concentration of *C. albicans* colony-forming units in the saliva of smokers was twice that observed in the saliva of non-smokers (Oliver et al., 1984). Similar observations were reported by others (Darwazeh et al., 2010).

The growth promotion of *C. albicans* in smokers can be due to the deleterious effects of smoking on the immune, inflammatory, and healing systems in periodontal tissues. Smoking may impair innate defenses against pathogens and negatively modulate the adaptive immune response (Lee et al., 2012). In addition, cigarette

smoking has been shown to decrease the surface expression of selectin in neutrophils, reducing their capacity to phagocyte the microbes (Ryder et al., 1998). Smoking may also impair the innate immunity through a deregulation of TLR and defensin expression. Indeed, exposure to cigarette smoke was shown to decrease the expression of certain key TLRs (2, 4, and 6) as well as hBD-2. However, pro-inflammatory cytokines, such as IL-6 and IL-8, were found to increase (Mahanonda et al., 2009; Semlali et al., 2012).

1.5. **Smoking cessation and health improvement**

Smoking cessation is associated with considerable health benefits, particularly for smokers (Rigotti, 2002). Indeed, smoking abstinence increases smokers' life expectancy and reduces the development of tobacco-related diseases. Strong evidence has shown that cessation following an acute coronary event results in a prevalent reduction of patient morbidity and mortality. With cessation, even a reduction of 5 smoked cigarettes/day was found to lead to an 18 % coronary mortality decrease in smokers (Gerber et al., 2009). In another literature review, smoking cessation was shown to contribute to reducing the risk of developing coronary heart disease by 36 % (Critchley et al., 2003).

Smoke abstinence reduces and reverses asthma-related symptoms in asthmatic smokers, confirming that smoking cessation has a positive impact on this group of asthmatics. Indeed, asthmatic smokers who stopped smoking were shown to have a better quality of life, with decreased hyperreactivity and a reduced amount of asthma medication (Tonnensen et al., 2005). Smoking cessation also reportedly improved asthma control, recovery to corticosteroid response (Chaudury et al., 2006), and airway hyperresponsiveness (Piccillo et al., 2008). In addition, smoking cessation was found to reduce the risk of developing a primary tumour in all major histological types of lung carcinoma (Khuder et al., 2001). Some evidence suggests that smoking cessation following diagnosis of early stage lung cancer can improve prognosis outcomes (Parsons et al., 2010).

Oral health is also affected by smoking cessation. For a long time, smoking cessation was reported to improve periodontal conditions (Haber et al., 1993; Bergstrom et al., 2000) and have a positive effect on periodontal treatment outcomes (Grossi et al., 1997). The link between smoking cessation and oral health improvement was later confirmed. Indeed, several studies have reported a reduction of probing pocket depth and improvement of clinical attachment in patients who had stopped smoking (Rosa et al., 2011; Preshaw et al., 2005). Overall research indicates that smoke abstinence improves the oral and general health of smokers.

1.6. **Tobacco cessation strategies**

Realistically, it is very difficult to analyse the addictive habit that is cigarette smoking. A cigarette smoker goes through different symptoms during the smoking cessation period. Early symptoms may include confusion, fatigue, sleep disturbance, moodiness, depression, and aggression, among others (Jarvis, 2004). These symptoms may disappear after one month. Late symptoms include cravings and increased appetite over time (Jarvis, 2004).

When this cessation is too difficult, it may require hospitalization for health management and to help smokers to effectively adopt a smoking cessation programme. For example, heart disease patients should monitor their smoking cessation with the help of physicians and appropriate medications (Gometz et al., 2011).

Any smoking cessation programme must involve active collaboration between the smoker and the health care system. Such programmes must be based on open discussion between the smokers and their health care professionals by clarifying the risks of continuing smoking and the smokers' ability to quit and to maintain their cessation goals. One approach called the "Five A" (Table 1) has been developed to help patients stop smoking (Coleman et al., 2004). To achieve smoking cessation, other coercive strategies can be proposed, such as a raising cigarette prices and

taxes and regulating cigarette use. However, these strategies have thus far not been successful (Forster et al., 2007). Several pharmaceutical strategies have emerged.

Table 1.1: The different possibilities to help patients stop smoking. Adapted from Coleman et al., (2004)

1	Ask about smoking at every opportunity
2	Assess smokers' interest in stopping
3	Advise against smoking
4	Assist smokers to stop
5	Arrange follow -up

1.6.1. **Pharmacotherapy for smoking cessation**

Randomized clinical trials involving smokers have shown that the use of pharmacotherapy promotes smoking cessation (Cahill et al., 2013). Indeed, different pharmacotherapy options are proposed to help smoking cessation. These include the use of bupropion, varenicline, nicotine vaccines and nicotine replacement therapy (NRT), among others.

1.6.1.1. **Bupropion**

Bupropion, a betaphenylethylamine derivative, preferentially interacts with norepinephrine and dopamine re-uptake (Wilkes, 2008). Bupropion also reportedly serves as an antagonist for nicotinic receptors. Several studies have reported the efficacy of bupropion on smoking cessation (George et al., 2002; Tidey et al., 2011). This, however, has not been confirmed by every study, as several authors have

reported no significance in sustained smoking cessation following bupropion treatment (Weiner et al., 2012; Evins et al., 2005). The recommended dose of bupropion for smoking cessation is 150 mg twice a day. The plasma concentration of this drug remains high even after 5 to 7 days, suggesting the possible smoking cessation after one week of bupropion use (Fiore et al., 2008; Mooney et al., 2006).

Bupropion has been shown to decrease nicotine/tobacco withdrawal symptoms and cigarette cravings (Mooney et al., 2006) and also reduce weight gain following smoking cessation (Farley et al., 2012). The combination of bupropion and standard nicotine patch therapy was shown to promote better smoking cessation (Fiore et al., 2008; Mooney et al., 2006). While the use of bupropion may be efficient in promoting smoking cessation, this drug does come with significant side effects. Patients using bupropion may suffer from insomnia, headaches, xerostomia, nausea, and anxiety. Some of the adverse effects, such as insomnia and xerostomia, can resolve quickly with no therapeutic intervention. Bupropion is not recommended for patients suffering from hypersensitivity to the drug and those who are undergoing rapid withdrawal from alcohol (Aubin, 2002).

1.6.1.2. **Varenicline**

Varenicline is a molecule that interacts with the a4b2 of nicotinic acetylcholine receptors (nAChRs), with a high affinity estimated to be three-fold and 16-fold greater than that of cytisine and nicotine, respectively (Jimenez-Ruiz et al., 2009; Rollema et al., 2007). Varenicline is also an agonist of homomerica7 in nAChRs (Mihalak et al., 2006). Varenicline reportedly helped patients with sustained abstinence, as measured by their carbon monoxide levels (Weiner et al., 2011). This was confirmed by another study showing a decrease in the number of cigarettes smoked, CO levels, and plasma nicotine level (Smith et al., 2016). The effect of varenicline involves the a4b2 of nAChRs. Studies have suggested a dual mechanism of action of varenicline. Indeed, it can act as (i) a partial agonist of a4b2 receptor, reducing the smoking cessation-induced drop in mesolimbic dopamine

concentrations and potentially relieving withdrawal symptoms; and (ii) as an antagonist of nicotine activity at a4b2 receptor by blocking the nicotine-induced dopaminergic activation, which may reduce the reward from smoking relapse (Jimenez-Ruiz et al., 2009; Tonstad et al., 2010).

Varenicline is efficient at 1 mg twice a day, with the suggested treatment period of one week for a smoker to quit cigarette smoking (Niaura et al., 2008), although treatment period may vary from one user to another. Some studies have suggested a greater efficacy if varenicline is used at 2 mg per day for 12 weeks (Rennard et al., 2012). The safety of varenicline is better than that of bupropion. Clinical results indicate that the use of varenicline at 3 mg in smokers and 1 mg in non-smokers is still tolerated, with limited adverse effects such as nausea and vomiting (Faessel et al., 2006). Other adverse effects such as insomnia and headaches have been reported with the use of varenicline (Motooka et al., 2018).

1.6.2. **Nicotine Replacement Therapy**

Nicotine replacement therapy (NRT) has been reported as an effective medication for the treatment of tobacco addiction. NRT functions as a direct agonist for nAChRs by providing the nicotine that is lost from the reduced smoking (Ferguson et al., 2006). It remains a first line pharmacotherapy to help smoking cessation (Edens et al., 2010) by increasing the chances of smoking cessation by 48 %, compared to no pharmacotherapy (Myung et al., 2012).

Other NRT include the nicotine patch and gum. The use of patches as nicotine therapy was shown to lead to a decrease in nicotine dependence, a significant reduction in the number of smoked cigarettes per day, and reduced levels of exhaled CO (Chou et al., 2004). The bioavailability of nicotine by NRTs was also found to be lower than that of cigarette smoke (Berlin, 2009). NRT in the form of a high-dose patch (31.2 mg) had the same effect on smoking cessation as did a low-dose patch (20.8 mg) (Chen et al., 2013). The transdermal patch system offers a continuous

release of nicotine over 16 or 24 h, thus it is not necessary to have high levels of nicotine in the patches. Nicotine gums, on the other hand, are short-acting, with self-titration of the dose according to the patient's needs (Nides, 2008).

The adverse effects of NRT are limited, compared to those of other smoking cessation therapies. The skin patches can cause skin irritation at the placement site. Oral NRTs cause more adverse effects such as mouth discomfort, dyspepsia, and pain, to name only these, while the NRT inhaler may cause mouth and throat irritation and coughing (Nides, 2008).

1.7. **New strategies for cigarette smoking replacement**

As aforementioned, the different smoking cessation pharmacotherapies have some positive effects, but also several limitations in terms of adverse effects (Aubin et al., 2014). The medical community and smokers are therefore looking for new strategies to prevent or at least decrease combustible cigarette smoking to improve smokers' health. Among these new strategies, we will concentrate on the most recent one, namely, the electronic cigarette (e-cigarette).

1.7.1. **Electronic cigarettes**

The e-cigarette device is composed of a cartridge serving as a reservoir for the smoking/vaping liquid. On one end of the e-cigarette device is the cartridge which ends with the mouthpiece for smoking/vaping. The e-cigarette device also contains an atomizer, which is a heating element to change the liquid into vapour. The third constituent of the e-cigarette device is a battery/wired USB device (Figure 1.8). The power source can be either manual or automatic. Manual e-cigarettes require the activation of the heating element by pushing a button, while automatic batteries are activated by sucking in air (Cheng et al., 2014).

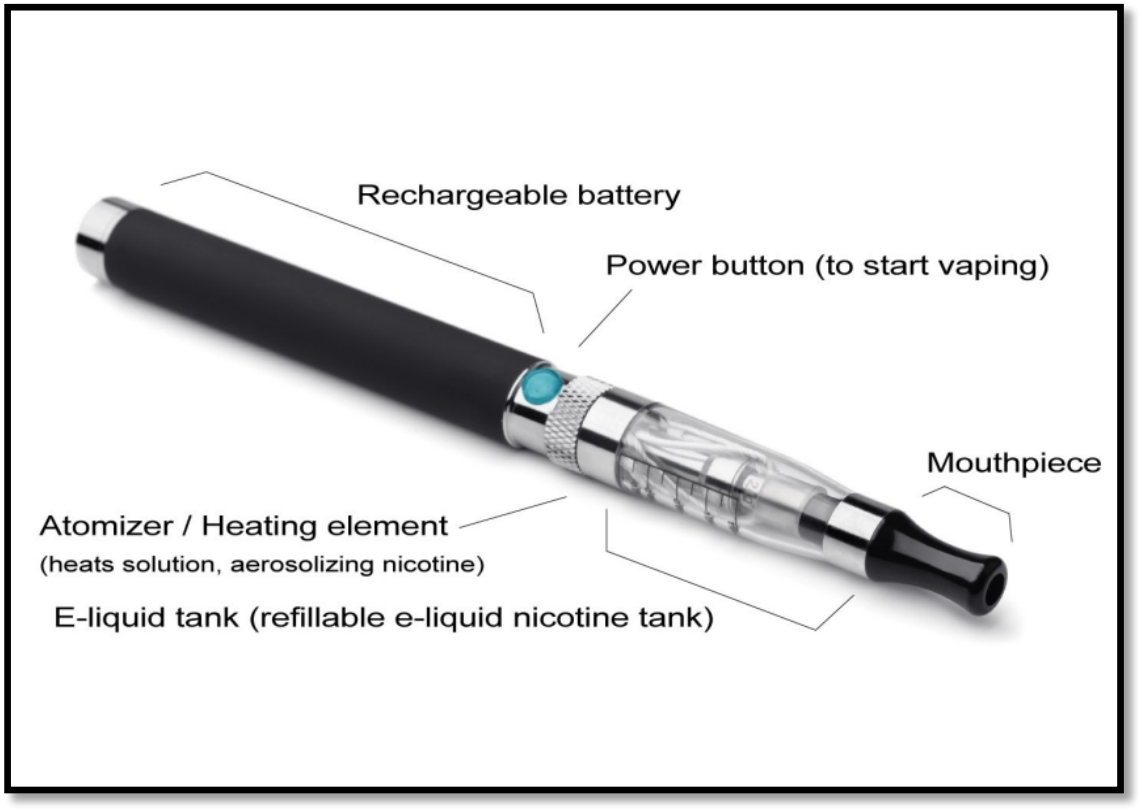

Figure 1.8: E- cigarette device. The basic e-cig components include; rechargeable battery, on/off button, atomizer, cartridge and mouthpiece. This figure is adapted from Harrell et al., (2014).

1.7.1.1. **E-cigarette cartridge**

The cartridge refers to the tank containing the e-cigarette liquid. The cartridge can be either already prefilled with the liquid or empty and ready to be filled (Figure 1.9). The user then pushes a button to squeeze drops of the e-liquid onto a wick connected to the heating element and atomizer. Different cartridges have been designed for the first, second, and third generation of e-cigarettes (Etter et al., 2010).

1.7.1.2. **E-cigarette atomizer**

The atomizer is used as a heating element that converts the liquid into vapour form (Figure 1.10). The vapour is generated by the heating element to temperatures that

may reach up to 200°C (Geiss et al., 2016), with a reported maximum atomizer temperature of approximately 250°C (Talih et al., 2015).

Figure 1.9: E-cigarette cartridge. Cartridge holds e-cigarette liquid that has mixture of different chemicals and flavours. This figure is adapted from *https://www.drugwatch.com/e-cigarettes/*

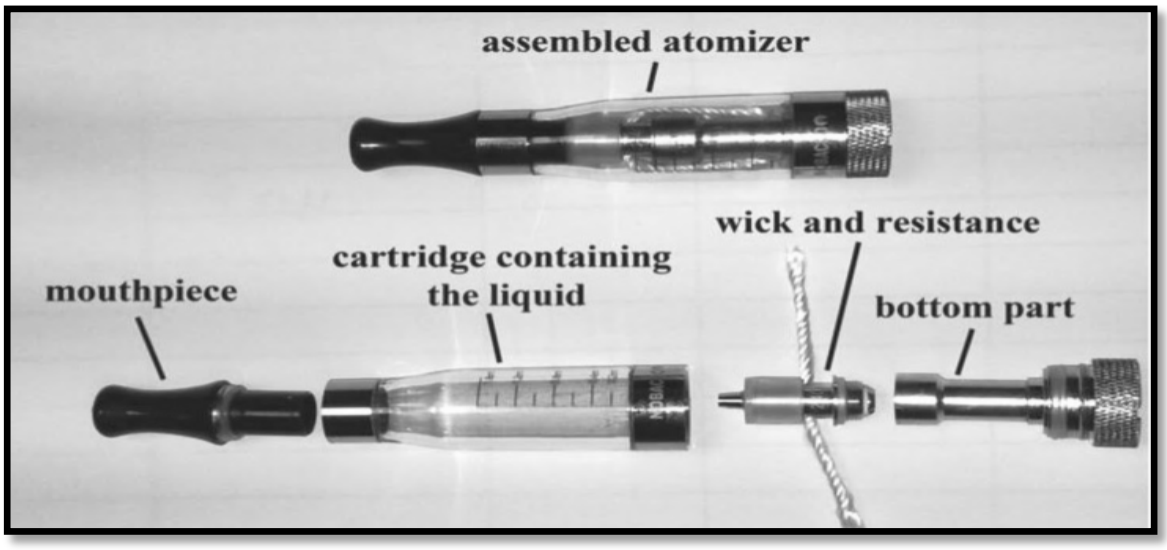

Figure 1.10: E-cigarette atomizer. This equipment converts the e-liquid to vapor by heating the e-liquid that is taken by wick and pulled inside the coil. This figure is adapted from Farsalinos et al., (2013).

1.7.1.3. **E-cigarette battery**

A battery-powered heating element is included in the e-cigarette device to heat the flavour elements without combustion. E-cigarette devices can be powered by a user-replaceable, rechargeable, or non-rechargeable battery (Figure 1.11). The battery can be a nickel-cadmium (NiCad), nickel-metal-hydride (NiMH), lithium ion (Li-ion), alkaline and lithium polymer (Li-poly), or lithium manganese (LiMn) (Brown and Cheng, 2014). A large number of e-cigarettes are powered by a lithium battery because it can store a large amount of energy in a compact space (Maraqa et al., 2018).

1.7.2. **E-cigarette generations**

Since its commercialization in 2004, e-cigarettes have gone through various improvements leading to different generations that are grouped as five generations in this section.

Figure1.11: E-cigarette battery: Different e-cigs have batteries with voltages ranging from 3 to 6 V. Some e-cigs offer the user the possibility to adjust device voltage. The e-cigs differ in the resistance of the heating element. Usually it is made from a nichrome wire (80% nickel, 20% chrome), but can be made from kanthal, an alloy mainly made of iron, chromium (20–30%), and aluminum (4–8%), or ceramic. This figure is adapted from Breland et al., (2017)

1.7.2.1. First generation

First-generation e-cigarettes, also referred to as cig-a-likes, contain a plastic cartridge to hold the e-liquid, a re-chargeable battery, and an atomizer. These e-cigarettes were designed to resemble combustible cigarettes in both size and shape (Figure 1.12). E-cigarettes of this first generation have a white body color with a tan mouthpiece, thereby mimicking combustible cigarettes (National Academies of Sciences, Engineering, and Medicine, 2018).

Figure 1.12: First generation of e-cigarette. Designed to look like conventional cigarette. It consists of plastic cartridge that holds e-liquid, a re-chargeable or non-re-chargeable battery, and an atomizer. This figure is adapted from https://ecigclopedia.com/the-4-generations-of-electronic-cigarettes/

1.7.2.2. Second generation

E-cigarettes of the second generation contain similar constituents as in the first generation, but with a larger battery and user-controlled output (i.e. variable voltage). These are considered mid-size e-cigarettes (Figure 1.13). They also have a large transparent refillable fluid cartridge.

1.7.2.3. Third generation

The third generation refers to a diverse set of products aesthetically different from the previous generations (Figure 1.14). Third-generation e-cigarettes can be shaped as squares or rectangles and feature customizable and rebuildable atomizers and

batteries. The system is designed to use different types of nicotine (National Academies of Sciences, Engineering, and Medicine, 2018).

Figure1.13: Second generation of e-cigarette. This is considered as the medium size e-cigs. The constituents of a second-generation of e-cig include larger battery, with controlled output, and large refillable fluid transparent cartridges. This figure is adapted from *https://ecigclopedia.com/the-4-generations-of-electronic-cigarettes/*

Figure1.14: Third generation of e-cigarette. This third generation of e-cigs consist of a vaped pod system shaped as square or big cylinder with customizable and rebuildable atomizers and batteries. This picture is adapted from *https://ecigclopedia.com/the-4-generations-of-electronic-cigarettes/*

1.7.2.4. **Fourth generation**

The fourth generation, called advanced personal vaporizers (APVs or Mods), is in the shape of a fat tube suggestive of a pack of cigarettes (Figure 1.15). This generation of e-cigarettes contains a replaceable battery depending on the voltage the user desires. This system can also be modified with a bigger reservoir for e-liquid storage.

1.7.2.5. **The most recent e-cigarette device: JUUL**

The JUUL e-cigarette is produced by Juul Labs, Inc., an American e-cigarette company located in San Francisco, CA. The JUUL e-cigarette contains nicotine salts from leaf tobacco into one-use cartridges. This e-cigarette is a temperature-regulated nicotine-vaping device with a USB flash drive appearance. The JUUL e-cigarette consists of a two-part device: a pre-filled, disposable e-liquid pod that clicks into a small battery. The e-liquid reservoir holds only 0.7 mL. This model of e-cigarette has been found to be very popular with teenagers (Vallone et al., 2020). JUUL devices do not fall into any of the four generations described previously, but are rather part of a new genre referred to as pod-mods (Figure 1.16). JUULs are similar to first-generation devices in that they do not provide control over power levels nor the customization of device components; only users can choose among the available flavoured liquids.

1.7.3. **Refill solutions for e-cigarettes (e-liquids)**

Typical e-cigarette device configuration allows smokers to obtain what they need in terms of nicotine amount without smoking a conventional cigarette (CC). Indeed, following aspiration through the e-cigarette mouth unit, an airflow sensor combined with a physical power button activates the e-cigarette devise battery to power an

atomizer to produce an aerosol from a refill solution frequently called e-liquid, containing nicotine and flavors.

Most e-liquids are composed of carrier solvents, such as vegetable glycerin (VG) and propylene glycol (PG) with and without various concentrations of nicotine, as well as various flavors (Bitze et al., 2018; Geiss et al., 2015). PG e-liquid is considered as a thinner, producing more of a throat hit to better simulate the feel of actual smoking (Li et al., 2016; DeVito et al., 2018), while VG e-liquid is thick and a bit sweet, producing clouds of vapour (Li et al., 2016; Zhang et al., 2013). For better vaping, a mixture of PG and VG is therefore used.

The vapour generated by the heated e-liquid refers to an aerosol composed of a suspension of fine particles of liquid, solid, or both in a gas of chemical substances used for vaping. The aerosol and the design of e-cigarettes are both strategically used to simulate combustible cigarette smoking. Following a puff, part of the e-liquid aerosol is delivered by inhalation into the user's upper and lower airways. The remaining aerosol is exhaled through the mouth and the nostrils (Trtchounian et al., 2010).

Figure1.15: Fourth generation of e-cigarette. This is the biggest e-cig in term of size and battery capacity. It consists of an advanced personal vaporizers with a fat tube shape, a replaceable battery that give the chance to control voltage, and large reservoir for e-liquid storage.This figure is adapted from https://ecigclopedia.com/the-4-generations-of-electronic-cigarettes/

Figure 1.16: JUUL e-cigarette: This pod-mods USB e-cig consists of a small pre- filled disposable e-liquid container that clicks in the battery. It is the most popular with adolescents. This figure is adapted from Strongin,(2019)

1.7.3.1. **Nicotine levels in e-cigarettes**

E-cigarette users have access to multiple e-liquids with varying concentrations of nicotine. E-liquids with a very low concentration of nicotine signifies less than 3 mg/ml, low nicotine concentration refers to 6 mg/ml, mid nicotine to 12 mg/ml, high nicotine refers to 18–20 mg/ml and finally, very high nicotine refers to a concentration greater than 21 mg/ml. E-liquid companies label nicotine concentration as milligrams, percentages, or descriptors (e.g., low, medium, high) (Zhu et al., 2014.).

Nicotine-free e-liquid is also available. However, a very low percentage of smokers use such e-liquids. The most popular nicotine-rich e-liquid appears to be the one with 18 mg/ml (Dawkins et al., 2013). A study by Kinnunen and colleagues (2016) showed that e-liquids with nicotine were more popular with ever smokers while nicotine-free e-liquids were more popular with never smokers (Kinnunen et al., 2016). Non-smoker teens were shown to be attracted by no nicotine and low nicotine e-cigarettes, while smokers favoured e-liquid with medium and high nicotine concentrations (Czoli et al., 2016).

1.7.3.2. **E-liquid pH**

Variable pH values are reported with different e-liquids. This pH variability is attributed to the concentration of nicotine in each e-liquid. Due to the alkalinity of nicotine, the greater its concentration in the e-liquid, the higher the pH of the e-liquid will be (Table 1.2; Lisko et al., 2015).

Studies have shown that the physiological response to nicotine doses and the rate of absorption of nicotine are dependent on pH value (Tomar et al., 1997; Richter et al., 2008). Indeed, the absorption of nicotine across mucosa or skin increases exponentially with pH value (Tomar et al., 1997). With regard to e-liquid, pH was shown to vary from 4.78 to 9.60 in nicotine-rich e-liquids, while nicotine-free e-liquids had neutral or slightly acidic pH varying between 5.1 and 6.4 (Lisko et al., 2015).

Of interest is that e-liquids containing menthol have a higher pH. Thus, nicotine as well as some flavours such as menthol may increase the pH of the e-liquids (Stepanov et al., 2015).

The measurement of pH in commercially available e-liquids which differed in flavour, nicotine content, brand and PG/VG ratio showed that the pH values varied from 5.35 to 9.26. The pH value was also associated with nicotine concentration in the aerosol (El-Hellani et al., 2018; Caldwell et al., 2012); with this pH variation, nicotine absorption may vary.

TABLE 2 THE PH OF THE E-LIQUIDS. ADAPTED FROM LISKO ET AL., (2015)

E-CIG	Flavor	Nicotine Label Conc.(mg)	Nicotine (mg/g)	% Difference from Label	pH	Free Nicotine (%)
SOUTH BEACH SMOKE	Vanilla	0	<LOD	NA	5.3	NA
	Tobacco	6	4.5	-25.0	8.3	65.9
	Tobacco Blue	6	4.2	-30.0	7.9	44.4
	Tobacco Gold	12	9.7	-19.2	8.4	68.8
	Peppermint	12	9.2	-23.3	8.7	81.9
	Menthol	16	13.1	-18.1	8.5	77.0
	Peach	16	12.2	-23.8	8.8	86.3
V2	Menthol	0	<LOD	NA	6.4	NA
	Sahara	6	5.4	-10.0	7.8	38.7
	Red	6	5.9	-1.7	8.4	69.7
	Sahara	12	11	-8.3	8.5	76.6
	Peppermint	12	9.6	-20.0	8.2	62.5
	Menthol	18	15.3	-15.0	8.7	83.8
	Red	18	16.7	-7.2	8.9	87.1
PREMIUM	Cherry	0	<LOD	NA	5.3	NA
	Coffee	0	<LOD	NA	5.8	NA
	blueberry	6	3.7	-38.3	7.3	14.7
	Watermelon	6	3.3	-45.0	7.7	32.9

1.7.3.3. **E-liquid flavours**

An extensive variety of flavours is currently available on the market. Some flavours are designed to mimic flavours found in combustible cigarettes (e.g., tobacco, menthol-tobacco) and cigars (sweet, fruit), while others mimic palatable foods (fruit, desserts, candy) or drinks (coffee, alcoholic drinks). Many contain names that provide little information on the flavour category (e.g., unicorn blood, truth serum, snake oil, etc.). There are more than 250 e-cigarette brands and 8000 different flavourings in the US market alone, with products rapidly evolving in the last few years (Kaur et al., 2018) and new flavours created every day to replace those showing toxic effects. These new flavours can even be personalised locally in a

vape/smoke shop by mixing flavouring chemicals with polyethylene glycerol/vegetable glycerin to make them more desirable to consumers.

1.7.4. **Prevalence of e-cigarette Use**

E-cigarettes thus provide smokers with an alternative means of nicotine intake. Today, e-cigarette use is widespread throughout smoker populations, as well as among teen smokers and non-smokers (Farsalinos et al., 2016).

1.7.4.1. **Prevalence in the U.S.**

In a 2013-2014 survey, it was reported that among all e-cigarette users, 2.6 % were adults and 1.21 % were adolescents (Jaber et al., 2018). In adult e-cigarette users, 68.1 % were current smokers, while 23.7 % were former smokers and 8.2 % were never smokers. In another study, the prevalence of e-cigarette users was 4.5 %, referring to over 10 million adult e-cigarette users in the U.S. Of these, 15 % were never cigarette smokers. Indeed, e-cigarette use was high among persons aged 18 to 24 years (9.2 %). It is important to note that over 50 % of e-cigarette users in the study were younger than 35 years of age (Mirbolouk et al., 2018).

1.7.4.2. **Prevalence in Europe**

From a study that included 28 European Union countries, we learned that 1.5 % of the EU population were regular e-cigarette users in 2014, with an increase to 1.8 % in 2017. During 2017, over 60 million Europeans aged 15 or older had ever used e-cigarettes, and over 7 million were regular users of e-cigarettes. Among the ever users of e-cigarettes, participants aged 15–24 years were less likely to be regular users than those aged over 55 years (16.9 vs. 38.1 %), as were never smokers compared to current and former smokers (12.8 vs. 27.0 vs. 41.3 %) (Laverty et al., 2018).

1.7.4.3. Prevalence in Australia

Among the general population, over 56 % of Australians considered e-cigarettes to be socially acceptable (Lee et al., 2018). Another study revealed that the prevalence of ever and past-year e-cigarette use among young Australian women was 11.1 % and 6.4 %, respectively. More than 25 % of past-year and ever e-cigarette users were never cigarette smokers. E-cigarette use in the last year was associated with teens and financial difficulty. Ever e-cigarette use showed similar associations and was also correlated with rural residence (Melka et al., 2019).

1.7.4.4. Prevalence in the United Kingdom

A 2015-2016 e-cigarette use survey showed that in teens aged between 11 and 16 years, ever smoking ranged from 11 to 20 %, while in regular e-cigarette users (at least weekly), it was between 1 and 4 % and between 7 and 18 % in ever users. Among never smokers, ever e-cigarette use ranged from 4 to 10 %, while among regular smokers, it ranged from 67 to 92 % (Bauld et al., 2017).

1.7.4.5. Prevalence in Canada

From a 2013 cohort study involving 1095 Canadians, we learned that approximately 79 % of younger non-smokers, 82 % of younger smokers, and 81 % of older smokers were aware of e-cigarettes. Those who ever tried e-cigarettes were reportedly 10 % of younger non-smokers, 42 % of younger smokers, and 27 % of older smokers. Moreover, current use of an e-cigarette (in the last 30 days) was reported by 0.3 % of younger non-smokers, 18 % of younger smokers, and 10 % of older smokers. Among ever tried e-cigarettes, 10 % of younger non-smokers, 46 % of younger smokers, and 43 % of older smokers reported having tried an e-cigarette containing nicotine (Shiplo et al., 2015). In another cohort study involving

42,094 Canadian students from 336 schools, over 17 % of students reported ever using e-cigarettes, while close to 6 % reported past 30-day use, with significant variations observed across provinces (Montreuil et al., 2017). In Québec, ever and past-30-day use of e-cigarettes among students was shown to be 34 % and 6 %, respectively (Lasnier et al., 2016). Lifelong e-cigarette use was reportedly 15 % among students in Ontario (Hamilton et al., 2014) and 10 % among students in the Niagara region (Khoury et al., 2016).

From this e-cigarette prevalence section, we understand that e-cigarette use is common, but differs across populations. Continuous and accurate estimates could inform health care policymakers and tobacco regulators on the demographic and geographic distribution of e-cigarette use to help develop safer e-cigarette regulations.

1.7.5. **E-cigarettes and smoking cessation**

One study suggested that e-cigarettes were more effective in cigarette smoking (CS) cessation than were other quitting strategies (nicotine gum and patch) (Margham et al., 2016). According to another study, controlling CS withdrawal symptoms was one effect of e-cigarette use, as the nicotine delivered by e-cigarettes to smokers contributed to enhancing mood and memory functions, which became increasingly better until completely relieving the withdrawal symptoms or most of them (Glasser et al., 2017).

In several other studies, e-cigarette users declared that e-cigarettes helped them to stop smoking better than the nicotine patch or bupropion did by reducing the withdrawal symptoms and cravings (Etter et al., 2010). A National Health Interview Survey showed that 22 % of ex-smokers who had used e-cigarettes had stopped smoking for 12 months (Glasser et al., 2017).

In addition, new smokers who quit combustible cigarettes were found to use e-cigarettes 4 times more than those who still smoked (Glasser et al., 2017). In a Polish study carried out by the Medical University of Silesia, 45 % of the cohort had completely stopped smoking after switching to ECs within two weeks, while 55 % did not. It should be noted that the level of nicotine was steady during the changing (Goniewicz et al., 2017). In another study, 60–76 % of smokers switched to ECs as a cessation strategy (Dawkins et al., 2013; Etter et al., 2011).

ECs with nicotine were found to be more effective than were other NRT for CS cessation. Indeed, 11 % of smokers reportedly stopped SC for 12 months by using nicotine-rich e-cigarettes (Rahman et al., 2014). In another study, 7 % of a cohort of smokers stopped smoking cigarettes by using e-cigarettes, while 6 % used nicotine (Rahman et al., 2014). All of these studies suggest that e-cigarettes helped smokers to smoke fewer cigarettes, thus supporting the cessation trial.

1.7.6. **Dual use of EC and SC**

Information on the impact of using EC and cigarette smoke together is rare. One study demonstrated that dual use could help smokers reduce the number of cigarettes and even quit smoking. This study reported that 67 % of smokers who could not stop smoking managed to reduce their daily conventional cigarette consumption (Siegel et al., 2011), compared to 92 % of ex-smokers who stated that e-cigarettes had helped them reduce the number of cigarettes per day in another study (Etter et al., 2011).

Dual use of the two products by smokers not necessarily intending to quit resulted in a 31 % abstinence rate at 6 months. This study concluded that e-cigarettes may help attenuate nicotine addiction (Siegel et al., 2011). In a British study, we learned that the success rate of quitting attempts increased by 0.098 % (95 % confidence interval 0.064 to 0.132; $P < 0.001$) and 0.058 % (0.038 to 0.078; $P < 0.001$) for every 1 % increase in the prevalence of e-cigarette use by smokers and e-cigarette users during a recent cessation attempt, respectively (Beard et al., 2016).

The effectiveness of e-cigarettes as a smoking cessation strategy was examined in another survey of 222 smokers who had tried e-cigarettes. Here, 31 % of e-cigarette users reported smoking abstinence at 6 months from their initial use of e-cigarettes (Siegel et al., 2011). The same study showed that 66.8 % of respondents reported a reduction in the number of cigarettes they smoked (Polosa et al., 2016). A Hungarian cross-sectional online study with over-18 participants suggested some advantages of using only e-cigarettes compared to dual use in terms of progression of mental health (mood, sleeping), sensory (smell, taste) and physical functions (breathing, well-being, stamina). The study also put forth that these improvements were greater with e-cigarettes than with dual use (Abafalvi et al., 2019).

The decrease in conventional cigarette use by using EC was also associated with a significant improvement in lung function (Polosa et al., 2014). In addition, as e-cigarette use reduced the number of burned cigarettes, blood pressure subsequently improved. Indeed, in patients using both types of smoking (EC and SC) systolic blood pressure improved, but not diastolic (Polosa et al., 2016). However, in a Health eHeart study carried out by Wang and colleagues (2018) testing four groups of participants (non-smokers, cigarette smokers only, e-cig only and dual users) with cardiopulmonary disease signs, the collected results differed. Indeed, it was shown that dual users expressed greater serious circumstances of breathing difficulty and poor health conditions, compared to the other groups. The authors concluded that these effects could be due to the exposure to both sources (SC and EC) of toxic chemicals along with the fact that more nicotine got into their blood (Wang et al., 2018). Another school survey study with young Chinese dual users revealed health problems related to the respiratory system (Wang et al., 2016).

In conclusion, the purpose of using both types of smoking (EC and SC) may help reducing cigarette smoke then lead to smoking cessation. However, the dual use may contribute to increase social relationships as compare to the use of SC only.

1.7.7. **Benefits of e-cigarette use**

As reported previously, the role of ECs in smoking cessation can be viewed as a health achievement due to the reduced use of conventional combustible cigarettes and thus the contact with toxic chemicals. Indeed, toxic substances from burning cigarettes, such as carbon monoxide and tobacco-related chemicals, are not found in ECs (Goniewicz et al., 2014). This is the main reason supporting the health benefits of using ECs. Numerous studies have reported that e-cigarettes have a lower level of toxic and carcinogenic elements, compared to those found in SC (Rom et al., 2015).

1.7.7.1. **Experimental data and animal model**

In a 2016 study, researchers investigated the effects of e-cigarette aerosol and cigarette smoke (CS) on human alveolar cell cultures (A549 cells) in mice and human alveolar cell cultures (A549 cells). Their results show that lung injury (as determined by wet-to-dry ratio) was higher in CS-exposed than in e-cigarette-exposed animals. An albumin leak in bronchoalveolar lavage fluid was evident in CS-exposed cells but not in e-cigarette-exposed ones. It should be noted that the exposure to e-cigarettes significantly increased the levels of IL-1β. However, exposure to CS was associated with a significant increase in IL-1β, IL-6, TNF-α expression and oxidative stress. Cell death evaluated by TUNEL staining showed a high level of dead cells with CS compared to that observed with e-cigarette aerosol. Furthermore, e-cigarettes were shown to inhibit cell growth, compared to non-exposed cells (Husari et al., 2016).

The authors thus concluded that despite higher exposure conditions, e-cigarettes exhibited fewer toxic effects on the lungs of experimental animals and on A549 cell cultures than CS did (Husari et al., 2016). Neilson and colleagues (2015) demonstrated that exposure of *in vitro*-engineered three-dimensional human airway tissue to e-cigarette aerosol for 6 h did not decrease cell viability, compared to that

observed with CS (Neilson et al., 2015). The interaction of e-cigarettes with osteoblasts has also been analyzed, showing that e-cigarettes, compared to CS, had less adverse effects on osteoblast interaction with dental implant material compared to SC (Rouabhia et al., 2019). All this existing experimental research suggests that e-cigarettes display lower toxicity than do conventional cigarettes.

1.7.7.2. **Beneficial effects of e-cigarettes on general health**

Certain clinical studies have indicated the health effects of e-cigarettes over conventional burning cigarettes. Indeed, it was reported that e-cigarette use contributed to reducing conventional tobacco cigarette consumption, which led to a significant reduction in COPD exacerbations ($p = 0.002$) from 2.3 ± 1) at baseline to 1.8 ± 1. The study also showed a significant reduction in COPD exacerbation in dual users. Finally, the authors noted that COPD symptoms and the ability to perform physical activities improved statistically in the EC group at both visits, with no change in the control group (Polosa et al., 2016).

In another study, involving 210 smokers divided into three groups, namely, nicotine e-cigarettes (8 mg/mL nicotine concentration), nicotine-free e-cigarettes (placebo), and controls, participants received a 3-month cessation programme that included a cognitive-behavioural intervention aimed at supporting people in changing their behaviour and improving motivation to quit. Data analyses showed that pulmonary health improved in the participants who stopped smoking compared to their own baseline. After 6 months, participants in the nicotine e-cigarette group smoked fewer cigarettes than any other group. Moreover, this group recorded the lowest level of exhaled carbon monoxide (CO) ($M = 12.012$, $S.D. = 8.130$) as well as the lowest level of dependence ($M = 3.12$, $S.D. = 2.29$), compared to that observed under the nicotine-free and control conditions (Lucchiari et al., 2020).

E-cigarettes have also been reported to improve heart health. Indeed, one study showed that within 1 month of switching from cigarettes to e-cigarettes, a significant

improvement in endothelial function and vascular stiffness was observed. Of interest is that females benefited more from switching than their male counterparts did and that those who complied best with the EC switch demonstrated the most significant improvement (George et al., 2019).

1.7.7.3. **Beneficial effects of e-cigarettes on oral health**

In a clinical study of smokers who switched from conventional cigarette smoking to e-cigarettes (Tatullo et al., 2016), participants were subjected to oral examinations and a self-assessment questionnaire regarding variations in certain parameters of general health and their need for SC. The respondents used e-cigarettes for 120 days. Clinical examinations at different times showed a reduced plaque index (PI) among the majority of participants who had used SC for less than 10 years. Switching from SC to e-cigarettes also showed a PI reduction in participants who had used SC for more than 10 years (Tatullo et al., 2016). The bleeding index also improved with e-cigarette use. The self-assessment questionnaire revealed that approximately 71 % of e-cigarette users felt an improvement of their general health, while less than one-third of participants felt no clear change in their general health status, either positive or negative, and only 2 participants indicated a worsening of their general health status (Tatullo et al., 2016). Although CC and e-cigarette users were not compared at the same time, this study did indicate oral health improvements following the use of e-cigarettes or from switching from CC to e-cigarettes.

In another clinical study, conventional cigarette (CC) smokers switched for 5 days to either (i) exclusively commercial e-cigarette use; (ii) dual use of commercial e-cigarettes and their usual cigarette brand; or (iii) discontinued use of all tobacco or nicotine products. Biochemical analyses showed a significant reduction in the number of detrimental urinary biomarkers with the use of e-cigarettes alone, while dual users exhibited a reduction (7–38 %) in 8 of 9 urinary biomarkers but had increased nicotine equivalents. Of interest is that every e-cigarette user showed a

significant decrease in exhaled CO (O'Connell et al., 2016). This observation is supported by Adriaens and colleagues (2018) who showed that short-time use of e-cigarettes significantly reduced exhaled CO, compared to that observed with CC. These studies suggest that partial or total CC substitution with e-cigarettes was able to reduce the exposure of smokers to hazardous products, thereby improving health.

E-cigarettes also reportedly promoted smoking cessation. A Malaysian cohort study reported that 20.5 % of previous cigarette smokers who switched to e-cigarettes quit smoking and that quitting was easier if smokers used e-cigarettes only, in contrast to dual use (Mohamed et al., 2018). These observations are supported by recent studies confirming the efficacy and safety of e-cigarettes over a short-term period, resulting in a high cessation rate (Hajek et al., 2019; Masiero et al., 2019). However, it is important to note that most available studies are generated from self-reported perceptions, which may not identify clinical manifestations or modifications that occur in the oral cavity of e-cigarette users. Furthermore, this reported safety was also based on short-term e-cigarette use. As such, the effects of the different chemicals in e-cigarettes (and their variable levels) on the oral cavity are as yet unknown, which continues to raise concerns regarding the safety and long-term effects of e-cigarette use.

1.7.8. **Concerns regarding e-cigarettes**

Evidently, e-cigarettes have advantages and disadvantages. They create various serious issues and raise a certain number of flags regarding different aspects of human health which could be either direct or indirect contact with the e-cigarette device itself or its constituents (e-liquid chemicals and flavours, batteries, cartridges, etc.). These concerns can extend to society by influencing people or triggering them to smoke, particularly youth. Moreover, e-cigarettes also represent an environment-polluting factor.

1.7.8.1. **E-cigarettes appear to target youth**

E-cigarettes were initially designed to help heavy smokers to quit the smoking habit. However, with the availability of e-cigarettes and the fierce publicity campaigns promoting them as safe devices, today's users are not only adult heavy smokers. Several studies indeed point out that e-cigarettes attract more and more youth.

A 2017 survey showed that among US youth, flavoured e-cigarettes without nicotine represented the most commonly vaped usage (Miech et al., 2017). These findings are supported by those of another study showing flavoured e-cigarettes to be the first e-cigarettes for most youth, young adult, and adult vapers (Harrell et al., 2017). In social media data, the most frequently discussed flavours mentioned were fruit, cream, tobacco, and menthol (Wang et al., 2015). Another study demonstrated that tobacco, menthol/mint, and fruit were the top three flavours preferred by consumers (Yingst et al., 2017). Longitudinal surveys with middle and high school students showed flavouring to be the second most important factor determining whether adolescents tried e-cigarettes, after curiosity (Bold et al., 2016; Kong et al., 2015). Regarding flavour and smoking initiation, flavoured e-cigarette use was found to be associated with a higher intention to initiate EC use (Dai et al., 2016). Furthermore, in a study based on a national sample of UK adolescents, fruit and sweet flavours were more likely to be tried by adolescents who had never smoked than by smokers who were trying to quit (Ford et al., 2016).

The new e-cigarette device called JUUL is reportedly highly used by youth. Indeed, in a recent study, JUUL devices appeared to be associated with a youth e-cigarette "epidemic" by attracting new users and facilitating frequent use by their highly addictive nicotine content and appealing flavours (Vallone et al., 2020). This study supports previous findings (Keamy-Minor et al., 2019) showing that JUUL use was higher among young people. The authors concluded that given the high nicotine content of the JUUL e-cigarette, there is concern over the potential for addiction as well as other serious health consequences among young people (Vallone et al.,

2020). Overall, the popularity of e-cigarettes in youth, whether smokers or non-smokers, raises a serious health concern related to e-cigarette use.

1.7.8.2. **Concerns regarding the e-cigarette device constituents**

1.7.8.2.1. **Possible harmful effects of e-cigarette batteries**

Since the commercialization of e-cigarette devices, several dangerous events including explosions and fires caused by these devices have been reported (US Fire Administration, 2014). These explosions or fires occurring during e-cigarette use may cause significant trauma to the user's face and oral cavity. In one case study, an explosion occurring during e-cigarette use caused extensive perioral and oral injuries. These injuries involved multiple mucosal surfaces, as well as avulsion and teeth fractures (Rogér et al., 2016). From another case study (Vaught et al., 2017), we learned that following an e-cigarette explosion, the user suffered fractures to the right naso-orbital-ethmoid complex as well as to the anterior and posterior frontal sinus tables, with frontal sinus outflow tract involvement. The resulting trauma required combined open and endoscopic repair, including open reduction internal fixation, with reconstitution and preservation of the frontal sinus and frontal sinus outflow tract. Damage caused by e-cigarette explosions can also affect the cervical spine (Norii et al., 2017). EC that exploded during use pushed the mouthpiece through the pharynx and into the first cervical vertebra, resulting in fractures of the first and second vertebrae. This required extensive surgeries to save the patient's life (Norii et al., 2017).

Yet another case study reported on an 18-year-old male whose e-cigarette exploded in his mouth, resulting in severe damage to the anterior dentition (fractured teeth, avulsions, luxation). The patient also suffered fractures to the premaxilla and anterior nasal spine, as well as sustained lacerations to the upper lip, labial mucosa, gingivae, tongue, hard palate, and facial skin (Brooks et al., 2017). Based on these findings, e-cigarettes should be handled with care and users should be made aware of the dangers and risks involved with device use.

1.7.8.2.2. **Concerns regarding the vaping solutions/e-liquids**

Typical e-cigarette device configuration enables smokers to obtain the desired amount of nicotine without having to burn SC. Indeed, following e-liquid refill of the EC, as the user inhales through the e-cigarette mouth unit, an airflow sensor combined with a physical power button activates the e-cigarette device battery, which in turn powers the atomizer to produce an aerosol from the e-liquid refill, which contains nicotine and flavourings. E-liquids contain carrier solvents, such as glycerol and propylene glycol. These chemicals are not designed to be heated/vapourized, which ultimately raises serious health concerns.

1.7.8.2.2.1. **Vaping propylene glycol**

Propylene glycol (PG) is one of the major carrier humectants in numerous e-cigarette e-liquids. The primary role of PG is to form vapour that resembles cigarette smoke. Being a synthetic substance, PG can absorb water and is thus used in different applications such as de-icing fluids, antifreeze/engine coolants, paints, coatings, and tobacco products (National Toxicology Program, 2004). PG was approved by the Food and Drug administration (2014) as a safe product that can be used by chemical, food, and pharmaceutical industries (Lim et al., 2016; Kaushik et al., 2010). It is generally used to absorb water to maintain moisture in certain medicines, cosmetics, and food products (Lim et al., 2016; Kaushik et al., 2010).

PG is also used to create artificial smoke or fog for use during fire-fighting training exercises (Magari et al., 2017). When PG enters the body, it takes approximately 2 days to break down (Ostrowski et al.,1999). Several studies have reported that repeated short-time exposure to PG may lead to irritations of the eyes, as well as of skin, nasal, and oral tissues (Werley et al., 2011). A rat model exposed for 90 days to various concentrations of PG displayed mild nasal hemorrhages and a high level of mucin (Suber et al., 1989). Clinical use of inhaled medications containing PG confirms the irritability of PG, as rhinitis patients treated with flunisolide nasal spray

containing a high concentration of PG reported nasal burning, stinging, and throat irritation (Trancik et al., 1982; Meltzer et al., 1990; Ratner et al., 1996).

While the FDA has approved PG for oral use with food, medicines, and cosmetics (2014), little is known regarding the effects of heated and inhaled PG on human health. It is well known that direct heating of PG leads to the formation of aldehydes, such as formalin and acrolein, which are known to be cytotoxic (Gillman et al., 2016). Of interest, aldehyde formation has been shown to increase with increased heating temperature related to the inadequate delivery of the e-liquid to the heating unit of e-cigarettes (Gillman et al., 2016). These findings thus suggest that vaping may harm the upper and lower airways of e-cigarette users. Further investigations are required to explore this area.

1.7.8.2.2.2. **Vaping vegetable glycerin (VG).**

The second humectant present in e-liquid is vegetable glycerin (VG, also known as glycerol). Following the FDA's approval Food and Drug Administration, 2014 (C.F.R 2014)) of VG of vegetable glycerin (VG) for food and pharmaceutical applications, e-cigarette companies incorporated VG into their e-liquids. Glycerin can act as a solvent, a humectant, and sometimes a sweetener. In e-cigarettes, VG is heated to help create thicker clouds and a smoke-like aerosol. The boiling point of VG is approximately 287°C (Baassiri et al., 2017). Overheating VG leads to its decomposition leading to the formation of acrolein, a known toxin and irritant (Stein et al., 1983). This chemical decomposition of VG following its use in e-cigarettes continues to raise health concerns. Indeed, inhalation of VG has been shown to potentially cause dry mouth, increased thirst, and sore throat (Polosa et al., 2014; Vardavas et al., 2012). These symptoms usually subside after a few weeks of smoking abstinence.

1.7.8.2.2.3. **Vaping PG-VG e-liquids.**

For better vaping, PG and VG humectants are frequently combined to trap and deliver nicotine and to form smoke-like clouds for the smoker's satisfaction.

Combining PG and VG can, however, have deleterious effects. An *in vitro* study using human bronchial epithelial cells showed that vapour generated with an equally mixed solution of PG/VG reduced metabolic activity compared to PG alone. It should be noted that VG alone was also found to reduce the metabolic activity of the bronchial cell line (Leigh et al., 2016). The adverse effects between PG, VG, and PG/VG may be attributed to the variation ratio of carbonyl following e-liquid vaping. Indeed, e-liquids with a varied PG/VG ratio were found to lead to higher carbonyl (formaldehyde, acetaldehyde, acetone) levels than did single PG-based or VG-based solutions (Kosmider et al., 2014). Carbonyl emissions by e-cigarette-vaped e-liquids have also been linked to the power setting. With a power setting ranging from 5–25 W, PG vaping was shown to produce more acetaldehyde, VG vaping to more acrolein, and PG /VG to high levels of formaldehyde at increasing power settings (Geiss et al., 2016). The presence of these chemicals could explain the reported adverse effects on the upper and lower airways.

1.7.8.2.3. **Concerns regarding e-liquid flavours**

Thousands of different flavours have been added to e-cigarette e-liquids (Krishnan-Sarin et al., 2017; Zhu et al., 2014). Some flavours resemble SC, including tobacco or menthol-tobacco, while others mimic sweet or fruity flavours, cigars, or even food tastes, such as fruits, desserts, candy, and drinks such as coffee. The clear reason for introducing flavours to e-cigarettes is not identified by the companies designing e-liquids. From e-cigarette promotional campaigns, we gather that these flavours are used as attractive elements to enhance e-cigarette use. Up to 2012, marketed e-liquids promoted the safety/advantages of e-cigarettes over traditional combustible cigarettes. Since that time, promotional campaigns have emphasized flavour diversity for consumers as an added bonus. The addition of flavours to combustible cigarettes is banned by regulatory measures in several countries, including Canada, the US, and the European Union, but not with e-cigarettes, thus giving e-cigarette industries free range to design and market various flavoured e-liquids. The inevitable question remains: Is vaped flavour safe for e-cigarette users?

The use of flavours in e-cigarettes raises several concerns. It is well documented that flavours encourage e-cigarette use (McKelvey et al., 2018; Chen et al., 2018). Indeed, in a study involving adolescents and young adults, one of the major reasons evoked for trying e-cigarettes (regardless of cigarette smoking status and school level) was the availability of appealing flavours, representing 43.8 % of participants (Kong et al., 2015). In another study, e-liquid taste was shown to be the main reason for approximately 40 % of respondents when choosing their preferred e-cigarette brand (Laverty et al., 2016). This preference was confirmed by other findings showing that the preferred flavours in adolescents were menthol, candy, or fruits (Pepper et al., 2016) and that they also perceived fruit-flavoured e-cigarette e-liquids as being less harmful than tobacco-flavoured ones.

To make their products more attractive, e-liquid companies have introduced new flavours, such as butter, with the presence of diacetyl (Jedlicka et al., 2018), mint, with the addition of camphor and cyclohexanone (Girvalaki et al., 2018), cherry, with added benzaldehyde (Loch et al., 2016), cinnamon, with added cinnamaldehyde (Behar et al., 2016), and even chocolate, with the presence of butyraldehyde (Jo et al., 2016). Some e-liquid flavours have been reported to be toxic. For example, at a microwave popcorn production facility, workers exposed to aerosolized flavouring agents containing diacetyl were shown to suffer from acute onset bronchiolitis obliterans, an irreversible obstructive lung disease (Kreiss et al., 2002; Barrington-Trimis et al., 2014; Farsalinos et al., 2015). The presence of diacetyl was reported in 110 out of 159 tested e-liquids (Farsalinos et al., 2015). This finding thus raises a serious health concern for e-cigarette users. Indeed, Clapp and colleagues (2017) suggested that e-cigarette users with an estimated consumption of 3 ml of e-liquid/day would be exposed to a level of diacetyl exceeding the 5-ppb established limit by the National Institute for Occupational Safety and Health (NIOSH) and the CDC. This limit is in fact exceeded by various e-liquids. The negative effect of diacetyl-rich e-liquid has also been confirmed by *in vitro* studies. Bronchial epithelial cells exposed to vaped flavoured e-liquids were indeed found to display cell toxicity

which was dependent on the level of diacetyl in each e-liquid (Leigh et al., 2016; Tierney et al., 2016).

In addition to diacetyl, *benzaldehyde* was found to be a potentially harmful chemical (Pankow et al., 2018; Kosmider et al., 2016). In an experimental animal model study, inhalation of volatilized benzaldehyde at 500 ppm resulted in irritation of the eyes and nasal mucosa of rabbits, while higher levels (750 ppm) led to increased animal mortality (Andersen, 2006). Because a vast number of e-liquids contain chemicals such as diacetyl and benzaldehyde, users and health regulation agencies should be better informed in this regard. Overall clinical and experimental results thus far recommend that we urgently consider e-cigarette flavours as serious health concerns and that they should be regulated.

1.7.8.2.4. **Concerns regarding nicotine dosing in e-cigarettes**

ECs were designed to deliver nicotine without the added toxic chemicals found in conventional SC. Nicotine delivery with first-generation e-cigarettes was reported to be lower than that of SC (Schroeder et al., 2014), while nicotine delivery with second- and third-generation e-cigarettes was found to be equal to or exceeding that delivered by SC (Vansickel et al., 2012; Ramôa et al., 2016).

Nicotine concentrations in manufactured e-cigarette refill liquids range from 0 to 36 mg/ml, thus providing users with easy access to a large selection (https://veppocig.com/my-burro-flavour-e-liquid-nicotine-30ml/). With this range of nicotine, users can create their own nicotine concentration for the desired effect by preparing high or mid-range nicotine-concentrated e-liquids. This open access to various concentrations of nicotine raises obvious health concerns. Studies have shown nicotine to increase airway mucus viscosity and the production of mucus by human bronchial epithelial cells (Chen et al., 2014; Gundavarapu et al., 2012) and to promote anti-inflammatory responses in the lung, leading to increased susceptibility to respiratory viral infections (Razani-Boroujerdi et al., 2004).

The presence of flavour in nicotine-rich e-liquid can also influence nicotine pharmacokinetics. Indeed, in a study involving young adult e-smokers, subjective reward value was reportedly higher with flavoured nicotine-rich e-cigarettes versus unflavoured products, as the participants worked harder for flavoured e-cigarette puffs than for unflavoured ones. Furthermore, the participants took twice as many flavoured puffs than they did unflavoured puffs. The authors concluded that flavouring enhanced the rewarding and reinforcing value of e-cigarettes with nicotine, leading to abuse liability in young adult smokers (Audrain-McGovern et al., 2016).

1.7.9. **Concerns regarding the effects of e-cigarettes on human health**

Because e-cigarettes do not burn, these devices are promptly endorsed as being a safe alternative to conventional cigarette smoking. It is also promoted as not containing tobacco products except nicotine. The actual fact is that e-cigarette liquid contains nicotine at various concentrations, in association with a wide range of flavours (Etter et al., 2011). The combination of nicotine and flavours may therefore represent health problems in e-cigarette users.

1.7.9.1. **Possible harmful effects of e-cigarettes on the respiratory system**

An increased risk of bronchitis symptoms by almost twofold was reported among past e-cigarette adolescent users compared to never users, and by 2.02-fold among current e-cigarette users, with the risk increasing with frequency of current use for 1–2 days and 2.52 for 3 or more days in the past 30 days, compared to that observed in never users (McConnell et al., 2017). The effect of e-cigarettes on the respiratory system was also confirmed by Lappas and colleagues (2018) who showed that immediately after e-cigarette use, healthy and mid-asthmatic participants experienced mechanical and inflammatory respiratory effects. Participants with mid-asthma exhibited higher baseline values and a more prominent effect immediately after e-cigarette use, compared to non-asthmatics. The non-asthmatics returned to

baseline values within 15 min post-exposure, while the mid-asthmatics returned to baseline values after 30 min (Lappas et al., 2018). The link between e-cigarette use and asthma was previously reported. A study with high school participants revealed that e-cigarette use was associated with asthma exacerbation leading to more days absent from school because of asthma (Cho & Paik, 2016). These findings are supported by another study showing a higher prevalence of current asthma among e-cigarette users (Choi & Bernat, 2016).

E-cigarette use was shown to result in reduced chronic obstructive pulmonary disease (COPD) exacerbations (Polosa et al., 2016). This observation is supported by an Internet survey of 1,190 regular COPD EC users claiming improvement (over 75 %) in respiratory symptoms after switching from combustible to e-cigarettes, whereas worsening was reported in 0.8 % (Farsalinos et al., 2014). However, these positive effects of e-cigarettes on patients with COPD have not been supported by experimental studies. Indeed, mice repeatedly exposed to inhaled nicotine-containing glycerol or PG were shown to develop COPD-like effects, airway hyperreactivity, and lung tissue destruction (Garcia-Arcos et al., 2016). COPD bronchial epithelial cells exposed to e-cigarettes were also found to secrete more IL-6 and CXCL8, compared to that observed in non-exposed cells (Higham et al., 2018).

1.7.9.2. **Possible harmful effects of e-cigarettes on the cardiovascular system**

It is recognised that combustible cigarettes have significant deleterious effects on the cardiovascular system of smokers. One compound harmful to heart function is nicotine. As e-cigarettes contain various concentration of nicotine, it is believed that they would also have adverse effects on the cardiovascular system. In one case study, a 70-year-old woman with a medical history of hypertension, hyperlipidemia, osteoarthritis, and allergic rhinitis developed paroxysmal atrial fibrillation following temporary use of e-cigarettes (Monroy et al., 2012). Online e-cigarette forums have

also reported that e-cigarettes have negative effects on the respiratory system, the mouth and throat, the sensorial system, and the digestive tract, not to mention the muscular/skeletal and cardio-circulatory systems. Negative cardiovascular effects include chest pain/pressure, arrhythmias, and abnormal blood pressure (hypertension). In addition, blood pressure changes have been reported by approximately 4 % of e-cigarette users (Hua et al., 2013). The use of e-cigarettes can also reportedly accelerate heart rate. Indeed, in one study, 5 min after the first puff of an e-cigarette, heart rate was shown to increase from 73 ± 2.0 to 78 ± 1.9 beats per minute (bpm). This increase persisted and elevated throughout the following period (1-h ad lib puffing). This effect was attributed to the presence of nicotine in the e-liquid (Vansickel et al., 2012).

In contrast, several studies have reported no adverse effects of e-cigarettes on the cardiovascular system. For example, participants who vaped second-generation e-cigarettes for 7 min showed no myocardial relaxation changes, compared to what was observed in combustible cigarette smokers (Riley et al., 2016). Therefore, further clinical and experimental studies are mandatory to shed light on the interaction between e-cigarettes and the cardiovascular system.

1.7.9.3. **Possible harmful effects of e-cigarettes on oral health**

Following e-cigarette use, the aerosol produced by the heated humectants comes in close contact with the oral cavity and its constituents (soft and hard tissues, saliva, and oral microbiome). This close contact raises significant oral health concerns.

1.7.9.3.1. **E-cigarettes and periodontal diseases**

It is well known that combustible cigarette smoking is an important risk factor for periodontitis, which affects the host immune-inflammatory response (Johnson et al., 2007). E-cigarettes are marketed as an innovation to prevent the adverse effects of traditional tobacco cigarettes. However, this enthusiasm should be tempered. In a pilot study involving participants with mild PD who had replaced their regular

smoking habits with e-cigarettes for two weeks, e-cigarettes were shown to lead to a significant increase in gingival bleeding on probing (Wadia et al., 2016). This observation is supported by other findings with combustible cigarette smokers, e-cigarette-only users, and non-smokers. Full-mouth plaque index (PI) and probing depth over 4 mm were found to be significantly higher in the combustible cigarette and e-cigarette groups than in the non-smoker group (Javed et al., 2017). This same study also reported higher bleeding on probing (BOP) in the non-smokers than in the CC smokers and e-cigarette users. Gingival pain was also more often reported in the combustible cigarette smokers than in the e-cigarette users (Javed et al., 2017). Although periodontal inflammation and self-perceived oral symptoms were higher with CCS, e-cigarettes also had a negative impact on the periodontal health of users. These clinical studies thus indicate that the close interaction between e-cigarettes and the oral periodontium may lead to compromised oral health. Further studies are warranted to confirm these observations and to establish the leading causes of these deleterious effects and the mechanisms involved in periodontal damage following e-cigarette use.

1.7.9.3.2. **Effects on peri-implant parameters**

Implant dentistry offers successful alternatives to many restorative problems, from replacing missing teeth or entire arches to simply stabilizing a moving denture. Unfortunately, stress factors such as smoking are now known to weaken implant functionalities and life implant duration (Sanchez-Perez et al., 2007; Levin et al., 2005). E-cigarettes contain different chemicals that may contribute to peri-implantitis. AlQahtani and colleagues (2018) demonstrated that peri-implant plaque index (PI), probing depth (PD) ≥ 4 mm, and total radiographic bone loss (RBL) were higher among combustible cigarette and e-cigarette users than in non-smokers. These clinical symptoms were accompanied with increased pro-inflammatory cytokine levels in saliva. Indeed, the levels of TNF-α, IL-6, and IL-1β were higher in combustible cigarette smokers and e-cigarette users than in non-smokers (AlQahtani et al., 2018). These observations are supported by another study in which

probing depth ≥ 4 mm and peri-implant bone loss were higher in e-cigarette users than in non-smokers. The measurements of cytokines forming peri-implant sulcular fluid showed increased levels of TNF-α and IL-1β in e-cigarette users compared to that observed in non-smokers (Al-Aali et al., 2018). These findings suggest that e-cigarettes can be associated with poor peri-implant health. Elevated levels of inflammatory cytokines in e-cigarette users (in peri-implant sulcular fluid) may lead to an increased peri-implant inflammatory process which could in turn contribute to dental implant failure. Thus, close clinical follow-up of e-cigarette users with dental implants should be mandatory to prevent dental implant failure.

1.7.9.3.3. **Effects on e-cigarette users' teeth**

E-cigarettes vapour comes in direct contact with the teeth. This contact may have adverse effects on tooth structure. A recent study conducted by Cho (2017) evaluating the association between e-cigarette use and oral symptoms among adolescents revealed a significantly increased risk of tooth damage, as 11.4 % of participants reported a "cracked or broken tooth" within the past 12 months. In addition, 18.5 % of adolescent e-cigarette users reported having experienced "gingival pain and/or bleeding" and 11.0 % reported tongue pain associated or not with inside-cheek pain (Cho, 2017). These findings are supported by observations in an experimental study performed with bovine enamel specimens exposed to aerosols from e-cigarettes using various e-liquid flavours (neutral, menthol, and tobacco) and various nicotine concentrations (0, 12, and 18 mg). The authors demonstrated that the e-cigarette aerosol from e-liquids with different nicotine contents and flavours actually altered enamel color. E-liquids flavoured with menthol and tobacco were in fact shown to alter enamel color by decreasing the yellowness of the enamel compared to that observed with neutral e-liquid (Pintado-Palomino et al., 2019). These studies thus confirm that e-cigarettes have negative effects on tooth structure and esthetics. Additional studies on the effect of e-cigarettes on tooth structure and esthetics could confirm these observations and shed light on the mechanisms leading to this tissue damage in the oral cavity.

1.7.9.3.4. **E-cigarette use and dry mouth/xerostomia**

In a clinical study in EC ever users, the most commonly reported disease symptoms were sores or ulcers in mouth (8.3 %) and having more than one cold (6.8 %) (Yao et al., 2017). These data support the findings of a previous study in which e-cigarette users reported sensitive teeth, mouth ulcers, headaches, and cold symptoms (Hua et al., 2013). In a prospective proof-of-concept study monitoring modifications in smoking behaviour of smokers who switched to e-cigarettes, the most frequently reported adverse events experienced by e-cigarette users were throat/mouth irritation (35.6 %), dry throat/mouth (28.9 %), headache (26.7 %), and dry cough (22.2 %) (Polosa et al., 2014). These studies therefore support the evidence that e-cigarettes have a negative impact on oral health by increasing mouth irritation, dry mouth and ulceration. Further studies are needed to inform dentists and other oral health professionals to better advise their patients and to prevent/treat e-cigarette-induced oral diseases.

1.7.9.3.5. **Effects on e-cigarette user saliva**

A comparative study of combustible cigarette smokers, e-cigarette users, and never smokers showed no difference in unstimulated whole salivary flow rate among the groups, although the levels of cotinine were found to be higher in the combustible cigarette smokers, followed by the e-cigarette users and non-smokers (Mokeem et al., 2018).

Pro-inflammatory cytokines (IL-1β and IL-6) levels were also shown to be higher in saliva of combustible cigarette smokers and e-cigarette users than in the never smokers. This study thus confirms that e-cigarettes can negatively the oral innate immunity played primarily by gingival epithelial cells and fibroblasts.

1.7.9.4. **Effects of e-cigarettes on oral microorganisms**

1.7.9.4.1. **Interaction with *Staphylococcus aureus***

As described previously, cigarette smoke condensate promotes caries, periodontal disease and oropharyngeal candidiasis. The question therefore is: Do e-cigarettes promote oral infectious diseases? This remains unanswered as very few studies have addressed this issue. One experimental study has shown that the exposure of human lung alveolar type II epithelial (A549) cells or alveolar macrophages to e-cigarette extract decreased antimicrobial activity against *Staphylococcus aureus* (SA). This observation was confirmed in a mouse model. Indeed, upon exposure to e-cigarette vapour extract, airway colonizer SA was found to increase SA biofilm formation, bacterial adherence and invasion of epithelial cells, resistance to human antimicrobial peptide LL-37 and up-regulation of virulent gene expression by SA (Hwang et al., 2016).

1.7.9.4.2. **Interaction with *Streptococcus mutans***

The presence of PG/GV gives e-liquid its high-viscosity properties. As a result, aerosols from PG/GV e-liquids are likely to adhere to exposed surfaces such as soft and hard tissues in the oral cavity and dental implants. This interaction may facilitate bacterial adhesion, thereby leading to oral infections such as caries. Furthermore, dental caries can be promoted by e-liquid flavours supplemented with sugars (Kubica et al., 2014, Kim et al., 2018). Sucrose/sucralose and sugar alcohol are known additives to e-liquid to enhance taste/fragrance (Tierney et al., 2016; Soussy et al., 2016).

In a recent study, e-cigarette aerosols were found to increase the adhesion of *Streptococcus mutans* (*S. mutans*) to enamel and that following this adhesion, flavoured e-cigarette aerosols increased biofilm formation. Indeed, enamel exposed to flavoured e-cigarette aerosols showed decreased hardness, compared to what was observed with unflavoured controls. Furthermore, this bacteria-initiated enamel demineralization was associated with high levels of esters (ethyl butyrate, hexyl

acetate, and triacetin) found in the e-liquids. The viscosity of the e-liquid thus promoted the adhesion of *S*. *mutans* to pits and fissures. Because commercial e-liquids contain several additives at various levels, including sucrose, sugar substitutes, and acids (Soussy et al., 2016; https://www.nudenicotine.com/product/sucralose-solutions-5-15/), interactions between e-liquids and teeth may vary from one e-liquid to another. This will require additional research to inform both users and dental professionals on the prevention of e-cigarette-induced caries. Further studies are also needed to shed light on the effect of e-cigarettes on other oral microorganisms such as ***C. albicans*** and oral candidiasis.

1.8. **Context**

Electronic cigarettes (e-cigarettes) were designed to provide smokers with the desired nicotine dosage without burning tobacco. E-cigarettes also house flavoured humectants which may or may not contain nicotine. These devices are widely available, with an increase in usage worldwide. They are promoted as both a “safe” alternative to combustible cigarette smoking and an efficient smoking cessation aid. Upon e-cigarette use, the vapour emanating from the heated humectants comes in close contact with the oral cavity, including the soft and hard tissues, saliva and oral microbiome. This proximity raises significant health concerns. The purpose of this research was to investigate the interactions between e-cigarettes and various constituents of the oral cavity.

1.9. **Hypotheses**

Previous studies including those from our research team have shown that e-cigarettes reduce gingival epithelial cell growth through an apoptotic-necrotic pathway. We thus hypothesise that:

1. Exposure of gingival fibroblasts to e-cigarettes may thus result in an impairment of gingival fibroblast functions.

2. The use of e-cigarettes may dysregulate the oral microbiome and oral microorganisms interactions with gingival epithelial cells.

1.10. **Objectives**

The objectives of the present study were:

1. To investigate the effects of e-vapour condensate with or without nicotine on normal human gingival fibroblast morphology, proliferation, migration and apoptosis. We studied the effects of e-vapour condensate on the adhesion, viability/proliferation, apoptotic process and migration of gingival fibroblasts. This study included nicotine-free and nicotine-rich

e-cigarettes. The effects of combustible cigarette smoke condensate and e-vapour condensate on gingival fibroblasts were also compared and analyzed.

2. To investigate the effects of ECs on *C. albicans* pathogenesis. We examined the effects of e-cigarette aerosol on growth and morphological changes of *C. albicans*. We also investigated the effects of e-cigarette aerosols on the expression of secreted aspartic proteases (SAPs) SAP2, SAP3, and SAP9 genes by *C. albicans* and the interaction between e-cigarette-exposed *C. albicans* and gingival epithelial cells were also evaluated.

CHAPTER 2: Comparative Study of the Effects of Cigarette Smoke and Electronic Cigarettes on Human Gingival Fibroblast Proliferation, Migration and Apoptosis.

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2.1 Résumé

Dans un effort pour réduire les maladies liées au tabagisme, des produits alternatifs tels que les cigarettes électroniques ont été proposés. Le but de cette étude est d'évaluer les effets de la cigarette électronique sur les fibroblastes gingivaux humains. Les fibroblastes ont été exposés de façon répétitive au condensés de la cigarette électronique en comparaison au condensé de la fumée de cigarette. Nous avons évalué la morphologie, la croissance, la migration et l'apoptose cellulaires. Nos travaux montrent que les cellules exposées aux condensés de la cigarette ou de la cigarette électronique ont une morphologie anormale et un taux de prolifération plus faible. Ces observations sont consolidées par un taux plus élevé de cellules apoptotiques comparativement aux cellules non exposées. L'analyse de la migration cellulaire montre que le condensé de la cigarette et de la cigarette électronique réduisent de façon significative la capacité de migration des fibroblastes. Il est à noter que les effets sont plus importants avec la cigarette, suivi de ceux de la cigarette électronique contenant la nicotine, puis de celle sans nicotine.

2.2 Abstract

In an effort to reduce smoking-related diseases, alternative products such as e-cigarettes have been proposed. However, despite their growing popularity, the potential toxicity of e-cigarettes remains largely unknown. In this study, human gingival fibroblasts were repeatedly exposed to cigarette smoke condensate (CSC) and to nicotine-rich (NR) or nicotine-free (NF) e-vapor condensates for 60 min once a day for various time periods. Results indicate that cells exposed to CSC or NR condensates showed an altered morphology and a reduced proliferation rate, as ascertained by MTT and BrdU assays. Fibroblast cultures exposed to either CSC or e-vapor condensates also showed increased levels of TUNEL-positive apoptotic cells, compared to that recorded in the control. Furthermore, the cell scratch test revealed that repeated exposures to CSC or to e-vapor condensates delayed both fibroblast migration and wound healing. It should be noted that CSC was much more damageable to gingival fibroblasts than were the NR and NF e-vapor condensates.

The representative chain of damage thus translates to CSC > NR e-vapor condensate > NF e-vapor condensate.

2.3 Introduction

Electronic cigarettes are now being proposed with the goal of preventing the adverse effects of combustible cigarettes and eventually encouraging smokers to quit smoking (McRobbie et al., 2014; Jo et al., 2018), because combustible cigarettes contain hundreds of harmful chemicals, including several carcinogens (Baker et al., 2004) incriminated in smoker-related health problems (Curry et al., 2009). The e-cigarette device combines a plastic tube, an electronic heating component, and a cartridge serving as a reservoir to hold the e-liquid solutions. The liquid solution is heated and vaporized to produce an aerosol that is then inhaled by smokers through their airways (Mikheev et al., 2016), thus initially entering in contact with the oral mucosa. The electronic cigarette (e-cigarette) is being marketed as a “safe alternative” because it does not require combustion (Wackowski et al., 2016; Cheney et al., 2016).

Many e-liquids contains a mixture of propylene glycol, glycerin, nicotine, and flavorings (Cheng, 2014; Uchiyama et al., 2016). However, following vaporization, studies have reported that not only glycerol, propylene glycol, nicotine, and flavors are present in the e-vapors, but also trace amounts of carcinogens, and heavy metals (Mikheev et al., 2016). The heated humectants (propylene glycol and glycerol) thus release various aldehydes, such as formaldehyde, acetaldehyde, and acrolein in the e-vapor (Jensen et al., 2015; Gillman et al., 2016; Farsalinos et al., 2015). Nickel content, notably, has been found to be much higher in EC vapor than in standard cigarette smoke (Williams et al., 2013), and airborne aluminum levels have been reported to be high following EC vaping (Schober et al., 2014). These chemicals may have serious adverse effects on human health.

EC vapor emissions have been shown to create harmful free radicals production and inflammation induction leading to tissue damage (Lerner et al., 2015, Rouabhia et al., 2017). After reaching the oral mucosa, the e-vapor may harm the oral tissue, as

does combustible cigarette smoke. Indeed, it is well recognized that combustible cigarette smoke can alter cell function and promote periodontal disease development and severity (Vogtmann et al., 2017). Periodontitis severity has been shown to increase with smoking intensity and duration (Lallier et al., 2017). Furthermore, cigarette smoke also reduces the host response to periodontopathic bacteria, resulting in a more aggressive periodontal breakdown (Goh et al., 2017). This situation may also occur with EC. Upon entering the oral cavity, e-vapor comes in direct contact with the oral mucosa where epithelial cells and fibroblasts interact to maintain tissue integrity and function (McCulloch, 1995).

Gingival fibroblasts, the predominant cell type in gingival connective tissue, play a critical role in remodeling and maintaining gingival structure and extracellular matrix (Cáceres et al., 2014). These fibroblasts are also key players in tissue repair and wound healing through their adhesion, proliferation, and migration. Exposure to e-vapor of gingival mucosa may result in impairment of the gingival fibroblast function. EC products, such as nicotine, may affect periodontal cells by inhibiting the growth/proliferation of human periodontal ligament fibroblasts through apoptotic mechanisms. The aim of this study was to investigate the effects of e-vapor condensate with or without nicotine on normal human gingival fibroblast adhesion, viability/proliferation, apoptotic process and migration following insult. The effects of combustible cigarette smoke condensate and e-vapor condensates were also compared and analyzed.

2.4 Material and Methods

2.4.1 Gingival fibroblast isolation and culture

Biopsies of lamina propria tissue (gingival connective tissue) were collected from healthy, never smoked donors (18–25 years of age, n = 10) following obtention of their informed consent. Before tissue collection, patient were screened by the dentist to ensure the absence of inflammatory status or periodontal diseases. This protocol was approved by the Université Laval Ethics Committee. To isolate the primary

gingival fibroblasts, the connective tissue was placed in a collagenase P solution (0.125 U/mL; Boehringer Mannheim, Laval, QC, Canada) for 45 min at 37°C under agitation. The isolated cells (2×10^5) were seeded in 75-cm^2 cell culture flasks and grown in Dulbecco's modified Eagle's (DME) medium containing 10% fetal calf serum (Invitrogen Canada Inc., Burlington, ON, Canada). Once the cells reached 90% confluence, they were subcultured and used between passages 4 and 5 in this study. Cells were used individually to perform the experiments.

2.4.2 Preparation of cigarette smoke condensate solution

1R3F cigarettes were purchased from the Kentucky Tobacco Research & Development Center (Orlando, FL, USA) and used to prepare the cigarette smoke condensate (CSC) solution. The preparation of the CSC was made as previously described (Yadav et al., 2016) with some modifications. Briefly, each cigarette was placed into one end of a silicone tube linked to an Erlenmeyer flask containing 20 mL of culture medium. On the other end, a second silicone tube linked to the Erlenmeyer was connected to a standard vacuum. The cigarette was attached to the cigarette holder and lit and the smoke was extracted by applying the vacuum which pulled the smoke directly into the culture medium. The procedure was repeated with a total of two whole cigarettes. The resulting CSC solution was then sterilized by filtration through a 0.22-μm filter and considered as a 100% stock solution (n = 6). It was aliquoted and stored at -20°C until use.

2.4.3 Preparation of the e-vapor condensate solutions

EMOW electronic cigarette devices were chosen to deliver the e-vapor (Kanger Tech Brand, Shenzhen, China (www.kangeronline.com). The disposable EC liquid (Flavor: Smooth Canadian tobacco; http://shop.juicyejuice.com/juicy-canadian-tobacco-e-liquid.ejuice) was selected for this study. Nicotine concentration in the e-liquid was 12 mg/ml. The EC device and e-liquid were chosen because they were advertised as a "starter" EC vaping kit. The EMOW EC and disposable cartomizer cartridges were purchased from local retailers. To prepare the e-vapor condensate,

500 µL of the e-liquid containing nicotine were introduced into the EMOW electronic cigarette reservoir. Two different EMOW EC devices were used, one for NR and one for NF e-vapor condensate preparations. Thereafter, the EC device was placed into one end of a silicone tube while the other end of the tube was linked to an Erlenmeyer flask containing 20 mL of culture medium prior to activating the peristaltic pump which activated the EC device system to produce the e-vapor through the silicone tube (see Fig.2.1). The e- vapor was drawn into the Erlenmeyer flask, and dissolves into the culture medium; this referees to e-vapor condensate. The vapor was drawn into the exposure chamber with a regime of 2 puffs every 60 sec: a 10-sec puff followed by a 20-sec pause, as previously described (Lerner et al., 2016). The vaping procedure stopped when the total volume (500 µL) of e-liquid was vaped. The same procedure was used to prepare nicotine-free e-vapor condensate using a separate EC device. Collected e-vapor condensate solutions were sterilized by filtration through a 0.22-*µ*m filter and considered as 100% stock solutions (n = 6). They were aliquoted and stored at -20°C until use.

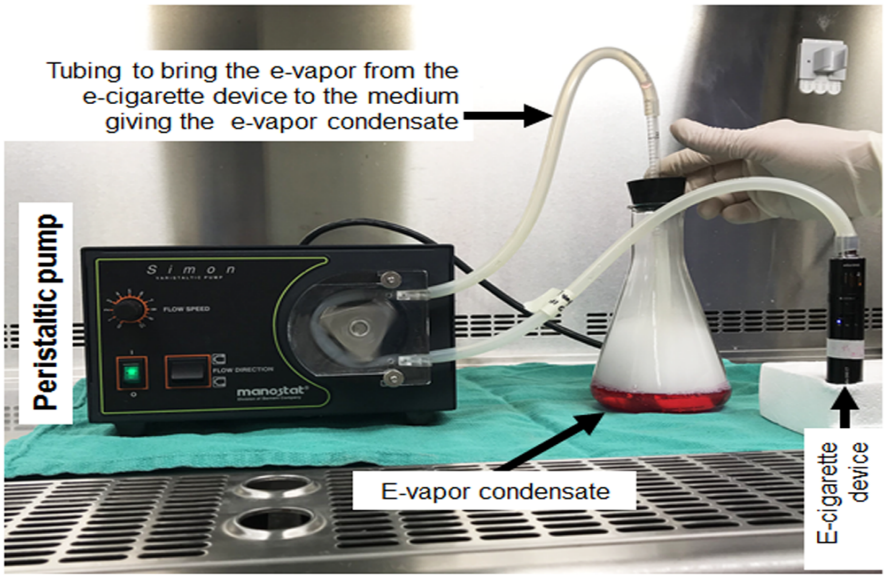

Figure 2.1: Schema showing the system used to generate the e-vapor condensates.

2.4.4 Effect of cigarette smoke and e-vapor condensates on human gingival fibroblast adhesion and morphology

Prior to cell seeding, five sterile glass slides (Bellco Glass Inc., Vineland, NJ, USA) (0.05 mm in diameter) were inserted into each well of a non-adherent 6-well plate (Sarstedt, Nümbrecht, Germany). Primary human gingival fibroblasts were then seeded at 10^5 cells/well in DMEM supplemented with 10% FBS. Immediately after seeding, the cells were incubated with different concentrations (0, 1, 5, or 10%) of either cigarette smoke or e-vapor condensate in triplicate at 37°C in a 5% CO_2 incubator for 24 h. Following incubation, the cells were fixed with methanol and glacial acetic acid (75/25, v/v) for 15 min, followed by three washes with PBS. The fixed cells were then incubated with 1 µg/mL of Hoechst 33342 (H42) (Riedel de Haen, Seele, Germany) in PBS for 15 min at room temperature in the dark. After three washes with PBS, the samples were observed under an epifluorescence light microscope (Axiophot, Zeiss, Oberkochen, Germany) and photographed. In a second set of experiments, fibroblasts were cultured for 24 h without smoke products then exposed or not for 60 min once a day for three days to either CSC or e-vapor condensates. At the end of the 3-day treatment regime, cells were observed under an inverted microscope and photographed (n = 6).

2.4.5 Effect of cigarette smoke and e-vapor condensates on cell growth

Fibroblasts were seeded (10^4 cells/well) in 6-well plates and cultured for 24 h prior to exposure to smoke products, after which time the culture medium was refreshed and supplemented with various concentrations (0, 1, 5, or 10%) of CSC, NF e-vapor condensate, or NR e-vapor condensate. Contact of the cells with the smoke condensate was maintained for 60 min at 37ºC in a 5% CO_2 humid atmosphere. Following each exposure, the medium containing the smoke condensate was replaced with a new smoke condensate-free medium. The cells were then incubated for 24 h at 37ºC. The smoke condensate exposure procedure was repeated once a day for 3, 5, and 7 days. Following each exposure period, cell growth was measured by MTT assay. Briefly, an MTT stock solution (5 mg/mL) was prepared in phosphate-

buffered saline, added to each culture at a final concentration of 1% (vol/vol), and subsequently incubated for 4 h at 37°C. At the end of this incubation period, the medium was removed and 1 mL of 0.04 M HCl in isopropanol was added to each culture, followed by incubation for 15 min to release the dye from the cells. Two hundreds µL of the reaction mixture was transferred in triplicate to wells of a 96-well plate and the absorbance was read at 550 nm using an ELISA reader (X-Mark microplate spectrophotometer; BioRad Laboratories, Mississauga, ON, Canada). Results were reported as the mean (SD) (n = 5).

2.4.6 Effect of cigarette smoke and e-vapor condensates on fibroblast proliferation

Fibroblasts were seeded (10^4 cells/well) in 6-well plates and allowed to adhere for 24 h. The culture medium was then supplemented with various concentrations (0, 5, or 10%) of CSC or nicotine-free/rich e-vapor condensate. The cells were in contact with the smoke condensates for 60 min once a day for 3 days. Following each exposure, the medium containing the smoke condensate was maintained for 1 h and replaced thereafter with a new smoke condensate-free medium. At the end of the 3-day treatment, cell proliferation was analyzed by DNA synthesis using a 5-bromo-2´-deoxyuridine (BrdU) labeling and detection kit (Roche Diagnostics GmbH, Mannjeim, Germany) according to the manufacturer's instructions. Briefly, cells were first incubated with 10 µM of BrdU labeling solution for 20 h at 37°C and were then fixed with Fixdent solution for 30 min at room temperature. Anti-BrdU-peroxidase working solution was added to each well, followed by incubation for 90 min at room temperature. The cells were then washed three times with PBS. A substrate solution (tetramethylbenzidine) was added to each well, followed by incubation for 15 min at room temperature. This substrate was used to detect the BrdU integrated into the proliferating cells. The reaction was stopped with 1 M of H_2SO_4. Finally, 3 x 200 µL of reaction solution were transferred to a 96-well plate. Absorbance was read at 450 nm on an automatic microplate reader (Bio-Rad Laboratories) with 690 nm as the reference wavelength. Results were reported as the mean (SD) (n = 4).

2.4.7 Apoptotic cell analysis by DNA fragmentation assay

Gingival fibroblasts (10^4 cells) were seeded onto 12-mm glass coverslips and allowed to reach 80% confluence before being exposed or not to either cigarette smoke or e-vapor condensates at various concentrations (0, 1, 5, or 10%). The exposure procedure was 60 min once a day for 3 days. Following each exposure, the medium containing the smoke condensate was maintained for 1 h and replaced thereafter with new smoke condensate-free medium. The cells were cultured for 24 h at 37°C in a humidified atmosphere of air containing 5% CO_2. At the end of the exposure regime, the cells were permeabilized with 4% paraformaldehyde for 60 min and then stained using the Trevigen TACS® 2 TdT-Blue Label *in situ* Apoptosis Detection Kit (Trevigen Inc., Gaithersburg, MD, USA) according to the manufacturer's instructions. Briefly, the cells were incubated with Proteinase K solution at 5 µg/mL for 30 min at 37°C and subsequently washed twice with deionized H_2O. The cells were then overlayered with terminal deoxynucleotidyl transferase (TdT) at 5 nM for 1 h at 37°C, followed by incubation for 5 min at room temperature with a stop solution. The cells were then washed twice with deionized H_2O, covered with Streptavidin-HRP conjugate (1/50) solution for 10 min at 37°C, followed by two washes with PBS. Cells were covered with TACS Blue Label (1/50) solution as a substrate for 2 min at room temperature. At the end of this incubation, the cells were washed several times with deionized H_2O. Counterstaining was performed using Nuclear Fast Red for 5 min at room temperature, after which time the cells were washed with deionized water prior to dehydration. Dehydration was performed using ethanol and the slides were mounted using histological mounting medium solution (Fisher Scientific, Ottawa, ON, Canada). The stained samples were then examined under a light microscope. The apoptotic and non-apoptotic cells were discriminated by blue- and red-stained nuclei, respectively. The blue-stained cells were counted under an optical microscope (at least 10-field from each slide, with three slides from each experiment). Results were presented as the mean (SD) (n = 4) of the percentage of blue-stained apoptotic cell/total cells in the observed microscope fields.

2.4.8 Effect of cigarette smoke and e-vapor condensates on gingival fibroblast migration

Human gingival fibroblasts were seeded (10^5/well) into 6-well plates, grown to confluence, and later exposed 60 min to either cigarette smoke or e-vapor condensate at various concentrations (0, 1, 5, or 10%). The medium containing the smoke condensate was replaced thereafter with a new smoke condensate-free medium and the cells were cultured thereafter for 24 h at 37°C in a humidified atmosphere of air containing 5% CO_2. Exposure to the smoke condensate was performed during 3 days at a rate of one exposure per day. At the end of the exposure procedure, crossed scratch wounds were created on each confluent monolayer using a 200 µL sterile pipette tip perpendicular to the bottom of the dish. The culture medium was then refreshed with new medium and the cells were incubated at 37°C in a CO_2 humid atmosphere. At the end of each incubation period (0, 10, 24, and 48 h), the smoke condensate-exposed and non-exposed cultures were examined under an optical microscope, where digital photographs of each wound were taken. Wound closure (cell migration) was then analyzed using the NIH ImageJ public domain image processing program to measure the non-covered surface between the opposite edges of the wound as a function of time. The smoke condensate-exposed and non-exposed cell cultures were compared, with the difference considered significant when $p < 0.05$. Results were reported as the mean (SD) ($n = 5$).

2.4.9 Statistical analyses

Each experiment was performed at least four independent times, with experimental values expressed as mean (SD). Statistical analyses were performed by comparing the control (absence of smoke condensate) and test cultures (presence of smoke condensate or e-vapor condensate). The statistical significance of differences between the values was determined using a one-way ANOVA. Subsequent statistical analyses (Tukey–Kramer Multiple Comparisons Test) were performed using InStat statistical software with P value declared significant at ≤ 0.05.

2.5 Results

2.5.1 Cigarette smoke and e-vapor condensates modulated fibroblast morphology but not early adhesion

Human gingival fibroblasts were exposed immediately after seeding to various concentrations of CSC and NR or NF e-vapor condensate and were subsequently used to determine cell adhesion. Fig. 2.2 shows that 24 h after seeding, none of the smoke condensates showed adverse effects on cell adhesion, as cell density (as ascertained by Hoechst-stained cells) was similar in all of the tested conditions (CSC, NF e-vapor/NR e-vapor condensate, and the control). However, the longer culture period (3 days) did reveal a reduced cell density and altered cell morphology (Fig. 2.3). Indeed, following contact with CSC or e-vapor condensate once a day for 3 days, a reduction in cell density was observed in the cultures placed in contact with CSC. This exposure led to altered cell shape. The fibroblasts went from a small, elongated cell shape (control) to a large-sized cell with a faint cytoplasm (Fig. 2.3). It should be noted that the change in cell morphology was greater in the CSC-treated than the NR e-vapor condensate-treated cells. Furthermore, cell density and cell morphology in the NR e-vapor condensate-treated cultures were different than that recorded in the control. These data thus suggest that both CSC and e-vapor condensate negatively affected the human gingival fibroblasts.

2.5.2 Cigarette smoke and e-vapor condensates decreased fibroblast growth

Fibroblast growth was analyzed at different time points (3, 5, and 7 days). As shown in Fig. 2.4, at as early as 3 days, the CSC decreased fibroblast growth, compared to that observed in the control (non-exposed cells). Of interest is that this negative effect was observed with 1, 5, and 10% of CSC; indeed, the higher the concentration of CSC, the greater the growth inhibition. Similar observations were made with the NR e-vapor condensate, with the most significant effect noted with 5 and 10% of NR e-vapor condensate. However, the reduction in fibroblast growth was greater with CSC than with NR e-vapor condensate. The NF e-vapor condensate slightly reduced fibroblast growth, compared to what was observed with the NR e-vapor condensate.

After 5 and 7 days of exposure, the decrease in fibroblast growth was maintained. CSC showed the most important inhibitory effect, followed by NR e-vapor condensate. It is interesting to note that even NR e-vapor condensate reduced fibroblast growth, specifically at high concentrations (5 and 10%). To support these observations, we performed a BrdU assay to assess fibroblast proliferation. As shown in Fig. 2.5, following exposure once a day for three days, fibroblast proliferation was significantly ($P < 0.001$) decreased in the presence of 1, 5 and 10% of CSC. Interestingly, the group exposed to NR e-vapor condensate also displayed a significant inhibition of the cell proliferation rate with 5 and 10% concentrations. Cell growth inhibition was greater with CSC than with NR e-vapor condensate, while a slight but significant reduction of fibroblast proliferation was obtained with NF e-vapor condensate (Fig. 2.5).

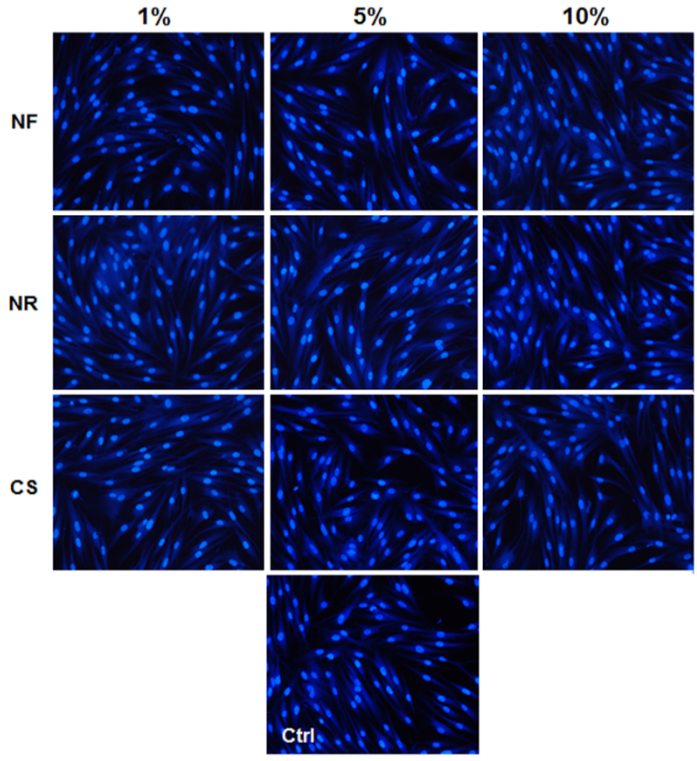

Figure 2.2. Early exposure to CSC and e-cigarette vapor condensate had no effect on fibroblast adhesion. Cells were first cultured in the presence or absence of CSC or e-vapor condensate for 24 h. Attached cells were then stained with Hoechst. (a) Untreated control cells; (b) cells cultured with 10% nicotine-free e-vapor

condensate; (c) cells cultured with 10% nicotine-rich e-vapor condensate; and (d) cells cultured with 10% CSC. Scale bar = 50 μm.

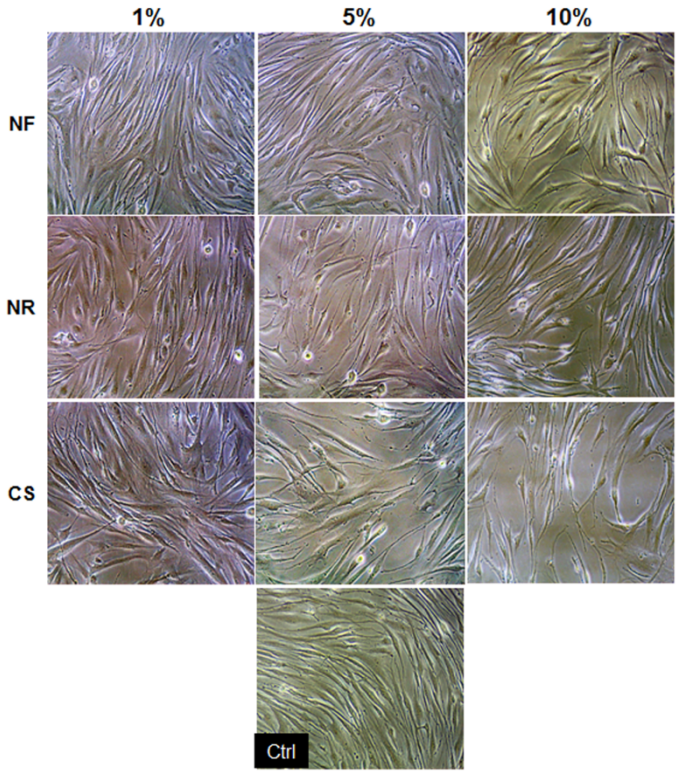

Figure 2.3. CSC and e-cigarette vapor condensate modulated human gingival fibroblast morphology. Cell shape/morphology after exposure of cells to CSC or e-vapor condensate for 60 min a day for 3 days. Photos were taken 24 h after the final exposure.

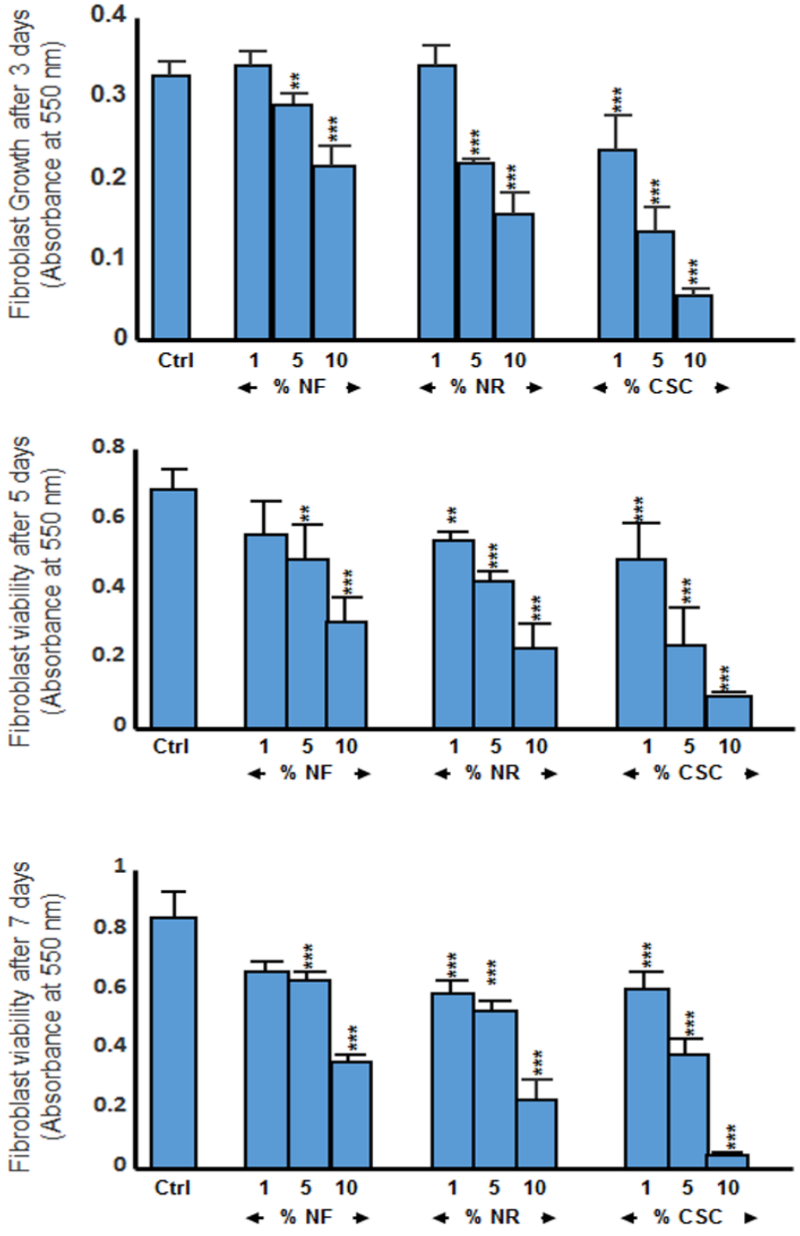

Figure 2.4. CSC and e-cigarette vapor condensate decreased human gingival fibroblast growth. Fibroblasts were exposed 60 min once a day for 3, 5, and 7 days to CSC, NR, or NF e-vapor condensate. Cell growth was assessed by MTT assay. Results are means ± SD. A significant difference was found between the exposed cells (CSC, NR, and NF e-vapor condensate) to the non-exposed control cells. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

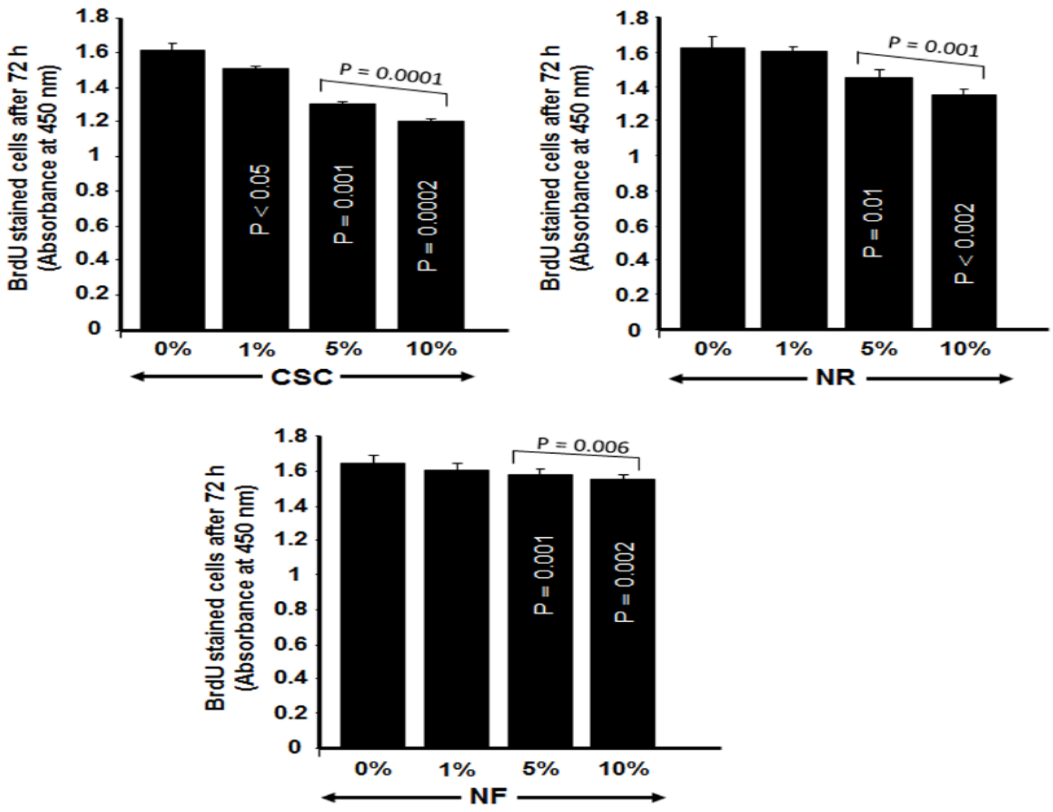

Figure 2.5. CSC and e-cigarette vapor condensate downregulated human gingival fibroblast proliferation. Following exposure for 60 min once a day for 3 days to either CSC or e-vapor condensate, fibroblasts were subjected to a 5-bromo-2′-deoxyuridine (BrdU) assay. Data are expressed as the means ± SD. Significance at $p < 0.05$ was determined by comparing the control (non-exposed cells) to exposed (CSC, NR or NF) cells.

2.5.3 Cigarette smoke and e-vapor condensates promoted gingival fibroblast apoptosis

Because CSC and the e-vapor condensates led to a decrease in gingival fibroblast proliferation, we analyzed cell apoptosis by DNA fragmentation using the TUNEL

assay. After exposure once a day for three days to CSC at 10%, the fibroblast cultures showed an increased number of TUNEL-positive apoptotic fibroblasts (Fig. 6), compared to what was observed in the non-exposed control cultures. Apoptotic cells were characterized by broken nuclei with a blue color (Fig. 2.6A). Exposure to either NR or NF e-vapor condensate also promoted gingival fibroblast apoptosis, as ascertained by the presence of TUNEL-positive cells (Fig. 2.6A, arrows). In the Fig. 2.6A, the proportion of apoptotic cells was higher in the presence of CSC than in the NR and NF e-vapor condensate-exposed cultures. This was confirmed by quantitative analyses (Fig. 2.6B) confirming that the percentage of apoptotic fibroblasts was highest in the CSC-exposed cultures, ranging from 1.6 ± 0.9 in the control to 22 ± 3% in the 10% CSC-exposed culture. The percentage of TUNEL-positive fibroblasts was also greater in the 10% NR e-vapor condensate-exposed cultures, ranging from 1.6 ± 0.9 in the control to 11 ± 2 in the NR e-vapor-exposed cultures. Finally, 10% NF e-vapor condensate also led to an increase of TUNEL-positive cells, reaching 6% ± 1%, compared to that observed in the control (non-exposed cultures). Similar effects were obtained with the 1 and 5% concentrations (Fig. 2,6B). It should be noted that CSC induced fibroblast apoptosis 2- to 3-fold, compared to NR e-vapor condensate. On the other hand, NR e-vapor condensate was 2 times more active than NF e-vapor condensate in leading to fibroblast apoptosis. Finally, even without nicotine, e-vapor condensate still promoted gingival fibroblast apoptosis.

2.5.4 Cigarette smoke and e-vapor condensates delayed gingival fibroblast migration and wound closure

We investigated the effects of cigarette smoke and e-vapor condensates on cell migration/wound healing. As shown in Fig. 2.7, CSC reduced fibroblast migration from both edges, thereby inhibiting total wound closure even after 48 h, as confirmed by the large uncovered area (132 ± 10 µm with 5% CSC and 287 ± 12 µm with 10% CSC, compared to 4.6 ± 2 µm recorded in the control); the inhibition of fibroblast migration and wound repair was thus greater with 10% CSC. Exposure to NR e-

vapor condensate also showed a delay in fibroblast migration/wound closure (Fig. 2.7). At as early as 10 h, the uncovered area ranged from 177 ± 38 μm in the control to 284 ± 37 μm in the 10% NR e-vapor condensate-exposed culture. Comparable results were obtained with 5% NR e-vapor condensate. After 48 h, the entire wound was repaired in the control group, while in the 10% NR e-vapor condensate-exposed culture, an uncovered area remained, at approximately 21.8 ± 5 μm. The NF e-vapor condensate-exposed culture also showed a delay in wound closure but to a lesser extent than that observed in the CSC- and NR e-vapor condensate-treated cultures (Fig. 2.7).

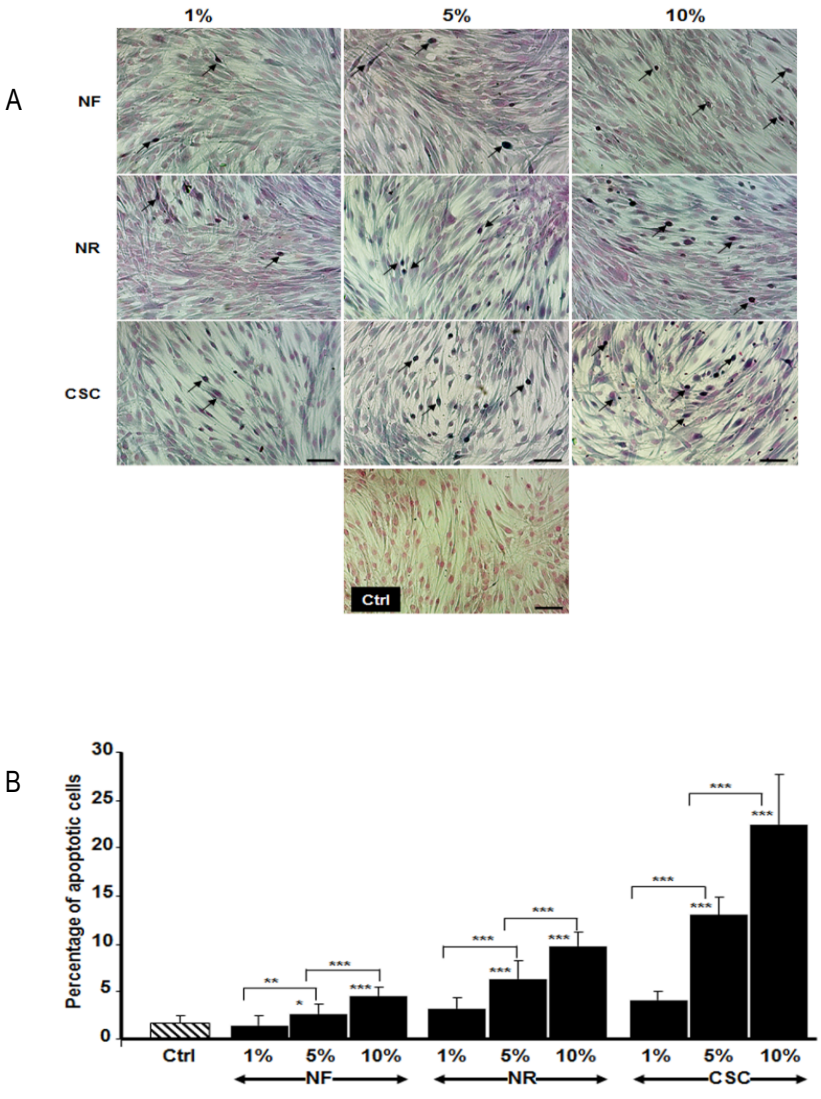

Figure 2.6. CSC and e-vapor condensate increased human gingival fibroblast apoptosis. Primary human gingival fibroblasts were cultured up to 80% confluence onto glass slides then exposed for 60 min once a day for 3 days to either 10% CSC or e-vapor condensate. Panel (A) Representative photos of four independent experiments. Scale bar = 50 μm. Panel (B) shows percentage of apoptotic cells in each condition. (*) $p \leq 0.05$, (**) $p \leq 0.01$, (***) $p \leq 0.0001$ when compared with the control (non-exposed cells); or the difference concentrations from the same agent (CSC, NR, NF).

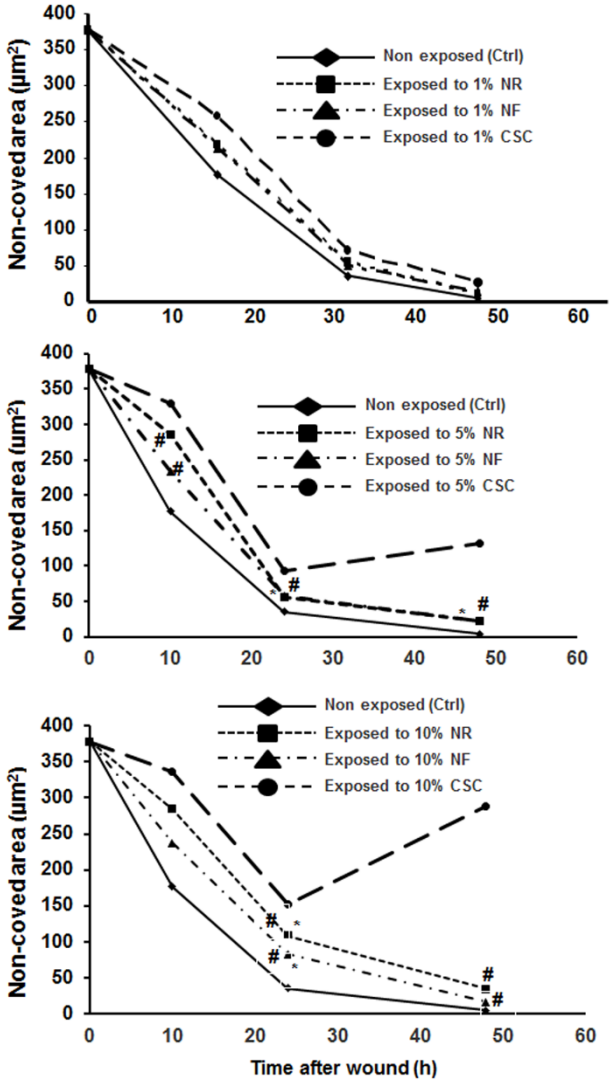

Figure 2.7. CSC and e-vapor condensate modulated human gingival fibroblast migration. Cells were cultured up to 100% confluence, then exposed or not to either

CSC or e-vapor condensate 60 min a day for 3 days. Scratches were then made on each monolayer and the medium was refreshed, with cell migration/would closure observed over time to assess the uncovered area. Values refer to non-covered surface area (μm^2) after each time point. (*) $p \leq 0.05$ when compared with the control (non-exposed cells); (#) $p \leq 0.05$ when compared with the CSC-exposed cells.

2.6 Discussion

With the well-known adverse effects of conventional cigarettes, smokers are turning to so-called “safer” nicotine-rich products, such as e-cigarettes. Some in vitro studies have reported that vaping e-cigarettes may be less harmful than inhaling conventional cigarette smoke (Misra et al., 2014; Romagna et al., 2013). The present study shows that exposure of gingival human fibroblasts to nicotine-rich and nicotine-free e-vapor condensate resulted in alterations in both cell shape and growth, although these negative effects were less significant than those evidenced with cigarette smoke condensate (CSC). These findings support those of other studies in which cell viability and oxidative stress were shown to be lower with e-vapor than with cigarette smoke (Anthérieu et al., 2017). However, it is important to note that e-vapor and e-vapor condensate have both been shown to adversely affect cell viability (Rubenstein et al., 2015, Rouabhia et al., 2017). Exposure of Kupffer cells to tobacco or e-cigarette extracts resulted in a marginal decrease in cell viability coupled with a significant decrease in cell density (Rubenstein et al., 2015). Another study reported that exposure of human epithelial alveolar cells to e-vapor condensate led to significant decrease in cell viability (Bengalli et al., 2017).

In light of these cell viability/growth results, one may speculate that e-cigarettes are safer than conventional cigarettes, but because they are not be totally harmless, a safety warning should be maintained with e-cigarette devices. Our study shows that both CSC and e-vapor condensate may interfere with the cell proliferation process. Indeed, while fibroblasts exposed to CSC showed a reduced level of BrdU-positive cells, the presence of e-vapor condensate led to a reduction in fibroblast proliferation. However, cell division as to BrdU level was greatly inhibited by CSC,

compared to what was observed with e-vapor condensate. It also should be noted that cell growth and proliferation were decreased by NR as compared to NF-e-vapors. The decrease is greater with the 10% concentration as compared to the 1% and 5% concentrations of NR compared to NF e-vapors. The difference we observed between NR and NF e-vapors could be attributed to nicotine, because this chemical was previously showed to decrease fibroblasts growth (Esfahrood et al., 2015). Cell proliferation is a key process for tissue structure and function and involves multiple cell types, including fibroblasts (Chiquet et al., 2015). Gingival fibroblasts actively participate in the tissue repair process by proliferating, migrating, and filling the wound beyond the synthesis of growth factors and extracellular matrix molecules (Hwang et al., 2009). Tobacco has been found to affect human gingival fibroblast adhesion, cytoskeletal structure morphology and cell proliferation, among others (Ghilarducci et al., 1995). This study shows that fibroblast proliferation can also be affected following repeated exposures to e-vapor condensate. Similar observations regarding the harmful effects of e-cigarettes on human periodontal ligament fibroblasts (Willershausen et al., 2009), the airway epithelial cell line (Rowell et al., 2017), primary gingival epithelial cells (Rouabhia, 2017) and human vascular endothelial cells (Putzhammer et al., 2016) have also been reported, thus confirming the deleterious effects of e-cigarettes on human cells, at least in vitro.

The reduced proliferation rate of gingival fibroblasts following the exposure to CSC or e-vapor condensate may be due to cell apoptosis. Indeed, both the CSC- and e-vapor condensate-exposed cultures showed a high percentage of TUNEL-positive apoptotic fibroblasts. These results are in agreement with those reported by Sancilio et al. (2016) who showed that nicotine-free and nicotine-rich e-liquids increased the production of ROS and Bax expression, followed by apoptosis occurrence in human gingival fibroblasts after 48 h of exposure. E-cigarettes were also shown to promote apoptosis in gingival epithelial cells (Rouabhia et al., 2017), human bronchial epithelial cells (Taylor et al., 2016) and human endothelial cells (Anderson et al., 2016). Of interest in the present study is that gingival fibroblast apoptosis was higher in the presence of CSC than with e-vapor condensate (with or without nicotine).

Modulating fibroblast proliferation and increasing cell apoptosis after cell exposure to smoke could thus have significant repercussions on wound healing.

The scratch assay has widely been used to assess the effects of different agents on wound healing (Derradjia et al., 2016; Martinotti et al., 2017). We used this assay to demonstrate that CSC significantly delayed gingival fibroblast migration. These data support those reported with gingival fibroblasts (Silva et al., 2012; Semlali et al, 2011), gingival epithelial cells (Rouabhia et al., 2017) and airway epithelial cells (Amatngalim et al., 2016). We also showed that when gingival fibroblasts were exposed to e-vapor condensate, cell migration and wound healing were delayed, compared to that observed in the non-exposed control. These findings are in agreement with those of Willershausen et al. (2014) who observed a noticeable inhibition of gingival fibroblast migration following exposure to a menthol-flavored e-liquid. While the delay in fibroblast migration was greater with CSC than with e-vapor condensate, both affected this migration. The CS, NR and NF condensates showed substantial adverse effects on human gingival fibroblasts. However, the mechanisms leading to such cell deregulations need to be investigated. Furthermore, in a native tissue, fibroblasts are in close contact with epithelial cells, thus future studies should include both cell type by using an engineered human oral mucosa as previously reported (Rouabhia and Allaire, 2010). Such system may better mimic what could happen when native tissue is in contact with e-vapors following e-cigarette use by smokers.

2.7 Conclusion

We demonstrated that exposure to conventional cigarette smoke and e-vapor condensate modulates gingival fibroblast activities. The damage to gingival fibroblasts was greater with conventional cigarette smoke condensate than with nicotine-rich e-vapor condensate. The nicotine-rich e-vapor condensate produced a significant effect on gingival fibroblast shape, proliferation, and migration/wound closure. Finally, the nicotine-free e-vapor condensate had a non-negligible effect on gingival fibroblasts but to a lesser extent. This suggests that chemicals other than nicotine present in the e-liquid present a certain level of toxicity to the cells. The

representative chain of damage thus translates to CSC > NR e-vapor condensate > NF e-vapor condensate. Overall results emphasize the need to further investigate e-cigarettes as a possible factor contributing to cell damage and delayed wound healing.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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CHAPTER 3: E-cigarettes vapour increase *Candida albicans* growth and modulate its interaction with gingival epithelial cells

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3.1. Résumé

La vapeur de cigarette électronique (e-cigarette) entre en contact avec les différents constituants de la cavité buccale, y compris des microorganismes comme Candida albicans. Le premier objectif de cette étude est d'évaluer les effets de la vapeur de cigarette électronique (e-cigarette) sur la croissance et l'expression de certains gènes appartenant à la famille de "secreted aspartyl protease (SAP)". Nos travaux montrent que la vapeur d'e-cigarette favorise la croissance et le changement de forme de *C. albicans*, et la production de taux plus élevés de chitine. Nous avons aussi montré que la vapeur de cigarette électronique augmente l'expression des gènes *SAP2, SAP3* et *SAP9* comparativement au contrôle (non exposé). La mise en contact de *C. albians,* préalablement exposé à l'aérosol de la cigarette électronique, avec les cellules épithéliales augmente l'adhésion de la levure aux cellules épithéliale, le taux de LDH et la différenciation cellulaire, mais entraîne une réduction de la prolifération des cellules épithéliales. L'ensemble de ces résultats suggère que l'usage de la cigarette électronique pourrait favoriser les infections à *C. albicans*, ce qui pourrait augmenter le risque de candidose *oropharyngée.*

3.2. Abstract:

Electronic cigarette (e-cigarette) vapor comes in contact with the different constituents of the oral cavity including microorganisms like *Candida albicans*. We examined the impact of e-cigarettes vapor on *C. albicans* growth and expression of different virulent genes, such as secreted aspartic proteases (SAPs), and the effect of e-cigarette vapor-exposed *C. albicans* on gingival epithelial cell morphology, growth and LDH secretion. An increase in *C. albicans* growth was observed with nicotine-rich e-cigarettes compared to that observed with non-exposed cultures. Following exposure to e-cigarette vapor, *C. albicans* produced high levels of chitin. There was alos an increase in *C. albicans* hyphal length and the expression of SAP2, SAP3 and SAP9 genes. E-cigarette-exposed *C. albicans* adhered better to epithelial cells than control. Indirect contact between e-cigarette vapor exposed *C. albicans* and gingival epithelial cells led to epithelial cell differentiation, reduced cell

growth and increased *lactate dehydrogenase (*LDH) activity. Overall results indicate that e-cigarettes vapor may interact with *C. albicans* to promote their pathogenesis, which may increase the risk of oral candidiasis in e-cigarette users.

3.3. Introduction

Cigarette smoking constitutes a well-established risk factor for oral infections [1]. Indeed, smokers are more prone to severe periodontal disease, caries, and *candidiasis* [2,3]. Data have shown that tobacco alters the interaction between *Porphyromonas gingivalis* and the host, leading to periodontitis [4]. Although periodontitis has been strongly associated with bacteria such as *P. gingivalis*, a variety of microorganisms, including *C. albicans*, have been detected in periodontal pockets [5,6]. *Candida albicans (C. albicans)* has thus been associated not only with oropharyngeal *candidiasis* but also with severe forms of periodontitis [5,7]. Patients with systemic disorders as diabetes mellitus, neutropenia, agranulocytosis, and acquired immunodeficiency syndrome (AIDS) have also been shown to harbor enteric *Staphylococcus aureus* and *Candida* sp. in their periodontal pockets [6,8]. Furthermore, studies have reported the presence of *C. albicans* in non-immunologically compromised patients suffering from severe chronic periodontitis [5,9].

Candida virulence was promoted by various exogenous factors, such as cigarette smoke [3] which has been shown to stimulate *C. albicans* adhesion and growth, as well as biofilm formation [3,10]. Conventional cigarette smoke (CCS) was also found to favor *C. albicans* growth, with an increased expression of enhanced adherence to polystyrene (*EAP1*), hyphal wall protein 1 (*HWP1*) and certain secreted aspartyl proteinase (*SAP*) genes known to be involved in the yeast's virulence [10].

To counter the adverse effects of cigarette smoke, an electronic cigarette (e-cigarette) was introduced on the market and promoted as a "safe alternative" to the smoking habit [11]. The e-cigarette combines a plastic tube, an electronic heating

component, and a reservoir for an e-liquid solution that contains propylene glycol and glycerol, with or without nicotine [12]. Following airflow detection by the internal sensor in the e-cigarette device, the heating component in contact with the e-liquid produces a vaping solution of a smoke-like aerosol that is subsequently inhaled into the upper airways [12].

During e-cigarette use, the first site in contact with the e-liquid vapor is the oral cavity, including the gingival tissues and the oral microbial community. E-cigarette use reportedly induces production of harmful free radicals and inflammation leading to gingival cell damage, which may affect the innate defense, thereby promoting oral infections [13].

As the effect of e-cigarettes on oral microorganisms such as *C. albicans* has not yet been fully elucidated, we sought to analyze the growth and expression of the *SAP2*, *SAP3* and *SAP9* genes by *C. albicans* following multiple exposures to conventional cigarette smoke (CCS), nicotine-rich (NR) e-cigarettes, and nicotine-free (NF) e-cigarettes vapors. We also investigated the interaction between e-cigarette vapor exposed *C. albicans* and gingival epithelial cells.

3.4. Material and Methods

3.4.1. Candida strain

C. albicans (ATCC-SC5314) was grown in Sabouraud liquid medium (Becton Dickinson, Cockeysville, MD, USA) supplemented with 1% glucose. The culture was grown to the stationary phase for 18 h at 30°C in a shaking water bath. The blastoconidia were collected, washed with phosphate-buffered saline (PBS), and counted by means of a hemacytometer (Reichert, Buffalo, NY, USA). The cell suspension was adjusted to 10^8 *C. albicans* cells/ml prior to being exposed or not to CCS or e-vapor.

3.4.2. E-cigarettes

eGo one CT electronic cigarette devices (www.joyetech.com) purchased from local retailers (Québec City, QC, Canada) were used to deliver the e-cigarette vapor. Disposable e-cigarette liquids with and without nicotine (Flavor: Smooth Canadian tobacco, http://shop.juicyejuice.com/juicy-canadian-tobacco-e-liquid.ejuice) were included in this study. Nicotine concentration in the e-liquid was 18 mg/ml. The selected e-cigarette devices and e-liquids were chosen because of their availability to users. For the conventional cigarette, we used 1R3F cigarettes purchased from the Kentucky Tobacco Research & Development Center (Orlando, FL, USA).

3.4.3. Effect of e-vapor on C. albicans growth

C. albicans (10^6 cells) were placed in a 50-ml sterile culture tube containing 2 ml of fresh Sabouraud liquid medium. The following four conditions were used in each *C. albicans* culture experiment: non-exposed or exposed to CCS, NR e-vapor, or NF e-vapor. The exposures to the e-cigarettes vapor were performed using a peristaltic pump and custom-made smoke chamber (See Fig. 3.1). Briefly, *C. albicans* cultures in 35 mm diameter petri dishes were aseptically placed inside the smoke chamber. The e-cigarette device was linked to one end of a silicone tube while the other end of the tube was linked to the smoke chamber. The peristaltic pump was used to deliver the e-cigarette vapor into the chamber. Following activation of the peristaltic pump, the e-cigarette device delivered the e-cigarette vapor through the silicone tube into the exposure chamber. The e-vapor (with and without nicotine) drawn into the chamber represented 2 puffs every 60 sec with a 5-sec puff followed by a 30-sec pause [14] with minor modifications. With this procedure, *C. albicans* cells were atmospherically exposed to the e-vapor. To promote contact of *C. albicans* cells with e-vapor, the cultures were gently agitated during the exposure process. The exposure procedure to CCS was identical to that used with the e-vapor. Briefly, a cigarette was linked to one end of a silicone tube while the other end of the tube was linked to the smoke chamber. The peristaltic pump allowed for the delivery of the CCS of one-half cigarette into the chamber, with an approximate 20-sec burning

time. For each condition, the exposure time consisted of 15 min a day twice a day for 2 and 3 days.

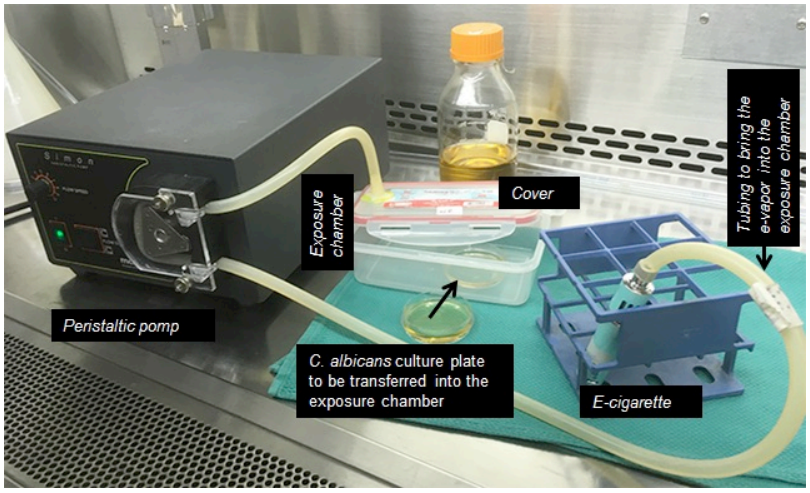

Figure 3.1: Exposure protocol of *Candida albicans* to e-cigarette vapor or combustible cigarette smoke.

Each *C. albicans* exposure condition was subsequently incubated for one additional hour prior to medium changing. The *C. albicans* pellets were then fed fresh Sabouraud medium and were cultured until the following exposure prior to undergoing various analyses. *C. albicans* cultures placed into a smoke-free/e-vapor-free chamber for the same duration as the assay conditions were included in the study as controls (Ctrl). Each exposure condition (CCS, NF, NR, and Ctrl) was performed in a separate exposure chamber to avoid culture cross-contamination. At the end of each exposure regime (2 or 3 days), *C. albicans* growth was determined by MTT assay, as previously reported [15]. Results were reported as the means ± SD, n = 5.

3.4.4. Effect of e-vapor on C. albicans cell wall chitin content

In a first set of experiments, the *C. albicans* cells (10^6) were seeded in Sabouraud liquid medium and exposed twice a day to CCS, NF e-vapor, or NR e-vapor for

15 min each, and were cultured thereafter for 24 h at 37°C. The cells were then collected and centrifuged, with the resulting pellets suspended in 500 µL of 4% paraformaldehyde solution to fix the cells. After 60 min of incubation at room temperature, the cells were washed twice with PBS and incubated thereafter for 5 min at room temperature with calcofluor white stain in the presence of 10% potassium hydroxide. The cells were then observed under an epifluorescence (UV) microscope and photographed. In a second set of experiments, *C. albicans* cells exposed twice a day to CCS, or to NF or NR e-vapor for 15 min and cultured for 16 h at 37°C were then collected and centrifuged, with the pellets subsequently suspended in 2 ml of fresh Sabouraud medium. The cell count was initiated on each condition using a *hemocytometer* counting protocol. An exact amount of *C. albicans* cells (400 x 10^6) from each condition (CCS, NR, NF, and Ctrl) was then centrifuged, with the resulting pellet resuspended in 500 µL of lysis buffer containing 200 µL of glass beads (0.425–0.6 mm in diameter). The cells were disrupted by means of a MiniBead-beater (Biospec Products, Bartlesville, OK, USA) for 2 min at 5,000 rpm for 10 cycles under cold conditions [16]. Each cell wall was hydrolyzed in 6N HCl for 16 h at 100°C, evaporated at 65°C, and dissolved thereafter in sterile water (1 mL). A small volume (100 *µ*l) of each solution was supplemented with 100 µL of 1.5N Na_2CO_3 in 4% acetylacetone and boiled (100°C) for 30 min, followed by the addition of 0.7 mL of 96% of ethanol to each sample. A volume of 100 µl of a solution containing 1.6 g of dimethylaminobenzaldehyde in 30 mL of HCl and 30 mL ethanol was added to each sample, with the resulting mixture incubated thereafter for 1 h at 37°C in a dark atmosphere. Absorbance was measured at 520 nm by means of an xMark microplate spectrophotometer (Bio-Rad, Hercules, CA, USA). Chitin concentrations were calculated using a standard curve of glucosamine (0–200 mg); (n= 4).

3.4.5. *Effect of e-vapor* on *C. albicans transition from blastospore to hyphal form*

Qualitative and quantitative assays were performed to determine the impact of e-cigarette vapor on *C. albicans* morphological changes (yeast to hyphae). *C. albicans* (10^5 cells) were grown in 3 mL of Sabouraud liquid medium supplemented with 1% glucose and 10% fetal bovine serum (FBS). The FBS promoted the *C. albicans* hyphae transition. The cultures were immediately exposed to CCS, NR e-vapor, or NF e-vapor for 15 min, followed by incubation for either 3 or 6 h at 37°C prior to assessing the cell morphological changes. CCS was considered as the positive control, while non-exposed *C. albicans* cultures were considered as the negative controls. Following incubation for 3 or 6 h, the cultures were observed microscopically and photographed to record the *C. albicans* morphology ($n = 5$), while hyphal length in each condition was measured by means of NIH-ImageJ software (Version 1.52j).

3.4.6. *Effect of e-vapor on the expression of SAP2, SAP3, and SAP9 genes by C. albicans*

C. albicans (5×10^6 cells) were first placed in a 50-mL sterile culture tube containing 2 mL of fresh Sabouraud liquid medium. The cells were then exposed or not to CCS, NR e-vapor, or NF e-vapor twice for 15 min, with a 6 h interval between each exposure. Exposure to the CCS and e-cigarettes vapors was performed using a peristaltic pump and a smoke chamber. Following each exposure, the cultures were incubated for 60 min before the culture medium was refreshed. Following the second exposure, the *C. albicans* cultures were incubated for 16 h at 37° C and subsequently used to extract total RNA, as previously reported [15]. The RNA (1 µg of each sample) was first reverse transcribed into cDNA by means of the iScript cDNA Synthesis kit (Bio-Rad) and used thereafter for quantitative PCR (qPCR). Reactions were performed using a PCR supermix (Bio-Rad; iQ SYBR Green supermix). Specific *SAP*2, *SAP*3, and *SAP*9 primers (Table 3.1) were added to the reaction mix at a final concentration of 250 nmol/L. Five microliters of each cDNA

sample were added to a 20-*µ*l PCR mixture containing 12.5 µl of the iQ SYBR Green supermix, 0.5 µl of each primer (*ACT1* (*housekeeping gene*), *SAP*2, *SAP*3, *and SAP*9) along with 7 µl of RNase/DNase-free water. Reactions were performed using a Bio-Rad MyCycler Thermal Cycler. The CT was automatically determined using the accompanying Bio-Rad CFX Manager. The thermocycling conditions for each gene were established as 5 min at 95°C, followed by 30 cycles of 15 s at 95°C, 30 s at 60°C, and 30 s at 72°C, with each reaction performed in triplicate. The specificity of each primer pair was determined by the presence of a single melting temperature peak. *ACT1* produced uniform expression levels varying by < 0.5 CTs between sample conditions and thus became the reference gene for this study. The results were analyzed using the $2^{-\Delta\Delta Ct}$ (Livak) relative expression method [17] (*n*=5).

Table 3.1: **Primer sequences used for the qRT-PCR. Primers were optimized previously, see [10, 15]**

Gene	Primer sequence (5' à 3')	Amp size (bp)
ACT-1	Forward: GACAATTTCTCTTTCAGCACTAGTAGTGA Reverse: GCTGGTAGAGACTTGACCAACCA	87
SAP2	Forward: TCCTGATGTTAATGTTGATTGTCAAG Reverse: TGGATCATATGTCCCCTTTTGTT	82
SAP3	Forward: GGACCAGTAACATTTTTATGAGTTTTGAT Reverse: TGCTACTCCAACAACTTTCAACAAT	87
SAP9	Forward: ATTTACTCCACAGTTTATCACTGAAGGT Reverse: CCACAAGAACCACCCTCAGTT	86

3.4.7. Adhesion of e-vapor-exposed C. albicans to gingival epithelial cells

Human gingival epithelial carcinoma cell line (Ca9-22) purchased from Health Science Research Resources Bank (HSRRB) (Osaka, Japan) was used for our *in vitro* experiments [18]. Cells were maintained in the Roswell Park Memorial Institute medium (RPMI)-1640 supplemented with L-glutamine and 10% fetal calf serum (FCS). In a first set of experiments, we analyzed the adhesion of smoke- or vapor-

exposed *C. albicans* to an epithelial cell monolayer. Briefly, *C. albicans* cells (10^6) were exposed twice a day for 15 min with a 6-h interval between the first and the second exposure to CCS, NR e-vapor, NF e-vapor, or not. Cultures were maintained at 37°C during 24 h. The following day, the smoke-exposed, vapor-exposed, or non-exposed *C. albicans* cultures were used to count the cell number, and then each one was used to infect the epithelial cell monolayers. For this purpose, Ca9-22 cells (10^5) were seeded in 6-well tissue culture plates and incubated at 37°C for 24 h prior to contact with the *C. albicans*. The epithelial cell cultures were then pulsed with 10^4 *C. albicans* that had been exposed or not to CCS, NF, or NR products. The contact periods between the epithelial cells and the *C. albicans* were 6 and 24 h. At the end of each contact period, the medium was removed, the culture were washed twice with fresh medium with gentel agitation to remove non-adherent *C.albicans* cells, then the cultures were fixed with 4% paraformaldehyde for 60 min and subsequently stained with crystal violet dye. After staining, the cultures were examined under an optical microscope and photographed. Each condition was run in triplicate (n = 4).

3.4.8. Growth of epithelial cells following contact with e-vapor-exposed C. albicans

Epithelial cells were seeded (5×10^5) in 6-well tissue culture plates and cultured for 24 h at 37ºC in a 5% CO2 incubator. The following day, the culture medium was refreshed, and the e-vapor-exposed and CCS-exposed *C. albicans* was put in contact with epithelial cells through a transwell culture system. The *C. albicans* recipient well received 10^6 cells. The porosity of the membrane was 0.4 um to allow for medium exchange but not *C. albicans*, which prevented the direct adverse effect of *C. abicans* on the epithelial cells. The transwell culture plates were then incubated at 37ºC in a 5% CO2 humid atmosphere for 24 h prior to analysis. The following day, the upper chamber was used to collect *C. albicans* cells, which were washed twice with Sabouraud medium, followed by a cell number count to discriminate the blastospores and hyphal forms. The culture supernatants were collected and used to measure LDH activity. For LDH activity measurement we also included positive

control which was obtained by culturing the gingival epithelial cells in the presence of 1% Triton X-100 (100% cell death). A negative control was obtained by culturing gingival epithelial cells under normal cell growth conditions. Epithelial cell shape was ascertained by inverted optical microscopy and subsequently photographed. At the end of this step, the epithelial cells were detached following incubation with a 0.05% trypsin-0.04% EDTA solution. Epithelial cell suspensions were used to determine the viable cell numbers in each condition, as determined by the trypan blue exclusion assay. The cell suspensions were then centrifuged, and the resulting cell pellets were lysed to extract total proteins to be used for subsequent analyses. Each experiment was performed in duplicate, and the means ± standard deviations of four separate experiments were calculated and plotted.

3.4.9. Statistical analysis

Continuous variables were expressed using mean ± SD. Data were analyzed using a two-way ANOVA. The CCS-, NF-, and NR-exposed, as well as the non-exposed conditions were merged for 15 min to define four conditions. All of the statistical analyses had a significant interaction factor ($p < 0.0001$) and expressed heterogeneous variances. The Satterthwaite's degree of freedom statement was added for unequal variance structures. Comparisons among the different conditions at different days (2 and 3 days for the *C. albicans* growth analyses) were performed by partitioning the interactions. The normality assumption was verified using the Shapiro-Wilk test following a Cholesky factorization. Results were considered significant with P values < 0.05. All of the analyses were conducted using the SAS 9.4 statistical package (SAS Institute Inc., Cary, NC, USA) and R (R Core Team (2016), Foundation for Statistical Computing, Vienna, Austria).

3.5. Results and Discussion

3.5.1. E-cigarette vapor promoted C. albicans growth

E-cigarettes are proposed as a “safe alternative” to conventional cigarettes and a possible option to quit smoking [11]. As a result, the number of e-cigarette users and consumer acceptability has increased, despite false safety recommendations regarding this smoking process [19]. Indeed, data have unfortunately confirmed that e-cigarettes/e-vapors are not as safe as users believe them to be. Following use, e-cigarette vapor first comes in contact with the oral cavity, which may affect the oral tissue. Indeed, studies report a definite adverse effect of e-cigarettes on gingival cells [20].

E-cigarettes could also modulate the oral microbial community. Our results in fact indicate that e-cigarette vapor promoted *C. albicans* growth. Exposure of *C. albicans* cultures to NR e-cigarette vapor for 15 min twice a day for 2 days showed significant ($p < 0.001$) *C. albicans* growth, compared to that observed in the controls (non-exposed cultures) (Fig. 3.2). Following the MTT assay, the absorbance increased from 0.37 ± 0.04 in the control to 0.79 ± 0.003 in the presence of NR e-vapor. It should be noted that NF e-vapor also increased the growth of *C. albicans*, with its absorbance increasing from 0.37 ± 0.04 to 0.6 ± 0.02 (Fig. 1). However, both the NR and NF e-vapor recorded low *C. albicans* growth, compared to that observed with the CCS. With the NR e-vapor, the absorbance was 0.79 ± 0.003, while it was 1.015 ± 0.04 with the CCS (Fig. 1a). Comparable results were obtained after 3 days of exposure (Fig. 1b) showing a significant ($p < 0.001$) growth increase when comparing the absorbance obtained with the NR or NF e-vapor and that in the control. A significant increase of *C. albicans* growth was also recorded when comparing the CCS results and the NR and NF e-vapor results. It should also be noted that NR e-cigarettes significantly ($p < 0.01$) promoted *C. albicans* growth, compared to that recorded by the NF e-vapor.

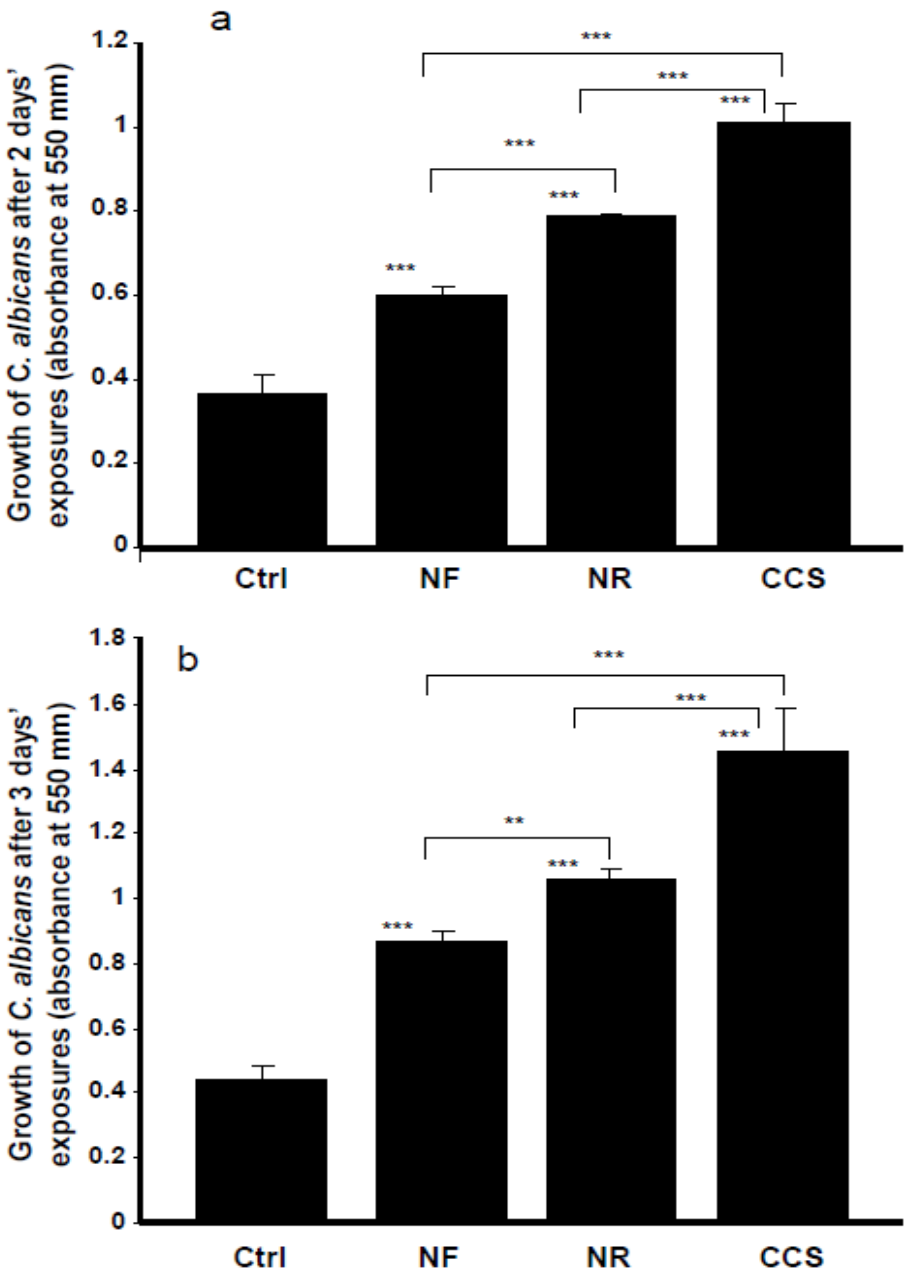

Figure 3.2. E-cigarette vapor promoted *C. albicans*. Cells were exposed or not for 15 min twice a day for 2 or 3 days, with the growth determined by MTT assay. Results are means ± SD, n = 5. A significant difference was observed when comparing the *C. albicans* cells exposed to CCS, NR e-vapor, or NF e-vapor and those of the control (non-exposed cells). We also compared NF to NR, NR to CCS, and NF to CCS. **P < 0.01; ***P < 0.001.

3.5.2. Chitin content was high in e-cigarette vapor-exposed C. albicans

C. albicans growth following exposure to e-cigarette vapor was accompanied by increased chitin production. The fluorescence intensity of the CCS-exposed and e-vapor-exposed cells was higher than that expressed by the non-exposed cells. Cell density with intense fluorescence was also higher in the CCS-exposed and e-vapor-exposed cultures than in the non-exposed cultures (data not shown). The effect of e-vapor on chitin production was supported by the quantitative analyses of the chitin content. Indeed, following exposure to NR e-vapor, *C. albicans* cells recorded significantly ($P < 0.01$) higher levels of chitin than did the control (Fig. 3.3). However, chitin expression was greater in the CCS-exposed *C. albicans* than it was in the NR and NF e-vapor-exposed cells.

This is the first study to report this modulatory effect of e-cigarette vapor on *C. albicans* chitin content. Similar observations were reported with standard cigarette-exposed *C. albicans*, showing high amounts of chitin in *C. albicans* exposed to cigarette smoke condensate compared to non-exposed *C. albicans* cells [16].

Cell wall proteins, including chitin, are known to be involved in the sensing of stressful agents such as changes in carbon source [21]. When exposed to e-vapor, *C. albicans* may consider this contact to be an abnormal situation, thereby promoting chitin production as a protective pathway against the possible deleterious effects of the e-vapor. Indeed, studies showed that *C. albicans exposed to antifungal molecules* increased chitin production to overcome the effect of the drug [16,22]. *C. albicans* may possibly develop a resistance mechanism against CCS and e-cigarette vapor through an increased expression of chitin, as is reported in the present study. This may translate to a clinical impact for e-cigarette users.

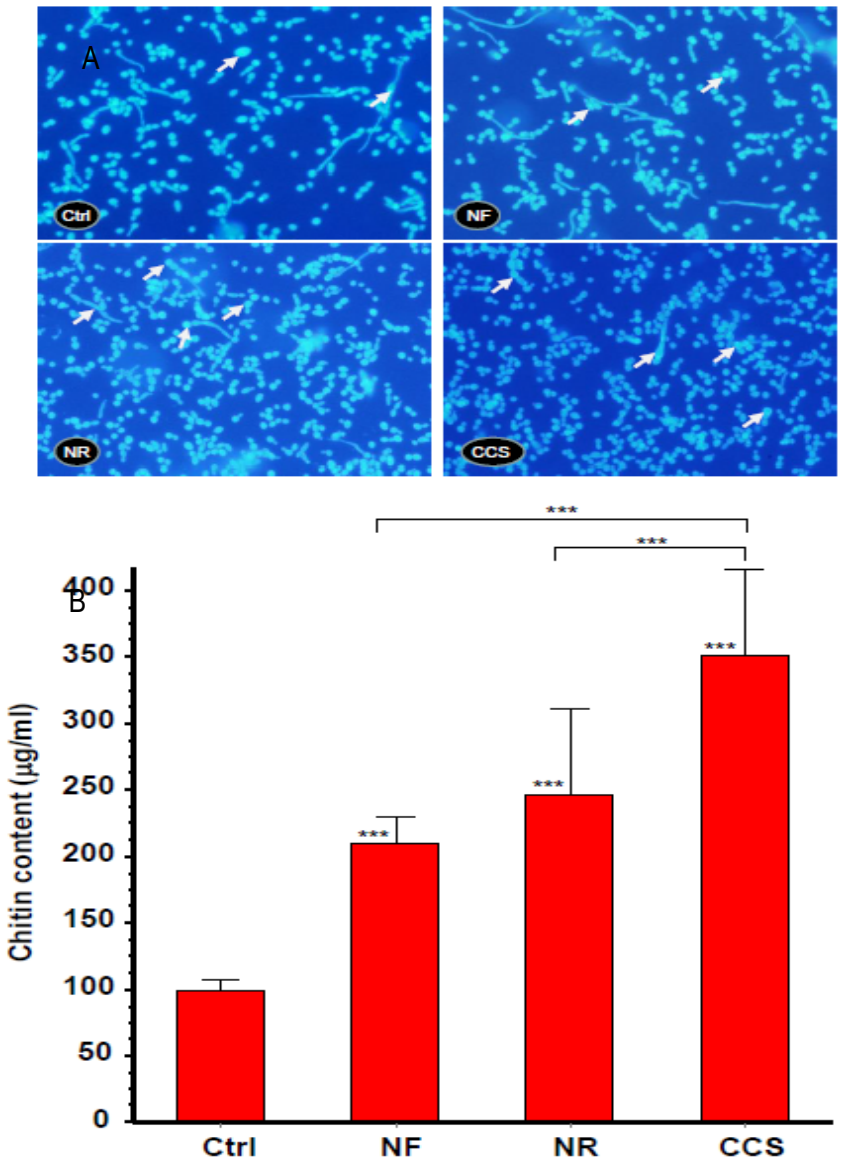

Figure 3.3. E-cigarette vapor increased the level of chitin produced by *C. albicans*. Following exposure or not to CCS, NR e-vapor, or NF e-vapor, *C. albicans* cells stained with calcofluor/potassium hydroxide were observed using epifluorescence microscopy and a UV filter (Panel A). Representative images are from 4 independent experiments, with each experiment performed in duplicate (Panel A). Scale bars = 50 µm. In a second set of experiments, cell wall proteins were extracted and subjected to chitin level quantification, as described in the M&M section. Chitin levels are presented (Panel B). Statistical significance was obtained by comparing the cells

exposed to CCS, NR or NF e-vapor and those of the control (non-exposed cells). ***P < 0.001.

3.5.3. E-vapor-exposed C. albicans displayed an increase in hyphal length

The ability of *C. albicans* to grow as yeast cells, pseudohyphae, and hyphae is a pivotal aspect of its capacity to move from the commensal to the pathologic phenotype. It has been demonstrated that *C. albicans* virulence can be altered by changing the morphology of the yeast [23]. Various forms of *C. albicans* have been found in both infected tissues and biofilms, which suggests a role for each form during infection [24]. Because e-cigarette vapors were capable of promoting *C. albicans* growth, we sought to determine whether e-cigarette vapor could modulate *C. albicans* morphology.

Our findings show that hyphal length was significantly greater in the e-vapor-exposed cultures than in the controls. As shown in Fig. 3.4, longer hyphal tubes were observed at 3 and 6 h in the NR e-vapor-exposed and CCS-exposed *C. albicans* cultures. It is important to note that both the NR e-vapor and the CCS induced a significantly ($P < 0.05$) longer hyphal size, even at 3 h of incubation, compared to that observed in the non-exposed controls. The hyphal tubes were more visible after 6 h of incubation.

Hyphae are considered necessary for *C. albicans* to invade its host [23]. The increased *C. albicans* growth and hyphal length following exposure to e-cigarette vapor may thus result in oral health issues. Indeed, smokers are reportedly more prone to caries [2], periodontal disease [25], and candidiasis [3]. Because e-cigarettes promoted *C. albicans* overgrowth and morphological changes, this may translate to an increased risk of candidiasis and periodontitis in e-cigarette users.

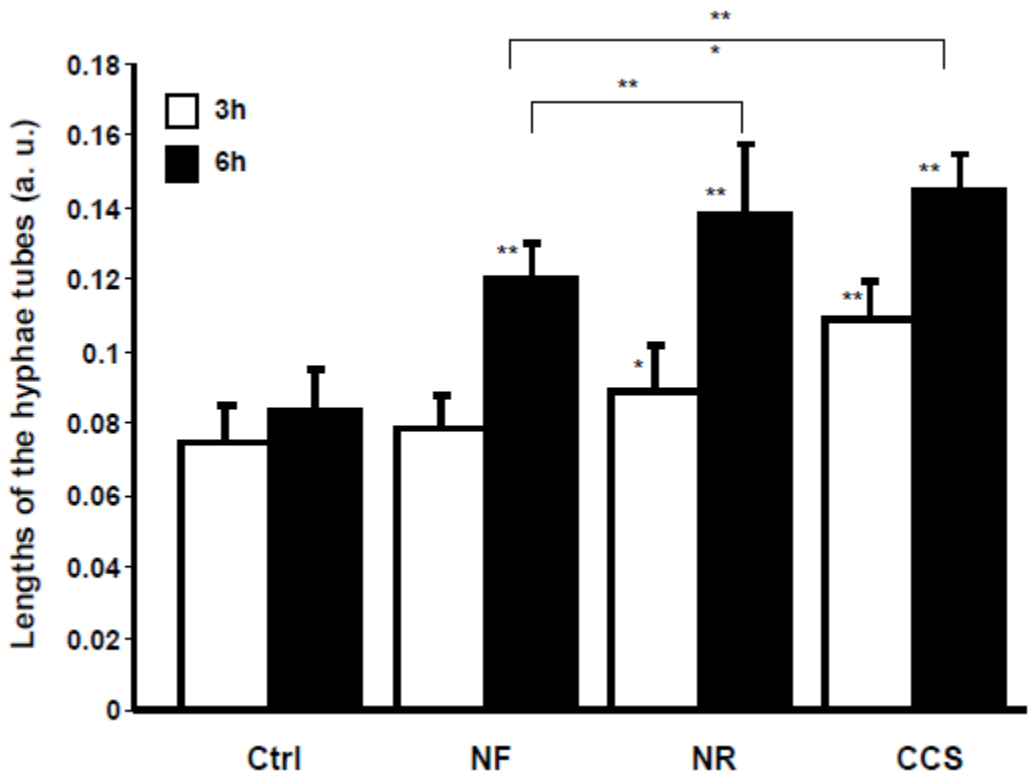

Figure 3.4. E-cigarette vapor increased the hyphal length of *C. albicans* cultured under cell morphology transition conditions. *C. albicans* cells were exposed or not to CCS, NF e-vapor, or NR e-vapor, then cultured at 37°C in the presence of 10% fetal calf serum. After 3 and 6 h, the hyphal tube length was measured by means of NIH-ImageJ software (n = 5). *P < 0.05; **P < 0.01 (r. u = relative unit).

3.5.4. E-vapor-exposed C. albicans expressed high virulent gene levels

Candida growth and form changing are under the control of various genes [26]. Among these is the *SAP* gene family, known to promote *C. albicans* adhesion, growth, and biofilm formation [27]. Our results show that *C. albicans* exposed to NR e-cigarette vapor expressed a high level of *SAP2*, compared to that observed in the non-exposed culture (Fig. 3.5). *SAP2* mRNA levels were also higher with exposure to NF e-vapor than in the non-exposed cultures (Fig. 3.5). However, the effects of

NR and NF e-vapor on *SAP2* gene expression were lower than those recorded by the CCS (Fig. 3.5). *SAP2* is associated with *C. albicans* growth and the yeast forms of *C. albicans* [28] *and* is also essential for mucosal *infections* [29]*. Because the e-cigarettes increased SAP2 expression, this may explain the growth of C. albicans observed following its exposure to NR and NF e-vapor and suggests that e-vapor-exposed C. albicans could be virulent in smokers.*

SAP3 gene was another aspartyl proteinase modulated through the exposure of *C. albicans* to cigarette smoke and e-vapor. As shown in Fig. 3.5, compared to the non-exposed cultures, NR e-vapor-exposed *C. albicans* expressed a high level of *SAP3* mRNA. It should also be noted that NF e-cigarettes also increased *SAP3* gene expression by *C. albicans*, compared to that observed in the control. However, the effect on *SAP3* expression was greater with the NR e-vapor than with the NF e-vapor, and greater with the CCS than with either e-cigarettes or the control (Fig. 3.5).

Because *SAP3* was shown to regulate *C. albicans* growth and phenotypic switching [30], the modulatory effect of e-cigarettes on *SAP3* expression may be a possible mechanism promoting *C. albicans* pathogenesis. The capacity of *C. albicans* to switch reversibly between the white phenotype and the opaque phenotype is required. *C. albicans* switching also promotes the yeast's dissemination, causing systemic candidiasis [31]. We thus suggest that e-cigarettes increase *SAP3* gene expression which may lead to *C. albicans* switching and thus to increase its virulence. Further studies are required to validate this hypothesis.

Our findings also indicate that the e-cigarettes promoted *SAP9* gene expression. Indeed, NR e-cigarette vapor produced a significant ($p < 0.001$) increase of *SAP9* gene expression by *C. albicans*, compared to that observed in the non-exposed cultures (Fig. 3.5). NF e-vapor also promoted *SAP9* expression, however, the effect was significantly ($P < 0.001$) greater with NR than with NF e-vapor. It should be noted that when *C. albicans* was exposed to CCS, the expression of *SAP9* was significantly ($p < 0.001$) higher than that observed in the control or with the NR or NF

e-vapor (Fig. 3.5). Similar to other secreted aspartic proteases, *SAP9* is associated with the fungal cell wall [27,32] and is reported to upregulate under biofilm-forming conditions [33]. *Sap9* thus contributes to the virulence of *C. albicans* [34] and the decrease of host innate immunity [35], which may favor the onset of *C. albicans* infection.

3.5.5. E-vapor-exposed C. albicans adhered better to gingival epithelial cells

The effect of e-cigarettes on *C. albicans* growth and *SAP* gene expression raised the following question: How do e-vapor-exposed *C. albicans* cells interact with gingival epithelial cells? Fig. 3.6 shows that *C. albicans* adhesion to the epithelial cell monolayer culture was greater following exposure to NR e-vapor than it was in the control. On the other hand, the adhesion observed of NR e-vapor-exposed *C. albicans* to epithelial cells was lower than that observed in the CCS-exposed cells. It should be noted that at 24 h, *C. albicans* adopted the hyphal form covering a larger area of the epithelial monolayer culture (Fig. 3.6). The cell density of the hyphae in the NR e-vapor-exposed *C. albicans* adhering to the epithelial cell culture was greater than that observed with the non-exposed *C. albicans*. This is the first study demonstrating the effect of e-cigarettes in modulating *C. albicans* adhesion to epithelial cells. This is also in agreement with other reported findings with conventional cigarette smoke showing greater *C. albicans* adhesion to gingival fibroblasts [15]. This may be explained by an increased contact of *C. albicans* with epithelial cells through cell wall proteins, as chitin content indeed increased in the e-vapor-exposed *C. albicans* (Fig. 3.3). As e-cigarettes were shown to promote *C. albicans* growth, as well as increased chitin content and *SAP* gene expression, we performed an indirect interaction study between e-vapor pre-exposed *C. albicans* and gingival epithelial cells using a transwell culture system.

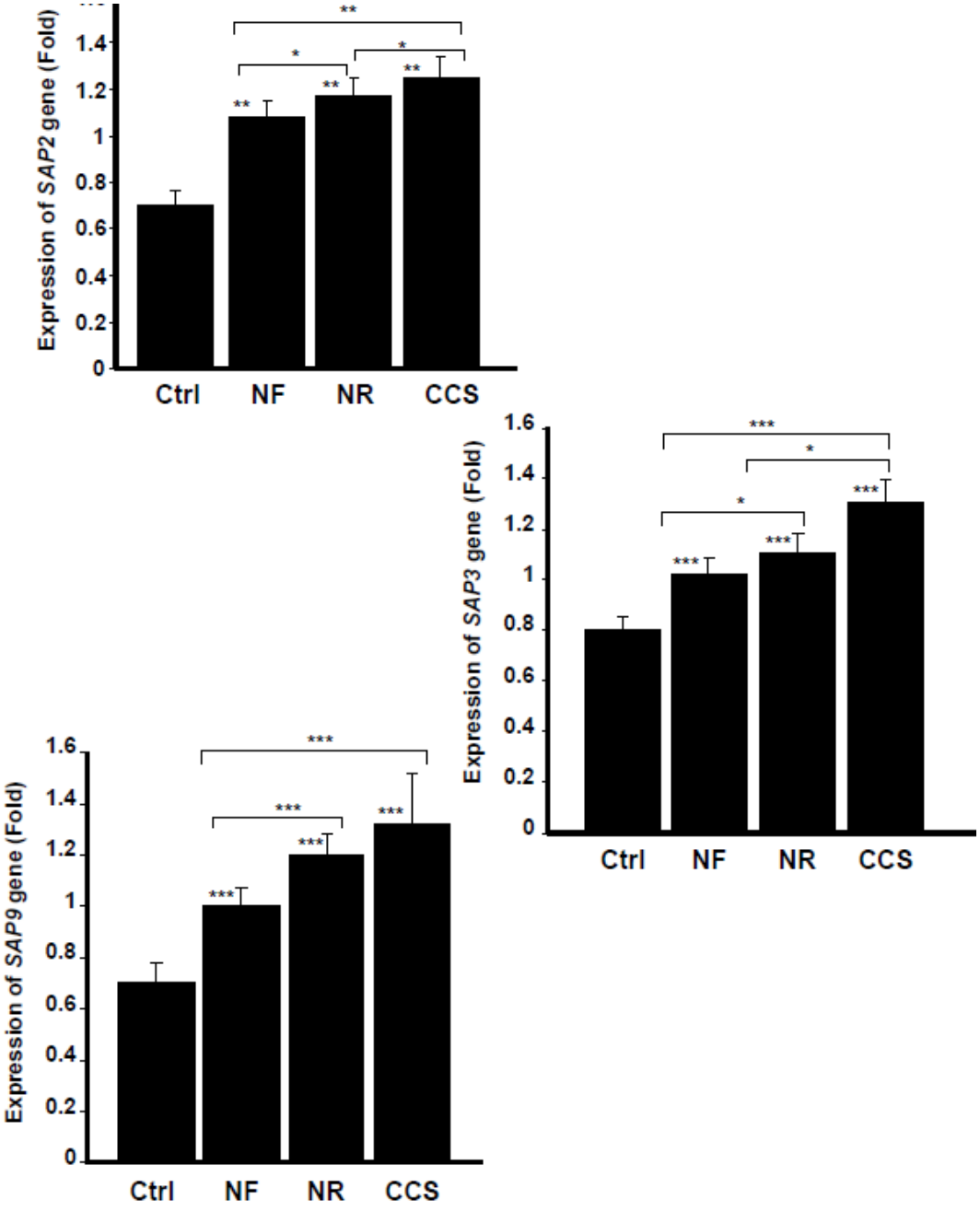

Figure 3.5. E-cigarette vapor increased the expression of secreted aspartyl proteinases *SAPs* 2, 3, and 9. *C. albicans* cells were exposed or not twice a day for 15 min to CCS, NF e-vapor, or NR e-vapor, then incubated for 16 h at 37°C prior to the extraction of total RNA and analysis by qRT-PCR (n = 5). The expression was normalized to the GAPDH (housekeeping gene). Statistical significance was obtained by comparing

the cells exposed to CCS or to NR or NF e-vapor and those of the control (non-exposed cells). *P < 0.05; **P < 0.01; ***P < 0.001.

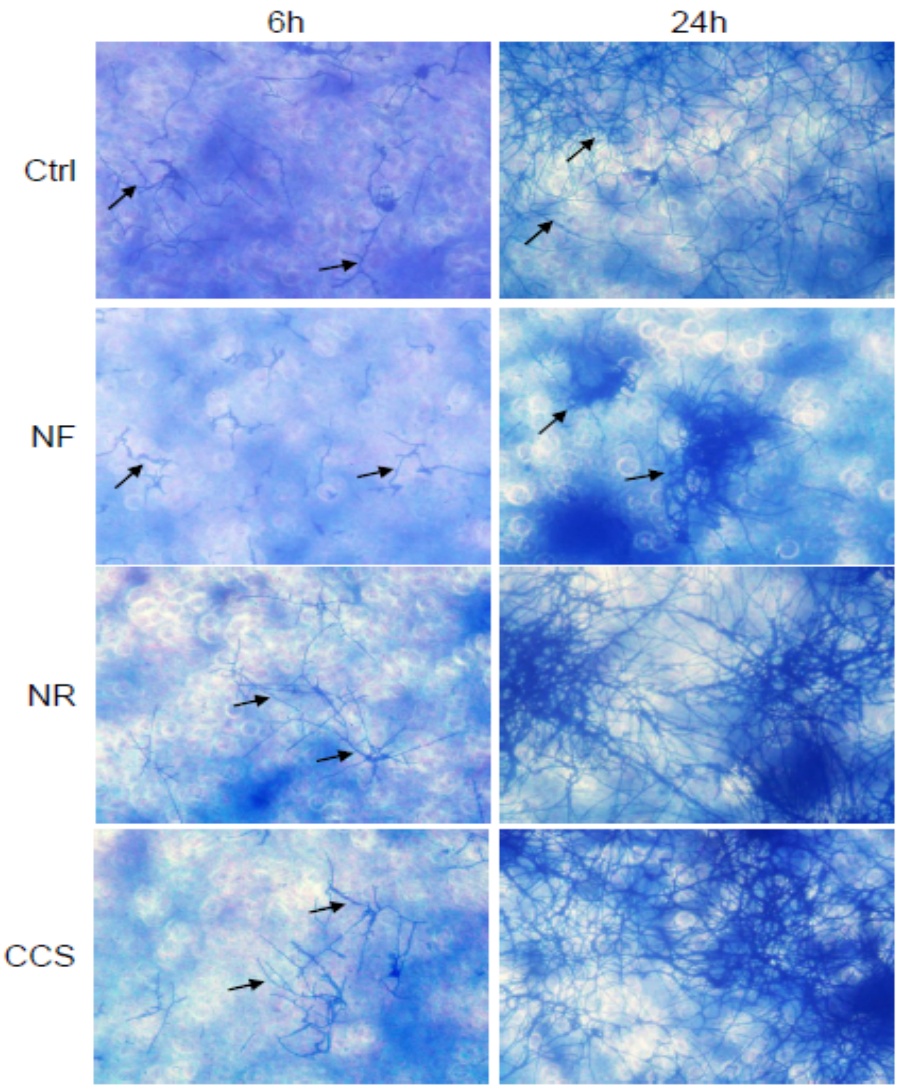

Figure 3.6. *C. albicans* pre-exposed to e-cigarette vapor adhered better to gingival epithelial cells cultures. Gingival epithelial cells were seeded in 6-well plates and cultured for 24 h. The cell monolayers were then co-cultured with e-vapor pre-exposed *C. albicans*. Adhesion of *C. albicans* to the gingival cells was assessed after 6 and 24 h using the crystal violet staining assay. Representative images are from four independent experiments, with each experiment performed in duplicate. Scale bars = 50μm.

3.5.6. Cross- talk interactions between e-vapor-exposed C. albicans and epithelial cells promoted yeast growth and morphological changes

As shown in Fig. 3.7, *C. albicans* growth and hyphal morphological changes were significantly increased in both the e-vapor and CCS pre-exposed *C. albicans* co-cultured with gingival epithelial cells, with the observed growth approximately two folds with NR e-vapor, compared to the control (Fig.3.7a). NR e-vapor-exposed *C. albicans* co-cultured with epithelial cells also showed a significant ($p < 0.01$) growth increase compared to that observed in the control. However, the greatest growth increase was obtained following *C. albicans* exposure to CCS and subsequent co-culture with epithelial cells for 24 h. *C. albicans* morphological change from blastospore to hyphal form was also modulated by exposure to e-vapor and to CCS and co-culture with gingival epithelial cells. As shown in Fig. 3.7b, both the NR and NF e-vapor-exposed *C. albicans* co-cultured with epithelial cells recorded a significant high transition against the control. Furthermore, the effect of NR e-vapor was significantly higher than that of NF e-vapor, while CCS represented the agent producing the greatest transition of *C. albicans* following co-culture with gingival epithelial cells (Fig. 3.7b). This is in agreement with previous studies showing increased bacterial adhesion to and colonization on epithelial cells in the presence of cotinine or nicotine [36]. The effect of e-cigarette vapor and CCS on *C. albicans* growth and form changing when in contact with epithelial cells may be due to the high expression of chitin, as this cell wall protein increased after exposure to e-cigarette vapor (Fig. 3.3). The high level of chitin could thus play a role in promoting the interaction of *C. albicans* with the host cells, as previously reported [16,37]. Because *C. albicans* exposed to e-vapor/CCS then co-cultured with gingival epithelial cells showed increased growth and morphological changes, we put forth that this may affect epithelial cell behaviors.

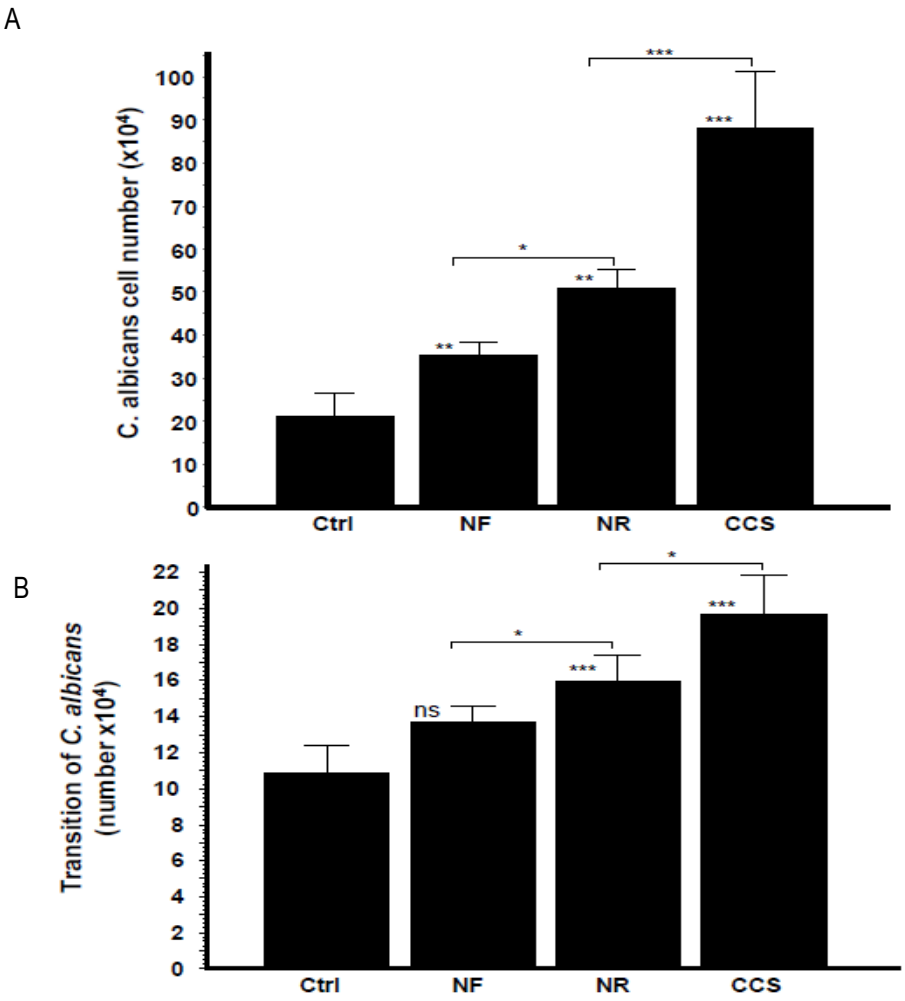

Figure 3.7. Growth and form changing of *C. albicans* pre-exposed to e-cigarette vapor then co-cultured with gingival epithelial cells. *C. albicans* cells were exposed twice a day for 15 min to CCS, NR e-vapor, or NF e-vapor, followed by co-culture with gingival epithelial cells in a transwell culture system. After 24 h, the *C. albicans* cells in the upper chamber were collected and used to determine their growth and transition by optical microscope analysis (n = 4). *P < 0.05; **P < 0.01; ***P < 0.001

3.5.7. E-vapor-exposed C. albicans promoted morphological changes in epithelial cells and reduced their growth

Microscopic observations of the epithelial cell monolayer following culture in the presence of either e-vapor-exposed or CCS-exposed *C. albicans* revealed the presence of differentiated epithelial cells (Fig. 3.8, arrows). These large-sized cells were characterized by a wide and faint nucleus, large cytoplasm, and the presence of vacuoles in the culture being pulsed with NR e-vapor-exposed *C. albicans*. Fewer differentiated cells were observed with the NF e-vapor and in the control compared to the NR e-vapor and CCS conditions. The greatest number of differentiated cells was observed with exposure to CCS (Fig. 3.8). Therefore, even with indirect contact, e-vapor-exposed *C. albicans* exerted some adverse effects on the gingival epithelial cells by modulating their cell shape. Comparable observations were reported with primary human gingival epithelial cells exposed to e-cigarettes [20], and skin keratinocytes exposed to ultraviolet radiation [38]. The morphological changes observed following epithelial culture in the presence of e-vapor-exposed or CCS-exposed *C. albicans* are supported by evidenced reduction in epithelial cell growth. As shown in Fig. 3.9, the viable epithelial cell number decreased significantly ($p < 0.05$) in the cultures pulsed with e-vapor-exposed or CCS-exposed *C. albicans*. Indeed, this viable cell number dropped from 13×10^5 cells in the control to 10×10^5 with the NR e-vapor and 8×10^5 with the CCS. Furthermore, the decrease in epithelial cell viability was accompanied by an increase in LDH activity. As shown in Fig.3.10, high levels of LDH activity were recorded by epithelial cells pulsed with NR e-vapor-exposed *C. albicans*.

This study is the first to demonstrate the possible adverse effects of e-cigarette-exposed *C. albicans* on gingival epithelial cells. It suggests that e-cigarette vapor may enhance the capacity of *C. albicans* to evade epithelial cell defenses by promoting overgrowth and transition (Fig. 3.7). E-cigarettes have already been shown to produce negative effects on different cell types, including gingival fibroblasts [39], epithelial cells [20], endothelial cells [40], and osteoblasts [41]. The present study supports this existing data by showing that e-cigarettes affect oral

microbial behaviors by stimulating their pathogenesis through overgrowth, transition, and the expression of virulent genes, such as *SAPs*.

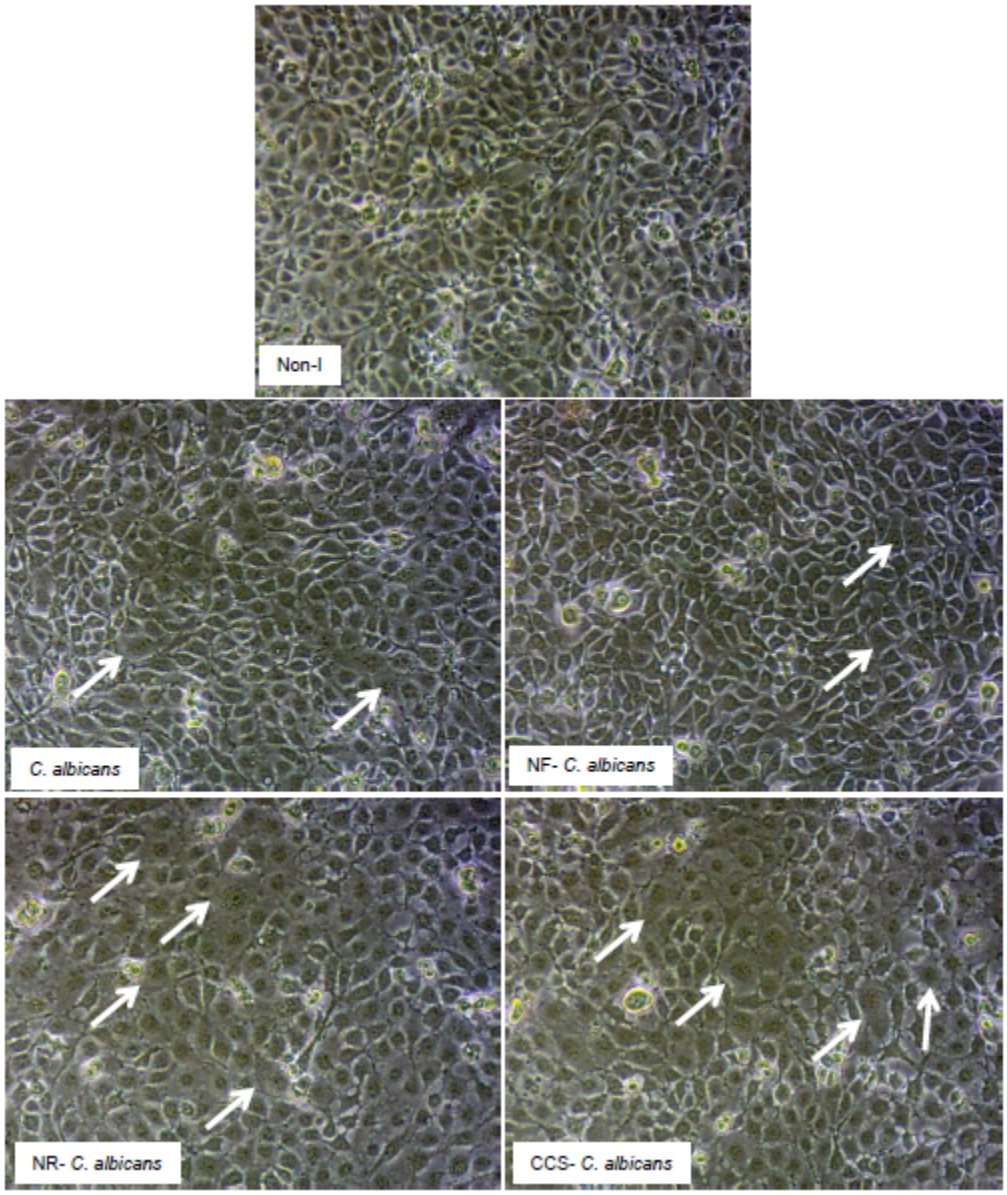

Figure 3.8. *C. albicans* pre-exposed to e-cigarette vapor promoted gingival epithelial cell differentiation. Gingival epithelial cell monolayers were co-cultured with *C. albicans* pre-exposed to e-vapor twice a day for 15 min. Following co-culture for 24 h in a transwell culture system, the epithelial cell monolayers in the lower culture chambers were observed under an inverted optical microscope and photographed. Representative

images are from four independent experiments, with each experiment performed in duplicate. Arrows indicate the differentiated cells. Scale bars = 50 µm.

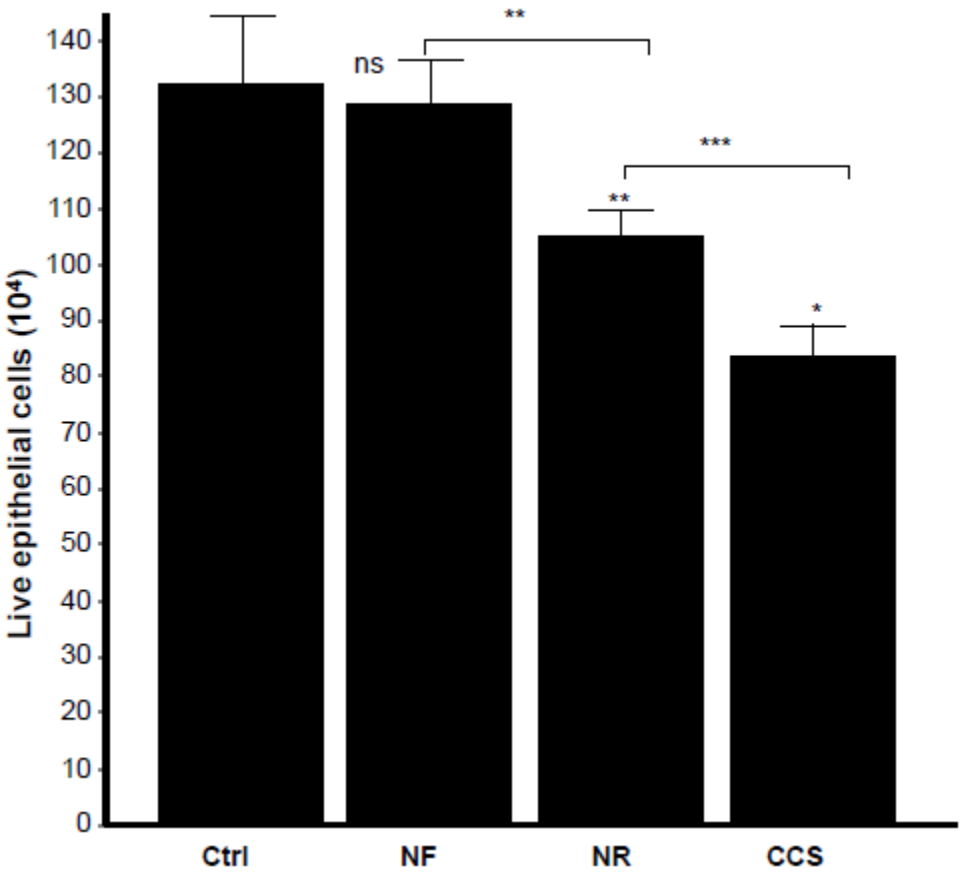

Figure 3.9. E-vapor pre-exposed *C. albicans* decreased gingival epithelial cell viability. Gingival epithelial cells were co-cultured for 24 h in the presence of e-vapor pre-exposed *C. albicans*. Epithelial cells were then detached and the viability was determined by trypan blue exclusion assay (n = 4). Statistical significance was obtained by comparing the cells exposed to CCS, NR e-vapor, or NF e-vapor and those of the control (non-exposed cells). *P < 0.05; **P < 0.01; ***P < 0.001; ns = non-significant.

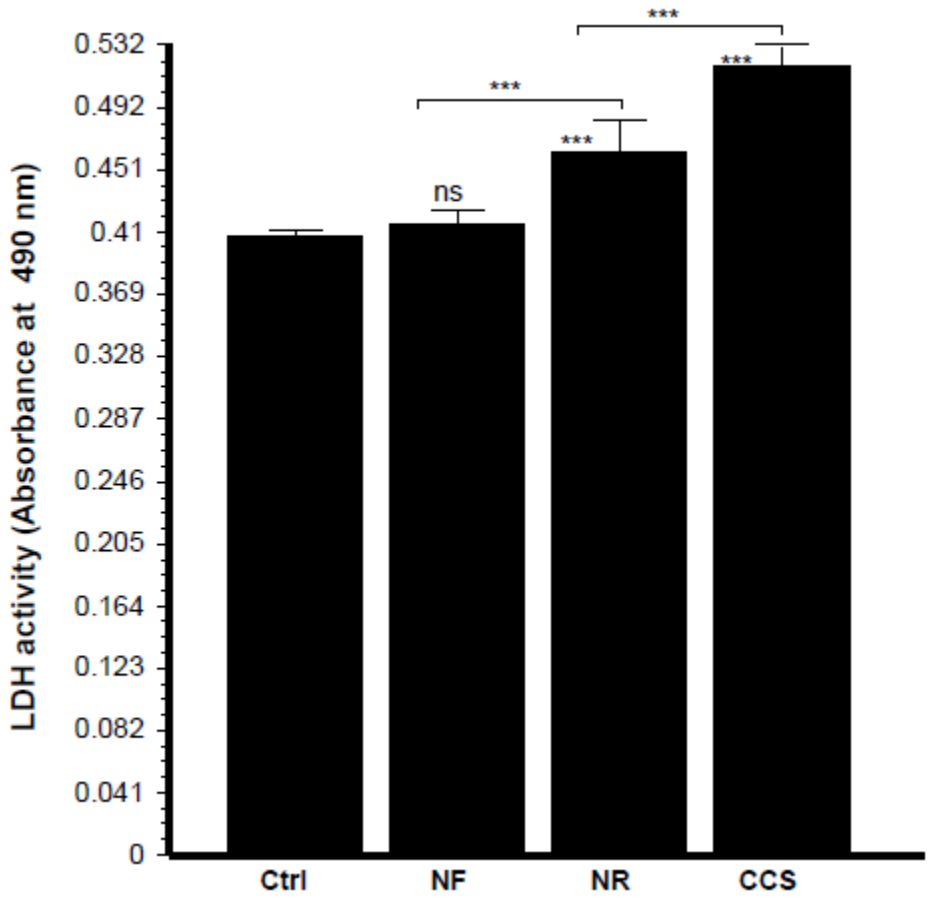

Figure 3.10. Epithelial cells co-cultured with e-vapor pre-exposed *C. albicans* displayed high levels of LDH activity. Gingival epithelial cells were co-cultured for 24 h in the presence of e-vapor pre-exposed *C. albicans*. Culture supernatants were collected and used to measure LDH activity, as described in the M&M section (n = 4). Statistical significance was obtained by comparing the cells exposed to CCS, NR e-vapor, or NF e-vapor and those of the control (non-exposed cells). ***P < 0.001; ns = non-significant.

3.6. Conclusion

This study demonstrates that e-cigarettes with or without nicotine promoted the growth and hyphal length of *C. albicans* and that both nicotine-free and nicotine-rich e-cigarettes increased the expression of different *SAP* genes, such as *SAP2*, *SAP3*, and *SAP9*, which are known to contribute to *C. albicans* growth and virulence. Our findings also confirm that co-culture with e-vapor-exposed *C. albicans* increased gingival epithelial cell differentiation and reduced their growth. The co-culture showed even higher growth and morphological change of e-vapor-exposed *C. albicans* than when placed in indirect contact with epithelial cells, compared to that observed with non-exposed *C. albicans*. Overall results show the contribution of e-cigarette exposure to *C. albicans* overgrowth, leading potentially to oral candidiasis.

Author Contributions: All of the authors contributed substantially to the conception and design of the study. HA and MR contributed to the data acquisition, analysis, interpretatio, and manuscript preparation, while WCh and AS validated the data analysis and interpretation. The authors also ensured the manuscript's critical revision for important intellectual content. Each author approved the final version of the manuscript for submission.

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CHAPTER 4: GENERAL DISCUSSION

ECs are marketed for smokers as a safe replacement for SC smoking and to help them reduce the damages caused by smoking (Wackowski et al., 2016). However, several surveys and *in vitro* studies report that e-cigarettes are not as healthy as they are claimed to be (Al Rifai et al., 2020; Osei et al., 2019). Our findings support these studies by showing that both nicotine-rich and nicotine-free condensates induced significant morphological alterations in human gingival fibroblasts. This study also concurs with other research demonstrating that e-vapour and CS extracts in contact with endothelial cells for 24 and 48 h altered cell morphology (Putzhammer et al., 2016). Frequent exposure to e-cigarette extracts was shown to shift mesenchymal stem cells into different abnormal shapes, compared to that observed in non-exposed cells (Shaito et al., 2017).

Collectively, these studies and ours confirm the potentially harmful effects of e-cigarettes on different types of cells, therefore suggesting that e-cigarette users should be better informed regarding these potential deleterious effects. In addition, affecting fibroblast morphology can lead to additional changes in behaviours. Our findings demonstrate this, as e-cigarette condensate decreased fibroblast viability and proliferation. Of interest is that both the nicotine-free and nicotine-rich e-cigarettes decreased this viability and proliferation in exposed fibroblasts. That said, the greatest deleterious effect on gingival fibroblast viability and proliferation was observed with combustible cigarette smoke condensate. Although e-cigarette condensate had a lower effect on fibroblast viability and proliferation than the CSC recorded, it did have a significant effect compared to that observed in non-exposed cells.

Many similar findings support our results. We reported that e-vapour caused epithelial cell death through the caspase-3 pathway (Rouabhia et al., 2017). E-vapour was shown to increase primary human bronchial epithelial toxicity and reduced cell viability, with higher values recorded by cells exposed to regular

cigarette smoke (Scheffler et al., 2015). Another study showed that A549 cell proliferation decreased in a nicotine-dependent manner following exposure to e-cigarette vapour (Rigg et al., 2019). Furthermore, neonatal mice exposed to e-cigarettes with nicotine recorded a reduction in alveolar cell proliferation, with impaired lung cell growth (McGrath-Morrow et al., 2015).

Reducing cell viability and proliferation may involve the apoptosis process. We therefore performed apoptosis analyses. The apoptotic activities confirm that CSC had a significant apoptotic effect on gingival fibroblasts, particularly at 5 and 10 %. It should be noted that the CSC had a greater apoptotic effect than did e-vapour condensate with and without nicotine. However, both nicotine-free and nicotine-rich e-vapour condensate promoted fibroblast apoptosis, compared to that observed in non-exposed cells. Of interest is that there was a greater number of apoptotic gingival fibroblasts with nicotine-rich e-vapour condensate than with nicotine-free condensate. The different effects observed with nicotine-free and nicotine-rich e-cigarettes may be explained by the presence of nicotine. Indeed, nicotine was found to be toxic to cells by reducing their proliferation (Chang et al., 2002). Comparable results were indicated in multiple incubations of human gingival fibroblasts with e-fluid (Sancilio et al., 2016). In other research, several incubations with e-vapour extract were found to promote apoptotic activity in normal epithelial cells (Yu et al., 2016) as well as human umbilical vein endothelial cells (Anderson et al., 2016).

Fibroblast cells, found in abundance in tissues and organs, are vital to cell function and structure by building extracellular matrix (ECM) with collagen, fibronectin, and proteoglycans to shape the cell framework, and by supporting cell motility and contraction for tissue homeostasis and wound repair (McAnulty et al., 2007). In this study, NF/NR e-vapour condensate and CSC appeared to negatively affect fibroblast migration, which may slow down the role of fibroblasts in wound healing processes. Similar results obtained by Semlali and colleagues (2011) show that cigarette smoke inhibited human gingival epithelial cell migration. The decreased fibroblast migration

supports these effects by e-vapour condensate in reducing fibroblast proliferation and increasing cell apoptosis.

In addition to oral cavity constituents, e-cigarettes can also affect the oral microbiome. Oral microbes are present in the oral cavity as commensal cells under the control of the host innate immunity. When the innate immunity is diminished or is in the presence of certain factors that stimulate the growth and pathogenesis of one or another oral microbe, this commensalism can morph into pathogenic microbes.

Several studies report that *C. albicans* is sensitive to internal or exogenous factors such as cigarette smoke, pH changes, etc. (Sampaio-Maia et al., 2016; Nagarajanet al., 2018). In the second part of our research, we demonstrate that repeated exposure to e-vapour increased *C. albicans* growth. It should be noted that both e-cigarettes with and those without nicotine increased *C. albicans* growth. However, the growth of this yeast was still greater with CC smoke exposure than with e-cigarette exposure and the control. Our study supports previous investigations with cigarette smoke (Semlali et al., 2014). A cross-sectional study detected *C. albicans* carriage was high in e-smokers, conventional smokers, and water-pipe smokers (Mokeem et al., 2019).

C. albicans growth may also involve certain membrane proteins such as chitin. Our study shows that chitin was significantly produced by *C. albicans* following exposure to e-vapour and to CS. Indeed, we were the first to uncover this relationship between chitin production and e-cigarette exposure. Chitin, a cell wall constituent in *C. albicans*, interacts with other cell wall molecules such as glucans, polysaccharides and mucopolysaccharides, waxes, and pigments to form a barrier that protects against any threat caused by environmental changes (Reyna-Beltrán et al., 2019). E-vapour can thus affect not only yeast growth but also its morphological changes.

Following exposure to e-vapour, under hyphae culture conditions, e-vapour and CS were shown to increase *C. albicans* hyphal form length. Indeed, the length of the hyphae was significantly increased at 3 and 6 h of culture. This increase was more easily observable with both nicotine-rich e-vapour and CS, compared to that observed in the control and with nicotine-free e-vapour. *C. albicans* was thus capable of changing from yeast to hyphae and became more virulent by invading tissues to form biofilms leading to oral candidiasis (Tsui et al., 2016). These results support other findings with cigarette smoke and cigarette smoke condensate (Alanazi et al., 2014; Ali & Karuppayil, 2018). *C. albicans* hyphae undermine innate immunity by destroying macrophages and inhibiting human defensin expression (Ghosh et al., 2009; Marcil et al., 2002). *C. albicans* pathogenesis is under the control of various genes (Verma-Gaur et al., 2016).

C. albicans expresses different genes involved in its adhesion, growth, morphological changes, and biofilm formation. From these genes comes a secreted aspartyl proteinase family (Sap) of genes (Kumar et al., 2015). It was revealed that *C. albicans* exposed to e-vapour expressed a high level of *Sap2*, *Sap3*, and *Sap9* genes. This increased expression was observed with e-cigarette vapour both with and without nicotine. As previously reported, *Sap2* is involved in the infection process by damaging the surface of oral epithelial mucosa and is has a protective function when *C. albicans* is threatened (Rahman et al., 2007). *Sap3* gene promotes *C. albicans* adhesion to mucosal tissue, contributing to oral tissue damage at the early stage of infection (Naglik et al., 2003). As for *Sap9*, this gene contributes to the formation of *C. albicans* biofilm and adhesion (Joo et al., 2013) and also regulates the fungal cell wall during pathogenesis (Albrecht et al., 2006). Cigarette smoke condensate was shown to produce an increase in *Sap2* (Semlali et al., 2014).

Because e-cigarettes increase *C. albicans* growth and virulent gene expression, this may contribute to *C. albicans* pathogenesis. We therefore investigated the interaction of gingival epithelial cells with e-cigarette-pretreated *C. albicans*. Our results show that e-cigarette-pre-exposed *C. albicans* adhered much more to

gingival epithelial cells than did non-exposed *C. albicans*. There were also elevated levels of hyphal forms in the e-cigarette pre-exposed *C. albicans* when put in contact with gingival epithelial cells. These experiments mimic what may happen in the mouth following the use of e-cigarettes. This study suggests that e-cigarettes may increase the interaction of *C. albicans* with gingival tissues, which may lead to candidiasis.

Previous studies report similar observations with *C. albicans* and gingival fibroblasts (Alanazi et al., 2014) as well as increased bacterial adhesion to epithelial cells (Gilpin et al., 2019). To confirm these data, we initiated an indirect contact between gingival epithelial cells and *C. albicans* pre-exposed to e-cigarettes. A trans-well culture system was used for this purpose. *C. albicans* pre-exposed to e-cigarettes were placed in an upper culture chamber, while the gingival epithelial cells were placed in the lower cell culture chamber. Both the *C. albicans* and the epithelial cells interacted through the culture medium that goes through a porous membrane. Using this culture system, we showed that the cross-talk between *C. albicans* and the epithelial cells promoted the growth of the yeast and led to the differentiation of the gingival epithelial cells. Both nicotine-free and nicotine-rich e-cigarettes were responsible for this yeast growth and epithelial cell differentiation. Similar observations were reported previously showing changes in cell morphology of gingival epithelial cells following exposure to e-cigarettes (Rouabhia et al., 2017).

Exposure to e-cigarettes was also found to induce morphological changes in lung epithelial cells (Lerner et al., 2015). The morphological changes we observed with the indirect contact between epithelial cells and e-cigarette-pretreated *C. albicans* were confirmed by the decreased epithelial cell viability and the increased LDH activity. Similar findings show that flavoured e-liquid decreased lung epithelial cell proliferation (Rowell et al., 2017). Furthermore, short- and long-term exposure of both primary and cancer cell lines to e-vapour were found to induce a reduction in cell viability and an increase in cell apoptosis (Yu et al., 2016). Further to this, e-

vapour was shown to have a negative effect on nasal epithelial cells (Martin et al., 2016).

CONCLUSION

Our studies confirm that e-cigarettes with and without nicotine are harmful to human gingival fibroblasts by decreasing their proliferation and increasing their death, and to fibroblasts by impairing their migration. In addition, *C. albicans* becomes more virulent when exposed to e-vapour. Indeed, exposure to e-cigarette vapor promotes the growth and morphological changes of *C. albicans*, which are features known to promote its pathogenesis. Our results also demonstrate that e-cigarettes increase the expression of different virulent genes (*Sap2*, *Sap3*, and *Sap9*), which suggests that e-cigarettes promote *C. albicans* virulence by activating genes involved in the yeast's adhesion, growth, and biofilm formation. Pre-exposed *C. albicans* also negatively interacts with gingival epithelial cells. In fact, both direct and indirect contact of epithelial cells with e-cigarette vapour-exposed *C. albicans* promotes the yeast's growth and form changing, increases epithelial cell differentiation, and reduces their viability.

Our overall findings show that e-cigarettes have definite deleterious effects on the oral cavity on two levels: The first effect refers to the decreased role of gingival cells/tissues in the host protection/defense and the second pertains to the increased *C. albicans* pathogenesis and virulent gene expression.

In conclusion, while e-cigarettes may be considered safe, compared to conventional combustible cigarettes, they are not harmless. Further investigations are thus required to examine the effects of e-cigarette vapour on human oral innate immunity. Ideally, long-term studies will shed light on the effect of e-cigarettes on the users' oral health.

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