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Evaluating in vitro toxicity of flavors in alternative nicotine delivery systems utilizing different in vitro testing strategies

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Ich habe eine Dissertation zum Thema:

„ Evaluating in vitro toxicity of flavors in alternative nicotine delivery systems utilizing different in vitro testing strategies"

an der Mathematisch-Naturwissenschaftlichen Fakultät der Universität Rostock angefertigt. Dabei wurde ich von Herrn Prof. Dr. Ralf Zimmermann betreut.

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List of original publications

First Authorships

The following manuscripts were written by Selina Rinaldi as first author and published in or submitted to peer-reviewed journals. The contribution of Selina Rinaldi is described below.

Title: Oral nicotine pouches with an aftertaste? Part 1: screening and initial toxicological assessment of flavorings and other ingredients

Authors: Nadja Mallock-Ohnesorg*, **Selina Rinaldi***, Sebastian Malke, Nadine Dreiack, Elke Pieper, Peter Laux, Thomas Schulz, Ralf Zimmermann & Andreas Luch

*Both authors contributed equally

Journal: Archives of Toxicology

Year: 2023

DOI: 10.1007/s00204-023-03538-9

Selina Rinaldi was involved in planning and performing the toxicological evaluation of the ingredients analytically identified in nicotine pouches. She wrote and revised the manuscript.

Title: Oral nicotine pouches with an aftertaste? Part 2: in vitro toxicity in human gingival fibroblasts

Authors: **Selina Rinaldi**, Elke Pieper, Thomas Schulz, Ralf Zimmermann, Andreas Luch, Peter Laux, Nadja Mallock-Ohnesorg

Journal: Archives of Toxicology

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DOI: 10.1007/s00204-023-03554-9

Selina Rinaldi designed the study and performed all biological assays and the majority of the analytical experiments. In detail, she was involved in establishing the extraction method for nicotine pouches, planned and performed MTT and LDH assay, and the qPCR experiments. She evaluated the data, wrote and revised the manuscript.

Title: Flavors in e-liquids and electronic cigarette aerosols elicit differential transcriptional changes in lung epithelial cell models exposed in submerged and air-liquid interface conditions

Authors: **Selina Rinaldi**, Anjana Khadka, Nina Hupf, Jan Heide, Sven Ehlert, Leonie Gloger, Johannes Becker, Sebastian Malke, Peter Laux, Ralf Zimmermann, Andreas Luch, Mathilde N. Delaval, Elke Pieper

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Selina Rinaldi designed the study and performed in part the biological assays in submerged conditions. In detail, she planned the transcriptome assay, the qPCR, FACS and proteome profiler experiments. She performed the MTT assay. Selina Rinaldi performed the bioinformatic analysis of all transcriptomic data using IPA and DAVID. She evaluated the data, wrote and revised the manuscript.

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Zusammenfassung

Das Rauchen herkömmlicher Tabakzigaretten verursacht nach Angaben der Weltgesundheitsorganisation jährlich 8 Millionen Todesfälle, obwohl das Bewusstsein für die Gesundheitsrisiken weit verbreitet ist. Rauchstoppversuche sind jedoch häufig nicht erfolgreich. Alternative Nikotinabgabesysteme (ANDS) wurden von der Tabakindustrie in den letzten Jahren auf den Markt gebracht, darunter orale Nikotinbeutel und elektronische Zigaretten (E-Zigaretten). Die charakteristischen Inhaltsstoffe dieser Produkte sind Nikotin und Aromastoffe, enthalten jedoch keinen Tabak. Allerdings steigern diese Aromastoffe die Attraktivität dieser Produkte vor allem für Kinder und Jugendliche, unabhängig von ihrem Konsumverhalten bezüglich anderer Tabakprodukte. Die *e-cigarette or vaping associated lung injury* (EVALI)-Krise in den Vereinigten Staaten im Jahr 2019 hat gezeigt, dass E-Zigaretten ein unterschätztes Problem für die öffentliche Gesundheit darstellen, und hat die Aufmerksamkeit besonders auf ihre Aromastoffe gelenkt. Während die verwendeten Aromastoffe für den oralen Konsum „*generally recognized as safe*“ sind, sind die gesundheitlichen Auswirkungen des inhalierbaren Aerosols von E-Zigaretten, nach wie vor kaum bekannt oder gar untersucht worden.

Das übergeordnete Ziel dieser Arbeit bestand darin, einen Beitrag zu einer Risikobewertungsstrategie für Aromastoffe in ANDS zu leisten, indem mehrere in vitro Methoden auf ihre Eignung für toxikologische Analysen untersucht wurden. Dafür wurden die toxikologischen Profile gängiger E-Liquid-Aromastoffe Zimtaldehyd, Eugenol und Vanillin sowie die Bestandteile von Nikotinbeuteln untersucht.

Im Rahmen der Studie wurden Nikotinbeutel mit Hilfe eines *non-targeted screenings* auf ihre Inhaltsstoffe analysiert. Zudem wurde der Nikotingehalt analytisch bestimmt. Nikotinbeutelextrakte wurden anschließend an einer humanen oralen gingivalen Zelllinie getestet, um die Lebensfähigkeit der Zellen, Entzündungsreaktionen und Marker für oxidativen Stress zu untersuchen. Typische E-Liquid Aromen wie Zimtaldehyd, Eugenol und Vanillin wurden an humanen bronchialen Epithelzellen (16HBE) unter submersen Bedingungen als auch im *Air-Liquid Interface* (ALI) auf transkriptomische Veränderungen untersucht. Ein Online-Aerosol-Überwachungssystem wurde im ALI integriert. Des Weiteren wurde eine gängig verwendete Abrauchmethode für E-Liquids optimiert, um die Aromakonzentration im konditionierten Medium zu erhöhen.

Die Ergebnisse zeigten, dass Nikotinbeutel insgesamt 186 Aromastoffe enthielten. Für einige dieser Aromastoffe existierte ein Grenzwert für die tägliche Aufnahmemenge, wurde eine Gefahreneinstufung gemäß der Verordnung (EG) Nr. 1272/2008 über Einstufung, Kennzeichnung und Verpackung vorgenommen, existierte eine Einstufung durch die Internationale Agentur für Krebsforschung oder sie waren nicht als Lebensmittelzusatzstoffe zugelassen. Nikotinbeutelextrakte verursachten unabhängig von der Nikotinkonzentration Entzündungen und oxidativen Stress in vitro, und korrelierten zudem mit der Zusammensetzung der Aromamischung. Die untersuchten E-Liquid Aromen lösten transkriptomische Veränderungen auf Zellebene aus, die auf mögliche schädliche Auswirkungen auf die Lunge durch unterschiedliche Mechanismen hindeuten. Unter submersen Bedingungen zeigten die untersuchten Aromastoffe Veränderungen in Zytokin-Signalkaskaden, während unter der ALI-

Exposition die Auswirkungen je nach Aromastoff variierten und zu einer gestörten Ionenhomöostase und zu einer veränderten Lungenmorphologie führten. Ein höherer Ertrag an Aromen in konditioniertem Medium wurde durch eine Veränderung des Volumens und durch das Hinzufügen von fetalem Kälberserum erreicht.

Diese Ergebnisse liefern wertvolle Erkenntnisse für die Entwicklung von Richtlinien zur Prüfung von aromatisierten ANDS. Weiterhin wird deutlich, dass dringend Strategien entwickelt werden müssen für die Sicherheitsbewertung von Aromastoffen in neuen Nikotinabgabesystemen.

Abstract

Smoking of traditional tobacco cigarettes causes 8 million deaths annually according to the World Health Organization, despite widespread awareness of their health risks. Attempts to stop using tobacco are however often not successful due to the high addictiveness of nicotine. Recently, the tobacco industry has developed alternative nicotine delivery systems (ANDS), including oral nicotine pouches (ONP) and electronic cigarettes (e-cigarettes). The characterizing ingredients of these products are nicotine and flavors but no tobacco. However, their flavoring components particularly attract young users, regardless of prior smoking history. The 2019 e-cigarette or vaping associated lung injury (EVALI) crisis in the United States highlighted e-cigarettes to be an underestimated public health concern, drawing particular attention to their flavoring ingredients. While these flavorings are “generally recognized as safe” for oral consumption, their health effects upon inhalation remain poorly understood.

The overall aim was to contribute to a risk assessment strategy for ANDS flavorings, by investigating several in vitro methods for their suitability to be used in toxicological assessments. Therefore, the toxicological profiles of common e-liquid flavoring compounds, cinnamaldehyde, eugenol and vanillin, and nicotine pouch constituents were investigated.

The study analyzed ONPs for flavor ingredients using a non-targeted screening and analyzed nicotine content, then conducted in vitro toxicology assays using human oral cells to examine cellular viability, inflammatory responses, and oxidative stress markers. Transcriptomic changes induced by commonly used e-liquid flavorings cinnamaldehyde, vanillin, and eugenol were evaluated under both submerged and Air-Liquid Interface (ALI) conditions. An online aerosol monitoring system was integrated at the ALI to monitor flavor output on a puff-by-puff basis. Lastly, the vaping machine method was optimized in order to enhance flavor concentration in conditioned medium to be used in toxicological assays.

Results revealed that ONPs contained a total of 186 flavoring substances, with some having established acceptable daily intakes, hazard classifications according to Regulation (EC) No. 1272/2008 on Classification, Labelling and Packaging, a classification by the International Agency for Research on Cancer, or not being authorized as food additives. ONP extracts caused inflammation and oxidative stress independently of nicotine but correlating with flavoring mixture composition. The studied e-liquid flavorings induced transcriptomic changes suggesting potential adverse pulmonary effects through differing mechanisms. Submerged conditions showed altered cytokine signaling cascades, while ALI exposure resulted in disrupted ion homeostasis and altered lung morphology, with effects varying by flavor compounds. A higher flavor yield in conditioned medium was mainly achieved through changing the volume of the cell culture medium and addition of fetal calf serum.

These findings provide valuable insights for developing regulatory testing guidelines for flavored ANDS. The results also highlight the need for more efforts on safety assessments of flavoring compounds in emerging nicotine delivery systems.

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List of Abbreviations

16HBE	Human bronchial epithelial cell line
ADI	Acceptable daily intake
ALI	Air-Liquid Interface
ANDS	Alternative nicotine delivery system
BALF	Bronchial alveolar lavage fluid
cDNA	Complementary DNA
CORESTA	Cooperation Centre for Scientific Research Relative to Tobacco.
DAD	Diode array detector
DAVID	Database for Annotation, Visualization, and Integrated Discovery
DCF	2',7'-dichlorofluorescein
DCFDA	2',7' –dichlorofluorescin diacetate
DEGs	Differentially expressed genes
E-cigarette	Electronic cigarette
EFSA	European Food Safety Authority
ELISA	Enzyme-linked immunosorbent assay
EU	European Union
EVALI	e-cigarette or vaping associated lung injury
FACS	Fluorescence-Activated Cell Sorting
FCS	Fetal calf serum
FDA	Food and Drug Administration
FSC	Forward scatter
GC	Gas chromatography
GHS	Globally harmonized system
GO	Gene ontology
GPx	Glutathione peroxidase
GRAS	Generally recognized as safe
HMOX1	Heme oxygenase 1
HGF-1	Human oral gingival fibroblasts
HPLC	High-performance liquid chromatography
HS-SPME	Headspace solid-phase microextraction
HTPs	Heated tobacco products
IARC	International Agency for Research on Cancer
IL	Interleukin
IPA	Ingenuity Pathway Analysis
JECFA	Joint FAO/WHO Expert Committee on Food Additives
LDH	Lactate dehydrogenase
LLE	Liquid-liquid extraction
MMP	Metalloproteinase
MS	Mass spectrometry
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide
NAD(P)H	Nicotinamide adenine dinucleotide (phosphhate)
NRT	Nicotine replacement therapy
OA-TOF MS	orthogonal acceleration time of flight mass spectrometer
ONP	Oral nicotine pouches
PG	Propylene glycol
qPCR	Quantitative real-time polymerase chain reaction
REMPI	Resonance enhanced multi-photon ionization
ROS	Reactive oxygen species
SPI	Single photon ionization
SSC	Side scatter
TEER	Transepithelial electrical resistance
TNFα	Tumor necrosis factor alpha
TPD	Tobacco Products Directive
VG	Vegetable glycerin
WHO	World Health Organization

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Introduction

1.1 Prevalence and consequences of tobacco smoking in Europe and Germany

Despite widespread awareness of the harmful health consequences [1, 2], the prevalence of tobacco use in the World Health Organization (WHO) European region was still high with a rate of 24.8%, in 2020 [2]. Specifically in Germany 28.3% of persons over 14 years of age were current smokers in 2024, which remained unchanged since 2017 [1]. Among individuals over 18 years of age, 27.1% of men and 19.1% of women were smokers [3]. Among the age group 11 to 17 years, 7.2% were smokers without any difference in gender [3]. Tobacco smoke exposure induces systemic adverse effects rather than being confined to localized sites of the body. It causes several types of cancer, cardiovascular diseases, stroke, lung diseases, adverse effects on fertility, neurological disorders and others [4, 5]. Consequently, worldwide more than 7 Million people die every year from exposure to all forms of tobacco [6]. In Germany, 13.3% of deaths were due to the consequences of smoking tobacco in the year 2018 [3].

1.2 Toxicants of tobacco smoke and their health consequences

Tobacco smoke consists of a vapor and a particulate phase, each containing distinct toxicants [7]. Overall, more than 5,000 chemicals have been identified in tobacco smoke. Since the 1990s, researchers have developed several comprehensive lists of these harmful substances. The initial classification, known as the “Hoffmann analytes” listed biologically active or carcinogenic compounds present in tobacco smoke [8]. Another approach was followed by Talhout et al. almost 15 years later publishing a list of 98 substances that are toxic to the respiratory and cardiovascular system [9]. Subsequently, the U.S. Food and Drug Administration (FDA) compiled a list of 93 harmful and potentially harmful constituents in tobacco smoke which are either carcinogenic, respiratory or cardiovascular toxicants, reproductive or developmental toxicants, or addictive substances [10]. This does not represent an exhaustive listing of published analytes [11].

The toxicants in tobacco smoke originate from two different sources: the combustion process during smoking and the tobacco plant itself [12]. The latter may accumulate metals, such as lead or cadmium [13] from the soil or retain pesticide residues from the cultivation process [14]. The WHO established a list of twelve most toxic compounds that warrant prioritized monitoring in cigarette smoke emissions: acetaldehyde, acrolein, formaldehyde, benzene, 1,3-butadien, carbon monoxide, benzo(a)pyrene (B(a)P), 4-(N-nitrosomethylamino)-1-(3-pyridyl)-1-butanone (NNK), and N’-nitrosonornicotine (NNN) [15]. The first three compounds belong to the classes of carbonyls and together with benzene, 1,3-butadien, and carbon monoxide are present in the vapor phase of the smoke. The latter three compounds are part of the particulate phase in tobacco smoke. All of these priority compounds are classified as carcinogenic by the International Agency for Research on Cancer (IARC), with B(a)P, NNK and NNN being the major contributors to lung cancer [16]. Carbon monoxide exerts its toxicity through a distinct mechanism by entering the blood stream and binding to hemoglobin, consequently impairing vital oxygen transport [17].

Under the Tobacco Products Directive (TPD) 2014/40/EU [18], regulatory limits have been established for tar, nicotine, and carbon monoxide. While mainstream smoke, being directly inhaled by the user, and sidestream smoke, being emitted from the cigarette at the tip, share similar compositions, the latter contains higher concentrations of toxins due to differences in temperatures during combustion [19]. Taken together, tobacco smoke is a highly toxic mixture and represents a public health concern.

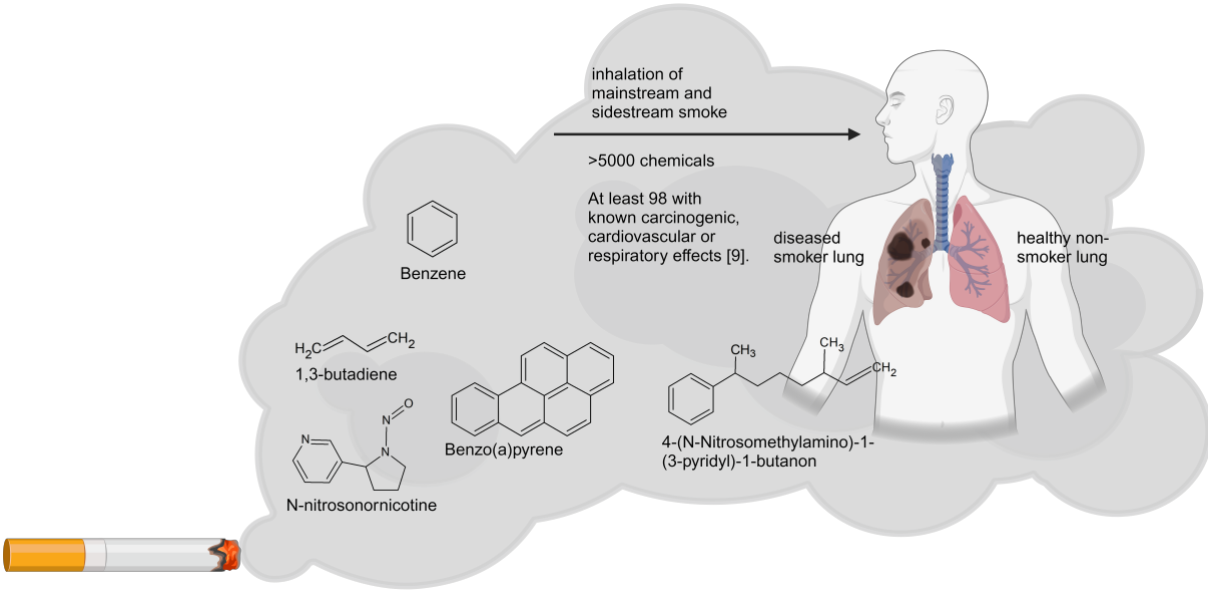

Figure 1. Examples of chemicals contained in cigarette smoke. Examples of toxic chemicals which are inhaled through mainstream and sidestream smoke. These are known to cause adverse health effects, particularly concerning the lung. Created with BioRender.com.

1.3 Alternative nicotine delivery systems

1.3.1 Product types

Due to the deleterious health effects of the exposure to tobacco constituents, several alternative nicotine delivery systems (ANDS) with lower toxicant emissions have been developed over the years. There are nicotine-replacement therapies (NRT) which belong to the group of medicinal products, heated tobacco products (HTPs), electronic cigarettes (e-cigarettes) and oral nicotine pouches (ONP) (Figure 2). These products can be classified in two categories: tobacco-containing products such as HTPs and snus, and non-tobacco products that solely contain nicotine such as NRTs (e.g. nicotine patches, gums, inhalers, nasal sprays, or Lozenges), e-cigarettes, and ONPs [20, 21], as well as new heat sticks containing tea leaves instead of tobacco [22]. However, NRTs are registered medicinal products developed by the pharmaceutical industry specifically as a smoking cessation tool [23]. In contrast, products such as HTPs, snus, e-cigarettes or ONPs are so-called “life-style” products invented and marketed by the tobacco industry. Among the tobacco-containing products, snus is a finely ground, pouched tobacco, which is typically placed under the upper lip to release its nicotine. Unlike cigarettes, it does not require burning and is therefore considered smokeless. The focus of this work is on the non-tobacco products, particularly e-cigarettes and ONPs, and their functioning is explained in detail in Chapter 1.3.3.

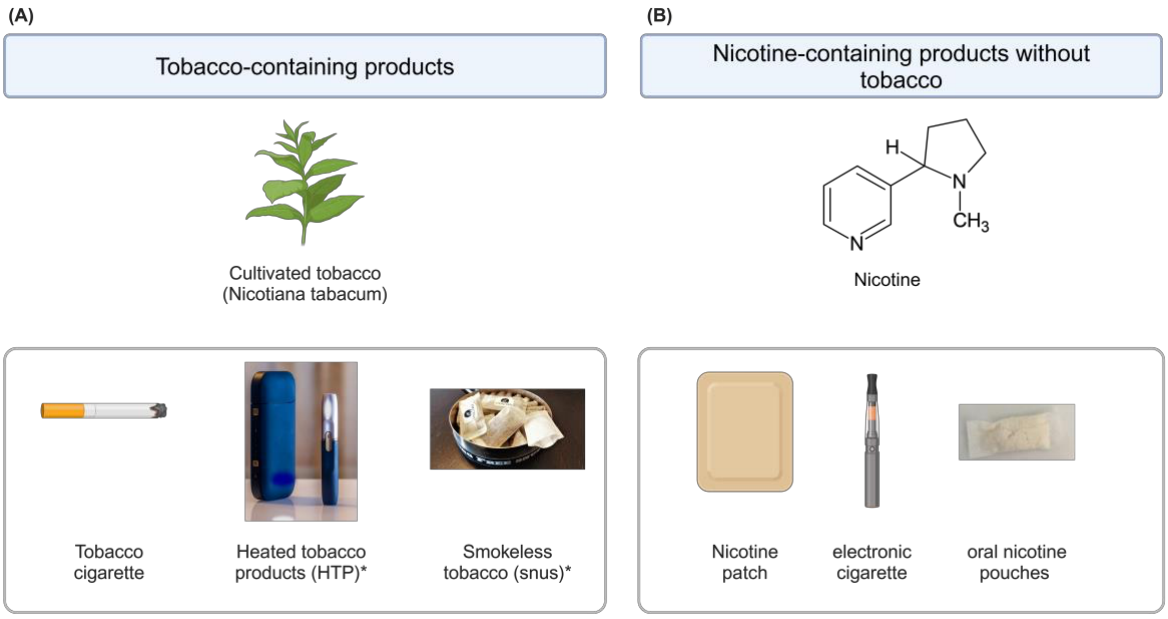

Figure 2. Overview of available tobacco-containing and non-tobacco-containing products. Panel (A) shows products containing tobacco with nicotine while panel (B) shows products containing non-tobacco products with nicotine. Created with BioRender.com. *Pictures obtained from www.pixabay.com

1.3.2 Prevalence of alternative nicotine delivery systems

In contrast to tobacco cigarettes, e-cigarettes and ONPs are mainly used by the younger generations [3, 24, 25]. However, exact data on the prevalence of e-cigarette and ONP use among younger age groups is contradicting and difficult to obtain. Young people nowadays are increasingly accessible through online platforms rather than traditional communication methods such as landlines [26]. In addition, self-reported information might be influenced by the presence of parents [27]. The estimated prevalence in Europe in 2019 varied greatly among the different countries between 2.8% in Kyrgyzstan and 41% in Monaco, among the 15-16 age group and has been observed to be further on the rise [24, 25, 28], without difference in prevalence between boys and girls [28]. Dual use of cigarettes and e-cigarettes is prevalent, with 6.4% of individuals in Europe [29], 42% in Nordic European countries [30] and 80% in Germany reporting this behavior [1]. In Germany, estimated prevalence of current e-cigarette use is higher among people aged between 18 and 24 years than for people aged between 14 and 17 years with 3.3% and 2.3%, respectively in 2020 [27]. The prevalence of e-cigarette ever-use was higher with 13.4% in the 14 to 17 age group and 22.8% in the 18 to 24 age group [27]. In 2023, 2.2% of the German population were current e-cigarette users [31]. In comparison, less than 1% of the population in the European Union (EU) were using ONPs in 2021 [32], with significant variation across individual countries. For example, in Poland, 4.3% were using ONPs in 2024 [33]. In contrast, the prevalence in the United Kingdom (UK) and the Netherlands was considerably lower with 0.3% of the UK adult population using ONPs in 2020 and 2021 with a rising tendency [34], and 0.06% in the Netherlands in 2020 [35]. Whether women or men are more likely to use ONPs is conflicting [33-35].

Overall, although exact numbers are challenging to obtain, conventional tobacco use maintains higher prevalence rates in the general population compared to e-cigarettes and ONPs, despite a gradual

decline in tobacco use in Europe and globally [2]. However, e-cigarettes and ONPs are mainly used by the younger generations in comparison to cigarettes and the numbers of users are constantly rising [24, 25, 28, 34]. This trend raises public health concerns, as nicotine addiction is occurring at a younger age and is often regarded as a gateway to the use of traditional tobacco cigarettes [36, 37]. This is in contrast to e-cigarettes' promotion as tobacco cessation tools, though the evidence remains contradictory. A crucial consideration concerns the distinction between nicotine-addicted and nicotine-naive populations. Overall, research demonstrates that e-cigarettes have detrimental health effects, increase youth smoking rates and serve as gateways to tobacco products [38-40]. However, US and UK studies suggest they may function effectively as harm reduction tools, helping heavy smokers reduce or abstain from tobacco use [41, 42].

1.3.3 Basic operating principles of electronic cigarettes and oral nicotine pouches

There are currently various types of e-cigarettes on the market. Essentially, these products are battery operated systems containing a tank which holds the e-liquid. Within the e-liquid tank is placed a wick surrounded by a metal coil, which commonly consists of kanthal or nichrome among other metals [43]. The wick, commonly made from cotton among other materials, absorbs the e-liquid which is then aerosolized by heating the coil with the power of the battery [44]. The aerosol can then be inhaled by the user. The e-liquid typically contains the base-solvents propylene glycol (PG) and vegetable glycerin (VG), water, flavors and optionally nicotine [45]. A schematic illustration of an e-cigarette is shown in Figure 3.

ONPs are small bags filled with cellulose, nicotine, flavors and other ingredients [21]. The pouches are placed between the upper lip and the gum where they release nicotine and flavors. Nicotine is then absorbed via the oral mucosa.

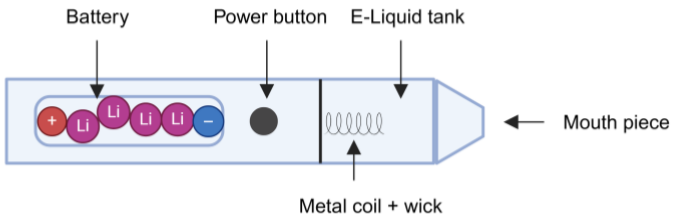

Figure 3. Schematic illustration of an electronic cigarette (e-cigarettes). The e-cigarette contains a battery, which is activated via a power button, leading to heating of the metal coil thereby aerosolizing the e-liquid in the e-liquid tank, leaving the device through the mouth piece. Created with BioRender.com.

1.3.4 Regulation of e-cigarettes, e-liquids and oral nicotine pouches in the European Union

In the EU, the safety requirements and obligations for packaging and labelling for e-cigarettes and nicotine-containing e-liquids are delineated in article 20 of the Tobacco Products Directive (TPD) 2014/40/EU [18]. This directive governs the regulation of ingredients in e-liquids, including flavorings. These ingredients must be of high purity and should not elicit adverse health effects in either unheated or heated forms, this includes those with carcinogenic, mutagenic or reproductive toxic properties. The directive prohibits misleading ingredients such as vitamins, stimulating agents like caffein, and coloring

agents for the aerosol. As a result, certain flavors are excluded. The implementation of the TPD into German law includes a list of substances that are restricted, with diacetyl being one of them. A ban on mentholated tobacco products came into force in May 2020 [46], which does not apply to e-cigarettes. Although e-liquid flavorings are regulated, these regulations do not take into account the complex mixture effects or the chemical transformations that occur when the flavorings are heated, such as the formation of degradation products and the emergence of new chemical reactions [47, 48].

ONPs do not contain tobacco and are therefore not regulated under the TPD. In Germany, these products are classified as novel food products, and nicotine is not authorized as a food additive. Consequently, the sale of ONPs is prohibited [49]. Nevertheless, they remain widely accessible through online retailers and current reports from Germany indicate that 15% of German high school students aged 16 to 17 were consuming nicotine pouches in 2023 [50].

1.4 Flavorings in alternative nicotine delivery systems

1.4.1 The role of flavorings in alternative nicotine delivery systems

Flavors constitute a fundamental component of e-cigarettes and ONPs, playing a pivotal role in generating the sensory experience and acting as a primary motivator for their consumption, irrespective of the consumer's smoking status [35, 51-54]. As discussed in Chapter 1.3.2, young people in particular are attracted by these products. Furthermore, the availability of flavors, particularly menthol, not only facilitates product initiation [55] but is also associated with more frequent use, dual use [51], and sustained consumption [56]. The sensory attraction of flavors, particularly those such as menthol, fruit and candy is a key factor in their popularity. This is underlined by the fact that a large majority would stop using these products if flavored products were taken off the market, with 78% of respondents aged 18-24 years and 74% of those aged 25-29 years [57]. Moreover, flavors alone contribute to a reduced risk perception [58] of ANDS compared to cigarettes [59, 60] or by masking the harshness of higher nicotine concentrations [61]. The latter may facilitate accessibility to high concentrations of nicotine among adolescents. This misperception of the inherent risks of e-cigarettes might also be driven by the advertisement of the tobacco companies which use the claim that ANDS are less harmful than tobacco products or are part of a "harm reduction strategy". On top of that, this advertisement is strategically targeted at young people and women in order to gain new customers and increase their profits [33, 62-64]. However, the risk perception of e-cigarettes might have experienced a change due to the e-cigarette or vaping associated lung injury (EVALI) crisis in the US in 2019. This caused 2807 hospitalized patients with a median age of 24 years and 68 deaths [65], with the diluting agent vitamin E acetate suspected to be the primary cause for EVALI [66]. This crisis highlights that adverse health incidences from e-cigarettes occur in a much younger age group compared to harmful effects from long-term cigarette smoking.

1.4.2 Commonly used flavorings in alternative nicotine delivery systems

The e-liquid market is confusing with more than 250,000 notified e-cigarettes and e-liquids in Germany alone, until 2022 [67]. In order to facilitate systematic categorization of flavored e-liquids, the flavor wheel was developed by Krüsemann et al. (2019) [68]. However, the labelling of e-liquids is often

insufficient for identification of the individual flavors used in a mixture. Several research groups have investigated this issue. Up to 50 individual flavoring agents have been identified in e-liquid products from the US market [69] with total flavor concentrations of up to 343 mg/mL [69, 70]. Some individual flavors in e-liquids from both the US and Dutch markets had concentrations of more than 10 mg/mL [69, 71]. Commonly identified flavoring agents include but are not limited to cinnamaldehyde, eugenol, vanillin, diacetyl, ethyl maltol, benzaldehyde, menthol, and limonene [69, 70, 72, 73]. Cinnamaldehyde, eugenol, vanillin, and diacetyl were used for toxicological investigations in the present study (Figure 4) due to their frequent usage in e-liquids. The most abundant flavors belong to the chemical groups of esters, terpenes, ketones, alcohols, and aldehydes [69, 74]. Esters, such as ethyl acetate, ethyl butyrate and ethyl hexanoate, are the main chemical class which is characterized by a fruity aroma and is therefore found in nearly all fruit-flavored e-liquids [75]. Terpenes including limonene and pulegone are often used to produce citrus [75] and minty flavors [76], respectively. Ketones like ethyl maltol or diacetyl impart a caramel taste [77]. Alcohols like l-menthol, which provides a mint flavor [75], and benzyl alcohol, known for its bitter and cherry taste, are also frequently used [70, 78]. Finally, vanillin and cinnamaldehyde, are responsible for the characteristic vanilla and cinnamon aromas found in e-liquids [75].

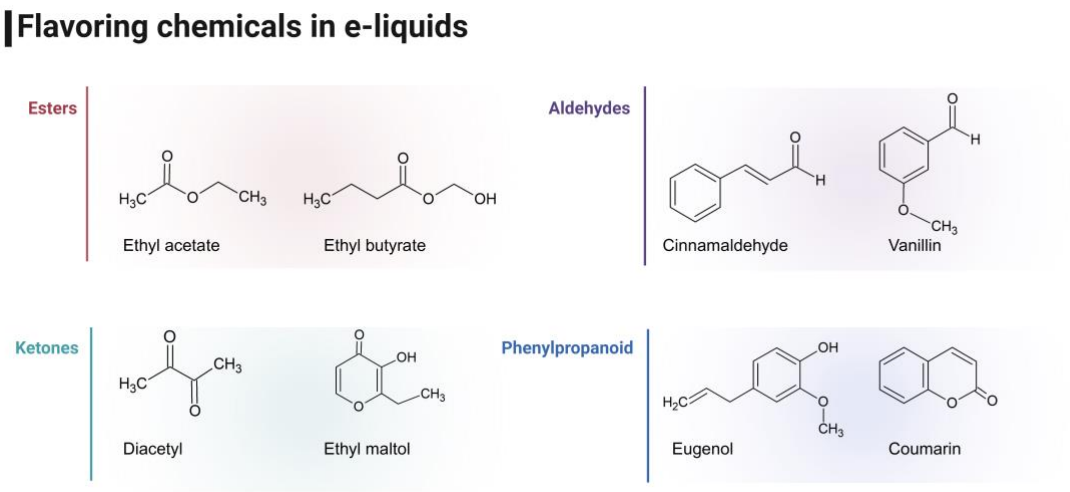

Figure 4. Examples for commonly used flavoring agents in e-liquids. Cinnamaldehyde, vanillin, eugenol, and diacetyl were also chosen for the experiments conducted in the study herein. Structures drawn with ChemSketch, version 2019.2, Advanced Chemistry Development, Inc. (ACD/Labs), Toronto, ON, Canada, www.acdlabs.com. Created with BioRender.com and adapted from “Simple phenolic acids (chemical structure).

As indicated above, these chemical classes are often mixed together in e-liquid formulations. For instance, Omaiye et al. identified a “cool mint” flavoring mixture that incorporates all of these classes, including p-menthone, corylone, triacetin, benzyl alcohol, menthol, vanillin, ethyl acetate, and ethyl maltol [79]. Similarly, Behar et al. identified an e-liquid mixture containing linalool, cinnamaldehyde, menthol, ethyl butyrate, ethyl maltol [70]. Importantly, when several flavor compounds are combined, they may undergo chemical transformations during the vaping process, potentially resulting in the formation of additional chemical classes (see Chapter 1.5.3) [80]. The flavors used in the e-liquids have the status of being generally recognized as safe (GRAS) by the FDA and are approved as food additives by the European Food Safety Authorization (EFSA). Both classifications are relevant for oral uptake.

However, the toxicity of the flavoring mixtures following heating and inhalation remains unknown and even more important, untested. [81]

1.5 Toxic properties of base-solvents, other ingredients and flavors in alternative nicotine delivery systems

1.5.1 Inhalation toxicity of base-solvents propylene glycol and vegetable glycerin in e-liquids

The base-solvents PG and VG are the primary ingredients of e-cigarettes with a fraction of 4 - 98% and 3 - 95%, respectively [82]. Although these substances have long been utilized by the pharmaceutical, cosmetics, and food industries and are considered safe for oral, dermal or intravenous administration, their safety profile upon inhalation remains a subject of concern [45]. Recent studies have highlighted the potential toxic effects of PG/VG, both in vitro and in vivo, revealing significant health risks concerning respiratory function, tumorigenesis, and cardiovascular effects [83-88]. Specifically, studies have shown that PG/VG exposure promotes tumor progression, facilitates lung metastasis [83, 88] and induces immunosuppression in mice [83]. Particularly the latter effect has been demonstrated in several in vivo studies, including increased protein expression in bronchoalveolar lavage fluid (BALF) in rats [84], neutrophil infiltration into mouse BALF and immunosuppression by cytokine decrease, alongside allergic inflammatory responses [87]. Increased mucus secretion and metalloproteinase-9 (MMP-9) levels have been found in sheep nasally exposed to PG/VG aerosols, which may indicate respiratory impairment [86]. Endothelial dysfunction, a biomarker for adverse cardiovascular events, was investigated in the aorta of a mouse model in response to inhalation of PG/VG aerosols. The results indicated a positive effect, likely attributable to formaldehyde formation during the vaping of PG/VG [85]. The formation of carbonyls during heating of the base-solvents has been demonstrated several times [89-94]. The base-solvents undergo thermal degradation through oxidation and contact with the metal coil, with VG primarily producing acrolein while PG forms formaldehyde and acetaldehyde [89]. The formation of these compounds is influenced by various parameters including power settings [90, 92], coil resistance [93], e-cigarette brand [90], concentrations of base-solvents, flavor content [94], or “dry-puffing” [91]. Notably, the predominant aldehyde in e-cigarette vapor is formaldehyde, which is also present in high concentrations in cigarette smoke. An association of formaldehyde inhalation and nasal cancer in humans has been established and is therefore classified as carcinogenic to humans (class 1) by IARC, respectively [95]. Acrolein and crotonaldehyde are classified as probably (class 2A) and possibly (class 2B) carcinogenic to humans by IARC [96]. This shows that PG/VG’s inherent toxicity and decomposition products need to be accounted for in a human health risk assessment of e-liquids.

In vitro investigations have demonstrated multiple cellular effects, including decreased cell viability and metabolic activity [97], decreased cell count [98] and increased cytotoxicity [99, 100] depending on the ratio of PG/VG [98, 101, 102]. Studies have documented decreased transepithelial electrical resistance (TEER), increased lactate dehydrogenase (LDH) [103], elevated inflammatory protein expression (IL6, IL8, MMP-9) in oral cells [104], transcriptomic changes in human middle ear cells [100], DNA damage and apoptosis in primary human lung cells, with PG showing a more pronounced effect [101, 105]. Additional findings include histological alterations, possibly by toxic crosslinking of carbonyls, thereby reducing important cell populations of the lung [101, 106]. Cytotoxicity and genotoxicity were reported

to be most likely caused by carbonyls [107] as these effects were absent in direct base-solvent exposure conditions.

While human data on PG/VG inhalation remains limited, documented effects include pulmonary irritation and cough [108]. Indoor exposure to PG has been associated with allergic symptoms and asthma [109].

In conclusion, in vitro and in vivo data has demonstrated a major involvement of the base-solvents PG and VG in e-liquid toxicity. However, a major knowledge gap exists regarding the inhalation toxicity of the added flavorings in e-liquids. This study therefore aimed at evaluating the contribution of the flavors in e-liquid toxicity.

1.5.2 Toxic ingredients in oral nicotine pouches

Limited independent research data on the toxicity of the ingredients in ONPs is available (see Chapter 1.5.3). Nicotine, the main active ingredient, may either be synthetically produced [110] or derived from tobacco-leaf material, although tobacco itself is not contained in the final product [111]. Tobacco-specific nitrosamines, which are genotoxic compounds formed during tobacco curation and fermentation, are consequently detected at low concentrations in some ONPs [111]. Furthermore, a research study conducted by the tobacco industry, reported the detection of the toxicants chromium and formaldehyde in ONPs at low levels, which are also present in other tobacco products and e-cigarette emissions [112].

1.5.3 Flavorings in oral nicotine pouches and electronic cigarettes

As discussed in Chapter 1.4.2, up to 50 individual flavoring agents have been detected in a singular e-liquid mixture, many of which (should) have GRAS status. However, the inhalation safety of these compounds is not well established. Some commonly used flavorings have been identified as potential respiratory allergens, skin sensitizers or irritants. For example, α-Ionone is classified as a respiratory allergen according to the globally harmonized system (GHS) [113], while isoeugenol [114] and eugenol [115] are known skin sensitizers. In addition, isoeugenol is able to cause irritation in the tract of the lower airways [114]. Linalool, although considered as a weak skin sensitizer, can autoxidize to form linalool hydroperoxides, which are stronger sensitizing compounds compared to linalool [116]. Cinnamon-flavored e-liquids commonly contain cinnamaldehyde, which is known for its irritating potential [117]. Some flavoring agents, containing a carbonyl group, can react with other e-liquid components, such as cinnamaldehyde with PG to cinnamaldehyde propylene glycol acetals (Figure 5) [118]. These have been demonstrated to be more cytotoxic than the flavoring agent itself. Additionally, the heating and vaporization process can lead to the formation of harmful degradation products. For instance, diacetyl, a flavor with a buttery aroma having GRAS status, is banned in e-liquids in the EU due to its potential to cause respiratory damage in the small airways when inhaled [119]. It has been found as a degradation product in the vapor of diacetyl-free e-liquids. This shows that not only the compounds in their original form should be considered for toxicity but also degradation or reaction products.

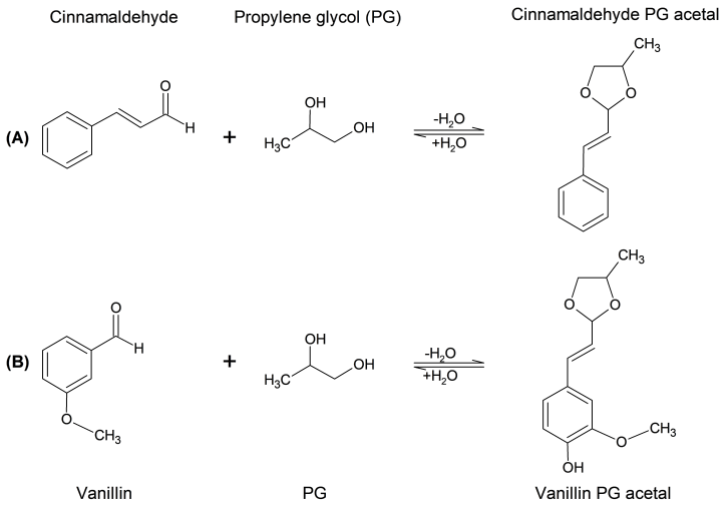

Figure 5. The chemical reaction of aldehydes with propylene glycol. Panel (A) shows the reaction of cinnamaldehyde with propylene glycol (PG), leading to the formation of cinnamaldehyde PG acetal and the elimination of water. Panel (B) shows the reaction of vanillin PG, resulting in the formation of vanillin PG acetal, with the elimination of water. Adapted from [120]. Structures drawn with ChemSketch, version 2019.2, Advanced Chemistry Development, Inc. (ACD/Labs), Toronto, ON, Canada, www.acdlabs.com.

Numerous in vitro and in vivo studies have been performed to shed light on the toxic potential of flavoring agents in e-liquids. These studies have examined various endpoints, including cytotoxicity, inflammation and transcriptomic changes [100, 121]. Cytotoxicity was found to be flavor-dependent by several research groups [97, 122-125], with cinnamon-flavored e-liquids causing high cytotoxicity [93, 122, 123]. However, results across studies have been inconsistent, with some showing reduced cell viability [126, 127], others showing no change [128, 129], and some producing inconclusive results [130]. Leigh et al. [97] suggested that menthol, coffee and strawberry-flavored e-liquids are most cytotoxic compared to other flavors. Inflammatory responses have also been observed in cells exposed at the Air-Liquid Interface (ALI) to e-liquid aerosol. For example, epithelial cells exposed to tobacco-flavored e-liquid aerosols showed increased release of the pro-inflammatory cytokines IL-8 and IL-6 [130]. Similar inflammatory responses have been observed with other flavors and other cell lines, particularly cinnamon [97, 124, 127]. Another study did not show release of cytokines in response to flavors [128].

It has been suggested that the toxicity might be driven by receptor binding of the flavorings vanillin, cinnamaldehyde, and menthol to TRPV1, TRPA1, and TRPM8, respectively [131, 132]. These receptors have been discussed to be involved in respiratory diseases and symptoms such as cough, allergy and asthma among others [133].

In vivo data on the inhalation toxicity of flavors is scarce, as animal testing on tobacco products is banned in the EU [134]. However, one rat study demonstrated that vanillin caused increased neutrophils in the BAL fluid and revealed proteomic changes related to downregulation of lipid metabolism [84].

The available data indicate that flavors can lead to adverse effects in cell and animal models. The implications for humans remain to be investigated with long-term epidemiological studies. However, pinning down effects on specific flavors will be challenging.

Research on the contribution of toxicity of flavors in ONPs is limited due to their novelty. However, Shaikh et al. found that ONPs induce cytotoxicity, reactive oxygen species and inflammatory cytokine release in oral epithelial cells depending on the flavor composition [135].

1.6 Challenges for the risk assessment of flavorings in alternative nicotine delivery systems

The information provided in Chapters 1.4 to 1.6 reveals several challenges that are encountered when conducting a risk assessment of e-cigarettes and ONPs, particularly regarding the flavors. A critical issue is the lack of comparability between existing studies, which can largely be attributed to the variability in study designs. These variations include differences in cell models (ALI versus submerged, type of cell lines), exposure methods (aerosolized versus e-liquid exposure), and the specific flavoring mixtures used. The choice of cell model is an important factor in in vitro research. The type of cells used, alongside culture conditions and mode of exposure, can significantly influence the study's outcome, potentially altering the interpretation of results [136]. Commonly used lung epithelial cell lines such as NCI-H292, A549, BEAS-2B, and 16HBE [70, 72, 93, 97, 123, 125, 128, 130, 137-140] are commercially available, well-characterized, and relatively cost-effective. These can be cultured under submerged or ALI conditions [141, 142]. While primary bronchial epithelial cells obtained from donors offer greater human relevance, they are more expensive and require specialized handling expertise. ALI culture systems, where cells are grown on inserts with nutrients supplied from the basal side and air exposure on the apical side, allow for cellular differentiation into various types present in vivo, including goblet, basal, ciliated, and club cells. This multi-cellular environment more closely mimics human conditions compared to submerged monocultures. The selection of the cell model should reflect the relevant lung region (alveolar or bronchial) [143] for the study objectives. Several research groups have highlighted the importance of carefully choosing an appropriate cell model, taking into account the specific lung region (alveolar or bronchial) [143] and the type of cell lines [70, 144]. Similarly, for ONPs, analogous considerations must be made, but with a focus on the context of oral exposure.

Realistic laboratory simulation of e-cigarette flavoring exposure presents another major challenge. Various approaches have been employed, including the use of commercial smoking machines to collect aerosols in cell culture medium for submerged exposure [70, 123], cloud chamber methodologies condition [102, 145], and direct ALI exposures to freshly generated e-cigarette aerosols [97, 144, 146]. The latter is considered most representative of real-life vaping conditions in an in vitro setting.

The high temperatures reached during vaping (up to 350°C [147]) may cause thermal decomposition of flavoring compounds (e.g., diacetyl). This thermal breakdown, along with the formation of new products such as PG acetals contribute to uncertainties regarding the actual compounds to which consumers are exposed. These factors must be taken into account in experimental setups.

The vast array of flavorings available on the market, often with publicly undisclosed ingredients, further complicates the risk assessment of e-liquids and ONPs. Given these complexities, this present work aims to address gaps in the current understanding and to develop a testing strategy for the risk assessment of e-cigarettes and ONPs, with a focus on flavoring components.

1.7 Aim of this work

Global smoking prevalence declined from 32.1% in 2000 to 21.7% in 2020 [2], while ANDS usage, such as e-cigarettes and ONPs, is experiencing an increase in popularity. This can be partly attributed to the variety of available flavors. The addition of flavors to ANDS has two crucial implications. They can facilitate the transition from traditional tobacco products to tobacco-free alternatives while simultaneously appealing to young non-smokers [53]. Moreover, certain flavors increase addiction [148] and potential abuse of e-cigarettes [61]. Despite the perception of these products as being free of risk [58], e-cigarette aerosols contain toxic chemicals, although fewer and at lower concentrations, similar to those in tobacco smoke and therefore pose a risk to human health. Given the novelty of ONPs, a comprehensive risk assessment is currently unfeasible, due to insufficient information concerning their ingredients. In both product categories, a significant uncertainty is associated with the flavors, which are generally recognized as safe for oral ingestion. However, the precise flavor composition of ONPs and e-liquids is not disclosed on the products. Therefore, mixture toxicity may play a role and the inhalation toxicity of flavors in the case of e-cigarettes has not been tested. Previous research on ANDS flavoring toxicity has primarily evaluated multi-flavor products. In order to conduct a comprehensive risk assessment and to regulate flavors in ANDS properly, it is essential to know the toxicity of individual flavors. Another main challenge for the risk assessment of flavors is the absence of standardized in vitro testing strategies which results in a lack of comparability among existing studies.

Overall, this PhD work addresses the knowledge gaps on local and respiratory health effects of flavorings in e-cigarettes and ONPs. The first aim was to identify ingredients in a sample set of 50 ONPs with a focus on flavors (Mallock-Ohnesorg & Rinaldi et al., 2023). The second aim was to determine in vitro toxicity of five selected nicotine pouch products on oral gingival fibroblasts (Rinaldi et al., 2023). The third aim was to investigate inhalation toxicity of individual flavor compounds in e-liquids on bronchial epithelial cells in a submerged and an ALI cell culture model (Rinaldi et al., submitted to Respiratory Research journal). The fourth aim was to evaluate the suitability of a commonly employed exposure method for conducting toxicity assessments of flavors in e-cigarettes, specifically the capture of e-cigarette aerosols in cell culture medium.

The following lists the research questions of particular relevance to this work:

1. What is the chemical profile of flavor compounds in oral nicotine pouches?
2. Can we correlate identified nicotine pouch ingredients or nicotine content with toxic effects observed in oral gingival fibroblasts?
3. What are the toxic effects from individual flavors on lung cells in two different cell models (submerged and ALI)?
4. Which similarities and differences can be identified between submerged and ALI exposure to individual flavors?
5. In what way does the impinger method demonstrate validity and reliability for toxicological analysis of e-liquid flavors?

2 Methodology

In this section, the principles of the used assays and methods are described. For a detailed description of the methodologies, please refer to the original publications Rinaldi et al. (2023), Mallock-Ohnesorg & Rinaldi et al. (2023) and Rinaldi et al. (*submitted*).

2.1 Basic principles of in vitro cell culture models: Submerged and Air-Liquid Interface

2.1.1 Submerged cell models

Submerged cell culture models are widely applied in toxicological in vitro studies. They offer the advantage of being cost-effective, and are easy to handle [142]. In the present study, human oral gingival fibroblasts (HGF-1) were used under submerged conditions to investigate the toxic potential of ONP extracts on the gingiva, which is the tissue surrounding the teeth [149]. As ONPs are typically placed between the upper lip and the gingiva where they release nicotine and flavors, the gingiva is the main contact point in this setup. Consequently, HGF-1 cells were chosen as the appropriate cell model. The cells were maintained in their cell culture medium during routine handling. For experimental purposes, they were harvested, quantified and seeded at their appropriate density and well plate size. They were subsequently allowed to adhere for 24 hours prior to experimental use. Figure 6 depicts a schematic overview of the extraction procedure and HGF-1 exposure. Briefly, five ONPs were extracted in 10 mL HBSS and HEPES, adapted from [150], with an adjusted pH-value to 6.8 ± 0.2 according to standard artificial saliva (ISO 53160–1) [151]. After 5, 10, 20, 30, and 60 min of extraction by shaking the ONPs in a flask at 37°C and 200 rpm, the whole extract was filtered using a syringe filter with a polyethersulfone membrane (0.22 µm, Merck KGaA).

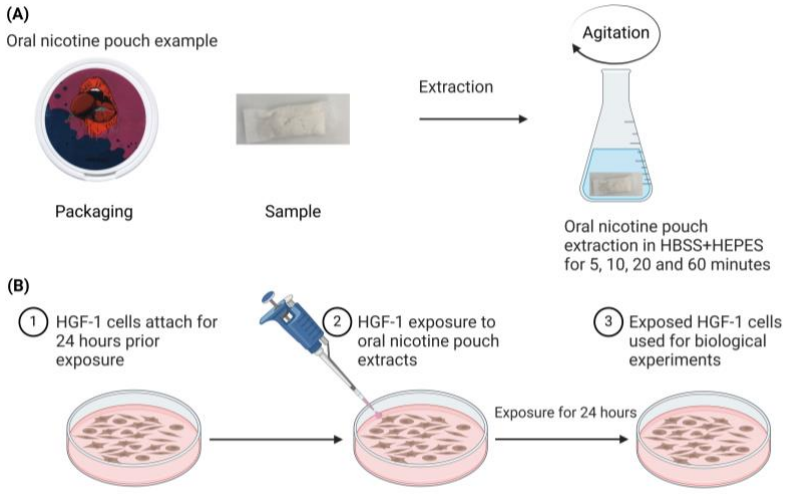

Figure 6. Schematic workflow of oral nicotine pouch extraction and human gingival fibroblasts (HGF-1) exposure. The upper part (A) depicts an example of oral nicotine pouches – the packaging and a sample as well as the method of extraction using Hank's balanced salt solution (HBSS) + 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) to obtain nicotine pouch extracts for cell exposure. The lower part (B) shows a schematic workflow of HGF-1 seeding and attachment over 24 hours (1), HGF-1 exposure to oral nicotine pouch extracts for 24 hours (2), followed by biological experiments to assess potential toxic effects (3). Created with BioRender.com.

In the third study presented in this manuscript (Rinaldi et al., submitted), the human bronchial epithelial cell line (16HBE) was utilized under submerged conditions for the toxicity assessment of the flavoring agents cinnamaldehyde, eugenol, vanillin, and diacetyl diluted in cell culture medium. Diacetyl served as a positive control. The exposure workflow is depicted in Figure 7. The cells were maintained in their cell culture medium during routine handling until exposure. The 16HBE cells represent an appropriate

cell model for toxicity testing, as they retain numerous features of differentiated normal bronchial epithelial cells, including tight junctions and the capacity for ion transport [152-154]. However, at ALI, they do not effectively produce mucins, which constitute a primary clearance factor in the lung [155]. Furthermore, conflicting reports exist regarding cilia formation in these cells [152, 155].

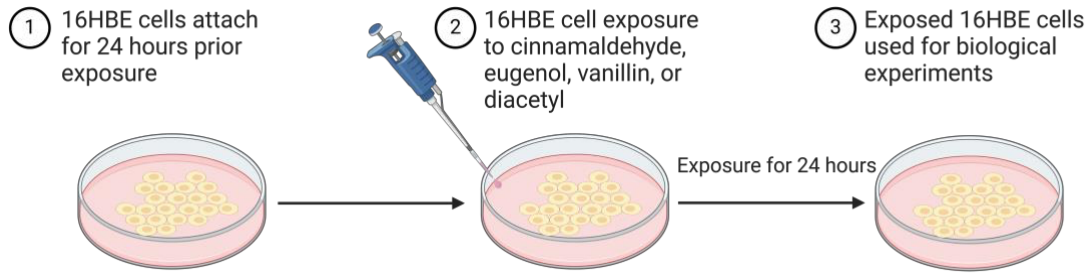

Figure 7. Schematic representation of the exposure workflow of human bronchial epithelial cells (16HBE) to flavoring agents under submerged conditions. The workflow consists of 3 main steps: (1) Cell seeding and attachment period, (2) cell exposure to cinnamaldehyde, eugenol, vanillin, or diacetyl as the positive control, (3) cells were used for biological experiments to assess potential toxic effects following 24 hours of exposure. Created with BioRender.com.

2.1.2 Air-Liquid Interface cell model

The ALI was chosen as a further cell model to investigate the toxic effects of commonly used flavoring agents in e-liquids, particularly cinnamaldehyde, eugenol and vanillin. In contrast to the submerged cell culture, the ALI model is more costly and time-consuming in handling. At the same time, it is more realistic in two ways. First, the model itself resembles physiological conditions, with the upper part of the cells in contact with air and the lower part in contact with the cell culture medium, resembling the blood barrier, which supplies the cells with nutrients and humidity [142]. Second, aerosol exposure is possible at ALI, which is more realistic when it comes to the toxicity testing of flavors in e-liquids [136]. Figure 8 depicts a schematic workflow of the ALI cell culturing.

2.1.3 Air-Liquid Interface cell model validation using transepithelial electrical resistance measurement

Tight junction proteins, such as occludins and claudins, are essential for connecting epithelial cells and forming a cohesive monolayer [152]. In order to validate the ALI cell model for having developed an intact barrier, TEER measurement was performed using a volt-ohm meter which was placed in either of the compartments, apical and basal, which are both filled with a salt solution. A current is applied between the electrodes thereby measuring the electrical resistance. Importantly, a blank without cells has to be measured and subtracted from the sample with cells to obtain the resistance of the cell layer itself. A higher electrical resistance translates into an intact barrier. [156]

2.1.4 Aerosol generation

Aerosols were generated connecting an e-cigarette (Wenax Stylus C Kit (Shenzen Geekvape Technology Co., Ltd., China) to a custom-made puffing system using a puff-topography scheme according to the standard of the International Organisation for Standardisation (ISO) 20768:2018 [157]. In total, 20 puffs were generated with a puff volume of 55 mL, a puff duration of 3 s, and a puff frequency of 30 s. The e-liquids utilized in this study were lab-made based on a standard for reference e-liquid (EN

17375:2020) with slight adaptions [158]. For the e-liquids, 30/70% PG/VG, 1% water, and 1% flavor (cinnamaldehyde, eugenol, or vanillin) in ethanol were used. The e-liquid contained a final flavor concentration of 70 µg/mL.

The generated emissions were monitored on a puff-by-puff basis to ensure the quality of the aerosol composition and the process reproducibility, which is described in Chapter 2.7.1.

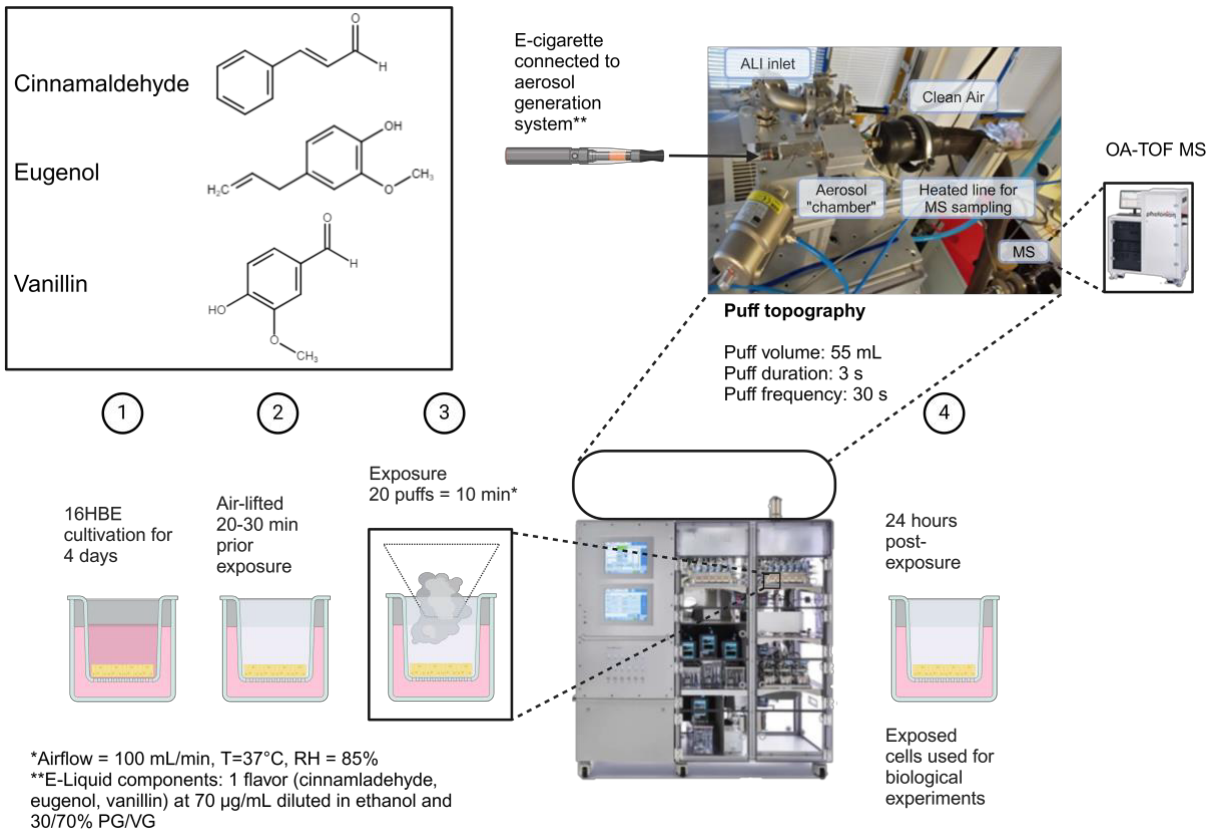

Figure 8. Overview of the exposure setup at Air-Liquid interface (ALI) condition. The highlighted box shows the chemical structures of the flavors (cinnamaldehyde, eugenol and vanillin) used. Below is a schematic workflow for the ALI exposure and the aerosol monitoring. Abbreviations: T – temperature; RH – relative humidity; OA-TOF MS – orthogonal acceleration time of flight mass spectrometer. Created with BioRender.com

2.1.5 Aerosol cell exposure

The cells at ALI were placed in the VITROCELL® Automated Exposure Station (AES) Extended Version (Vitrocell Systems GmbH, Germany). The generated flavored e-cigarette aerosols were directed to the AES. Figure 8 depicts the workflow of cell culturing and exposure including the aerosol monitoring. In order to validate the usability of the cell model for the purpose of this study, pre-tests were performed concerning the formation of tight junctions of 16HBEs at ALI and a dose-response of cinnamaldehyde e-liquid of 16HBEs at the ALI to determine the duration of exposure. All parameters of exposure are described in Figure 8. The cellular effects in response to flavors under submerged condition were compared to the effects under ALI condition.

2.2 Toxicological assessment of identified ingredients in oral nicotine pouches

In order to classify the substances identified in 50 ONPs (see Chapter 2.7.3), a comprehensive toxicological assessment was performed utilizing multiple sources. The assessment approach involved: (1) examining whether the ingredients possessed a harmonized classification according to Regulation (EC) No 1272/2008 on Classification, Labelling and Packaging (CLP) [159]; (2) determining if substances were classified as carcinogens by IARC;(3) consulting the databases maintained by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) [160] and the food flavorings database provided by the European Commission based on the Annex I to Regulation (EC) No. 1334/2008 [161]; (4) evaluating recommendations and opinions given by EFSA. Regarding the harmonized classification, only specific hazard statements were considered relevant for the exposure route of ONPs such as H315 (skin irritation cat. 2), H317 (skin sensitizing cat. 1), H361d (reprotoxic cat. 2), and H372 (repeated toxicity to organs cat. 1)

2.3 In vitro toxicity assays

2.3.1 LDH

Cytotoxicity was evaluated by measuring LDH in the supernatant of HGF-1 cells in response to ONPs and in apical washes as well as the basolateral medium of 16HBEs following ALI exposure to flavored aerosols. LDH is abundant in the cytoplasm of all cells. Upon damage of the cell membrane, it is quickly released and can be measured in the cell culture supernatant. Therefore, it is used as a measure of cytotoxicity. The LDH assay relies on a two-step conversion which results in the reduction of a yellow tetrazolium salt to a red formazan. The first step is the oxidation of lactate to pyruvate, catalyzed by LDH, in the presence of nicotinamide adenine dinucleotide (NAD^+)/NADH. The second step is the electron transfer from NADH to the yellow tetrazolium salt which is reduced to red formazan. The formazan can be colorimetrically measured at a wavelength of 490 nm and is directly proportional to the LDH released by damaged cells. [162]

2.3.2 Metabolic activity Assays

Metabolic activity assays were used to assess cell viability. The underlying principle is based on a colorimetric reaction within cells. A dye is internalized by cells and subsequently reduced by NAD(P)H to a quantifiable dye. The concentration of the measured product is proportional to the number of viable cells. Figure 9 illustrates the colorimetric reaction at the example of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT). [163]

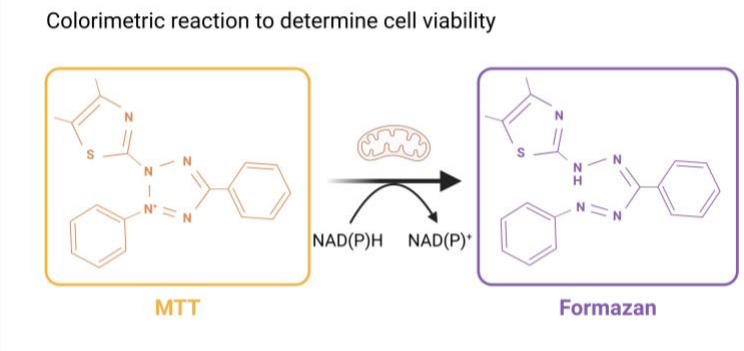

Figure 9. Illustration of the colorimetric reaction used in the metabolic activity assay. Shown is the metabolic activity assay and its colorimetric reaction of the yellow substrate 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) to the purple formazan in the mitochondria using nicotinamide adenine dinucleotide (phosphate) (NAD(P)H) for electron transfer. Created with BioRender.com and adapted from “MTT assay”.

The PrestoBlue assay was used to measure cell viability of 16HBE cells at ALI following exposure to flavored aerosols. It uses the water soluble and non-fluorescent dye resazurin which is reduced by NADPH or NADH to the red fluorescent dye resorufin. Resorufin can be spectrophotometrically measured at a wavelength of 530 nm excitation and 590 nm emission. [164]

To determine cell viability in HGF-1 cells in response to ONP extracts and in 16HBE cells following submerged flavoring exposure, the MTT assay was used. MTT is light-yellow and can cross the cell and mitochondrial membrane due to its positive charge and lipophilicity, where it is reduced to the purple formazan by NAD(P)H (Figure 9) [163]. The formazan's optical density can be spectrophotometrically measured at a wavelength of 570 nm.

2.3.3 2',7' –dichlorofluorescin diacetate assay

The DCFDA assay was used to measure intracellular reactive oxygen species (ROS), specifically hydrogen peroxide and hydroxyl radicals [165] in oral gingival fibroblasts following nicotine pouch exposure. The principle of the assay is based on the non-fluorescent dye (2',7'-dichlorodihydrofluorescein diacetate (DCFDA)), which is able to enter the cells due to the ester groups [166]. Within the cells, it is deacetylated by intracellular esterases resulting in the non-fluorescent substance 2',7'-dichlorofluorescein (DCF). When DCF is oxidized by ROS it becomes fluorescent and can be measured photometrically at 485 nm excitation and 530 nm emission. The measured fluorescence is proportional to ROS activity. [165, 166]

2.4 Detection of changes on gene level

2.4.1 Microarray

To identify the molecular mechanisms in 16HBE cells in response to flavorings in e-liquids in submerged and ALI conditions, transcriptomic alterations were measured using a microarray analysis. Specifically, a Clariom™ S assay (Affymetrix, St. Clara, CA, USA) was used. This technique allows the simultaneous measurement of approximately 20,000 genes. The fundamental principle involves the hybridization of the fluorescently tagged sample cDNA with the probe on a solid chip [167]. In detail, the microarray chip is typically fabricated from glass, silicon or quartz among other materials. The chip surface is arranged in a grid, where each spot contains a unique nucleic acid sequence corresponding to a specific known gene. First, the RNA of the sample is extracted, converted to cDNA and purified (Figure 10). In a next step, the cDNA is cut into fragments which are fluorescently labelled with biotin and transferred to the DNA chip, where the matching nucleic acid sequences from the chip and the sample cDNA are allowed to hybridize. In order to minimize non-specific hybridization, the chip is washed several times. The detected fluorescence intensity corresponds to the amount of bound cDNA and can be quantified. [168]

The transcriptomic changes obtained can be processed by various bioinformatic tools to explore the implications of differentially expressed gene (DEG) profiles and to identify possible molecular mechanisms. In this study, the Database for Annotation, Visualization, and Integrated Discovery (DAVID) and Ingenuity Pathway Analysis (IPA) (Qiagen, Hilden, Germany) were employed. Gene

ontology (GO) terms were retrieved from DAVID to gain insights into the function of the DEGs, while IPA was used for pathway analysis.

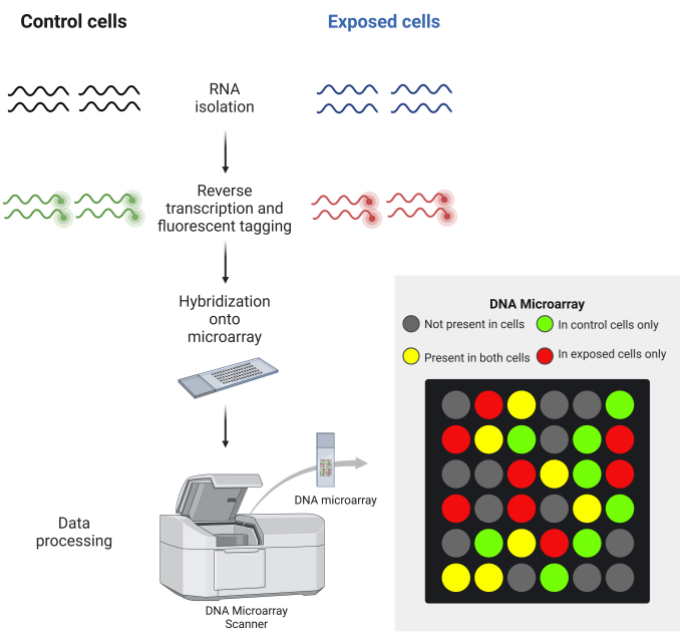

Figure 10. Schematic representation of the microarray analysis workflow. The RNA is isolated from cells under various experimental conditions, then converted into complementary DNA (cDNA) using reverse transcriptase. The cDNA is fluorescently labeled, followed by hybridization onto a microarray and scanning. Finally, the resulting data are analyzed using bioinformatics tools for further processing and interpretation. Created with BioRender.com and adapted from "DNA microarray" and retrieved from "Microarray Editable Results (Layout)".

2.4.2 Quantitative real-time polymerase chain reaction

In order to investigate the gene expression modulation of genes related to oxidative stress (*glutathione peroxidase* (*GPx*), *superoxide dismutase* (*SOD*) *2*, and *heme oxygenase* (*HMOX*) *1)*, and inflammation (*interleukin* (*IL*) *6*, *IL8,* and *tumor necrosis factor alpha* (*TNFα*)) in oral gingival fibroblasts following ONP extract exposure and to validate the findings of the transcriptomic analysis, the quantitative real-time polymerase chain reaction (qPCR) technique was used. This involves the amplification of the targeted DNA sequence over several cycles and the simultaneous quantification by measuring the fluorescence signal. In order to quantify gene expression of target genes, they were normalized against the housekeeping gene hypoxanthine-guanine phosphoribosyltransferase (HPRT). Exposed samples were then compared to the respective negative control.

2.5 Detection of changes on protein level

2.5.1 Fluorescence-activated cell sorting

In order to further validate the findings of the transcriptomic analysis on protein level of 16HBE cells in submerged condition, the release of selected cytokines into the cell culture medium was examined using fluorescence-activated cell sorting (FACS). Therefore, a bead-based cytokine panel kit was used, which allows the simultaneous detection of several cytokines in the cell supernatant.

Figure 11 depicts a schematic representation of the FACS analysis principle. The beads are coated with capture antibodies that bind to specific cytokines. To distinguish the different cytokines, each bead type is uniquely color-coded with a fluorescent dye. In a subsequent step, the sample is incubated with a detection antibody that binds to the captured cytokine. The detection antibody is also labelled with a fluorescent dye, allowing for detection by the photo detector following excitation by the laser beam of

the flow cytometer [169]. Side scatter (SSC) and forward scatter (FSC) are the two key measurements obtained, of which FSC measures the size of the target and SSC measures the target complexity [170]. Further, each cytokine is differently fluorescently labeled and has to be detected by a different detector [169, 170]. The obtained signals can be translated into the quantity of the abundant cytokines by comparing the fluorescence intensity to a standard curve [169].

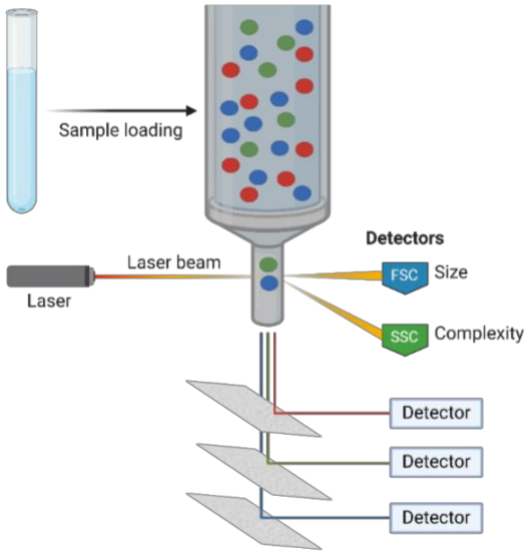

Figure 11. Schematic representation of the fluorescence-activated cell sorting (FACS) principle. The fluorescently labelled cytokines are individually excited by a laser beam and the resulting fluorescence is detected by the respective detector. Abbreviations: FSC – Forward scatter; SSC – Side scatter; Created with BioRender.com and adapted from "FACS sorting principle".

2.5.2 Enzyme-linked immunosorbent assay

In order to validate the findings of the transcriptomic analysis on protein level of 16HBE cells at ALI, the release of selected cytokines into the basal cell culture medium was examined using enzyme-linked immunosorbent assay (ELISA). For this purpose, a sandwich ELISA was used. This assay is performed on a microtiter plate and involves four essential steps (Figure 12). Firstly, the capture antibody is added to the plate followed by addition of the cell culture supernatant containing the cytokine of interest, which the detection antibody binds. In a third step, the detection antibody, specific to the cytokine is added. Finally, the detection antibody is conjugated to an enzyme that catalyzes a reaction with substrate, resulting in a change of color. The color change can be photometrically measured and is proportional to the amount of cytokine present in the sample. [171]

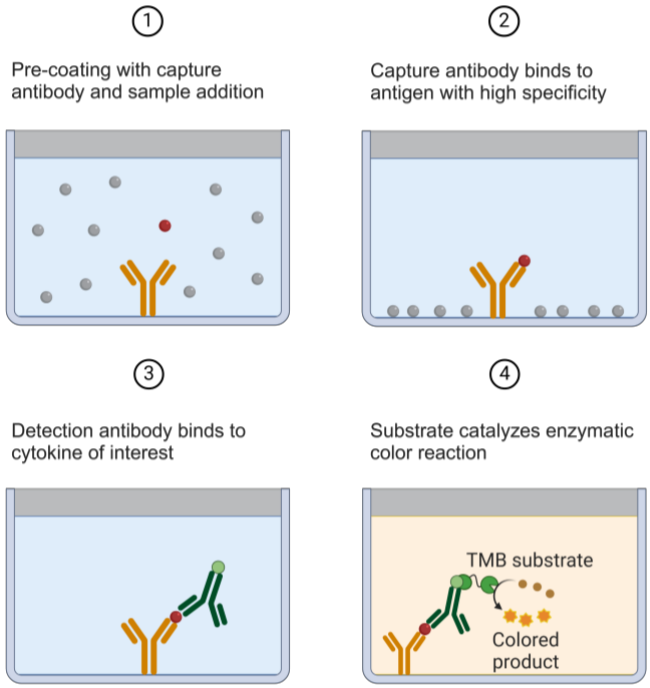

Figure 12. Schematic representation of the enzyme-linked immunosorbent assay (ELISA) workflow. The workflow consists of four main steps with (1) showing the pre-coating with the capture antibody and the sample addition which is bound to the capture antibody in step (2). Panel (3) shows the addition and binding of the labeled detection antibody which is catalyzed by a substrate in step (4) to a colored product which can be measured photometrically. Created with BioRender.com and adapted from "Sandwich Elisa".

2.6 Optimization of the impinger method for the collection of flavored e-liquid aerosols in cell culture medium (unpublished data)

The impinger method on the linear Borgwaldt LM4E vaping machine (Borgwaldt GmbH, Germany) was optimized for the following parameters: Type of impinger, volume of cell culture medium, use of fetal calf serum (FCS) - as contained proteins might bind flavors [172] - and power setting on the e-cigarette (LYNDEN Play, Germany) (Table 1) in order to increase the flavor yield in cell culture medium.

Table 1. Optimized parameters for collecting flavored e-liquid aerosols in cell culture medium using a linear vaping machine.

Parameter	Options
Type of impinger	Borgwaldt, Simax (frit), Sarstedt
Volume of cell culture medium	15 and 30 mL
% of FCS	0% FCS and 5% FCS
Power setting on e-cigarette	Low (20 W) and high (30 W)

The experimental design is schematically depicted in Figure 13. The vaping machine consists of an e-cigarette holder with an activation button on one side and a pump module on the other side. In between two impingers filled with cell culture medium are connected. When the e-cigarette is activated, the pump module draws the e-liquid aerosols through the cell culture medium. The e-cigarette was filled with 2 mL of lab-made e-liquid (Chapter 2.1.2) and operated at 20 W. For aerosol generation, the standard ISO 20768:2018 was used with a puff volume of 55 mL, a puff frequency of 1 puff every 30 s, a puff duration of 3 s, and a rectangular puff profile [157]. In total, 100 puffs were collected in 15 or 30 mL cell culture medium. The tank containing the e-liquid was weighed before and after vaping in order to determine the

mass of consumed e-liquid. Three independent experiments were performed for each of the options within the parameters.

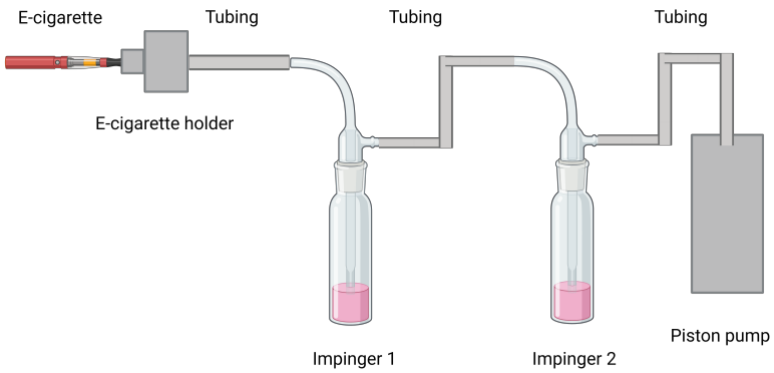

Figure 13. Schematic representation of the linear vaping machine. This setup connects the electronic cigarette (e-cigarette) to the e-cigarette holder, the impingers filled with cell culture medium, and the piston pump. Created with BioRender.com.

2.7 Analytical experiments

2.7.1 Aerosol monitoring during 16HBE Air-Liquid Interface exposure with lab-made e-liquids

During ALI aerosol exposure, an online aerosol monitoring was performed to control the quality of the aerosol composition and the process reproducibility. The e-liquid components might undergo thermal stress reactions during the vaping process. Up to the author's knowledge, it is the first time that simultaneous online e-cigarette aerosol exposure and online aerosol monitoring was combined with a focus on a puff-by-puff output of flavors and base-solvents. Therefore, an orthogonal acceleration time of flight mass spectrometer (OA-TOF MS, Photonion GmbH, Germany) was used. It was combined with single photon ionization (SPI) for the analysis of PG/VG and resonance enhanced multi-photon ionization (REMPI) for the analysis of the flavors both in sub second time resolution as described in Heide et al. [173].

2.7.2 High-performance liquid chromatography coupled with a diode array detector for the determination of nicotine in oral nicotine pouches extracts

To determine the nicotine concentration in ONP extracts, a high-performance liquid chromatography (HPLC) coupled with a diode array detector (DAD) was utilized. This technique enables the separation of individual analytes within a mixture, which are subsequently detected by the DAD. The system consists of a mobile and a stationary phase. With the help of a pump, the mobile phase carries the analytes over the stationary phase, where interactions between the analyte and the stationary phase result in differential retention. As a result, the analytes are eluted at distinct time intervals, allowing for their individual detection and quantification. [174] The interactions between the analytes and the stationary phase in the here employed reverse HPLC, are mainly dependent on polarity [175]. A DAD was connected to the instrument for the quantification of nicotine, which was detected at 260 nm.

2.7.3 Gas chromatography coupled with mass spectrometry for the determination of flavors in oral nicotine pouches and cell culture medium

Sample processing

Appropriate sample preparation is crucial before detection and quantification can be performed. Two different extraction methods were applied in this study. Liquid-liquid extraction (LLE) was employed for

extraction of flavors from ONP extracts. For extraction of flavors in cell culture medium instead, headspace solid-phase microextraction (HS-SPME) was used.

LLE is based on the partitioning of the analyte between an aqueous phase and an organic, water-insoluble phase. Extraction is also influenced by the pH value, which is why flavors were extracted under acidic and basic conditions. [176]

For identification and quantification of flavors in cell culture medium, HS-SPME was employed as the extraction technique. Unlike LLE, HS-SPME offers the advantage of not requiring solvents. Additionally, manual sample processing is not needed. Therefore, the loss of volatile substances is minimized. In this technique, analytes from the aqueous solution, in this case the cell culture medium, are extracted using a coated silica fiber which is positioned above the solution without direct contact. This extraction method relies on the diffusion of the analytes between the three phases: the aqueous phase, the gaseous phase (headspace) and the absorbing phase (the coated fiber). Once equilibrium is reached, the analytes are adsorbed onto the fiber. The extraction process can be optimized by adjusting parameters such as temperature, pH, agitation, salt addition to the aqueous phase, and the type of fiber coating, among others. The fiber is placed into the GC injector, where the analytes are thermally desorbed for further analysis. [177, 178]

Gas chromatography/Mass spectrometry

To identify flavor compounds in 50 ONPs or cell culture medium, gas chromatography (GC) coupled with a mass spectrometer (MS) for detection was utilized. This analytical technique is widely used for the separation, detection and quantification of analytes in complex mixtures, particularly for volatile substances such as flavors. The sample is introduced to the column using helium as carrier gas. The sample is volatilized by heating, and the individual analytes interact with the column and are retained based on their specific chemical properties. Key factors that influence the retention time of the analytes include the column temperature, analyte volatility and column dimensions. [179] For detection and quantification of the analytes, an MS was connected to the GC. In the MS, analytes are fragmented by electron ionization, where high-energy electrons cause the analytes to ionize. The resulting charged ions are then directed to the quadrupole mass filter which sorts them by their mass-to-charge ratio using an electric field. [180] The identification of flavor compounds in ONPs was based on their specific EI-MS-spectra using NIST 11, FFNSC 3, and an in-house aroma library. The quantification of flavors in cell culture medium was achieved using the peak area, an internal standard and a calibration curve.

3 Results and discussion

3.1 Oral nicotine pouches - Analytical identification of ingredients with a focus on flavors and toxicological screening

The goal of the studies (Mallock-Ohnesorg & Rinaldi et al., 2023 and Rinaldi et al., 2023) was a qualitative flavoring screening in 50 ONP market samples and the toxicological assessment of five selected ONP extracts as well as the reference snus CRP1.1. Further, the nicotine content in ONP extracts was quantified. Together with the information from EFSA and JECFA regarding acceptable daily intake (ADI) assessments and the toxicological screening approach used in this study, a risk estimation of nicotine pouch products was performed. Up to the author's knowledge, it is the first time that the ONP ingredients with a focus on flavoring compounds were analytically determined. Altogether, in the 50 nicotine pouches investigated, a total of 186 chemicals were identified (Mallock-Ohnesorg & Rinaldi et al., 2023). Out of the 186 identified substances, 86 are depicted in Figure 14, which were either found in at least 3 pouches or had a toxicological concern such as an established ADI (28 substances), a classification according to IARC (3 substances), or a hazard classification according to the CLP Regulation (EC) No. 1272/2008 (8 substances) [181]. In addition, 13 substances were unauthorized for the use as food additives in the EU.

Methyl eugenol, benzophenone, and β-myrcene were found in ONPs and were assigned a 2B classification by IARC, indicating potential carcinogenicity to humans based on sufficient evidence in animal studies but limited evidence in humans. While methyl eugenol is not authorized as food additive in the EU, there is no restriction for benzophenone and β-myrcene in terms of authorization. The relevant CLP hazard statements were limited to H317 (may cause an allergic skin reaction), H361d (suspected of damaging the unborn child), and H372 (causes damage to the lung through prolonged or repeated exposure). Several compounds detected such as isoeugenol, geraniol, citral, limonene, linalool, and carvone, carry the H317 classification. With the exception of carvone, they are additionally listed as fragrance allergens in Annex III of the Cosmetics Regulation (EC No. 1223/2009) [182]. As elaborated in Chapter 1.5.3, linalool, when oxidized, exhibits significantly enhanced skin sensitizing potential compared to its non-oxidized form [116]. In conclusion, the direct application of ONPs containing these flavor compounds to the oral mucosa presents a potential risk for localized adverse effects due to allergic reactions.

In total, 28 compounds with established ADIs by EFSA or JECFA were identified in ONPs, are discussed below only for nicotine pouch products 1 to 5, in the context of the toxicological screening.

Another safety concern is presented by detected compounds that are not authorized for addition in food items (Figure 14). Six of these substances are no flavoring substances and are suspected impurities from the production process. Myosmine is a tobacco alkaloid, an impurity of nicotine, and has been previously found in ONPs [183]. Similarly, tobacco-specific nitrosamines, which are carcinogenic compounds and impurities associated with nicotine extraction, have been detected in ONPs, albeit at low concentrations [184]. Other identified compounds, such as isomenthol, isomenthyl acetate,

neoisomenthol, pulegone, and ledol could be impurities from plant extracts [185-189]. Tris(2-butoxyethyl) phosphate could have been incorporated intentionally in the pouch material as it is commonly used as a flame retardant [190]. However, it has been associated with hepatotoxicity and carcinogenicity [191]. Saccharin is a sweetening agent that is regulated under the Regulation (EC) No. 1333/2008 on food additives but does not fall under the Regulation (EC) No. 1334/2008 on flavoring substance. All of these compounds should not be present in ONPs as they have no function, contribution to toxicity is unknown and are not allowed to be added to food items. In order to be sure, whether these substances are impurities or occur more frequently in ONPs, these should be monitored by responsible health authorities.

The analytical screening of flavors in pouches was conducted qualitatively. It offered new insights into the ingredients contained in ONPs, representing a novel contribution to the field. This non-targeted screening analysis enabled the detection of unexpected compounds, particularly substances neither authorized as food additives nor possessing flavoring properties. The identification of these substances potentially introduced into ONPs, whether intentionally or unintentionally, may be crucial for surveillance authorities' monitoring activities. Furthermore, this analysis provided a foundation for an initial risk assessment based on the identified substances and their toxicological properties.

No detection limits could be determined for the numerous identified substances. Compounds present below detection limits may have been missed or obscured by matrix effects. For a comprehensive risk assessment, a quantitative screening would be necessary to determine the concentrations and detection limits of these compounds. This would enhance understanding of whether ADIs could be exceeded during consumption and to what extent. The absence of quantification represents a limitation of this study. Nevertheless, this study was an initial investigative step to lay a foundation for the understanding of ONP characteristics.

In conclusion, questions emerge regarding the necessity of including numerous flavorings in lifestyle products such as ONPs, particularly those classified as possibly carcinogenic or capable of causing adverse skin reactions at the site of product use. This concern is amplified by potential mixture effects, which introduce greater uncertainty the more compounds are combined. From a health perspective, it is advisable to restrict compounds of concern, which would still leave sufficient flavorings available for ONPs, ensuring variety across flavor categories. From the perspective of reducing ONP attractiveness to young consumers and non-smokers, restrictions on additional flavors beyond those of toxicological concern might be warranted.

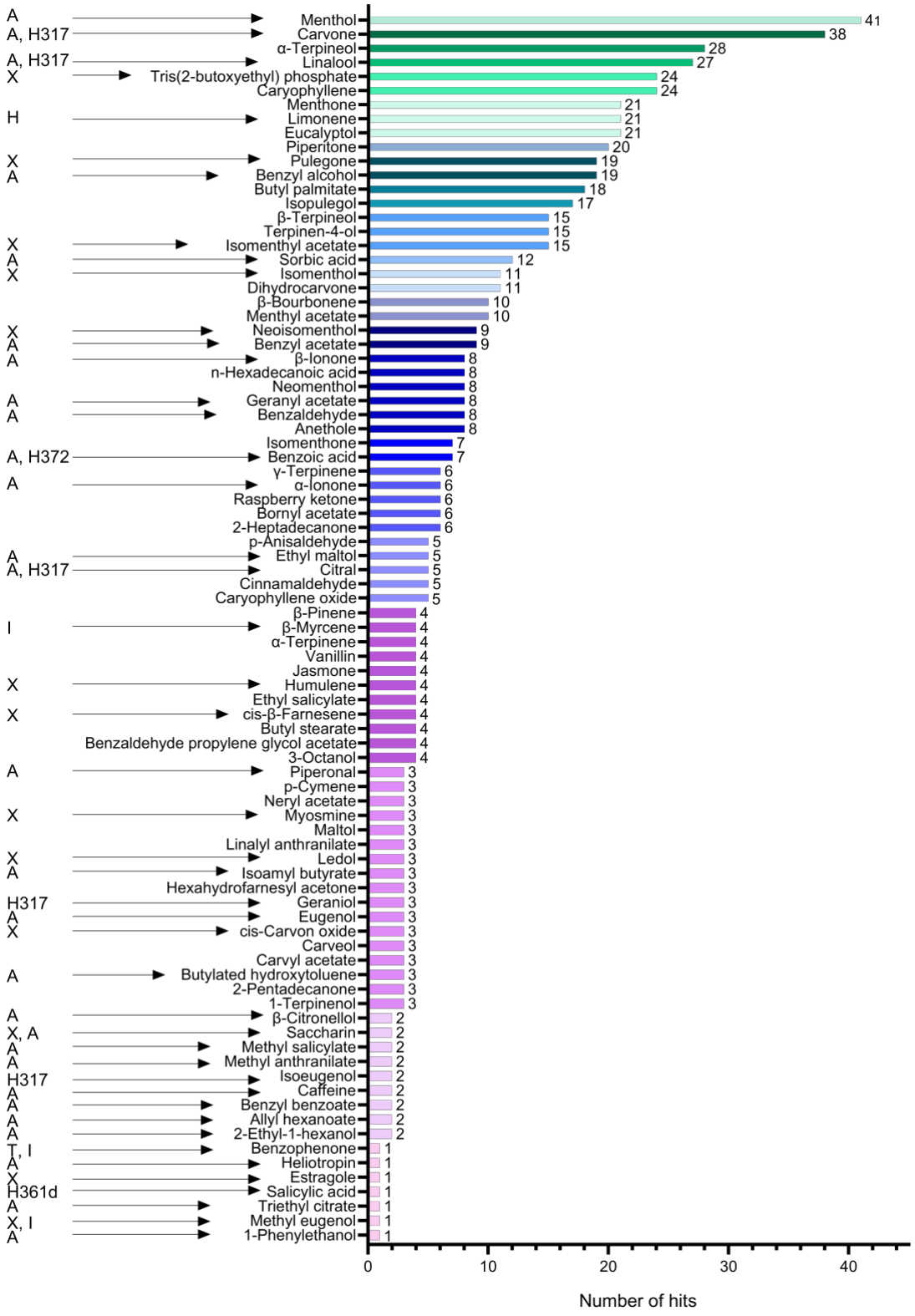

Figure 14. Identified flavors in oral nicotine pouches. The graph illustrates the 86 substances that were detected in at least 3 oral nicotine pouches or had at least one toxicological concern. The arrows indicate substances with a toxicological concern and the letters specify the remark such as (I) a classification according to the International Agency for Research on Cancer (IARC), (A) an established acceptable daily intake (ADI), (T) a tolerable daily intake, (H) a hazard classification according to the European Regulation on Classification, Labelling and Packaging (CLP), or (X) were not authorized by the European Food Safety Authority as food additives.

Following the analytical screening of ONPs, a toxicological screening was performed with five chosen nicotine pouch products and the reference snus CRP1.1 (Rinaldi et al., 2023). Those were selected based on the indicated nicotine content and flavor description on the packaging in order to cover a range of both. The results of the toxicological screening in HGF-1 cells revealed multiple adverse cellular responses to ONP extract exposure (Figure 15A). Specifically, certain ONP extracts induced elevated expression of the proinflammatory marker *IL6*, enhanced oxidative stress, increased LDH release, and decreased metabolic activity. Oral nicotine pouch products 2 and 3 demonstrated the most pronounced effects, causing dose-dependent decreases in metabolic activity, elevated oxidative stress and augmented *IL6* expression. The latter was observed in response to oral nicotine pouch product 2 only. Oral nicotine pouch products 4 and 5 showed reduced metabolic activity for the 60-min extracts and oxidative stress. These effects appear to be independent of nicotine content, as oral nicotine pouch products 4 and 5 contained higher nicotine concentrations across all extraction times compared to oral nicotine pouch products 2 and 3, with the exception of the 10-min extract of oral nicotine pouch products 3 and 5 (Figure 15B).

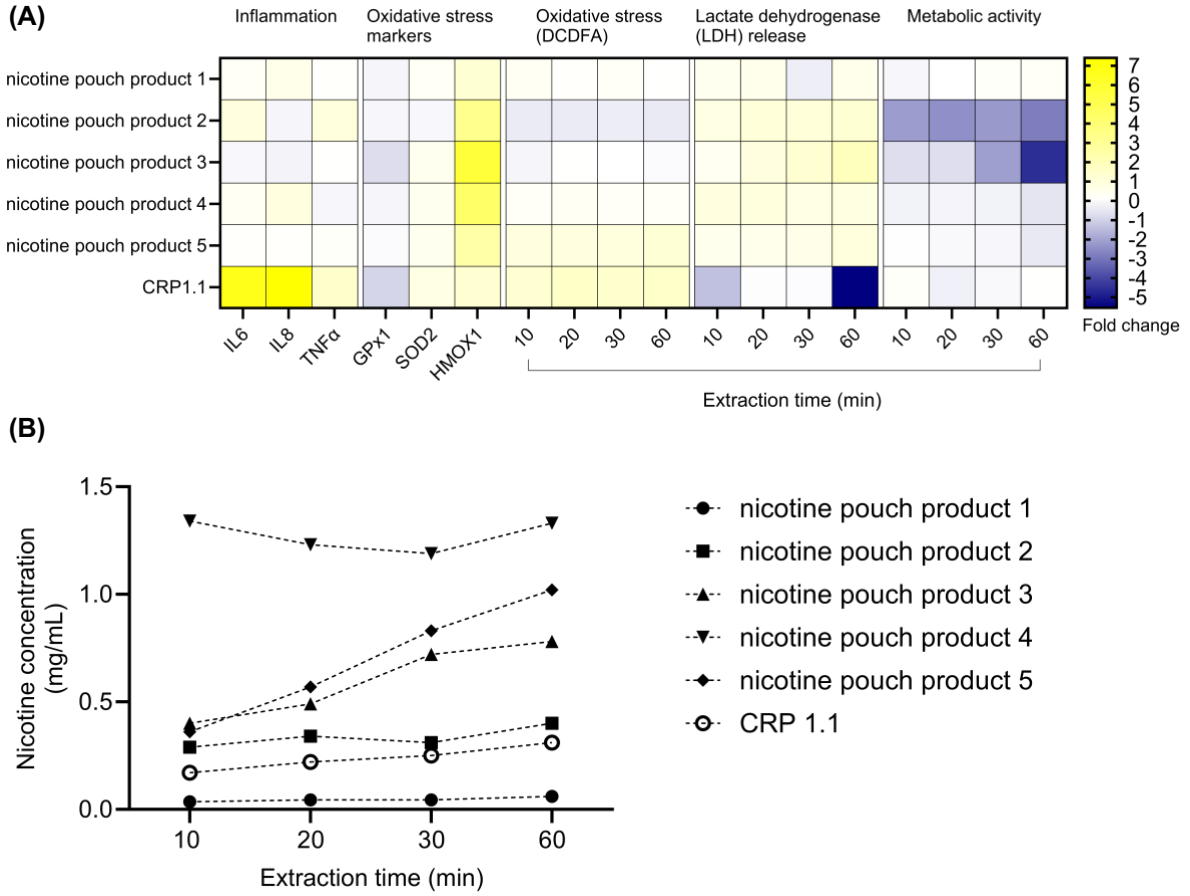

Figure 15. The toxicological screening of oral nicotine pouches in human gingival fibroblasts. The screening consisted of (A) measuring inflammation, oxidative stress and cytotoxicity/cell viability following exposure of oral gingival fibroblasts to oral nicotine pouch product extracts. Panel (B) shows the quantified nicotine content for each nicotine pouch product extract at 10, 20, 30, and 60 min.

The observed toxicity was hypothesized to derive from specific flavoring compounds rather than nicotine content. Notably, benzyl benzoate and benzaldehyde, which were present exclusively in oral nicotine

pouch products 2 and 3, respectively (Table 2), possess JECFA-established ADIs of 5 mg/kg bw by JECFA and a harmonized classification of the acute oral toxicity category 4 according to the Regulation ((EC) No. 1272/2008) for Classification, Labelling and Packaging [159]. Benzaldehyde demonstrates cytotoxicity against human peripheral blood mononuclear cells at concentrations exceeding 10 µg/mL [192] and disrupts normal ROS homeostasis through GPx inhibition [193]. In the present study, oral nicotine pouch product 3, containing benzaldehyde, exhibited a trend towards *GPx* downregulation (Figure 15A).

Table 2 lists the flavors that have an established ADI attributed by EFSA or JECFA, and were found in at least one of the five nicotine pouch products used for the toxicological assessment. In those products, 53 chemicals were detected in total, of which 17, 16, 24, 23, and 12 substances were found in nicotine pouch products 1, 2, 3, 4, and 5, respectively.

Table 2. List of identified flavors in oral nicotine pouches. Shown are the identified flavors in at least one of the nicotine pouches used for the toxicological screening with an acceptable daily intake (ADI) allocated by either the European Food Safety Authority (EFSA) or the Joint FAO/WHO Expert Committee on Food Additives (JECFA). Lastly, other identified flavors are listed that were used in the study concerning inhalation toxicity of flavors.

Total number of chemicals identified: 186

Flavors with established ADI by (Nicotine pouch product number)	**EFSA (mg/kg bw)**	**JECFA (mg/kg bw)**	**"Average scenario" (5 pouches per day) – limits of substance per pouch (mg)**	**"Worst-case scenario" (20 pouches per day) – limits of substance per pouch (mg)**
Benzyl alcohol (1, 2, 3, 5)	4 [194]	5	35	8.75
Carvone (1, 2, 3, 4, 5)		D-Carvone: 1	7	1.75
Citral (1)		0.5	3.5	0.88
Geranyl acetate (1)		0.5	3.5	0.88
Linalool (1, 2, 3, 4)		0.5	3.5	0.88
Benzyl benzoate (2)		5	35	8.75
Menthol (1, 2, 3, 4, 5)		4	28	7
Benzaldehyde (3)		5	35	8.75
Ethyl maltol (5)		2	14	3.5
Methyl anthranilate (5)		1.5	10.5	2.63
Other identified flavors used in the e-cigarette study (Nicotine pouch product number)				
Cinnamaldehyde (1)	-			
Eugenol (1)	-	ADI 2.5 (JECFA)	17.5	4.38
Vanillin (5)	-			

It is likely that specific flavors or the mixture of flavoring chemicals might induce adverse systemic or local effects such as oral lesions. Our calculations utilizing ADI values demonstrate potential safety threshold exceedance. Based on a patent documentation by Phillip Morris [195, 196], flavoring compounds constitute 1% of total pouch mass. With a mean pouch weight of 0.64 g [184], this translates to a flavoring agent exposure of 6.4 mg per pouch. Assuming a 50% ADI exhaustion to accommodate dietary and cosmetic exposure, consumption of 5-20 pouches per day [35, 197] exceeds safety thresholds for compounds with ADIs up to 1-4 mg/kg bw (Table 2), respectively, without taking into account any further dietary sources. Therefore, adverse effects cannot be ruled out to be induced by ONP use containing these substances. Further, repeated placement at the same part of the gum, represents additional concerns regarding local adverse effects. Concentrations at application sites likely exceed those compared to ingestion and might contribute to local adverse effects. Additionally, if consumers use several differently flavored pouches a day, this increases toxicant exposure. As 22 flavors were found in at least 10 pouches (Figure 14), it can be expected that ADIs might be exceeded although different pouches are used.

Local adverse effects are the main concern regarding the use of ONPs. Clinical studies, although limited, provide supporting evidence of adverse effects following ONP use [198]. A study assessing adverse effects in ONP users reported that 48%, 37%, and 21% experienced oral lesions, throat soreness, and mouth discomfort, respectively [199]. Histological examination revealed white oral lesions and parakeratosis following two years exclusive ONP use [200]. Furthermore, an observational study reported frequent gum complications associated with ONP use [201]. The potential progression of these oral mucosal alterations to malignant lesions following chronic exposure requires further investigation. However, the present investigation revealed elevated *IL-6* expression following exposure to oral nicotine pouch product 2 and reference snus CRP1.1, concurrent with oxidative stress (Figure 15). Inflammatory cytokines, including IL-6, IL-8, and TNFα, are associated with both oral cancer [202] and mucosal changes induced by smokeless-tobacco use [198]. Inflammation and oxidative stress are key factors in the development of oral lesions and carcinogenicity [203], and these processes are interrelated. A well-established link to smoking and tobacco use further highlights their role in oral pathogenesis [204]. In agreement with the present study, Shaik et al. [135] showed that flavored and unflavored ONPs induced cytotoxicity, ROS, and the release of inflammatory cytokines IL-6, IL-8 and TNFα, however depending on flavor composition. Some of the ONPs elicited a more toxic response than snus products [135], which is supported by the present study.

In conclusion, ONPs should not be considered harmless, although the tobacco industry showed that they contain fewer harmful substances compared to tobacco-containing snus [112, 205], and it has also been reported that oral lesions from tobacco-containing snus improved with the use of ONPs [206].

3.2 Optimization of a commonly used vaping method for submerged cell exposure – is it suitable for flavor toxicity assessment? (Unpublished data)

The aerosol collection method using commercially available or self-built [124] smoking machines is commonly used in toxicological and analytical studies investigating e-cigarette toxicity [70, 72, 123, 125,

126, 207, 208]. This method has also been employed by the tobacco industry, specifically to compare the in vitro effects of e-cigarette aerosols with tobacco smoke, both collected in cell culture medium, which is then referred to as conditioned medium. However, these studies often lack analytical characterization of the conditioned medium. To the best of the author's knowledge, no study has yet analyzed the cell culture medium for trapped flavors or chemicals of interest. To address this critical gap in methodology, a systematic investigation of flavor transfer from the e-liquid to the conditioned medium, method optimization, and subsequent cytotoxicity were conducted.

Optimization of the impinger method

The impinger method was optimized for three key parameters. First, three different impingers were compared (Borgwaldt, Sarstedt, and a Simax impinger with frit), using 15 mL and 30 mL of cell culture medium, with either 0% or 5% FCS. Table 3 summarizes the optimized vaping method parameters. The flavor concentration in the conditioned medium was measured for each of the parameters. The condition leading to the highest flavor outcome was considered the optimum.

Table 3. List of the optimized parameters for the impinger method and the optimal outcomes. The parameters shown were optimized to increase the flavor yield in the conditioned medium after vaping to be used for toxicological assays. Abbreviations: FCS – fetal calf serum; e-cigarette – electronic cigarette.

Parameter	Options	Optimal outcome
Impinger	Borgwaldt, Simax (frit), Sarstedt	Sarstedt
Volume of cell culture medium	15 and 30 mL	15 mL
Use of FCS	0% FCS and 5% FCS	5% FCS
Power setting on e-cigarette	Low (20 W) and high (30 W)	No difference

For each of the parameters, except the power setting, Figure 16A1 – C1 show the mean concentration of collected flavor in the conditioned medium, while Figure 16A2 to C2 show the fraction of collected flavor in conditioned medium compared to the consumed amount of e-liquid. Since e-liquid consumption was not consistent for each vaping run, the collection rate seems consistent across the different conditions within one parameter (Figure 16A2-C2). Importantly, the mean concentration of collected flavor differs significantly across the different conditions within the same parameter (Figure 16A1-C1). This inconsistent e-liquid consumption suggests that commercial e-cigarette devices, which are not standardized, do not function consistently.

The mean flavor content was highest in the Sarstedt impinger for all flavors (Figure 16A). In addition, the Borgwaldt and Simax (frit) impinger demonstrated higher standard deviations in flavor concentrations, while the Sarsted impinger consistently showed the lowest variation. Therefore, it was continued with the Sarstedt and Borgwaldt impinger. The results confirmed that Sarstedt had the lowest variation within all conditions tested (Figure 16B). Testing the Borgwaldt and Sarstedt impinger filled with 15 mL and 30 mL of cell culture medium, confirmed Sarstedt's superior consistency across all flavors. In addition, the impingers filled with 15 mL showed a higher flavor collection rate compared to 30 mL (Figure 16B). Building on these results, different FCS contents were tested using the Sarstedt impinger, revealing that medium with 5% FCS demonstrated the highest collection rate for all flavors

tested (Figure 16C). It was observed, that flavor transfer efficiency varied, correlating with their water solubility: Linalool (1.59 g/L) showed the poorest transfer efficiency, followed by cinnamaldehyde (1.42 g/L), and eugenol (0.7 g/L) with the highest transfer efficiency [209-211]. Limonene, being insoluble, consistently yielded values below the limit of quantification (data not shown) [212]. Interestingly, while many parameters affected the collection rate of flavors, changing power settings on the e-cigarette led to higher e-liquid consumption yet did not influence flavor transfer efficiency. However, the power setting might affect carbonyl formation [90, 92] or other thermodynamic processes not examined in this study.

In conclusion, the present study demonstrated that certain parameters had an influence on the concentration of captured flavor. The concentration varied especially depending on the volume of cell culture medium used and the addition of FCS. The impact of the impinger was minor compared to the other parameters, while the power setting of the e-cigarette had no influence. These findings emphasize how methodological choices can significantly impact experimental outcomes.

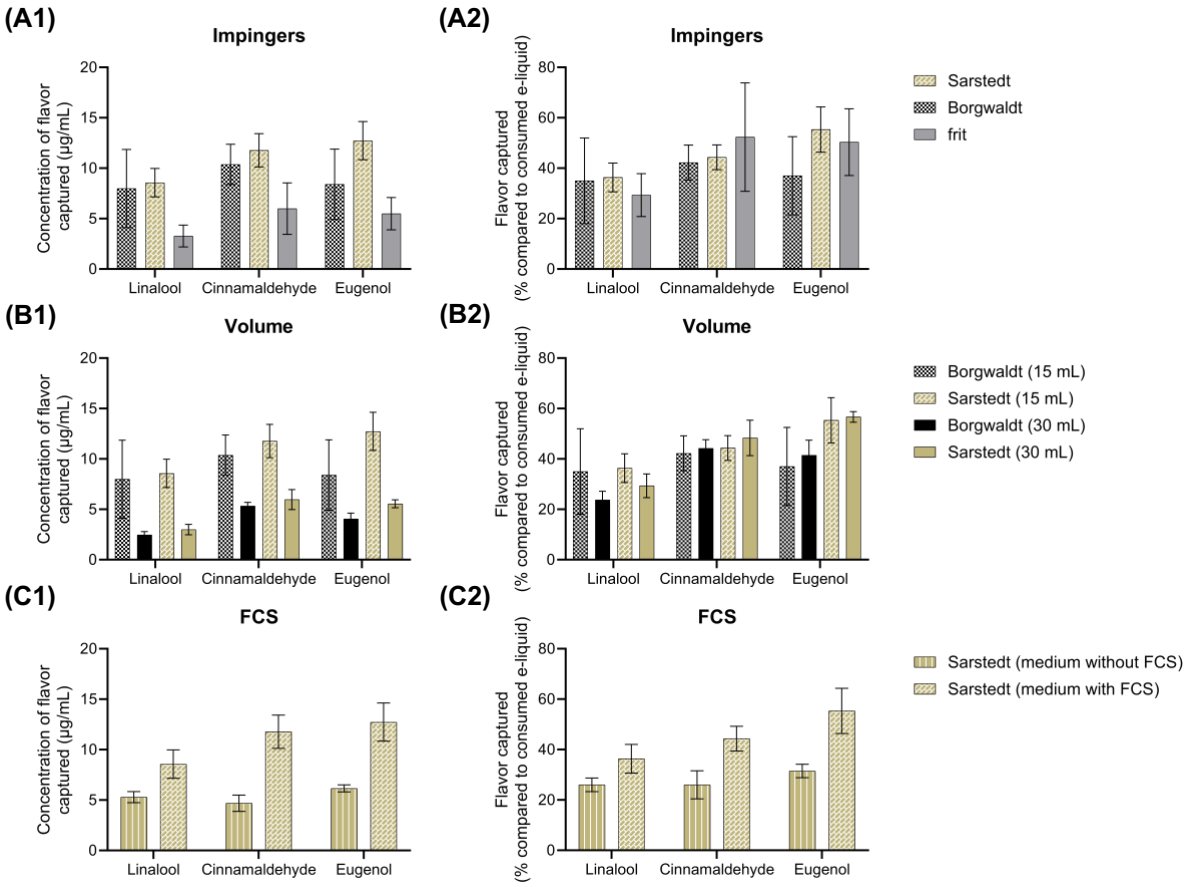

Figure 16. Flavor yields for each parameter using the impinger method. The aerosol collection method was optimized by adjusting (A) the type of impinger, (B) the volume of cell culture medium, and (C) the concentration of fetal calf serum (FCS). The graphs (A1, B1, C1) depict the mean concentration of collected flavors linalool, cinnamaldehyde, and eugenol. The graphs (A2, B2, C2) depict the collected flavor in % compared to the amount of aerosolized e-liquids. The parameter that resulted in the highest flavor yield was chosen as the optimum (refer to Table 3). Abbreviations: FCS – fetal calf serum.

Cytotoxicity findings

First, the cytotoxicity of the flavoring agent cinnamaldehyde diluted in cell culture medium was investigated, without aerosolization. A549 and 16HBE cells were exposed in submerged conditions

(Figure 17). Dose-dependent cytotoxicity was observed starting from 10 µg/mL in both cell lines, with metabolic activity dropping below 5% at 10 and 30 µg/mL concentrations in A549 cells (Figure 17A). Next, custom-made e-liquids containing varying concentrations of cinnamaldehyde (100, 250, or 700 µg/mL) were investigated, by vaping them using the Borgwaldt smoking machine under standard ISO conditions. The generated aerosol was captured in 30 mL cell culture medium, theoretically yielding concentrations of 0.4, 10, or 30 µg/mL assuming 100% transfer efficiency. Surprisingly, when this conditioned medium was applied to A549 cells, no cytotoxicity was observed (Figure 17B).

It was suspected that the transfer efficiency from flavor in the e-liquid to the conditioned cell culture medium was less than 100%. Previous experiments demonstrated that flavors captured in isopropanol transferred efficiently, though at less than 100% and varying with power settings and specific flavors analyzed [70]. Given the low water solubility of the flavoring compounds, it was hypothesized that isopropanol would show better transfer efficiency compared to cell culture medium [213] .

Having established optimal parameters for flavor transmission, the improved methodology was further validated. Using the optimized vaping method parameters, cinnamaldehyde e-liquid significantly decreased metabolic activity in 16HBE cells (Figure 17D), consistent with the measured concentration of approximately 10 µg/mL in conditioned medium (Figure 16C1). These results verified the earlier findings, which had shown significant decrease in metabolic activity at this same concentration when using pure cinnamaldehyde diluted in cell culture medium (Figure 17C). This alignment between the results of the optimized method and applying the pure flavor in cell culture medium demonstrated the success of the methodological improvements.

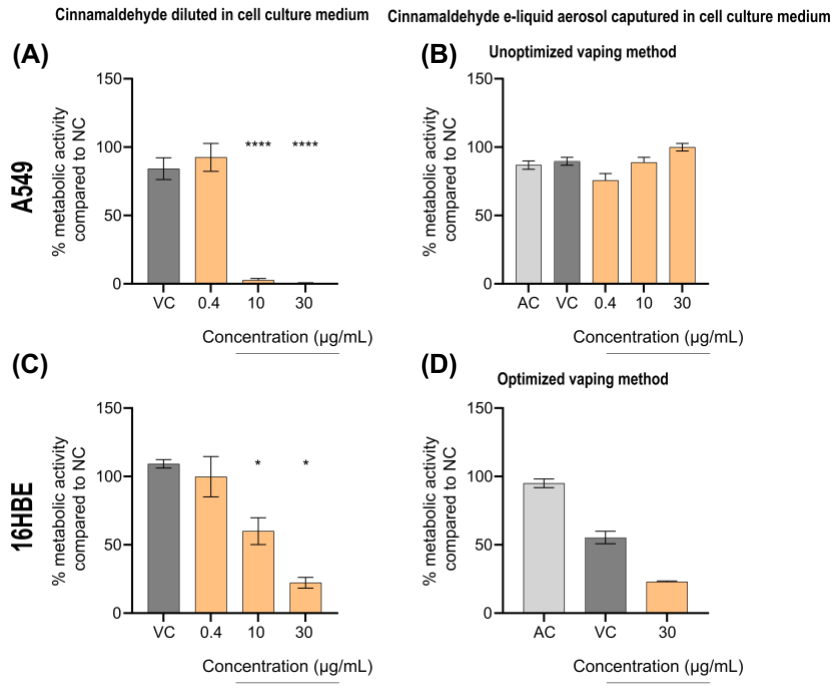

Figure 17. Metabolic activity of A549 and 16HBE cells following exposure to pure cinnamaldehyde in cell culture medium and cinnamaldehyde in conditioned medium using the optimized and unoptimized impinger method. In panels (A) A549 cells and (C) 16HBE cells were exposed to cinnamaldehyde diluted in cell culture medium at three different concentrations. In panels (B) A549 cells and (D) 16HBE cells were exposed to conditioned medium (cinnamaldehyde e-liquid or propylene glycol (PG)/vegetable glycerin (VG) e-liquid aerosolized and captured in cell culture medium). In panel (B) the unoptimized vaping method was used while in graph (D) the optimized vaping method was used.

The transfer rate of cinnamaldehyde into conditioned medium was improved from 5.3 µg/mL to 11.7 µg/mL (Figure 16B1). Notably, the outcomes of this present study align closely with the existing

body of literature. Industry studies using 20 mL of cell culture medium with 0% FCS typically report minimal or no (cyto-) toxicity from e-cigarettes compared to conventional cigarettes. This could be explained with the results from the present study, in which flavor transmission is significantly poorer with higher volume of cell culture medium and 0% FCS (Figure 16B1 + C1) [214-216]. This further strengthens the need for more research on flavor toxicity, since the tobacco industry has often shown, using this unoptimized method, that e-cigarettes have a minor effect in vitro compared to tobacco smoke. The methodological variations identified in the optimization process suggest that these industry findings may need to be reevaluated using standardized, optimized protocols. In contrast, other researchers using 10 mL complete cell culture medium demonstrated substantial cytotoxicity, correlating with flavorings rather than nicotine content, supporting the findings discussed in Chapter 3.1 [217]. Particularly noteworthy was the work of Behar et al., which used similar number of puffs/mL cell culture medium as the present study and also demonstrated flavor-dependent cytotoxicity [70]. Importantly, none of these previous studies documented method optimization, contributing to uncertainties in e-liquid toxicity assessment. The variations in methodology – different vaping machines, impingers, medium compositions, vaping regimes, and medium volumes- make direct comparisons challenging and highlight the need for standardization in the field.

Drawing conclusions from the method optimization and follow-up cytotoxicity assessment, several important recommendations emerge for future research and standardization efforts. These findings emphasize the urgent need for standardized guidelines in e-cigarette toxicological testing, particularly regarding flavors. Such guidelines should address multiple critical aspects: the optimization of vaping methodology, including impinger selection, standardization of cell culture medium constitution and volume, and the evaluation of different vaping regimes such as CORESTA, ISO, or new standards better reflecting user behavior. Additionally, analytical screening requirements for conditioned medium should be clearly defined.

Most importantly, our results underscore a crucial consideration for future studies: When assessing e-liquid mixture toxicity using the impinger method, results require critical review since not all flavors transfer efficiently to the conditioned cell culture medium. This fundamental understanding should be incorporated into future safety testing guidelines for e-liquids, ensuring more reliable and reproducible assessments of e-cigarette safety. When the impinger method is used, the parameters should be optimized for the specific compound. At least a quantification of the compound in the conditioned medium should be performed. This could be regulated in a future guideline for the safety testing of e-liquids. Further, parameters that need optimization, such as the impingers, the volume of cell culture medium, and the composition of cell culture medium should be defined.

3.3 Understanding the e-liquid flavors toxicity using two cell exposure methods

3.3.1 Aerosol monitoring at the Air-Liquid Interface

An online aerosol monitoring was connected to the custom-made aerosol generation system to validate the constant puff production and the presence of flavors in the aerosol. PG/VG was detected using SPI,

while cinnamaldehyde, eugenol, and vanillin were detected using REMPI. This setup also served as a quality control step, ensuring the reliable functioning of the e-cigarette over the whole exposure time of 20 puffs. The results were obtained on a puff-by-puff basis. Cinnamaldehyde was not detected at its nominal mass of 132 m/z, but ions with the mass signal of 104 m/z (Figure 18A) and 206 m/z (not shown) were identified. The latter suggests a chemical reaction of cinnamaldehyde with a thermal fragment of VG. In contrast, low relative intensities of eugenol and vanillin were detected (Figure 18B+C). The detection of vanillin, eugenol, and signal mass of 104 m/z were relatively stable over the 20 puffs and across the four replicates. PG's relative intensities were stable over the 20 puffs, whereas VG took the first two puffs to stabilize (Figure 18D-E).

Although the output of PG and VG remained consistent throughout a single experiment, the underlying cause for the variation between experiments for VG remains unidentified and the method needs further optimization. Yet, the results underscore the importance of online aerosol measurement for toxicological analysis of e-cigarettes at the ALI, serving as a quality measure to reduce the uncertainties in in vitro experiments with e-cigarettes. It was recently recommended in a review paper, that aerosols for exposure should be characterized for their main components and to use a quality control system by monitoring the aerosols [136]. In the present study, not all possible components in the aerosol were characterized, such as the formation of carbonyls, but the main compounds of interest, the flavors and PG/VG. The example of cinnamaldehyde illustrates the potential for new compounds to form during vaping, which must be considered in assessing the toxic potential of e-liquid flavors. Moreover, the low intensity of measured vanillin underscores that transmission efficiency can vary for each flavor as demonstrated in Chapter 3.2.

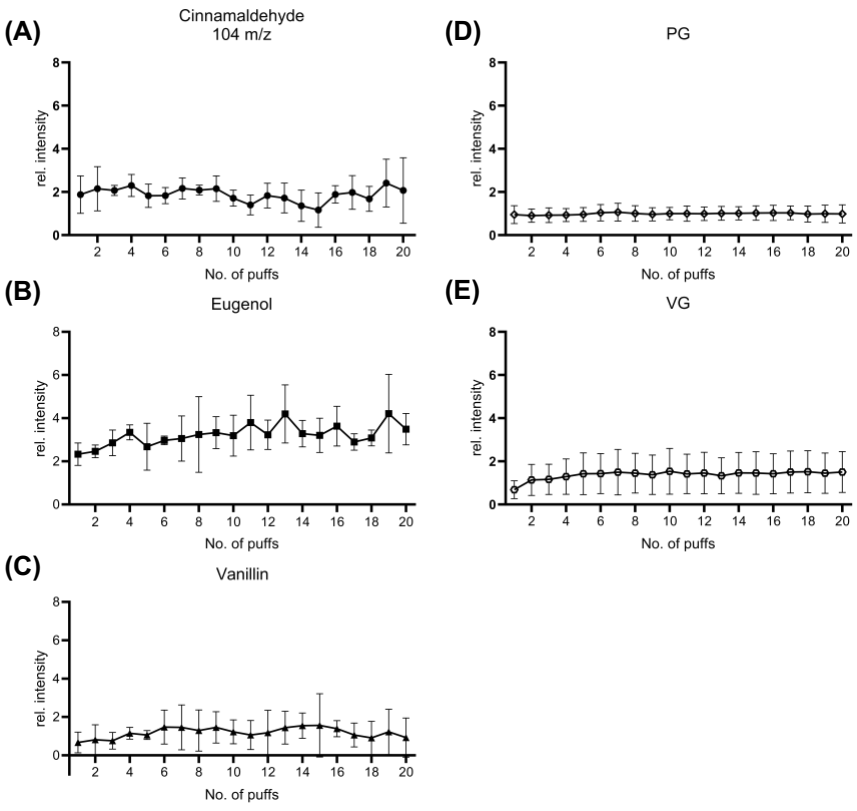

Figure 18. Results of the aerosol monitoring at the Air-Liquid Interface. The graphs show the measured relative intensity by each puff for (A) cinnamaldehyde fragment with a mass of 104 m/z, (B) eugenol, (C) vanillin, (D+E) the base-solvents (D) propylene glycol (PG), (E) vegetable glycerin (VG). The base-solvents were detected by single photon ionization and the flavors by resonance enhanced multi-photon ionization.

3.3.2 Transcriptomic changes following exposure to individual flavors using a submerged cell model and to aerosolized flavored e-liquids using an Air-Liquid Interface cell model

The study addresses the critical health concern regarding the inhalation of flavorings in e-liquids, which has not yet been systematically assessed. Two cell culture systems were employed – submerged and ALI – to systematically assess the toxicity of three common flavorings, cinnamaldehyde, eugenol, and vanillin on 16HBE lung cell. The results were compared with regards to the identified mechanistic pathways. Finally, a concluding recommendation for a testing strategy for flavorings in e-liquids is given.

In the submerged condition, 16HBE cells were exposed to flavors alone diluted in cell culture medium to investigate their intrinsic toxicity without introducing additional influencing factors. Only cinnamaldehyde showed dose-dependent cytotoxicity, while eugenol, vanillin and the positive control diacetyl exhibited no cytotoxicity (data not shown). The highest non-cytotoxic doses (0.4 µg/mL cinnamaldehyde, 30 µg/mL eugenol, vanillin, and diacetyl) were used for the transcriptomic analysis. The number of differentially expressed genes (DEGs) varied among flavors, with vanillin showing the highest number, followed by eugenol, diacetyl, and cinnamaldehyde, ranging between 190 and 97 genes (Figure 19A). These numbers were comparable to previous studies of liquid diacetyl exposure in primary human bronchial epithelial cells at the ALI [121].

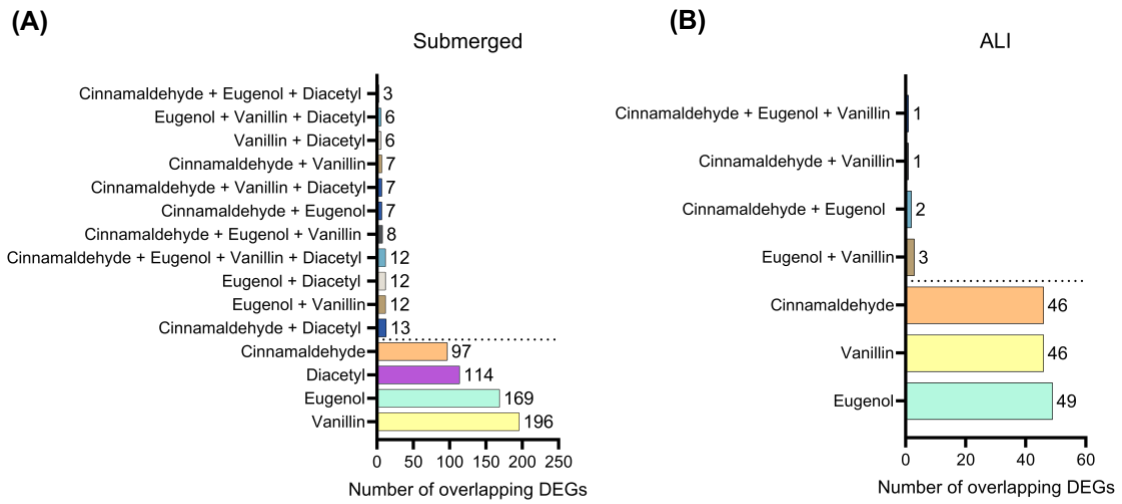

Figure 19. Differentially expressed genes (DEGs) for cinnamaldehyde, eugenol, vanillin, and diacetyl. Bar diagrams showing the number of DEGs in comparison to the control in 16HBE cells following (A) submerged and (B) ALI exposure to cinnamaldehyde, eugenol, vanillin, and diacetyl (only submerged).

Functional analysis using IPA and DAVID revealed that all three flavors influenced similar mechanistic pathways involving growth factor activity, cytokine activity, and neutrophil chemotaxis, ultimately possibly affecting immune function (Figure 20). The normal function of the lung is strictly dependent on this complex and tightly orchestrated system of growth factors and cytokines for maintaining defense mechanisms against external pathogens and stimulants [218]. The herein downregulated genes *IL6*, *IL1α*, *CXCL2*, and *TGFβ* (data not shown), among others play a crucial role in pathogen clearance. Mice deficient in these genes exhibited defective pathogen clearance, leading to prolonged lung inflammation or death. [219-224] Although eugenol, vanillin, and cinnamaldehyde have demonstrated anti-

inflammatory activity in diseased mouse tissue [225, 226], this characteristic might be detrimental in a healthy environment when disrupting cytokine homeostasis. Their elicited responses in 16HBEs were similar to the positive control diacetyl, and although only 12 genes were commonly changed by all flavors (Figure 19A), other altered genes might also impact immune function.

Immune function plays a pivotal role in maintaining healthy lung function and homeostasis. Immune dysfunction has been linked to chronic lung diseases such as COPD, a condition associated with chronic cigarette smoke exposure [227]. All flavors downregulated the protein IL6 compared to the control, while eugenol specifically downregulated the proteins IL1α, and CXCL1, CSF2 (data not shown). While most publications on flavor toxicity have focused on e-liquid mixtures, making it difficult to attribute effects to specific flavorings, many have demonstrated general flavor-dependent toxicity with the involvement of inflammatory cytokines, which is supported by the present study. Moreover, this present study identified effects from these three specific flavorings.

IPA analysis revealed that all flavors inhibited pathways including “Interleukin-10 signaling” and “pathogen induced cytokine storm” (data not shown). In addition, cinnamaldehyde and diacetyl had the most overlapping pathways, suggesting similar potential long-term toxicity upon inhalation.

Cinnamaldehyde inhibited additionally “IL-12 signaling and production in macrophages”, and “Role of macrophages, fibroblasts and endothelial cell in rheumatoid arthritis” (data not shown), similar to effects previously observed in mice transcriptome following acrolein exposure [228]. This suggests, potential synergistic effects between acrolein and certain e-cigarette flavors upon inhalation, as acrolein is also present in e-cigarette vapor.

In the ALI model, the flavors showed similar numbers of DEGs (vanillin: 41, eugenol: 42, and cinnamaldehyde: 41), with only 1 gene overlapping between all flavors (Figure 19B). Functional analysis revealed that eugenol affected voltage-gated potassium channel activity (Figure 20C), crucial for normal lung function, consistent with previous observations of altered ion homeostasis by e-cigarette flavors [229]. Cinnamaldehyde influenced endocytosis (Figure 20D), specifically changing RABEP2, which has been described to be involved in cilium assembly [230]. The adverse effects of cinnamaldehyde on ciliary function have been previously reported [231]. Cilia are important for clearing foreign particles from the lung and therefore represent an important defense mechanism [121]. Vanillin impacted anatomical structure morphogenesis (Figure 20D), suggesting potential disruption of lung development with chronic inhalation of vanillin-containing e-liquids. This is particularly concerning for younger individuals who are more prone to using flavored vaping products and whose lungs are especially vulnerable due to continued development.

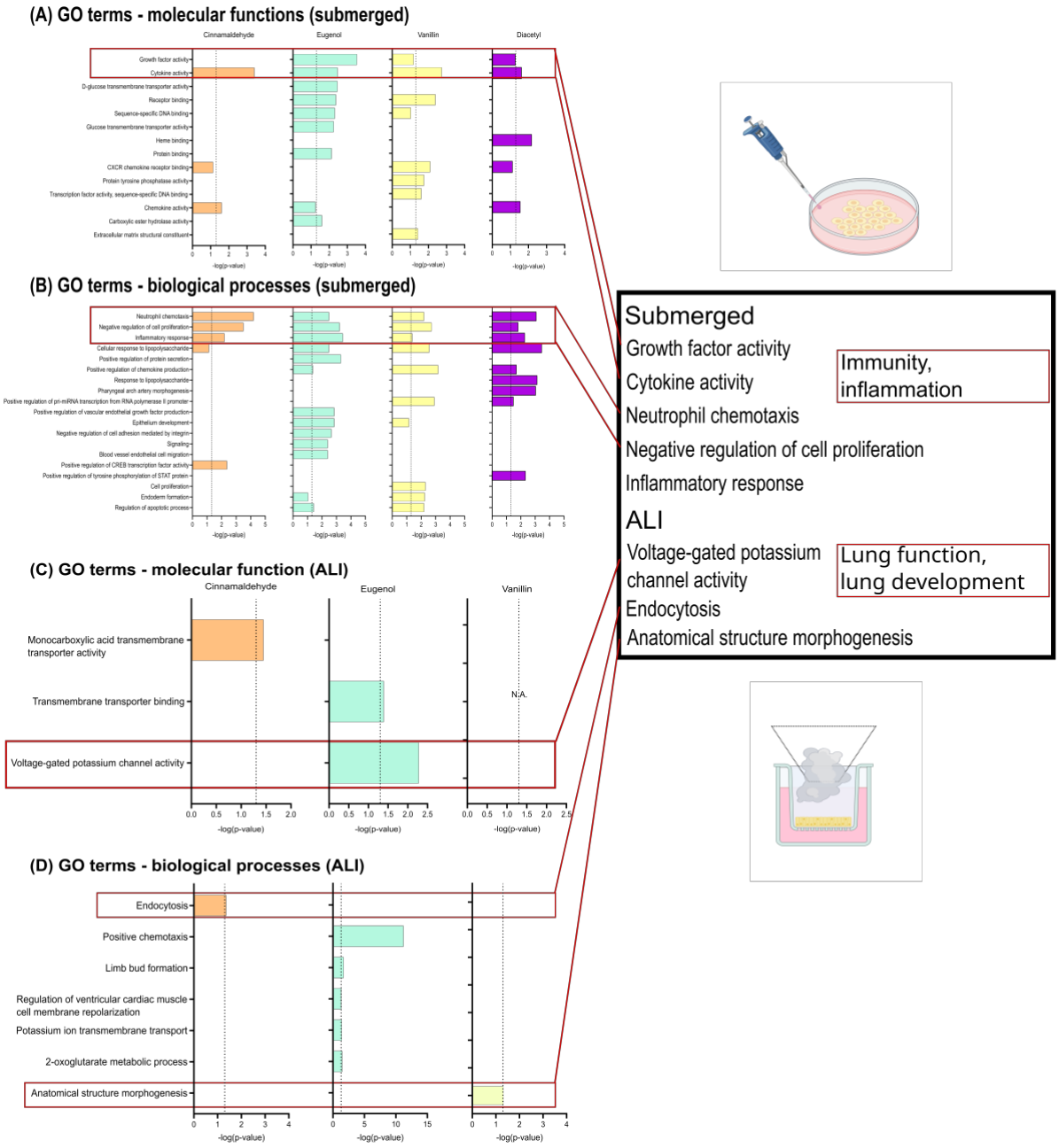

Figure 20. Functional analysis of the transcriptomic changes in 16HBE cells following submerged and Air-Liquid Interface (ALI) exposure to cinnamaldehyde, eugenol, vanillin, and diacetyl (only in submerged condition). Bar diagrams showing the top 10 enriched gene ontology (GO) terms by their -log(p-value) related to (A) molecular function (submerged), (B) biological function (submerged), (C) molecular function (ALI), or (D) biological processes (ALI) for cinnamaldehyde (orange), eugenol (turquoise), vanillin (yellow), and diacetyl (pink). The dotted line represents -log(p-value) = 1.3. Created with BioRender.com.

While submerged and ALI exposures led to different transcriptomic changes, both models revealed pathways leading to impaired lung function – through immune function in submerged exposure and disturbed ion homeostasis and morphological abnormalities in ALI exposure. The subtle ALI response corresponded with the aerosol monitoring data, where vanillin and eugenol signals were low. This is in line with the results obtained in Chapter 3.2, in which it was observed that the collection rate of flavors from e-liquid to cell culture medium varied depending on the flavor. For cinnamaldehyde, for which only

a fragment and an adduct were detected in the aerosol, the cellular response may be attributed to newly formed compounds rather than cinnamaldehyde itself (see Chapter 3.3.1).

The findings have important implications for current legislation, which only regulates ingredients such as parent compounds in e-liquids. The present study implies that current regulations warrant revision considering further chemical constituents in the aerosol. However, understanding parent compounds toxicity remains crucial due to potential mixture effects and varying e-liquid concentrations, where not all compounds may react with the base-solvents, meaning the parent compound is still present. Therefore, both cell models are valuable: the submerged model for quick, cost-effective flavoring screening and inherent toxicity assessment, and the ALI model for higher physiological relevance and better representation of chemical reactions during vaping.

3.3.3 Implications for risk assessment

This study presents the first combined experimental approach to test e-liquid flavorings using two cell models. The research revealed distinct transcriptomic patterns between the two cell models, influenced by several factors including the cell model itself, different modes of application (liquid form versus aerosol), varying exposure composition (flavors in cell culture medium versus flavors in PG/VG), and the potential formation of new compounds during vaping. While the results initially appear divergent between the two cell models, both ultimately indicate adverse effects on lung tissue. This phenomenon of model-dependent differences has been previously documented in nanoparticle research [142, 232]. Yet, the value of using both approaches in combination was demonstrated herein and can be advantageous for a comprehensive risk assessment.

However, each model has its (dis-) advantages. Certain key points have to be followed in order to gain a comprehensive understanding of the toxic mechanisms. A tiered testing strategy is recommended, beginning with submerged conditions testing where the flavor is diluted in cell culture medium. This initial approach offers advantages as it reveals inherent toxicity without confounding factors such as base-solvents or vaporization. It also enables simultaneous testing of multiple substances while maintaining cost-effectiveness and requiring only standard laboratory equipment.

To determine toxicity, several endpoints can be investigated. Omics analyses, including transcriptomics and proteomics, are particularly valuable for detecting subtle changes at non-cytotoxic doses. These methods can identify potential molecular mechanisms of toxicity at low doses, which is especially relevant for e-cigarette exposure in humans. While some flavorings might be present in low doses that don't exhibit acute adverse effects, they may still cause morbidity after long-term use. Although in vitro data cannot be directly translated to human effects, these studies help to identify potential mechanisms that could lead to adverse effects during long-term exposure.

Once potential toxic flavors have been identified, the next phase of the testing approach involves progressing to more physiologically relevant testing using the ALI model and aerosol exposure. Aerosol monitoring is crucial during ALI exposures to track thermal degradation products like diacetyl [233], new product formation such as aldehyde PG acetals [120], and variations in flavor transmission. This was

demonstrated in the present study using an online aerosol monitoring system, where vanillin showed lower output compared to other compounds, and cinnamaldehyde likely formed new products with base-solvents. A similar tiered testing approach has been suggested earlier however in the context of assessing e-liquid mixtures [131].

From a regulatory perspective, this study addresses two crucial components of risk assessment: hazard identification and hazard characterization. The in vitro approach is particularly valuable given EU restrictions on animal testing for e-cigarettes, aligning with the 3Rs principles [134]. The findings regarding specific compounds, could inform potential restrictions on certain flavorings. Based on the example of cinnamaldehyde's adverse effects, it has been proposed that e-liquid flavors require more stringent regulatory measures [69, 72]. In order to reduce vaping among youth, some states in the US and the Netherlands have taken a special regulatory approach by prohibiting all flavoring compound in e-cigarettes with the exception of tobacco and menthol variants. An investigation into user adaptation in the US demonstrated that consumers responded to flavor restrictions by transitioning to other available flavored products or even cigarettes [234]. Therefore, selectively prohibiting specific flavoring compounds may constitute a different strategy toward establishing tobacco alternatives with reduced health risks, ensuring that their ingredients and associated toxicological profiles are thoroughly characterized. At the same time, having a few flavored ANDS products available next to menthol and tobacco, might contribute to reduced smoking rates.

This structured approach enables a comprehensive toxicity assessment while addressing practical constraints and regulatory requirements. The combination of different testing methods provides a more complete understanding of potential health impacts, particularly for long-term exposure scenarios that are most relevant to actual e-cigarette use.

4 Conclusion and Outlook

This work contributes to the understanding of complex flavoring toxicity in ANDS and establishing an in vitro testing approach for the risk assessment of flavors.

First, the comprehensive non-targeted screening of 50 different ONPs provides valuable insights into their chemical flavor composition and presence of impurities. Out of the 186 identified substances, 46 had at least one toxicological remark. For five chosen ONPs and the reference snus CRP1.1, biological effects including cytotoxicity, inflammatory responses, and oxidative stress markers were investigated. While our experimental data were correlated with calculated ADIs of the flavor compounds, the establishment of definitive associations was limited by uncertainties in exact flavor concentrations and the complex nature of mixture effects. Notably, our findings eliminated dose-dependent nicotine toxicity as a contributing factor. To conclude, depending on the flavor composition of ONPs, they might cause local adverse effects through inflammatory and oxidative stress mechanisms, similar to CRP1.1. Therefore, classifying ONPs as a safer alternative might be misleading for consumers. Future studies should focus on determining the concentrations of specific identified compounds of toxicological concern in ONPs. Furthermore, more stringent regulations on ONP flavorings are necessary, particularly restricting those with an established ADI, hazard classification or classification of carcinogenicity.

Evaluating methodological approaches for e-liquid flavor risk assessment, the investigation of the impinger method revealed significant challenges. The differential transfer efficiency of flavors to cell culture medium highlighted that crucial methodological factors must be considered. For example, systematic parameter optimization achieved enhanced flavor concentrations in conditioned medium. Additionally, not all flavors could be detected in the conditioned medium, such as limonene, due to its water insolubility. However, these results emphasize the urgent need for standardized testing protocols and quality control measures to ensure reproducibility between laboratories and facilitate meaningful cross-study comparisons (Figure 21).

Given the limitations of the impinger method for assessing flavoring toxicity in vitro, a dual-approach in vitro testing strategy was adopted to investigate the toxicological mechanisms of popular flavors like cinnamaldehyde, eugenol, and vanillin at low, non-cytotoxic doses. The analysis employed both submerged and ALI conditions using human bronchial epithelial cells. While transcriptomic patterns differed between cell models and flavors, bioinformatic analysis of DEGs revealed that triggered pathways were potentially involved in lung impairment through distinct mechanisms. The submerged condition indicated immune system modification, potentially increasing susceptibility to respiratory infections and compromised pathogen clearance, with all flavors showing similar effects to the known inhalation toxicant diacetyl, despite belonging to different chemical classes. At the ALI, although vanillin and cinnamaldehyde share the same functional group, their cellular effects were found to differ. Transcriptomic changes revealed distinct alterations in ciliary function, ionic homeostasis and morphology in response to cinnamaldehyde, eugenol, and vanillin, respectively. The latter is a finding of particular concern given the high prevalence of e-cigarette use among youth and the fact that their

lungs are not fully developed. While the submerged lung cell model is relatively simple to implement and cost-effective, it lacks physiological relevance regarding human e-cigarette aerosol exposure and lung physiology. In contrast, the ALI lung cell model is more physiologically relevant for humans, despite requiring greater expertise in handling and exposure. Additionally, it allows for aerosol exposure, which is of importance for assessing the risks associated with e-cigarette aerosol exposure. The aerosol monitoring employed in the present work revealed differential flavor transfer to the aerosol and the potential formation of new products and thermal degradation during vaping. These findings underscore the importance of integrating an aerosol monitoring system during flavored e-liquid exposure at the ALI. However, the aerosol monitoring requires further technical optimization and determining the concentrations of flavors in aerosol that actually reach the cells could enhance the usability for human risk assessment even more. Additionally, monitoring the chemical reactions of flavorings during the vaping process and their associated toxicity could help identify specific compounds responsible for toxicity. These findings could then inform regulatory guidelines aimed at restricting harmful flavors.

Based on these findings, a tiered toxicological testing approach is proposed for future risk assessments of e-liquid flavorings (Figure 21). This framework entails a submerged cell model to identify potentially toxic flavors, followed by ALI aerosol exposure testing, incorporating online aerosol monitoring for enhanced quality control. The submerged cell exposure approach using conditioned medium generated by the vaping machine introduces many uncertainties and requires careful methodological consideration and standardization. Until comprehensive guidelines are established, thorough characterization of conditioned medium for compounds of interest, particularly flavors, is advised. Developing suitable in vitro methods is of particular relevance for the safety assessment of ANDS flavorings given the EU's restrictions on animal studies for these products [134]. The toxicity assessment methodology developed in this study also offers a systematic approach to evaluate the toxicity of ONPs. First, screening products for toxicity, subsequently analyzing their flavoring composition, and finally conducting targeted toxicological evaluations of the individual flavoring compounds, is a recommended approach. Overall, applying this recommended methodological approach could help identify flavorings of health concern leading to restriction of these specific compounds. This represents an alternative approach to general restriction of all flavors apart from menthol and tobacco, which is the path that the US and the Netherlands chose, although for a different motivation.

Looking ahead, significant knowledge gaps remain in the field of e-liquid flavoring safety. Future research priorities should be devoted to identifying specific flavoring compounds of concern, potentially leading to targeted restrictions in order to minimize user exposure to toxicants. Further, synergistic effects of flavoring mixtures represent challenges for future e-liquid risk assessment.

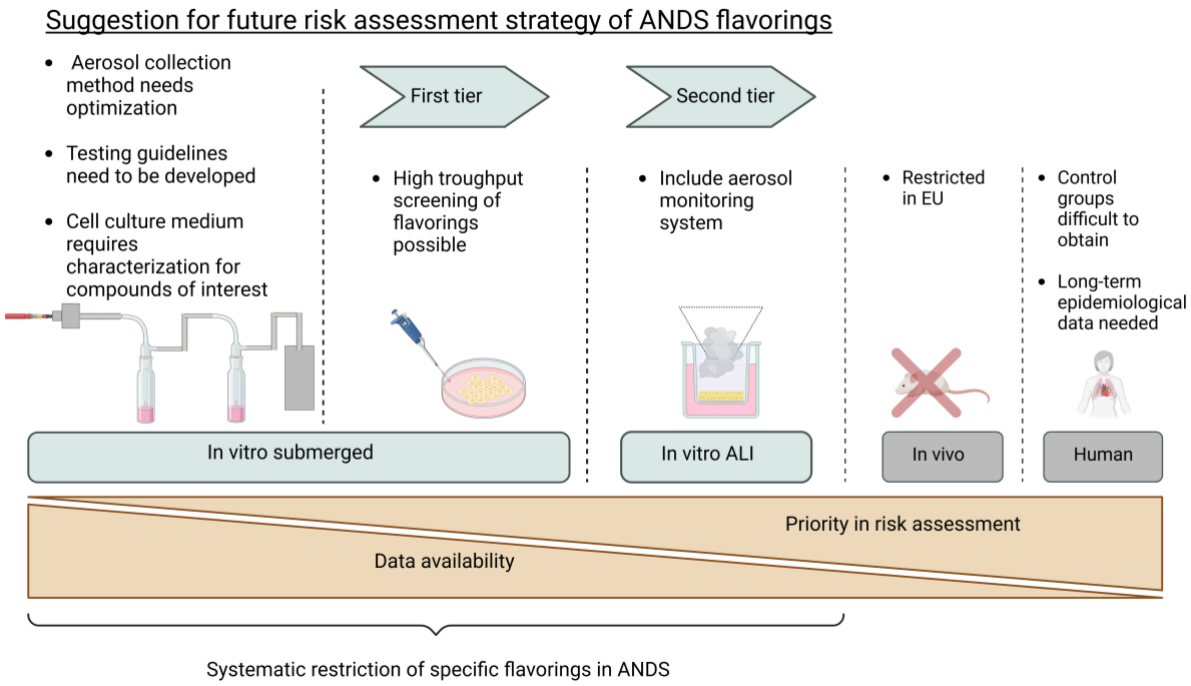

Figure 21. Graphical summary of the contributions of this work to the field. A graphical illustration of the herein recommended testing strategy for flavorings in ANDS such as e-cigarettes and oral nicotine pouches, based on the data obtained from the three conducted studies. Created with BioRender.com.

References

1. DEBRA. *Deutsche Befragung zum Rauchverhalten*. 2024 [cited 2024 December]; Available from: https://www.debra-study.info/.
2. WHO, *Global report on trends in prevalence of tobacco use 2000-2023*. 2024: Geneva: World Health Organization.
3. DKFZ. *Tabakatlas Deutschland 2020*. 2020 [cited 2024 December]; Available from: https://www.dkfz.de/de/krebspraevention/Downloads/pdf/Buecher_und_Berichte/2020_Tabakatlas-Deutschland-2020.pdf.
4. Dai, X., et al., *Health effects associated with smoking: a Burden of Proof study.* Nat Med, 2022. **28**(10): p. 2045-2055.
5. Centers for Disease Control and Prevention. *Cigarette Smoking*. 2024 [cited 2024 December]; Available from: https://www.cdc.gov/tobacco/about/index.html.
6. WHO. *Tobacco*. 2023 [cited 2025 January]; Available from: https://www.who.int/news-room/fact-sheets/detail/tobacco.
7. Hecht, S.S., *Tobacco smoke carcinogens and lung cancer.* J Natl Cancer Inst, 1999. **91**(14): p. 1194-210.
8. Hoffmann, D. and I. Hoffmann, *The changing cigarette, 1950-1995.* J Toxicol Environ Health, 1997. **50**(4): p. 307-64.
9. Talhout, R., et al., *Hazardous compounds in tobacco smoke.* Int J Environ Res Public Health, 2011. **8**(2): p. 613-28.
10. U.S. FDA. *Harmful and potentially harmful constituents in tobacco products and tobacco smoke: Established list*. 2012 2019 [cited 2025 February]; Available from: https://www.fda.gov/tobacco-products/rules-regulations-and-guidance-related-tobacco-products/harmful-and-potentially-harmful-constituents-tobacco-products-and-tobacco-smoke-established-list.
11. Li, Y. and S.S. Hecht, *Carcinogenic components of tobacco and tobacco smoke: A 2022 update.* Food Chem Toxicol, 2022. **165**: p. 113179.
12. Hecht, S.S., *Tobacco carcinogens, their biomarkers and tobacco-induced cancer.* Nat Rev Cancer, 2003. **3**(10): p. 733-44.
13. Angelova, V., K. Ivanov, and R. Ivanova, *Effect of Chemical Forms of Lead, Cadmium, and Zinc in Polluted Soils on Their Uptake by Tobacco.* Journal of Plant Nutrition, 2004. **27**(5): p. 757-773.
14. McDaniel, P.A., G. Solomon, and R.E. Malone, *The tobacco industry and pesticide regulations: case studies from tobacco industry archives.* Environ Health Perspect, 2005. **113**(12): p. 1659-65.
15. WHO, *WHO TobLabNet methods for measuring priority contents and emissions in tobacco and related products*. 2020: Geneva: World Health Organization.
16. Humans, I.W.G.o.t.E.o.C.R.t., *Tobacco smoke and involuntary smoking.* IARC Monogr Eval Carcinog Risks Hum, 2004. **83**: p. 1-1438.
17. Dorey, A., et al., *Acute and Chronic Carbon Monoxide Toxicity from Tobacco Smoking.* Mil Med, 2020. **185**(1-2): p. e61-e67.
18. European Parliament and the Council of the European Union, *Directive 2014/40/EU of the European Parliament and of the Council of 3 April 2014 on the approximation of the laws, regulations and administrative provisions of the Member States concerning the manufacture, presentation, and sale of tobacco and related products and repealing Directive 2001/37/EC*. 2014.
19. DKFZ, *Tabakrauch - ein Giftgemisch*. 2008, Deutsches Krebsforschungszentrum: Heidelberg.
20. Drope, J., et al., *Key issues surrounding the health impacts of electronic nicotine delivery systems (ENDS) and other sources of nicotine.* CA Cancer J Clin, 2017. **67**(6): p. 449-471.
21. Robichaud, M.O., A.B. Seidenberg, and M.J. Byron, *Tobacco companies introduce 'tobacco-free' nicotine pouches.* Tob Control, 2020. **29**(e1): p. e145-e146.
22. Tobacco Tactics. *Heated Tobacco Products*. 2025 08 April 2024 [cited 2025 January]; Available from: https://www.tobaccotactics.org/article/heated-tobacco-products/.
23. Leventhal, A.M., et al., *Differences in intention to use flavored oral nicotine products among young adult e-cigarette users and non-users.* Prev Med Rep, 2022. **30**: p. 102027.
24. Kinnunen, J.M., et al., *Electronic cigarette use among 14- to 17-year-olds in Europe.* Eur J Public Health, 2021. **31**(2): p. 402-408.
25. WHO, *Prevalence of tobacco and e-cigarette use by young people in the WHO European Region*. 2024: Geneva: World Health Organization.

26. Cantrell, J., et al., *Recruiting and retaining youth and young adults: challenges and opportunities in survey research for tobacco control.* Tob Control, 2018. **27**(2): p. 147-154.
27. Gali, K., et al., *Trends and consumption patterns in the use of e-cigarettes among adolescents and young adults in Germany (the DEBRA study).* Addict Behav, 2022. **133**: p. 107375.
28. Drugs, E.M.C.f., et al., *ESPAD 2019 methodology – Methodology of the 2019 European School Survey Project on Alcohol and other Drugs*. 2021: Publications Office of the European Union.
29. Ollila, H., et al., *Exclusive and dual use of electronic cigarettes among European youth in 32 countries with different regulatory landscapes.* Tob Control, 2024. **33**(5): p. 622-627.
30. Raitasalo, K., et al., *Single, Dual, and Triple Use of Cigarettes, e-Cigarettes, and Snus among Adolescents in the Nordic Countries.* Int J Environ Res Public Health, 2022. **19**(2).
31. Klosterhalfen, S., W. Viechtbauer, and D. Kotz, *Disposable e-cigarettes: Prevalence of use in Germany from 2016 to 2023 and associated user characteristics.* Addiction, 2024.
32. Lietzmann, J. and M. M., *Novel Tobacco Products and their effects on health - HWG workshop*. 2023, Publication for the Committee on Environment, Public Health and Food Safety, Policy Department for Economic, Scientific and Quality of Life Policies, European Parliament: Luxembourg.
33. Jankowski, M. and V.W. Rees, *Awareness and use of nicotine pouches in a nationwide sample of adults in Poland.* Tob Induc Dis, 2024. **22**.
34. Tattan-Birch, H., et al., *Tobacco-free Nicotine Pouch Use in Great Britain: A Representative Population Survey 2020-2021.* Nicotine Tob Res, 2022. **24**(9): p. 1509-1512.
35. Havermans, A., et al., *Awareness, use and perceptions of cigarillos, heated tobacco products and nicotine pouches: A survey among Dutch adolescents and adults.* Drug Alcohol Depend, 2021. **229**(Pt B): p. 109136.
36. Banks, E., A. Yazidjoglou, and G. Joshy, *Electronic cigarettes and health outcomes: epidemiological and public health challenges.* Int J Epidemiol, 2023. **52**(4): p. 984-992.
37. Gaiha, S.M. and B. Halpern-Felsher, *Public Health Considerations for Adolescent Initiation of Electronic Cigarettes.* Pediatrics, 2020. **145**(Suppl 2): p. S175-S180.
38. Al-Hamdani, M. and E. Manly, *Smoking cessation or initiation: The paradox of vaping.* Prev Med Rep, 2021. **22**: p. 101363.
39. Famiglietti, A., J.W. Memoli, and P.G. Khaitan, *Are electronic cigarettes and vaping effective tools for smoking cessation? Limited evidence on surgical outcomes: a narrative review.* J Thorac Dis, 2021. **13**(1): p. 384-395.
40. Liber, A.C., et al., *The role of flavored electronic nicotine delivery systems in smoking cessation: A systematic review.* Drug Alcohol Depend Rep, 2023. **7**: p. 100143.
41. Warner, K.E., et al., *Nicotine e-cigarettes as a tool for smoking cessation.* Nat Med, 2023. **29**(3): p. 520-524.
42. Lindson, N., et al., *Electronic cigarettes for smoking cessation.* Cochrane Database Syst Rev, 2025. **1**(1): p. CD010216.
43. Farsalinos, K.E., V. Voudris, and K. Poulas, *Are metals emitted from electronic cigarettes a reason for health concern? A risk-assessment analysis of currently available literature.* Int J Environ Res Public Health, 2015. **12**(5): p. 5215-32.
44. Pinto, M.I., et al., *Chemical characterisation of the vapour emitted by an e-cigarette using a ceramic wick-based technology.* Sci Rep, 2022. **12**(1): p. 16497.
45. National Academies of Sciences, Engineering, Medicine, *Public Health Consequences of E-Cigarettes*, ed. K. Stratton, L.Y. Kwan, and D.L. Eaton. 2018, Washington, DC: The National Academies Press. 774.
46. European Network for Smoking and Tobacco Prevention, *Ban on menthol cigarettes: European Union member states shall prohibit the placing on the market of tobacco products with a characterising flavour.* Tob Prev Cessat, 2020. **6**: p. 40.
47. Kassem, N.O.F., et al., *A Review of the Toxicity of Ingredients in e-Cigarettes, Including Those Ingredients Having the FDA's "Generally Recognized as Safe (GRAS)" Regulatory Status for Use in Food.* Nicotine Tob Res, 2024. **26**(11): p. 1445-1454.
48. Strongin, R.M., et al., *Chemical and physiological interactions between e-liquid constituents: cause for concern?* Tob Control, 2024.
49. Wissenschaftliche Dienste des Deutschen Bundestages, *Kurzinformation Zur Legalität von tabakfreien Nikotinbeuteln in Deutschland*. 2024.
50. dpa, *Sucht und Drogen: Schüler konsumieren Nikotinbeutel.* Dtsch Arztebl International, 2025. **24**(1): p. [5]-[5].
51. Lindpere, V., et al., *Reasons for E-cigarette Use, Vaping Patterns, and Cessation Behaviors Among US Adolescents.* Nicotine Tob Res, 2023. **25**(5): p. 975-982.

52. Ou, T.S., et al., *E-cigarette use reasons and associated e-cigarette use dependence among college students: A longitudinal examination.* Addict Behav, 2024. **155**: p. 108039.
53. Romijnders, K.A., et al., *E-Liquid Flavor Preferences and Individual Factors Related to Vaping: A Survey among Dutch Never-Users, Smokers, Dual Users, and Exclusive Vapers.* Int J Environ Res Public Health, 2019. **16**(23).
54. Tosakoon, S., K.F. Romm, and C.J. Berg, *Nicotine pouch awareness, use and perceptions among young adults from six metropolitan statistical areas in the United States.* Tob Prev Cessat, 2023. **9**: p. 19.
55. Meernik, C., et al., *Impact of non-menthol flavours in e-cigarettes on perceptions and use: an updated systematic review.* BMJ Open, 2019. **9**(10): p. e031598.
56. Leventhal, A.M., et al., *Flavored E-cigarette Use and Progression of Vaping in Adolescents.* Pediatrics, 2019. **144**(5).
57. Harrell, M.B., et al., *Flavored Tobacco Product Use among Youth and Young Adults: What if Flavors Didn't Exist?* Tob Regul Sci, 2017. **3**(2): p. 168-173.
58. Morean, M.E., et al., *"Tobacco-free" Nicotine Pouches: Risk Perceptions, Awareness, Susceptibility, and Use Among Young Adults in the United States.* Nicotine Tob Res, 2023. **25**(1): p. 143-150.
59. Cooper, M., et al., *Flavorings and Perceived Harm and Addictiveness of E-cigarettes among Youth.* Tob Regul Sci, 2016. **2**(3): p. 278-289.
60. Ford, A., et al., *Adolescents' responses to the promotion and flavouring of e-cigarettes.* Int J Public Health, 2016. **61**(2): p. 215-24.
61. Gades, M.S., et al., *The Role of Nicotine and Flavor in the Abuse Potential and Appeal of Electronic Cigarettes for Adult Current and Former Cigarette and Electronic Cigarette Users: A Systematic Review.* Nicotine Tob Res, 2022. **24**(9): p. 1332-1343.
62. Hendlin, Y.H., S. Small, and P.M. Ling, *'No-Barriers' tobacco product? Selling smokeless tobacco to women, people of colour and the LGBTQ+ community in the USA.* Tob Control, 2023. **32**(3): p. 330-337.
63. Pang, R.D., et al., *Sex differences in the appeal of flavored e-cigarettes among young adult e-cigarette users.* Psychol Addict Behav, 2020. **34**(2): p. 303-307.
64. Pepper, J.K., K.M. Ribisl, and N.T. Brewer, *Adolescents' interest in trying flavoured e-cigarettes.* Tob Control, 2016. **25**(Suppl 2): p. ii62-ii66.
65. Centers for disease control and prevention. *Outbreak of Lung Injury Associated with the Use of E-Cigarette, or Vaping, Products*. 2020 25th February 2020 [cited 2025 January]; Available from: https://archive.cdc.gov/#/details?url=https://www.cdc.gov/tobacco/basic_information/e-cigarettes/severe-lung-disease.html.
66. Cao, D.J., et al., *Review of Health Consequences of Electronic Cigarettes and the Outbreak of Electronic Cigarette, or Vaping, Product Use-Associated Lung Injury.* J Med Toxicol, 2020. **16**(3): p. 295-310.
67. Seidel, J. *Rechtliche Grundlagen von E-Zigaretten*. 2022 [cited 2025 February]; Available from: https://www.bfr-akademie.de/media/wysiwyg/2022/EZIG22/rechtliche-grundlagen-von-e-zigaretten.pdf.
68. Krusemann, E.J.Z., et al., *An E-Liquid Flavor Wheel: A Shared Vocabulary Based on Systematically Reviewing E-Liquid Flavor Classifications in Literature.* Nicotine Tob Res, 2019. **21**(10): p. 1310-1319.
69. Omaiye, E.E., et al., *High concentrations of flavor chemicals are present in electronic cigarette refill fluids.* Sci Rep, 2019. **9**(1): p. 2468.
70. Behar, R.Z., et al., *Analytical and toxicological evaluation of flavor chemicals in electronic cigarette refill fluids.* Sci Rep, 2018. **8**(1): p. 8288.
71. Krusemann, E.J.Z., et al., *GC-MS analysis of e-cigarette refill solutions: A comparison of flavoring composition between flavor categories.* J Pharm Biomed Anal, 2020. **188**: p. 113364.
72. Behar, R.Z., et al., *Distribution, quantification and toxicity of cinnamaldehyde in electronic cigarette refill fluids and aerosols.* Tob Control, 2016. **25**(Suppl 2): p. ii94-ii102.
73. Tierney, P.A., et al., *Flavour chemicals in electronic cigarette fluids.* Tob Control, 2016. **25**(e1): p. e10-5.
74. Omaiye, E.E., et al., *Electronic Cigarette Refill Fluids Sold Worldwide: Flavor Chemical Composition, Toxicity, and Hazard Analysis.* Chem Res Toxicol, 2020. **33**(12): p. 2972-2987.
75. Dinu, V., et al., *Policy, toxicology and physicochemical considerations on the inhalation of high concentrations of food flavour.* NPJ Sci Food, 2020. **4**: p. 15.
76. The good scents company. *(R)-(+)-pulegone*. 2025 [cited 2025 February]; Available from: https://www.thegoodscentscompany.com/data/rw1006501.html.

77. Paravisini, L., et al., *Caramel flavour perception: Impact of the non-volatile compounds on sensory properties and in-vitro aroma release.* Food Res Int, 2017. **100**(Pt 1): p. 209-215.
78. The good scents company. *Benzyl alcohol*. 2025 [cited 2025 February]; Available from: https://www.thegoodscentscompany.com/data/rw1001652.html.
79. Omaiye, E.E., et al., *Disposable Puff Bar Electronic Cigarettes: Chemical Composition and Toxicity of E-liquids and a Synthetic Coolant.* Chem Res Toxicol, 2022. **35**(8): p. 1344-1358.
80. Salam, S., et al., *Flavor-Toxicant Correlation in E-cigarettes: A Meta-Analysis.* Chem Res Toxicol, 2020. **33**(12): p. 2932-2938.
81. Scientific Committee on Health, Environmental and Emerging Risks. *Scientific Opinion on electronic cigarettes*. 2021; Available from: https://health.ec.europa.eu/document/download/50b3adac-5451-47c1-8427-ea67e874c970_en.
82. Hahn, J., et al., *Electronic cigarettes: overview of chemical composition and exposure estimation.* Tob Induc Dis, 2014. **12**(1): p. 23.
83. Arias-Badia, M., et al., *E-cigarette exposure disrupts antitumor immunity and promotes metastasis.* Front Immunol, 2024. **15**: p. 1444020.
84. Day, N.J., et al., *Rat bronchoalveolar lavage proteome changes following e-cigarette aerosol exposures.* Am J Physiol Lung Cell Mol Physiol, 2023. **324**(5): p. L571-L583.
85. Jin, L., et al., *Formaldehyde and the transient receptor potential ankyrin-1 contribute to electronic cigarette aerosol-induced endothelial dysfunction in mice.* Toxicol Sci, 2024. **201**(2): p. 331-347.
86. Kim, M.D., et al., *The combination of propylene glycol and vegetable glycerin e-cigarette aerosols induces airway inflammation and mucus hyperconcentration.* Sci Rep, 2024. **14**(1): p. 1942.
87. Muthumalage, T. and I. Rahman, *Pulmonary immune response regulation, genotoxicity, and metabolic reprogramming by menthol- and tobacco-flavored e-cigarette exposures in mice.* Toxicol Sci, 2023. **193**(2): p. 146-165.
88. Pham, K., et al., *E-cigarette promotes breast carcinoma progression and lung metastasis: Macrophage-tumor cells crosstalk and the role of CCL5 and VCAM-1.* Cancer Lett, 2020. **491**: p. 132-145.
89. Bekki, K., et al., *Carbonyl compounds generated from electronic cigarettes.* Int J Environ Res Public Health, 2014. **11**(11): p. 11192-200.
90. El-Hellani, A., et al., *Nicotine and Carbonyl Emissions From Popular Electronic Cigarette Products: Correlation to Liquid Composition and Design Characteristics.* Nicotine Tob Res, 2018. **20**(2): p. 215-223.
91. Farsalinos, K.E., V. Voudris, and K. Poulas, *E-cigarettes generate high levels of aldehydes only in 'dry puff' conditions.* Addiction, 2015. **110**(8): p. 1352-6.
92. Geiss, O., I. Bianchi, and J. Barrero-Moreno, *Correlation of volatile carbonyl yields emitted by e-cigarettes with the temperature of the heating coil and the perceived sensorial quality of the generated vapours.* Int J Hyg Environ Health, 2016. **219**(3): p. 268-77.
93. Noel, A., et al., *Sub-ohm vaping increases the levels of carbonyls, is cytotoxic, and alters gene expression in human bronchial epithelial cells exposed at the air-liquid interface.* Respir Res, 2020. **21**(1): p. 305.
94. Qu, Y., K.H. Kim, and J.E. Szulejko, *The effect of flavor content in e-liquids on e-cigarette emissions of carbonyl compounds.* Environ Res, 2018. **166**: p. 324-333.
95. IARC, *Formaldehyde, 2-Butoxyethanol and 1-tert-Butoxypropan-2-ol*, in *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans*. 2006: Lyon, France: International Agency for Research on Cancer.
96. IARC, *Acrolein, Crotonaldehyde, and Arecoline*, in *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans*. 2021: Lyon, France: International Agency for Research on Cancer.
97. Leigh, N.J., et al., *Flavourings significantly affect inhalation toxicity of aerosol generated from electronic nicotine delivery systems (ENDS).* Tob Control, 2016. **25**(Suppl 2): p. ii81-ii87.
98. Effah, F., et al., *In Vitro high-throughput toxicological assessment of E-cigarette flavors on human bronchial epithelial cells and the potential involvement of TRPA1 in cinnamon flavor-induced toxicity.* Toxicology, 2023. **496**: p. 153617.
99. Sassano, M.F., et al., *Evaluation of e-liquid toxicity using an open-source high-throughput screening assay.* PLoS Biol, 2018. **16**(3): p. e2003904.
100. Song, J.J., et al., *Transcriptomic analysis of tobacco-flavored E-cigarette and menthol-flavored E-cigarette exposure in the human middle ear.* Sci Rep, 2020. **10**(1): p. 20799.

101. Komura, M., et al., *Propylene glycol, a component of electronic cigarette liquid, damages epithelial cells in human small airways.* Respir Res, 2022. **23**(1): p. 216.
102. Mercier, C., et al., *In vitro toxicological evaluation of aerosols generated by a 4th generation vaping device using nicotine salts in an air-liquid interface system.* Respir Res, 2024. **25**(1): p. 75.
103. Effah, F., et al., *Toxicological assessment of E-cigarette flavored E-liquids aerosols using Calu-3 cells: A 3D lung model approach.* Toxicology, 2023. **500**: p. 153683.
104. Beklen, A. and D. Uckan, *Electronic cigarette liquid substances propylene glycol and vegetable glycerin induce an inflammatory response in gingival epithelial cells.* Hum Exp Toxicol, 2021. **40**(1): p. 25-34.
105. Li, Z., et al., *Investigation of the in vitro toxic effects induced by real-time aerosol of electronic cigarette solvents using microfluidic chips.* Food Chem Toxicol, 2024. **188**: p. 114668.
106. Manna, V.J., et al., *Utilizing primary human airway mucociliary tissue cultures to model ramifications of chronic E-cigarette usage.* Toxicol In Vitro, 2024. **94**: p. 105725.
107. Ruth, T., et al., *Inhalation toxicity of thermal transformation products formed from e-cigarette vehicle liquid using an in vitro lung model exposed at the Air-Liquid Interface.* Food Chem Toxicol, 2023. **182**: p. 114157.
108. Wieslander, G., D. Norback, and T. Lindgren, *Experimental exposure to propylene glycol mist in aviation emergency training: acute ocular and respiratory effects.* Occup Environ Med, 2001. **58**(10): p. 649-55.
109. Choi, H., et al., *Sources of propylene glycol and glycol ethers in air at home.* Int J Environ Res Public Health, 2010. **7**(12): p. 4213-37.
110. Jordt, S.E., *Synthetic nicotine has arrived.* Tob Control, 2023. **32**(e1): p. e113-e117.
111. Mallock, N., et al., *Levels of nicotine and tobacco-specific nitrosamines in oral nicotine pouches.* Tob Control, 2024. **33**(2): p. 193-199.
112. Azzopardi, D., C. Liu, and J. Murphy, *Chemical characterization of tobacco-free "modern" oral nicotine pouches and their position on the toxicant and risk continuums.* Drug Chem Toxicol, 2022. **45**(5): p. 2246-2254.
113. Barhdadi, S., et al., *Toxicity assessment of flavour chemicals used in e-cigarettes: current state and future challenges.* Arch Toxicol, 2021. **95**(8): p. 2879-2881.
114. Api, A.M., et al., *RIFM fragrance ingredient safety assessment, isoeugenol, CAS Registry Number 97-54-1.* Food Chem Toxicol, 2016. **97S**: p. S49-S56.
115. Api, A.M., et al., *RIFM fragrance ingredient safety assessment, Eugenol, CAS Registry Number 97-53-0.* Food Chem Toxicol, 2016. **97S**: p. S25-S37.
116. Brared Christensson, J., et al., *Air-oxidized linalool: a frequent cause of fragrance contact allergy.* Contact Dermatitis, 2012. **67**(5): p. 247-59.
117. PubChem. *Cinnamaldehyde*. 2024 [cited 2024 December]; Available from: https://pubchem.ncbi.nlm.nih.gov/compound/637511.
118. Jabba, S.V., et al., *Chemical Adducts of Reactive Flavor Aldehydes Formed in E-Cigarette Liquids Are Cytotoxic and Inhibit Mitochondrial Function in Respiratory Epithelial Cells.* Nicotine Tob Res, 2020. **22**(Suppl 1): p. S25-S34.
119. McKernan, L., et al., *Criteria for a recommended standard: occupational exposure to diacetyl and 2, 3-pentanedione.* Cincinnati, OH: NIOSH, 2016.
120. Erythropel, H.C., et al., *Formation of flavorant-propylene Glycol Adducts With Novel Toxicological Properties in Chemically Unstable E-Cigarette Liquids.* Nicotine Tob Res, 2019. **21**(9): p. 1248-1258.
121. Park, H.R., et al., *Transcriptomic response of primary human airway epithelial cells to flavoring chemicals in electronic cigarettes.* Sci Rep, 2019. **9**(1): p. 1400.
122. Bahl, V., et al., *Comparison of electronic cigarette refill fluid cytotoxicity using embryonic and adult models.* Reprod Toxicol, 2012. **34**(4): p. 529-37.
123. Behar, R.Z., Y. Wang, and P. Talbot, *Comparing the cytotoxicity of electronic cigarette fluids, aerosols and solvents.* Tob Control, 2018. **27**(3): p. 325-333.
124. Bengalli, R., et al., *Lung Toxicity of Condensed Aerosol from E-CIG Liquids: Influence of the Flavor and the In Vitro Model Used.* Int J Environ Res Public Health, 2017. **14**(10).
125. Omaiye, E.E., et al., *High-Nicotine Electronic Cigarette Products: Toxicity of JUUL Fluids and Aerosols Correlates Strongly with Nicotine and Some Flavor Chemical Concentrations.* Chem Res Toxicol, 2019. **32**(6): p. 1058-1069.
126. Otreba, M., et al., *E-cigarettes: voltage- and concentration-dependent loss in human lung adenocarcinoma viability.* J Appl Toxicol, 2018. **38**(8): p. 1135-1143.

127. Vasanthi Bathrinarayanan, P., et al., *An investigation into E-cigarette cytotoxicity in-vitro using a novel 3D differentiated co-culture model of human airways.* Toxicol In Vitro, 2018. **52**: p. 255-264.
128. Leigh, N.J., et al., *Cytotoxic effects of heated tobacco products (HTP) on human bronchial epithelial cells.* Tob Control, 2018. **27**(Suppl 1): p. s26-s29.
129. Moses, E., et al., *Molecular Impact of Electronic Cigarette Aerosol Exposure in Human Bronchial Epithelium.* Toxicol Sci, 2017. **155**(1): p. 248-257.
130. Lerner, C.A., et al., *Vapors produced by electronic cigarettes and e-juices with flavorings induce toxicity, oxidative stress, and inflammatory response in lung epithelial cells and in mouse lung.* PLoS One, 2015. **10**(2): p. e0116732.
131. Effah, F., et al., *Pulmonary effects of e-liquid flavors: a systematic review.* J Toxicol Environ Health B Crit Rev, 2022. **25**(7): p. 343-371.
132. Paschke, M., et al., *Activation of the cold-receptor TRPM8 by low levels of menthol in tobacco products.* Toxicol Lett, 2017. **271**: p. 50-57.
133. Liu, M., et al., *Role of TRPV1 in respiratory disease and association with traditional Chinese medicine: A literature review.* Biomed Pharmacother, 2022. **155**: p. 113676.
134. European Parliament and the Council of the European Union, *Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010on the protection of animals used for scientific purposes*. 2010.
135. Shaikh, S.B., et al., *Flavor Classification/Categorization and Differential Toxicity of Oral Nicotine Pouches (ONPs) in Oral Gingival Epithelial Cells and Bronchial Epithelial Cells.* Toxics, 2022. **10**(11).
136. Muthumalage, T., et al., *Challenges in current inhalable tobacco toxicity assessment models: A narrative review.* Tob Induc Dis, 2024. **22**.
137. Escobar, Y.H., et al., *In Vitro Toxicity and Chemical Characterization of Aerosol Derived from Electronic Cigarette Humectants Using a Newly Developed Exposure System.* Chem Res Toxicol, 2020. **33**(7): p. 1677-1688.
138. Gerloff, J., et al., *Inflammatory Response and Barrier Dysfunction by Different e-Cigarette Flavoring Chemicals Identified by Gas Chromatography-Mass Spectrometry in e-Liquids and e-Vapors on Human Lung Epithelial Cells and Fibroblasts.* Appl In Vitro Toxicol, 2017. **3**(1): p. 28-40.
139. Raduka, A., et al., *Electronic cigarette exposure disrupts airway epithelial barrier function and exacerbates viral infection.* Am J Physiol Lung Cell Mol Physiol, 2023. **325**(5): p. L580-L593.
140. Muthumalage, T., et al., *E-cigarette flavored pods induce inflammation, epithelial barrier dysfunction, and DNA damage in lung epithelial cells and monocytes.* Sci Rep, 2019. **9**(1): p. 19035.
141. He, R.W., et al., *Optimization of an air-liquid interface in vitro cell co-culture model to estimate the hazard of aerosol exposures.* J Aerosol Sci, 2021. **153**: p. 105703.
142. Lenz, A.G., et al., *Inflammatory and oxidative stress responses of an alveolar epithelial cell line to airborne zinc oxide nanoparticles at the air-liquid interface: a comparison with conventional, submerged cell-culture conditions.* Biomed Res Int, 2013. **2013**: p. 652632.
143. Ganguly, K., et al., *Addressing the challenges of E-cigarette safety profiling by assessment of pulmonary toxicological response in bronchial and alveolar mucosa models.* Sci Rep, 2020. **10**(1): p. 20460.
144. Scheffler, S., et al., *Evaluation of E-cigarette liquid vapor and mainstream cigarette smoke after direct exposure of primary human bronchial epithelial cells.* Int J Environ Res Public Health, 2015. **12**(4): p. 3915-25.
145. Nair, V., et al., *Menthol in electronic cigarettes: A contributor to respiratory disease?* Toxicol Appl Pharmacol, 2020. **407**: p. 115238.
146. Delaval, M., et al., *Novel instrument to generate representative e-cigarette vapors for physicochemical particle characterization and in-vitro toxicity.* Journal of Aerosol Science, 2019. **129**: p. 40-52.
147. Schripp, T., et al., *Does e-cigarette consumption cause passive vaping?* Indoor Air, 2013. **23**(1): p. 25-31.
148. Landry, R.L., et al., *The role of flavors in vaping initiation and satisfaction among U.S. adults.* Addict Behav, 2019. **99**: p. 106077.
149. National Cancer Institute. *Gingiva*. 2024 [cited 2024 December]; Available from: https://www.cancer.gov/publications/dictionaries/cancer-terms/def/gingiva.
150. Delvadia, P.R., W.H. Barr, and H.T. Karnes, *A biorelevant in vitro release/permeation system for oral transmucosal dosage forms.* Int J Pharm, 2012. **430**(1-2): p. 104-13.

151. International Organisation for Standardisation, *ISO 53160–1: Determination of the colourfastness of articles for common use - part 1: test with artificial saliva*. 2010-10, International Organisation for Standardisation: Geneva.
152. Callaghan, P.J., et al., *Epithelial barrier function properties of the 16HBE14o- human bronchial epithelial cell culture model.* Biosci Rep, 2020. **40**(10).
153. Forbes, B., et al., *The human bronchial epithelial cell line 16HBE14o- as a model system of the airways for studying drug transport.* Int J Pharm, 2003. **257**(1-2): p. 161-7.
154. Sangiorgi, C., et al., *HSP60 activity on human bronchial epithelial cells.* Int J Immunopathol Pharmacol, 2017. **30**(4): p. 333-340.
155. Silva, S., et al., *Air-liquid interface (ALI) impact on different respiratory cell cultures.* Eur J Pharm Biopharm, 2023. **184**: p. 62-82.
156. Srinivasan, B., et al., *TEER measurement techniques for in vitro barrier model systems.* J Lab Autom, 2015. **20**(2): p. 107-26.
157. International Organisation for Standardisation, *ISO 20768: Vapour products – Routine analytical vaping machine – Definitions and standard conditions*. 2018-09, International Organisation for Standardisation: Geneva.
158. DIN e.V. (Hrsg.), *DIN EN 17375:2020-09 Elektronische Zigaretten und Liquids für elektronischen Zigaretten-Referenz-E-Liquids*. 2020, Beuth Verlag: Berlin.
159. European Parliament and the Council of the European Union, *Regulation (EC) No 1272/2008 of the European Parliament and of the Council of 16 December 2008 on classification, labelling and packaging of substances and mixtures, amending repealing Directives 67/548/EEC and 1999/45/EC, and amending Regulation (EC) No 1907/37/EC*. 2008b.
160. World Health Organization. *Evaluations of the Joint FAO/WHO Expert Committee on Food Additives (JECFA)*. 2024 [cited 2025 March]; Available from: https://apps.who.int/food-additives-contaminants-jecfa-database/.
161. European Parliament and the Council of the European Union, *Regulation (EC) No. 1334/2008 of the European parliament and of the Council of 16 December 2008 on flavourings and certain food ingredients with flavouring properties for use in and on foods and amending Council Regulation (EC) No. 1601/91, Regulation (EC) No. 2232/96 and (EC) No. 110/2008 and Directive 2000/13/EC*. 2008a.
162. Kumar, P., A. Nagarajan, and P.D. Uchil, *Analysis of Cell Viability by the Lactate Dehydrogenase Assay.* Cold Spring Harb Protoc, 2018. **2018**(6).
163. Ghasemi, M., et al., *The MTT Assay: Utility, Limitations, Pitfalls, and Interpretation in Bulk and Single-Cell Analysis.* Int J Mol Sci, 2021. **22**(23).
164. Sonnaert, M., et al., *Quantitative Validation of the Presto Blue Metabolic Assay for Online Monitoring of Cell Proliferation in a 3D Perfusion Bioreactor System.* Tissue Eng Part C Methods, 2015. **21**(6): p. 519-29.
165. Degli Esposti, M., *Measuring mitochondrial reactive oxygen species.* Methods, 2002. **26**(4): p. 335-40.
166. Ortega-Villasante, C., et al., *Fluorescent in vivo imaging of reactive oxygen species and redox potential in plants.* Free Radic Biol Med, 2018. **122**: p. 202-220.
167. Miller, M.B. and Y.W. Tang, *Basic concepts of microarrays and potential applications in clinical microbiology.* Clin Microbiol Rev, 2009. **22**(4): p. 611-33.
168. Falciani, F., *Microarray Technology Through Applications*. 2007: Taylor & Francis.
169. van der Heyde, H.C. and I. Gramaglia, *Bead-based multiplexed analysis of analytes by flow cytometry.* Methods Mol Biol, 2011. **699**: p. 85-95.
170. Adan, A., et al., *Flow cytometry: basic principles and applications.* Crit Rev Biotechnol, 2017. **37**(2): p. 163-176.
171. Hornbeck, P.V., *Enzyme-Linked Immunosorbent Assays.* Curr Protoc Immunol, 2015. **110**: p. 2 1 1-2 1 23.
172. Qian, R., et al., *Recent advances and challenges in the interaction between myofibrillar proteins and flavor substances.* Front Nutr, 2024. **11**: p. 1378884.
173. Heide, J., et al., *Simultaneous on-line vacuum single- and multi-photon ionization on an orthogonal acceleration time-of-flight mass spectrometer platform.* Analyst, 2022. **147**(16): p. 3662-3674.
174. Malviya, R., et al., *High performance liquid chromatography: A short review.* Journal of Global Pharma Technology, 2010. **2**: p. 22-26.
175. Rusli, H., R.M. Putri, and A. Alni, *Recent Developments of Liquid Chromatography Stationary Phases for Compound Separation: From Proteins to Small Organic Compounds.* Molecules, 2022. **27**(3).

176. Bressolle, F., O. Nicolas, and M.T. Kelly, *CLINICAL ANALYSIS | Sample Handling*, in *Encyclopedia of Analytical Science (Second Edition)*, P. Worsfold, A. Townshend, and C. Poole, Editors. 2005, Elsevier: Oxford. p. 133-141.
177. Hübschmann, H.-J., *Fundamentals*, in *Handbook of GC-MS*. 2015. p. 7-354.
178. Lancioni, C., et al., *Headspace solid-phase microextraction: Fundamentals and recent advances.* Advances in Sample Preparation, 2022. **3**: p. 100035.
179. AL-Bukhaiti, W.Q., et al. *Gas Chromatography : Principles , Advantages and Applications in Food Analysis*. 2017.
180. Lovestead, T. and K. Urness, *Gas Chromatography - Mass Spectrometry (GC-MS)*. 2019, ASM Handbook, Volume 10: Materials Characterization.
181. European Parliament and the Council of the European Union, *Regulation (EC) No 1272/2008 of the European Parliament and of the Council of 16 December 2008 on classification, labelling, and packagind of substances and mixtures, amending and repealing Directives 67/548/EEC and 1999/45/EC, and amending Regulation (EC) No 1907/2006*. 2008b.
182. European Parliament and the Council of the European Union, *(EC) No 1223/2009 of the European Parliament and of the Council of 30 November 2009 on cosmetic products (recast)*. 2009.
183. Avagyan, R., M. Spasova, and J. Lindholm, *Determination of Nicotine-Related Impurities in Nicotine Pouches and Tobacco-Containing Products by Liquid Chromatography–Tandem Mass Spectrometry.* Separations, 2021. **8**(6): p. 77.
184. Mallock, N., et al., *Levels of nicotine and tobacco-specific nitrosamines in oral nicotine pouches.* Tob Control, 2022. **33**(2): p. 193-199.
185. Boelens, M.H., *Chemical characterization of cornmint oils.* Perfum Flavorist, 1993. **18**: p. 27-31.
186. Dampc, A. and M. Luczkiewicz, *Rhododendron tomentosum (Ledum palustre). A review of traditional use based on current research.* Fitoterapia, 2013. **85**: p. 130-43.
187. Tsai, M.-L., et al., *Chemical composition and biological properties of essential oils of two mint species.* Tropical Journal of Pharmaceutical Research, 2013. **12**(4): p. 577-582.
188. Anju, et al., *Chemical composition, in vitro and in silico evaluation of essential oil from Eucalyptus tereticornis leaves for lung cancer.* Nat Prod Res, 2023. **37**(10): p. 1656-1661.
189. Gallon, C., et al., *Chemistry matters: biological activity of Eucalyptus essential oils on mosquito larval mortality.* Entomologia Experimentalis et Applicata, 2020. **168**(5): p. 407-415.
190. Van Esch, G. and World Health Organization and International Programme on Chemical Safety, *Flame retardants: tris (2-butoxyethyl) phosphate, tris (2-ethylhexyl) phosphate, tetrakis (hydroxymethyl) phosphonium salts*. 2000: World Health Organization.
191. Saquib, Q., et al., *Tris(2-butoxyethyl) phosphate (TBEP): A flame retardant in solid waste display hepatotoxic and carcinogenic risks for humans.* Chemosphere, 2022. **296**: p. 133977.
192. Ulker, Z., L. Alpsoy, and A. Mihmanli, *Assessment of cytotoxic and apoptotic effects of benzaldehyde using different assays.* Hum Exp Toxicol, 2013. **32**(8): p. 858-64.
193. Tabatabaie, T. and R.A. Floyd, *Inactivation of glutathione peroxidase by benzaldehyde.* Toxicol Appl Pharmacol, 1996. **141**(2): p. 389-93.
194. Additives, E.P.o.F., et al., *Re-evaluation of benzyl alcohol (E 1519) as food additive.* EFSA J, 2019. **17**(10): p. e05876.
195. Bruun, H., *Nicotine Pouch*. 2018, Philip Morris Products SA.
196. Stahl, M., et al., *Nicotine pouch composition and pouch comprising such*. 2020, Philip Morris Products SA.
197. Prasad, K., et al., *Assessing consumer use and behaviour patterns of oral nicotine pouches in a multi-country study.* International Journal of Scientific Reports, 2022. **8**(6): p. 173-176.
198. Miluna, S., et al., *The Correlation of Swedish Snus, Nicotine Pouches and Other Tobacco Products with Oral Mucosal Health and Salivary Biomarkers.* Dent J (Basel), 2022. **10**(8).
199. Dowd, A.N., et al., *A Cross-Sectional Survey on Oral Nicotine Pouches: Characterizing Use-Motives, Topography, Dependence Levels, and Adverse Events.* Nicotine Tob Res, 2024. **26**(2): p. 245-249.
200. Miluna-Meldere, S., et al., *Oral mucosal changes caused by nicotine pouches: case series.* Diagn Pathol, 2024. **19**(1): p. 127.
201. Shao, Y., et al., *Perceptions of Oral Nicotine Pouches on Reddit: Observational Study.* J Med Internet Res, 2022. **24**(7): p. e37071.
202. Chiamulera, M.M.A., et al., *Salivary cytokines as biomarkers of oral cancer: a systematic review and meta-analysis.* BMC Cancer, 2021. **21**(1): p. 205.
203. Sardaro, N., et al., *Oxidative Stress and Oral Mucosal Diseases: An Overview.* In Vivo, 2019. **33**(2): p. 289-296.

204. Caliri, A.W., S. Tommasi, and A. Besaratinia, *Relationships among smoking, oxidative stress, inflammation, macromolecular damage, and cancer.* Mutat Res Rev Mutat Res, 2021. **787**: p. 108365.
205. Back, S., et al., *Harmful and potentially harmful constituents (HPHCs) in two novel nicotine pouch products in comparison with regular smokeless tobacco products and pharmaceutical nicotine replacement therapy products (NRTs).* BMC Chem, 2023. **17**(1): p. 9.
206. Alizadehgharib, S., et al., *The effect of a non-tobacco-based nicotine pouch on mucosal lesions caused by Swedish smokeless tobacco (snus).* Eur J Oral Sci, 2022. **130**(4): p. e12885.
207. Bozhilova, S., et al., *Optimization of aqueous aerosol extract (AqE) generation from e-cigarettes and tobacco heating products for in vitro cytotoxicity testing.* Toxicol Lett, 2020. **335**: p. 51-63.
208. Breheny, D., et al., *Comparative tumor promotion assessment of e-cigarette and cigarettes using the in vitro Bhas 42 cell transformation assay.* Environ Mol Mutagen, 2017. **58**(4): p. 190-198.
209. The Human Metabolome Database. *Showing metabocard for 3,7-Dimethyl-1,6-octadien-3-ol (HMDB0036100)*. 2025 [cited 2025 January]; Available from: https://hmdb.ca/metabolites/HMDB0036100.
210. The Human Metabolome Database. *Showing metabocard for Eugenol (HMDB0005809)*. 2025 [cited 2025 January]; Available from: https://hmdb.ca/metabolites/HMDB0005809.
211. The Human Metabolome Databse. *Showing metabocard for Cinnamaldehyde (HMDB0003441)*. 2025 [cited 2025 January]; Available from: https://hmdb.ca/metabolites/HMDB0003441.
212. National Center for Biotechnology Information. *PubChem Compound Summary for CID 22311, Limonene, (+/-)-.* 2025 [cited 2025 January]; Available from: https://pubchem.ncbi.nlm.nih.gov/compound/Limonene.
213. Morris, A.M., et al., *Effects of E-Cigarette Flavoring Chemicals on Human Macrophages and Bronchial Epithelial Cells.* Int J Environ Res Public Health, 2021. **18**(21).
214. Smart, D.E., et al., *Application of ToxTracker for the toxicological assessment of tobacco and nicotine delivery products.* Toxicol Lett, 2022. **358**: p. 59-68.
215. Taylor, M., et al., *E-cigarette aerosols induce lower oxidative stress in vitro when compared to tobacco smoke.* Toxicol Mech Methods, 2016. **26**(6): p. 465-476.
216. Tsolakos, N., et al., *Comparative toxicological assessment of cigarettes and new category products via an in vitro multiplex proteomics platform.* Toxicol Rep, 2024. **12**: p. 492-501.
217. Leslie, L.J., et al., *A comparative study of electronic cigarette vapor extracts on airway-related cell lines in vitro.* Inhal Toxicol, 2017. **29**(3): p. 126-136.
218. Deng, J.C. and T.J. Standiford, *Growth factors and cytokines in acute lung injury.* Compr Physiol, 2011. **1**(1): p. 81-104.
219. Chen, G.H., et al., *Local GM-CSF-Dependent Differentiation and Activation of Pulmonary Dendritic Cells and Macrophages Protect against Progressive Cryptococcal Lung Infection in Mice.* J Immunol, 2016. **196**(4): p. 1810-21.
220. Gou, X., et al., *IL-6 Prevents Lung Macrophage Death and Lung Inflammation Injury by Inhibiting GSDME- and GSDMD-Mediated Pyroptosis during Pneumococcal Pneumosepsis.* Microbiol Spectr, 2022. **10**(2): p. e0204921.
221. Huang, F.F., et al., *GM-CSF in the lung protects against lethal influenza infection.* Am J Respir Crit Care Med, 2011. **184**(2): p. 259-68.
222. Kulkarni, A.B., et al., *Transforming growth factor beta 1 null mutation in mice causes excessive inflammatory response and early death.* Proc Natl Acad Sci U S A, 1993. **90**(2): p. 770-4.
223. Lauder, S.N., et al., *Interleukin-6 limits influenza-induced inflammation and protects against fatal lung pathology.* Eur J Immunol, 2013. **43**(10): p. 2613-25.
224. Shull, M.M., et al., *Targeted disruption of the mouse transforming growth factor-beta 1 gene results in multifocal inflammatory disease.* Nature, 1992. **359**(6397): p. 693-9.
225. Barbosa-de-Oliveira, M.C., et al., *Modulation of Alveolar Macrophage Activity by Eugenol Attenuates Cigarette-Smoke-Induced Acute Lung Injury in Mice.* Antioxidants (Basel), 2023. **12**(6).
226. Liu, X., et al., *Vanillin Attenuates Cadmium-Induced Lung Injury Through Inhibition of Inflammation and Lung Barrier Dysfunction Through Activating AhR.* Inflammation, 2021. **44**(6): p. 2193-2202.
227. Gopallawa, I., et al., *A four-part guide to lung immunology: Invasion, inflammation, immunity, and intervention.* Front Immunol, 2023. **14**: p. 1119564.
228. Alewel, D.I., et al., *Differential transcriptomic alterations in nasal versus lung tissue of acrolein-exposed rats.* Front Toxicol, 2023. **5**: p. 1280230.

229. Lin, V.Y., et al., *Vaporized E-Cigarette Liquids Induce Ion Transport Dysfunction in Airway Epithelia.* Am J Respir Cell Mol Biol, 2019. **61**(2): p. 162-173.
230. The Human Protein Atlas. *RABEP2*. 2025 [cited 2025 January]; Available from: https://www.proteinatlas.org/ENSG00000177548-RABEP2].
231. Clapp, P.W., et al., *Cinnamaldehyde in flavored e-cigarette liquids temporarily suppresses bronchial epithelial cell ciliary motility by dysregulation of mitochondrial function.* Am J Physiol Lung Cell Mol Physiol, 2019. **316**(3): p. L470-L486.
232. Bessa, M.J., et al., *In Vitro Toxicity of Industrially Relevant Engineered Nanoparticles in Human Alveolar Epithelial Cells: Air-Liquid Interface versus Submerged Cultures.* Nanomaterials (Basel), 2021. **11**(12).
233. Sleiman, M., et al., *Emissions from Electronic Cigarettes: Key Parameters Affecting the Release of Harmful Chemicals.* Environ Sci Technol, 2016. **50**(17): p. 9644-51.
234. Li, D., et al., *Impact of the FDA flavour enforcement policy on flavoured electronic cigarette use behaviour changes.* Tobacco Control, 2022. **31**(Suppl 3): p. s176-s183.

Appendix

Contribution to conferences

1. Poster presentation at the ESTIV Congress 2022, 21 – 25 November, Sitges, Spain

   Title: Flavors in e-cigarettes – a tasty hazard?

2. Oral presentation at the European Aerosol Conference 2024, 25 – 30 August, Tampere, Finland

   Title: The transcriptomic changes of 16hbe14o lung cells in response to e-cigarette flavors in two different exposure settings – submerged and ALI

Curriculum Vitae

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Publications

Publication 1

Title: Oral nicotine pouches with an aftertaste? Part 1: screening and initial toxicological assessment of flavorings and other ingredients

Authors: Nadja Mallock-Ohnesorg*, Selina Rinaldi*, Sebastian Malke, Nadine Dreiack, Elke Pieper, Peter Laux, Thomas Schulz, Ralf Zimmermann & Andreas Luch

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Publication 2

Title: Oral nicotine pouches with an aftertaste? Part 2: in vitro toxicity in human gingival fibroblasts

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Journal: Archives of Toxicology, Year: 2023, DOI: 10.1007/s00204-023-03554-9

Publication 3

Title: Flavors in e-liquids and electronic cigarette aerosols elicit differential transcriptional changes in lung epithelial cell models exposed in submerged and air-liquid interface conditions

Authors: Selina Rinaldi, Anjana Khadka, Nina Hupf, Jan Heide, Sven Ehlert, Leonie Gloger, Johannes Becker, Sebastian Malke, Peter Laux, Ralf Zimmermann, Andreas Luch, Mathilde N. Delaval, Elke Pieper

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