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Exposure assessment to harmful compounds emitted by Electronic Nicotine Delivery System (ENDS) – from emission characterization to urinary biomarker determination

Sambiagio Nicolas

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par

Nicolas SAMBIAGIO

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Jury

Prof. Gerhard Gmel, Président
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Exposure assessment to harmful compounds emitted by Electronic Nicotine Delivery System (ENDS) – from emission characterization to urinary biomarker determination

Lausanne, le 7 mars 2022

pour le Doyen
de la Faculté de biologie et de médecine

Prof. Gerhard Gmel

À ma Maman que j'aurais tellement voulu rendre fière

À Jack

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Abstract

Electronic Nicotine Delivery Systems (ENDS or e-cigarettes) have been marketed as safer alternative to conventional cigarettes for smokers as their emissions have been reported to contain less harmful compounds than cigarette smoke. However, there is a lack of data concerning the toxicity of ENDS in the context of smoking cessation, which prevents them from being recommended by healthcare professionals.

This thesis was part of a large clinical trial: the ESTxENDS study ("Efficacy, Safety, and Toxicology of Electronic Nicotine Delivery Systems as an aid for smoking cessation; the ESTxENDS multicenter randomized controlled trial"). We hypothesized that urinary concentrations of biomarkers of exposure (BoE) to tobacco smoke and oxidative stress biomarkers decrease significantly from smoking to ex-smoking or vaping status. The thesis objectives were to 1) quantify known harmful compounds in ENDS emissions; 2) develop and validate a new liquid chromatography – tandem mass (LC-MS/MS) analytical method for the simultaneous analysis of two urinary oxidative stress biomarkers, 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) and 8-iso-prostaglandin $F_{2\alpha}$ (8-isoprostane); 3) to define the relative importance of BoE on oxidative stress levels (calculated with effect size indicators); and 4) assess the effects of smoking cessation with and without the use of ENDS on BoE to tobacco smoke and oxidative stress biomarkers over a 6-month period.

ENDS emissions generated in the laboratory using an in-house built vaping machine, contained aldehydes and heavy metals at concentrations lower than conventional cigarettes. The new developed LC-MS/MS analytical method for 8-oxodG and 8-isoprostane was specific and robust. Positive associations between these urinary biomarkers and BoE to tobacco smoke were observed in smokers. Concentrations of BoE to tobacco smoke decreased over the 6-month period for smoking participants who became ex-smokers or ENDS users. Furthermore, the urinary BoE concentrations in ex-smokers and ENDS users did not differ, except for nicotine metabolites. Urinary concentrations of 8-oxodG and 8-isoprostane did not change over the 6-month period.

In conclusion, smokers greatly benefited from the switch from cigarettes to ENDS. Thus, ENDS should be recommended as part of smoking cessation programs if they can lead to complete cigarette abstinence. However, non-smokers should be discouraged from using these products as ENDS emissions contain harmful compounds. Changes of oxidative stress levels associated with reduced exposure to harmful compounds following smoking cessation could not be demonstrated with the two selected oxidative stress biomarkers.

Résumé

La vaporette (cigarette électronique ou vapoteuse) est souvent présentée comme une alternative plus sûre au tabac comme ses émissions contiennent moins de composés nocifs que la fumée de cigarette. Cependant, des données manquent sur la toxicité des vaporettes dans le contexte de l'arrêt du tabac, ce qui empêche leur recommandation par les professionnels de la santé.

Le travail de thèse faisait partie de l'étude ESTxENDS (« Efficacy, Safety, and Toxicology of Electronic Nicotine Delivery Systems as an aid for smoking cessation: the ESTxENDS multicentre randomized controlled trial »). L'hypothèse était que les concentrations urinaires des biomarqueurs d'exposition (BdE) à la fumée du tabac et des biomarqueurs de stress oxydant diminuent significativement après l'arrêt tabagique avec ou sans la vaporette. Les objectifs de la thèse étaient de 1) quantifier les composés nocifs dans les émissions de la vaporette ; 2) développer et valider une nouvelle méthode analytique par chromatographie en phase liquide avec spectrométrie de masse en tandem (LC-MS/MS) pour l'analyse simultanée de deux biomarqueurs de stress oxydant urinaires, 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) et 8-iso-prostaglandin $F_{2\alpha}$ (8-isoprostane) ; 3) définir l'importance relative des BdE sur les niveaux de stress oxydant (calculée avec des indicateurs de taille d'effet) ; et 4) déterminer les effets de l'arrêt tabagique sur les BdE à la fumée du tabac et des biomarqueurs de stress oxydant sur une période de six mois.

Les émissions de la vaporette générées en laboratoire contenaient des aldéhydes et des métaux lourds à des concentrations inférieures à celles de la fumée de cigarette. La nouvelle méthode LC-MS/MS était spécifique et robuste. Des associations positives entre les biomarqueurs de stress oxydant et les BdE à la fumée du tabac ont été observées chez les fumeurs. Les concentrations des BdE diminuaient significativement sur la période de six mois pour les fumeurs devenus ex-fumeurs et vapoteurs. De plus, les concentrations des BdE chez les ex-fumeurs et vapoteurs étaient similaires, à l'exception des métabolites de la nicotine. Les concentrations de 8-oxodG et de 8-isoprostane n'ont pas varié sur la période de six mois.

En conclusion, les fumeurs qui abandonnent la cigarette au profit de la vaporette réduisent significativement leur exposition aux composés nocifs. La vaporette devrait donc être proposée lors de l'arrêt tabagique si elle permet de quitter complètement la cigarette. Cependant, il faut informer les non-fumeurs de ne pas utiliser la vaporette comme ses émissions contiennent des composés nocifs. Les changements de niveau de stress oxydant associés à la diminution de l'exposition aux composés nocifs suivant l'arrêt du tabac n'ont pas pu être montrés avec les biomarqueurs de stress oxydant sélectionnés.

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Abbreviations

ATSDR	Agency for Toxic Substances and Disease Registry (U.S.)
AUDIT	Alcohol use disorders identification test
BoE	Biomarker of exposure
BoPH	Biomarker of potential harm
BMI	Body mass index
BSD	Backscattered scanning electron
CORESTA	Cooperation Centre for Scientific Research Relative to Tobacco (F)
CRM	CORESTA recommended method
CVD	Cardiovascular disease
DTL	Direct-to-lung (vaping style)
E-liquid	Electronic cigarette liquid
EBC	Exhaled breath condensate
EDX	Energy-dispersive X-ray spectroscopy
ELISA	Enzyme-linked immunosorbent assay
EMA	European Medicines Agency (Europe)
ENDS	Electronic nicotine delivery systems
ESI	Electron spray ionization
ESTxENDS	Efficacy, Safety and Toxicology of Nicotine Delivery Systems (clinical study)
FDA	Food and Drug Administration (U.S.)
FSO	Swiss Federal Statistical Office (CH)
GC	Gas chromatography
HPHC	Harmful and potentially harmful compounds
HTP	Heated tobacco product
IARC	International Agency for Research on Cancer (F)
ICP-MS	Inductively coupled plasma mass spectrometry
IPAQ	International physical activity questionnaire
LC	Liquid chromatography
LOD	Limit of detection
LOQ	Limit of quantification
MRL	Minimal risk level
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MTL	Mouth-to-lung (vaping style)

NIDA	National Institute on Drug Abuse (U.S.)
NIOSH	National Institute for Occupational Safety and Health (U.S.)
NRT	Nicotine replacement therapy
OSHA	Occupational Safety and Health Administration (U.S.)
PAH	Polycyclic aromatic hydrocarbon
PEL	Permissible exposure limit
PDE	Permitted daily exposure
PSQI	Pittsburgh sleep quality index
REL	Recommended exposure limit
ROS	Reactive oxygen species
SCC	Smoking cessation counselling
SEM	Scanning electron microscope
SPE	Solid-phase extraction
STEL	Short-term exposure limit
TNE	Total nicotine equivalent
TSNA	Tobacco-specific nitrosamine
TWA	Time weighted average
UBA	German Environment Agency (D)
VOC	Volatile organic compound

Chemical compounds

1,2-DCVMA	N-acetyl-S-(1,2-dichloroethenyl)-L-cysteine
1-MHBMA	N-acetyl-S-(1-hydroxymethyl-2-propenyl)-L-cysteine
1-OHP	1-Hydroxypyrene
2,2-DCVMA	N-acetyl-S-(2,2-dichloroethenyl)-L-cysteine
2-HPMA	N-acetyl-S-(2-hydroxypropyl)-L-cysteine
2-MHA	2-Methylhippuric acid
2-MHBMA	N-acetyl-S-(2-hydroxy-3-butenyl)-L-cysteine
3-HPMA	N-acetyl-S-(3-hydroxypropyl)-L-cysteine
3-MHA	3-Methylhippuric acid
3-MHBMA	N-acetyl-S-(4-hydroxy-2-buten-1-yl)-L-cysteine
3-OH-cotinine	Trans-3'-hydroxycotinine
4-MHA	4-Methylhippuric acid

8-isoprostane	8-Iso-prostaglandin $F_{2\alpha}$
8-oxodG	8-Oxo-7,8-dihydro-2'-deoxyguanosine
AAMA	N-acetyl-S-(2-carbamoylethyl)-L-cysteine
AMCC	N-acetyl-S-(N-methylcarbamoyl)-cysteine
ATCA	2-Aminothiazoline-4-carboxylic acid
BMA	Benzylmercapturic acid
BPMA	N-acetyl-S-propyl-L-cysteine
CEMA	N-acetyl-S-(2-carboxyethyl)-L-cysteine
CMEMA	N-acetyl-S-(3-carboxy-2-propyl)-L-cysteine
CYHA	N-acetyl-S-(1-cyano-2-hydroxyethyl)-L-cysteine
CYMA	N-acetyl-S-(2-cyanoethyl)-L-cysteine
DHBMA	N-acetyl-S-(3,4-dihydroxybutyl)-L-cysteine
DNPH	2,4-Dinitrophenylhydrazine
GAMA	N-acetyl-S-(2-carbamoyl-2-hydroxyethyl)-L-cysteine
HEMA	N-acetyl-S-(2-hydroxyethyl)-L-cysteine
HPMMA	N-acetyl-S-(3-hydroxypropyl-1-methyl)-L-cysteine
IPMA3	N-acetyl-S-(4-hydroxy-2-methyl-2-trans-buten-1-yl)-L-cysteine
MA	Mandelic acid
MDA	Malondialdehyde
NAB	N-nitrosoanabasine
NAT	N-nitrosoanatabine
NNAL	4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol
NNN	N-nitrosonornicotine
NNK	4-(N-nitrosomethylamino)-1-(3-pyridyl)-1-butanone
PDO	Propane-1,3-diol
PG	Propylene glycol
PHEMA	N-acetyl-S-(2-hydroxy-1-phenylethyl)-L-cysteine + N-acetyl-S-(2-hydroxy-2-phenylethyl)-L-cysteine
PGA	Phenylglyoxylic acid
SPMA	N-acetyl-S-(phenyl)-L-cysteine
TTCA	2-Thiothiazolidine-4-carboxylic acid
tt-MA	Trans, trans-muconic acid
VG	Vegetal glycerin

Definitions[1]

Biological monitoring (biomonitoring):

Efficient tool for assessing exposure to a xenobiotic or its effects on the organism by measuring this xenobiotic, its metabolite(s) or reaction product(s) in biological samples of individuals. Biological samples include, but are not limited to, urine, blood, saliva, exhaled breath condensate, or hair.

Biomarker of effect:

A measurable biological alteration in an organism that may be associated with a possible or established health effect or a disease. Biomarkers of potential harm, including biomarkers of oxidative stress measuring oxidative stress level in individuals, are biomarkers of effect.

Biomarker of exposure:

A xenobiotic, its metabolite(s), or the product(s) resulting from an interaction between the xenobiotic and cellular constituents. Ideal biomarker of exposure is specific to the xenobiotic of interest and its concentration is proportional to the degree of exposure.

Biological half-life:

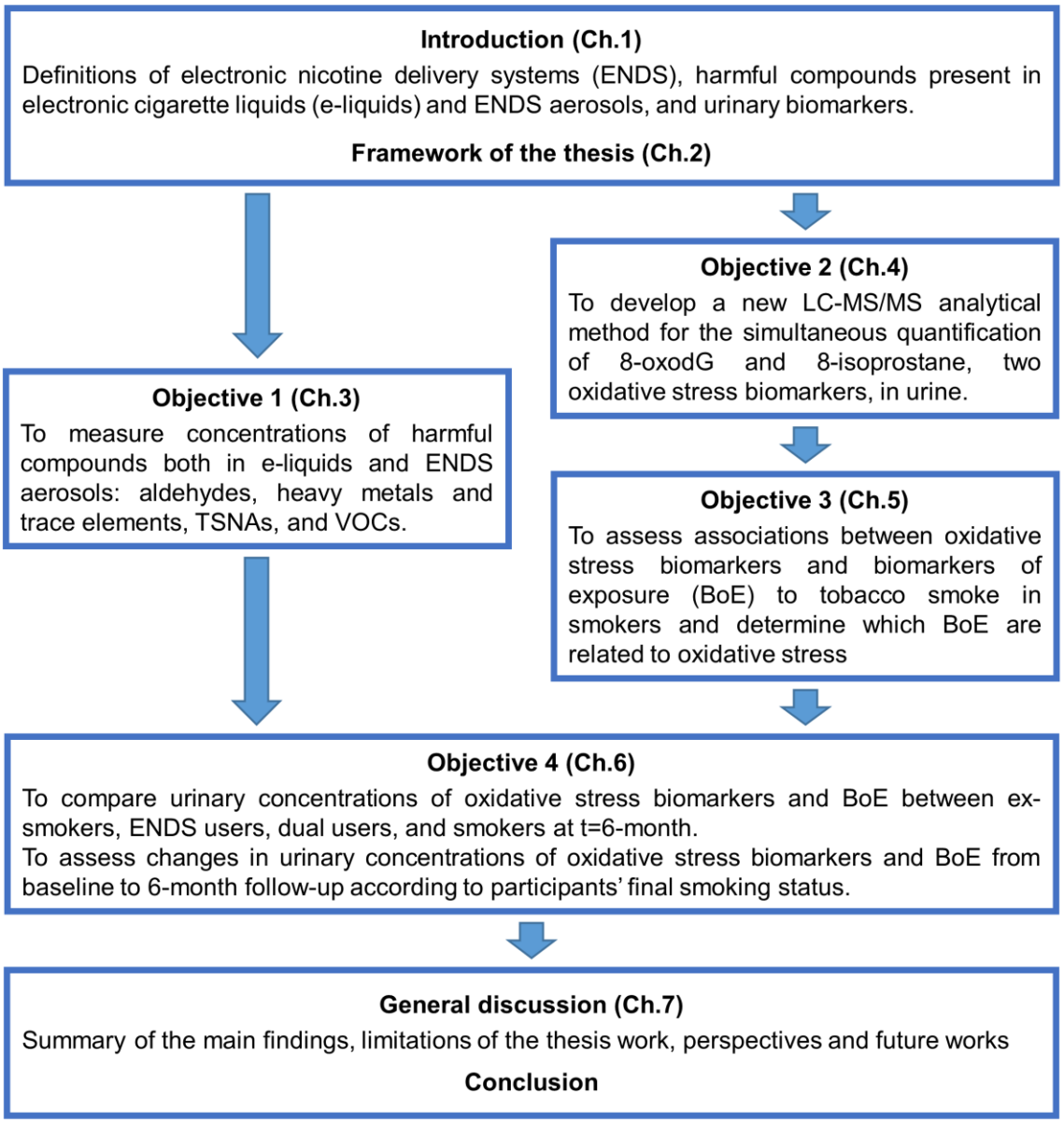
Time it takes for the concentration of a biomarker to drop in half in blood and is an indication of the peak dose. Elimination half-life is an estimate of the time it takes for a compound to be eliminated from the body (seven half-lives corresponds to elimination of about 99%).

Biotransformation:

Detoxification process used by an organism to eliminate a xenobiotic. It involves metabolism, often by enzymes, to increase the polarity of the xenobiotic to favor excretion.

[1] Definitions were modified from the Public Health Toxicology (open) course of The Johns Hopkins University (Creative Commons BY-NS-SA).

Thesis overview



Chapter 1 – Electronic Nicotine Delivery Systems

Electronic Nicotine Delivery Systems (ENDS), also known as electronic cigarettes, have become popular over the past decade and have conquered millions of users worldwide (Euromonitor International, 2017). While they were originally intended for smokers wishing to stop smoking, they have also seduced younger people (including non-smokers) by their high-tech designs and their sweet flavors. A part of the research in recent years has focused on identifying and quantifying harmful compounds in ENDS emissions, as well as their effects on cells, animals or humans, in order to assess short-term health risks. These results also provide insight into potential long-term health effects, although these remain largely unknown. More studies are needed to support recommendations and regulations for these rapidly evolving products.

Chapter 1 is divided into two main parts: the description of ENDS (Subchapters 1.1 to 1.4) and the toxicological considerations of these products (Subchapters 1.5 to 1.8). It concludes with a brief overview of the current debates surrounding ENDS (Subchapter 1.9).

1.1 A brief history of ENDS

The story begins in 1927 when Joseph Robinson filed a patent for an invention he named “Electric Vaporizer” (Robinson, 1930). This device designed to vaporize medical products was never commercialized. More than 30 years later, in 1965, Herbert A. Gilbert invented the very first electronic cigarette (Gilbert, 1965). He approached chemical, pharmaceutical, and tobacco companies with his “smokeless non-tobacco cigarette” in the hope of marketing his invention. He was unsuccessful and declared later that “these companies did what they had to do to try to protect their markets” (Dunworth, 2013). That time was not favorable to new products because combustible cigarette was then very popular, and tobacco industries were still striving to hide and deny the harmfulness of tobacco smoke. A few decades later, Phil Ray and Norman Jacobson marketed a (non-electronic) device that allowed for the inhalation of nicotine by evaporation, without combustion. They introduced for the first time the term “to vape” (Ling and Glantz, 2005). However, the U.S. Food and Drug Administration (FDA) banned the product in 1987 because it was considered as a new drug (Dunworth, 2014). Sixteen years later, in 2003, Hon Lik developed the very first modern electronic cigarette based on the

technology of ultrasonic nebulization to deliver nicotine (Hon, 2010). Nicotine was introduced in a mixture of propylene glycol and glycerol, and this composition is still the basis of the overwhelming majority of current electronic cigarette liquids (e-liquids). Finally, David Yunqiang Xiu invented and patented the technology of vaporization by electrical resistance heating under the name of Electronic Nicotine Delivery Systems in 2009 (Leduc and Quoix, 2016). This technology is the basis of all ENDS commercially available today.

1.2 How the electronic cigarettes work

Despite their name, electronic cigarettes have very little in common with combustible cigarettes, except that they are both means of delivering nicotine by inhalation. This generic term is sometimes used for other products such as heated tobacco products (HTPs; originally marketed as “heat-not-burn” products), but these are beyond the scope of this thesis. The term electronic nicotine delivery systems (ENDS) will be used to avoid confusion.

The technology of these devices is rather simple, but highly effective: an e-liquid is vaporized on contact with a heated element connected to a battery, and the vapor condensates rapidly to e-liquid droplets when the temperature of the gas phase decreases (David et al., 2020). The suspension of liquid droplet in a gas phase is called aerosol. The users inhale ENDS aerosols via a mouthpiece. The heating element is a coiled resistive wire (commonly named coil or resistance) composed of metal. Large varieties of coil composition exist: pure nickel, pure titanium, nichrome (alloy of nickel and chromium), stainless steel (alloy of iron, carbon, chromium and nickel), and kanthal (alloy of iron, chromium, and aluminum) (Vaping360, 2021). The coil is wrapped with an absorbent material, most often cotton wick. Other wicking materials include silica wick, ceramic wick, stainless steel mesh, and rayon wick (My Vape Box, 2020). The coil is alimented by a battery and it produces heat following Joule’s first law. Temperatures are usually comprised between 150°C and 250°C, depending on the power of the device (Geiss et al., 2016).

1.3 The different types of ENDS

ENDS have evolved rapidly since their introduction on the market, and the different models have been classified in four generations according to their characteristics (McRobbie, 2014;

Martin B., 2015; CDC, 2019; Williams and Talbot, 2019). One version of this classification is presented below.

- First generation: the “cig-a-like”

The first models of ENDS were designed to look like cigarettes and consisted of three components: the battery, the atomizer (part containing the resistive wire and the wicking material), and the cartridge (e-liquid reservoir). The battery part worked with a low and fixed voltage (around 3.7 V) and could contain either an electronic airflow sensor or a power button to activate the device. The models of this generation were further separated into two categories: disposable and reusable. The disposable models consisted of a single unit consisting of a battery, an atomizer and a cartridge. They worked until the battery was discharged and were then discarded. The reusable models, on the other hand, consisted of two separated parts: the cartomizer and the battery. The cartomizer is the fusion of the atomizer part with the cartridge, which is pre-filled with e-liquid. While the cartomizer has to be exchanged when the e-liquid runs out, the battery can be recharged, allowing multiple uses. More recent cartomizers can also be refilled by the users.

- Second generation: the “clearomizers”

The main constituents of ENDS remain the same compared to the first generation. Clearomizers, similarly to cartomizers, are the fusion between the atomizer and the cartridge. However, the two main differences are that the atomizer can be replaced (replaceable coil or resistance) and that the cartridge can be refilled with e-liquid. Second generation models are also called “tank”. The devices are generally larger and have lithium ion batteries that have a higher capacity. The voltage can be adjusted (between 3 V and 6 V) in several models. Users usually have to press manually the power button when using these devices.

- Third generation: the “mods”

ENDS from third generation are modifiable devices (“mods”), which are adapted for advanced ENDS users. They are sometimes also called Advanced Personal Vaporizers (APV). These devices differ from previous generations because users can select or modify ENDS components and vary the voltage, wattage, power, or even the operating temperature. Regulated mods and mechanical mods are the two main types of mods. Regulated mods contain an integrated circuit that allows the selection of voltage and/or wattage output. Mechanical mods are reserved for advanced users only as they do not contain integrated circuit: they are composed solely of a battery part, a connector and a power button. Using mechanical mods requires some knowledge in electricity as there is no security to prevent

overheating. Both mechanical and regulated mods can be combined with different kinds of atomizers. Modern devices also include box mods (square-shaped design or box-like shape) and squonk mods (adapted for dripping; also called bottom-fed devices as e-liquid is pumped, on demand, to the bottom of the atomizer).

The third generation includes several categories of atomizers: customizable clearomizers, rebuildable dripping atomizers (RDAs), and sub-ohm atomizers. Customizable clearomizers contain atomizer with different compositions and shapes. Advanced ENDS users can build their own atomizers by choosing the coils and wicking materials (rebuildable tank atomizers). RDAs are used without e-liquid reservoir: users have to drop e-liquids in the atomizer every few minutes ("direct dripping"). Compared to the clearomizers, RDAs offer stronger throat hit (i.e., satisfying sensations when smoke/aerosol containing nicotine reaches the back of the throat) and stronger aerosol production. Moreover, alternate flavors can be used without having to switch or wash the tank. However, RDAs can reach higher temperatures due to insufficient e-liquid supply, which might favor pyrolysis and emissions of harmful compounds. Sub-ohm atomizers have low resistance (<1 Ω), allowing an increased current passing through the coil that heat it up faster and generate more aerosols. They can be used in combination with RDAs. The latest models, those with temperature control or those with sub-ohm atomizers, are sometimes referred to as fourth generation.

- Fourth generation: the "pods"

Pods, ultra-portable systems, are similar to cartomizers: they are the fusion of an atomizer and a cartridge, and they can be either prefilled or refillable. The main advantage of pods is their user-friendliness: they do not required any knowledge of vaping. Unlike mods, there are no modifiable parameters in pods, and they are mostly auto-draw (buttonless) devices (i.e., they are activated upon inhalation). They are generally small in size (e.g., like an USB stick). Most pods are filled with nicotine salts, which allows a higher nicotine delivery per inhalation. Disposable pods, such as puff bars, are sometimes considered as the 5th generation. Users are seduced by the convenience of an easy-to-use ENDS that does not require charging, regular coil changes or refilling with e-liquid.

1.4 Electronic cigarette liquids

Electronic cigarette liquids (e-liquids) are mainly composed of propylene glycol (PG) and vegetable glycerin (VG) at different proportions (usually more than 95% of total volume), as

well as various flavorings and nicotine. Most e-liquids (99%) sold in the United States contain nicotine (CDC, 2021). In Europe, the proportion of ENDS users consuming e-liquid with nicotine varies from 63 to 96% (Kapan et al., 2020).

Propylene glycol, or propane-1,2-diol, is a colorless, viscous liquid (12.78 mPa·s at 45°C) with a slight sweet taste (Sigma-Aldrich, 2021a). PG has been widely used in cosmetic, pharmaceutical and food products (E1520). The current acceptable daily intake (ADI) of PG is 25 mg/kg per day, and PG is generally recognized as safe by oral, dermal and inhalation routes (EFSA Panel on Food Additives and Nutrient Sources added to Food (ANS) et al., 2018). Manifestation of PG toxicity appears at high doses (≥1 g/kg per day) in adult, which is much higher than the daily use of ENDS (0.7–34 mL per day) (Aherrera et al., 2017; EMA, 2017). However, throat irritation may happen in some individuals following exposure to PG aerosol at lower concentrations (Wieslander et al., 2001). Propane-1,3-diol (PDO) was proposed as a substitute to PG in e-liquids as it showed better thermal stability and flavoring properties (Bertrand et al., 2018).

Glycerol, glycerin or propane-1,2,3-triol, is also a colorless, odorless liquid that has a sweet taste, and has low toxicity. It is more viscous than PG (612 mPa·s at 30°C), and is therefore not suitable to all ENDS (Sigma-Aldrich, 2021b). Glycerol is also widely used in cosmetic, pharmaceutical and food products (E422). There is no ADI for glycerol as no adverse effect was observed in toxicological studies in animals (EFSA Panel on Food Additives and Nutrient Sources added to Food (ANS) et al., 2017). The most common source of glycerol found in e-liquid composition is vegetable-derived, which is why it is usually called vegetable glycerin.

In addition to PG/VG, a large variety of flavorings is added to e-liquids. A study described the flavored e-liquids sold in the Netherlands in 2017 and identified nearly 20,000 e-liquids and 245 unique flavoring compositions (Havermans et al., 2021). The most popular categories were fruits (34%), tobacco (16%), and dessert (10%). In the U.S. population, the results from the Population Assessment of Tobacco and Health Study (2014-2015 and 2015-2016) indicated that the most common flavors for both adults and youths were fruits, tobacco, dessert, and menthol (Schneller et al., 2018, 2019). The same flavor categories were reported by ENDS users from Canada and the United States in 2018 (Gravely et al., 2020). Most of the flavorings are also used in the food industry. While their oral toxicity is low, the effects of inhaling these flavorings have not always been tested.

In Europe, and in Switzerland, the concentration of nicotine in e-liquids is limited to 20 mg/mL according to the EU Tobacco Products Directive (European Union, 2016). No limit exists in the

United States, and e-liquids with nicotine concentration up to 50 mg/mL can be bought. Nicotine was present as free base, not protonated, in the first commercialized e-liquids, which limited the maximum concentration to avoid strong irritation of the throat. In pods, nicotine is present in salt form, mainly nicotine benzoate, which allows increasing the maximum concentration of nicotine as it is better tolerated by the user in this form (O'Connell et al., 2019).

1.5 Toxicants in e-liquids

The analysis of impurities in e-liquids gives insights to harmful compounds that ENDS users may inhale. Some harmful compounds are already present in e-liquids, while others are found almost exclusively in their aerosols. Stability of e-liquids over time is not well known, and degradation products could be formed from different e-liquid constituents, including flavorings. A recent meta-analysis classified the flavorings present in e-liquids according to their chemical groups (Salam et al., 2020). Twenty-two chemical classes were identified, the most frequent being alkenes (33%), esters (33%), aryls (24%), alcohols (18%), ketones (14%), aldehydes (10%), and lactones (9%). Different chemical transformations have been predicted and used to guide studies in further identifying new potential harmful compounds. Reactions between aldehydes and PG leading to acetals (acetalization) have been reported (Erythropel et al., 2019). No toxicological data on inhalation of these acetals is available. Cytotoxic and metabolic effects of PG acetals by in vitro studies have been reported, but further toxicological studies are recommended (Jabba et al., 2020). Therefore, information given by the manufacturers regarding e-liquid composition might be insufficient to perform a risk assessment.

The list of constituents identified in e-liquids has been established in a recent review (Eshraghian and Al-Delaimy, 2021). Firstly, most research focused on the compounds from the list of harmful and potentially harmful constituents (HPHCs) in tobacco products and tobacco smoke issued by the Food and Drug Administration (FDA) to compare ENDS and cigarettes (The Food and Drug Administration (FDA), 2012). Research then expanded to include impurities present in the solvents or other constituents and flavorings potentially harmful by inhalation. Most of the harmful compounds identified in e-liquids belong to the following chemical families: aldehydes, heavy metals and trace elements, polycyclic aromatic hydrocarbons (PAHs), tobacco-specific nitrosamines (TSNAs), and volatile organic compounds (VOCs). Tables 1 to 5 summarize the compounds identified in e-liquids. Flavorings with no known adverse effects have not been included in these tables. Moreover, studies that reported concentrations below the limit of quantification (LOQ) were not included in the tables.

Table 1 presents aldehydes identified in e-liquids. Formaldehyde is a Group 1 carcinogen (carcinogenic to human – IARC) and acetaldehyde is a Group 2B carcinogen (possibly carcinogenic to human – IARC) or a Group 1 carcinogen (IARC) when present in alcoholic beverages. Concentrations of acetaldehyde and formaldehyde quantified in e-liquid were lower than in aerosols. Most recent studies focused exclusively on ENDS emissions, as both compounds can be formed as a result of thermal degradation of the main components of e-liquids, PG and VG (Farsalinos and Gillman, 2018). Some common flavorings are also aldehydes, such as benzaldehyde, cinnamaldehyde, ethylvanillin, and vanillin. These can induce irritation responses upon inhalation.

Table 1 – List of aldehydes previously identified in electronic cigarette liquids (e-liquids)

Compounds	References
Acetaldehyde	(Farsalinos et al., 2015b; Varlet et al., 2015; Han et al., 2016; Sleiman et al., 2016; LeBouf et al., 2018)
Formaldehyde	(Farsalinos et al., 2015b; Varlet et al., 2015; Han et al., 2016; Sleiman et al., 2016)

Table 2 presents metals identified in e-liquids. Heavy metals include both essential (e.g., Fe, Zn, Se, Cu, Cr, Mn, and Mo) and non-essential (e.g., Cd, As, and Pb) trace elements (Marcovecchio et al., 2013). Above normal physiological concentrations, both can cause several acute and chronic adverse health effects (Balali-Mood et al., 2021). They were linked to cancer, kidney toxicity, neurotoxicity, cardiovascular diseases, respiratory symptoms and oxidative stress (Gaur and Agnihotri, 2019). The analysis of metals in e-liquids was the subject of a systematic review, which showed that measured concentrations vary greatly between studies (Zhao et al., 2020). In general, e-liquids samples that were in contact with the heating element (e.g., in clearomizers or pods) have higher metal concentrations than those of e-liquids still in bottles.

Table 2 – List of heavy metals and trace elements previously identified in electronic cigarette liquids (e-liquids)

Compound	References
Aluminum	(Beauval et al., 2016, 2017; Palazzolo et al., 2016; Olmedo et al., 2018)

Compound	References
Antimony	(Beauval et al., 2016, 2017; Olmedo et al., 2018)
Arsenic	(Beauval et al., 2016; Palazzolo et al., 2016; Olmedo et al., 2018; Song et al., 2018)
Cadmium	(Hess et al., 2017; Talio et al., 2017; Song et al., 2018)
Chromium	(Beauval et al., 2016, 2017; Hess et al., 2017; Kamilari et al., 2018; Olmedo et al., 2018)
Copper	(Beauval et al., 2016, 2017; Kamilari et al., 2018; Olmedo et al., 2018)
Lead	(Talio et al., 2015; Hess et al., 2017; Dunbar et al., 2018; Kamilari et al., 2018; Olmedo et al., 2018; Song et al., 2018)
Manganese	(Beauval et al., 2016; Palazzolo et al., 2016; Hess et al., 2017; Olmedo et al., 2018)
Nickel	(Palazzolo et al., 2016; Hess et al., 2017; Talio et al., 2017; Kamilari et al., 2018; Olmedo et al., 2018; Song et al., 2018)
Tin	(Olmedo et al., 2018)
Zinc	(Olmedo et al., 2018)

Table 3 presents PAHs identified in e-liquids. PAHs are formed during the pyrolysis processes or the incomplete combustion of organic matter. They are composed of carbon and hydrogen atoms and have between two and more fused aromatic rings. They present a low acute toxicity to human, but many of them are carcinogenic, especially the heavier ones (i.e., with an increased number of aromatic rings) (Moorthy et al., 2015; ATSDR, 2021). PAHs found in e-liquids are low molecular weight PAHs, and their concentrations are generally very low.

Table 3 – List of polycyclic aromatic hydrocarbons (PAHs) previously identified in electronic cigarette liquids (e-liquids)

Compound	References
Acenaphthene	(Han et al., 2016; Beauval et al., 2017)
Acenaphthylene	(Han et al., 2016; Beauval et al., 2017; Larcombe et al., 2021)
Chrysene	(Han et al., 2016; Beauval et al., 2017; Larcombe et al., 2021)

Compound	References
Fluoranthene	(Han et al., 2016; Beauval et al., 2017)
Fluorene	(Han et al., 2016; Beauval et al., 2017; Larcombe et al., 2021)
Naphthalene	(Han et al., 2016; Beauval et al., 2017; Barhdadi et al., 2021)
Phenanthrene	(Han et al., 2016; Beauval et al., 2017; Larcombe et al., 2021)
Pyrene	(Larcombe et al., 2021)
1-Methyl naphthalene	(Czoli et al., 2019)
2-Methyl naphthalene	(Czoli et al., 2019)

Table 4 presents the harmful VOCs identified in e-liquids. VOCs are defined as substances with a high vapor pressure and containing one or more carbon atoms. They include a large diversity of compounds, such as solvents, carbonyls, terpenoids, and alcohols. Not all VOCs cause adverse health effects; however, exposure to VOCs has been linked to respiratory symptoms, irritation, damage to the liver, kidney or central nervous systems, and cancers (US EPA, 2014). Flavorings are also part of the VOC family. The vast majority has a priori a low toxicity, at least by ingestion (Dinu et al., 2020). Others, such as diacetyl or acetylpropionyl were shown to be harmful to health upon inhalation. There is growing evidence that high concentrations of some flavorings via inhalation can cause deleterious effects to health, particularly because of their cytotoxic nature.

Table 4 – List of volatile organic compounds (VOCs) previously identified in electronic cigarette liquids (e-liquids)

Compound	References
Acetylpropionyl	(Farsalinos et al., 2015c; LeBouf et al., 2018; Barhdadi et al., 2021)
Benzene	(Han et al., 2016; LeBouf et al., 2018; Wagner et al., 2018)
Diacetyl	(Farsalinos et al., 2015c; Varlet et al., 2015; LeBouf et al., 2018; Barhdadi et al., 2021)
Ethyl benzene	(Han et al., 2016; LeBouf et al., 2018; Barhdadi et al., 2021)
Propylene oxide	(Sleiman et al., 2016)

Compound	References
Toluene	(Han et al., 2016; LeBouf et al., 2018; Wagner et al., 2018)
m-Xylene + *p*-xylene	(Han et al., 2016; LeBouf et al., 2018)
o-Xylene	(Han et al., 2016; LeBouf et al., 2018; Barhdadi et al., 2021)

Table 5 shows TSNAs identified in e-liquids. TSNAs are impurities found in tobacco products, which are formed during the curing and processing of tobacco by nitrosation reactions. TSNAs are strong carcinogens (Konstantinou et al., 2018). They can be found in nicotine extract from tobacco if it has been insufficiently purified. However, they are mostly undetectable or present at trace levels in pharmaceutical grade nicotine, which is also used in nicotine replacement therapy (NRT) products.

Table 5 – List of tobacco-specific nitrosamines (TSNAs) identified in electronic cigarette liquids (e-liquids)

Compound	References
N-Nitrosoanabasine (NAB)	(Han et al., 2016)
N-nitrosonornicotine (NNN)	(Farsalinos et al., 2015b)
4-(N-Nitrosomethylamino)-1-(3-pyridyl)-1-butanone (NNK)	(Farsalinos et al., 2015b; Han et al., 2016)

The vast majority of harmful compounds detected in e-liquids were present in trace amounts (with the exception of the flavorings) and have not been detected by all research groups. This is because e-liquid formulations are relatively simple, with few ingredients. The presence of impurities therefore depends on the quality of raw materials and the cleanliness of production lines and packaging equipment.

1.6 Puffing topography

The use of ENDS is subject to wide inter-individual variations, as is cigarette smoking. Several parameters define the puffing topography: puff duration, puff volume, puff number, and inter-puff interval. The daily or weekly e-liquid consumption also provides an insight of ENDS use.

Different methods were applied to characterize puffing topography: video recording of ENDS users, detection of hand movements, analysis of videos from the internet (especially on social networks), and use of commercial and non-commercial topography monitors (Robinson et al., 2021). Most studies were performed in laboratory conditions with fixed parameters, while only a few were done in the natural environment (i.e., the everyday life of the ENDS users) to estimate the actual use of ENDS. Puffing topography gives an insight of the puffing behavior of real ENDS users, and is the basis to establish standardized vaping machine protocol. However, many factors have an influence on puffing topography, such as the device type, the device power, the nicotine content of e-liquid, the ratio PG/VG, the e-liquid flavor, and ENDS user experience (Lee et al., 2015; Dawkins et al., 2016; Hiler et al., 2017; Farsalinos et al., 2018b; Lee et al., 2018b; Spindle et al., 2018; St Helen et al., 2018; Lee et al., 2019; Hiler et al., 2020; Kimber et al., 2021; Wagener et al., 2021). Table 6 presents the parameters of puffing topography (puff duration, puff volume, puff number, and inter-puff interval) reported in the literature.

Table 6 – Puffing topography parameters previously reported in literature: puff duration (s), puff volume (mL), inter-puff interval (s), and puff number. For each study, the generation of ENDS, the length of the session (directed or free), and the measurement device are reported. Several studies have also assessed the effect of some parameters on puffing topography such as ENDS devices, power settings, coil resistances, nicotine concentrations, flavors, propylene glycol/vegetable glycerin (PG/VG) ratios, and ENDS user experience.

Study	Conditions		Puff duration [s]	Puff volume [mL]	Inter-puff interval [s]	Puff number [-]
1st generation ENDS						
(Behar et al., 2015) [1]	10-min session (*ad libidum*); measurement with a CReSS pocket device		2.75 (0.96)	56 (22)	16.9 (8.2)	33 (8)
(Robinson et al., 2015) [1]	24-hour session (*ad libidum*); measurement with a wPUM		3.5 (1.8)	133 (90)	-	-
(Robinson et al., 2016) [1]	1-week session (*ad libidum*); measurement with a wPUM		2 (0.6)	65.4 (24.8)	-	-
(Lee et al., 2015) [2,3]	Free session (*ad libidum*); measurement with a CReSS pocket device	Baseline	2.2±0.1	64.0±4.8	19.2±2.7	19.3±2.5
		Week 1	3.1±0.3	66.5±3.7	15.2±2.2	23.7±2.4
		Week 2	2.9± 0.29	63.3± 5.2	22.1± 4.9	21.3±2.4

Study	Conditions		Puff duration [s]	Puff volume [mL]	Inter-puff interval [s]	Puff number [-]
(Lee et al., 2018b) [4]	1-week session (*ad libidum*); measurement with a (wPUM)	Established smokers	3.3 [2.3–4.3]	110.3 [10.4–150.3]	38.1 [24.7–51.4] [5]	13.7 [9.4–18.0] [5]
		Established non-smokers	1.8 [1.5–2.1]	54.7 [41.5–67.9]	21.7 [12.1-31.4] [5]	11.9 [9.1–14.7] [5]
2nd generation ENDS						
(Hiler et al., 2017) [1]	Directed session (10 puffs, each 30 s); parameters recorded by a mouthpiece-based topography recording device Experienced users used ENDS for ≥ 3 month with ≥ 1 mL per day (nicotine concentration ≥ 8 mg/mL) and smoked ≤ 5 cigarettes daily (CO ≤ 10 ppm) Naïve users were smokers: ≥ 10 cigarettes daily (CO ≥ 15 ppm) and < 5 ENDS use	0 ng/mL [6]				
		Experienced	5.9 (2.4)	175.7 (149.7)	-	-
		Naïve	3.3 (1.7)	100.0 (64.8)	-	-
		8 ng/mL [6]				
		Experienced	5.7 (2.2)	181.0 (139.6)	-	-
		Naïve	3.0 (1.5)	101.5 (66.6)	-	-
		18 ng/mL [6]				
		Experienced	5.0 (1.9)	127.0 (80.8)	-	-
		Naïve	2.8 (1.3)	86.5 (59.4)	-	-
		38 ng/mL [6]				
		Experienced	4.7 (3.9)	123.3 (168.1)	-	-
		Naïve	2.2 (0.8)	68.3 (64.1)	-	-
(Kosmider et al., 2018) [1]	24-h session (*ad libidum*); measurement with a CReSS pocket device		3.1 (1.2)	73.4 (51.5)	15.4 (22.0) [5]	156.2 (10.3)
(Spindle et al., 2018) [1]	Directed session (10 puffs, each 30 s); parameters recorded by a mouthpiece-based topography recording device	PG/VG 2:98	5.26 (1.95)	115.45 (58.28)	-	-
		PG/VG 20:80	4.99 (1.99)	108.85 (51.34)	-	-
		PG/VG 55:45	4.47 (1.52)	96.81 (51.61)	-	-
		PG/VG 100:0	4.32 (1.35)	100.25 (47.12)	-	-
(Lee et al., 2019) [7]	2-week session (*ad libidum*); measurement with a wPUM	Light	2.0	59.9	-	16.7 [5]
		Heavy	4.4	290.9	-	14.7 [5]

Study	Conditions		Puff duration [s]	Puff volume [mL]	Inter-puff interval [s]	Puff number [-]
3rd generation ENDS						
(Dawkins et al., 2016) [1]	1-h session (*ad libidum*); parameters recorded by the battery	24 ng/mL [6]	3.84 (1.02)	-	-	48.36 (22.86)
		6 ng/mL [6]	5.20 (1.39)	-	-	70.73 (34.45)
(Farsalinos et al., 2018b) [1]	30-min session (*ad libidum*); parameters recorded by the battery	6 W [8]	4.6 (1.0)	-	-	57 (20)
		10 W [8]	3.8 (0.8)	-	-	46 (16)
(St Helen et al., 2018) [1]	90-min session (*ad libidum*); measurement via video recording	Usual flavor	4.3 (1.6)	-	70.2 (44.7)	106 (67)
		Strawberry flavor	3.2 (1.3)	-	91.3 (48.4)	73 (35)
		Tobacco flavor	2.8 (1.1)	-	106.9 (65.9)	69 (46)
(Hiler et al., 2020) [1]	1-hour session (*ad libidum*); parameters recorded by a mouthpiece-based topography recording device [9]	8 mg/mL [6], 0.5 Ω [10], 40.5 W [8]	2.2 (0.9)	363.2 (147.3)	122.8 (88.6)	33.0 (16.4)
		3 mg/mL [6], 0.5 Ω [10], 40.5 W [8]	2.7 (0.7)	519.6 (252.3)	85.5 (40.7)	45.2 (17.1)
		8 mg/mL [6], 1.5 Ω [10], 13.5 W [8]	3.3 (1.1)	384.4 (185.2)	107.1 (78.8)	35.4 (18.3)
		3 mg/mL [6], 1.5 Ω [10], 13.5 W [8]	3.8 (1.5)	481.1 (275.4)	81.0 (39.8)	48.3 (29.7)
(Kimber et al., 2021) [3,4]	1st and 3rd generation ENDS; 20-min session (*ad libidum*); measurement via video recording [11]	Cig-a-like 18 mg/mL [6] Week 2	3.86 [3.07–4.65]	33.36 [26.62–40.07]	36.34 [8.13–64.55]	-
		Tank 18 mg/mL [6] Week 2	2.45 [1.86–3.04]	18.15 [13.18–23.12]	92.22 [71.30–113.14]	-
		Tank 6 mg/mL [6] Week 2	2.77 [2.17–3.37]	23.16 [18.06–28.26]	72.28 [50.81–93.74]	-

[1]Parameters presented as mean (standard deviation; SD); [2]Parameters presented as mean ± standard error of measurement (SEM); [3]Parameters reported for smokers who switched from tobacco to ENDS (baseline = initial use); [4]Parameters presented as mean [95% confidence interval; CI]; [5]For a typical session; [6]Nicotine concentration in e-liquid; [7]Parameters presented as mean (retrieved from a figure); [8]Battery power setting expressed in watts (W); [9]Sessions of 10-min (directed; 10 puffs every 30 s) were also recorded but not reported here; [10]Resistance of the coil; [11]Baseline and week 1 data were not included in the table to make it clearer. Topography devices: Clinical Research Support System (CReSS Pocket or CReSSmicro), wireless personal use monitor (wPUM).

Several studies investigated the so-called compensatory puffing behavior by varying nicotine levels (see Table 6). Individuals suffering from nicotine addiction need a certain dose to satisfy the craving, which varies between individuals (SCENIHR, 2010). Therefore, they will adjust their consumption of nicotine accordingly, as it was previously shown for smokers (Scherer, 1999). This effect was showed with a 3rd generation ENDS device and different nicotine content and battery parameters (Cox et al., 2021). It was also observed when smokers switched from tobacco to ENDS: individuals gradually changed their puffing behavior (Wagener et al., 2021).

The parameters observed in previous studies (Table 6) indicate that there is no typical puffing topography profile. This is particularly important considering that toxicological studies rely on standard puffing regimes to generate ENDS aerosols under controlled conditions with vaping machines. In 2015, the Cooperation Centre for Scientific Research Relative to Tobacco (CORESTA) E-cigarette Task Force wrote a report (2014 Electronic Cigarette Aerosol Parameters Study) that led to the publication of a CORESTA recommended method (CRM) defining the standards conditions of ENDS aerosol generation and collection parameters of an analytical vaping machine (Cooperation Centre for Scientific Research Relative to Tobacco (CORESTA), 2015a). Parameters defined in this CRM n°81 are the following: puff duration of 3 s, puff volume of 55 mL, and puff frequency of 1 puff every 30 s (Cooperation Centre for Scientific Research Relative to Tobacco (CORESTA), 2015b). In 2018, an international ISO standard (ISO 20768:2018) was published based on the CRM n°81 with the same puffing regime (ISO, 2018). However, this puffing regime was criticized as it was representative of the mouth-to-lung (MTL) vaping profile, but not of the direct-to-lung (DTL) vaping profile, during which longer puff durations and larger puff volumes were observed (Soulet et al., 2019). It was recommended adjusting the concentrations measured in aerosol by the mass of vaporized e-liquid as this is more reliable than the expression of these concentrations per puff (Farsalinos et al., 2018b).

1.7 Toxicants in ENDS aerosols

The main chemical families of compounds identified in aerosols are similar to those found in e-liquids: aldehydes, heavy metals and trace elements, PAHs, TSNAs, and VOCs. Free radicals and reactive oxygen species (ROS) were also measured in ENDS aerosols. Harmful compounds in ENDS aerosol were subject to a systematic review (Ward et al., 2020). The selected studies reported that concentrations of harmful compounds were globally higher with devices that were more powerful or with higher voltage, and that flavorings and/or thermal

degradation might be the sources of these harmful compounds. More than 90 publications were included in that review.

The interpretation of studies on ENDS emissions is challenging because multiple factors can influence the concentrations of harmful compounds: power, voltage, resistance, temperature, coil material, device, e-liquid, puffing regime, collection method, and analysis method. A standardized research e-cigarette (SREC) was developed by the National Institute on Drug Abuse (NIDA) in partnership with NJOY LLC for clinical studies (National Institute on Drug Abuse, 2021). The advantage is that the emissions of this device have been fully characterized, which facilitates the interpretation of the results and especially the comparison between different clinical studies using this device.

Table 7 presents the observations and conclusions of studies on aldehyde emissions. Several authors reported concentrations of carbonyls (including aldehydes, ketones, and other compounds with a C=O double bond), although the majority focused primarily on formaldehyde, acetaldehyde, and acrolein that are thermal degradation products of PG and VG. The addition of sweetener additives and flavorings in e-liquids increased the generation of aldehydes. The increase extent depended on the type of flavorings (e.g., fruity flavored e-liquids emitted more aldehydes) and the concentrations of flavorings in the mixture. Most powerful devices (i.e., with high power per unit heating coil surface area) were those leading to the formation of more aldehydes. Direct dripping is often associated to higher temperature and therefore to a greater thermal degradation of PG/VG. The proportion of PG in e-liquids positively correlated with the levels of aldehyde in aerosols. Finally, the puffing regime had a great influence on aldehyde emissions: concentrations increased with the puff number, the puff duration, and the puff volume. It was shown that the correction by mass of aerosolized e-liquid did not remove the observed increase.

Table 7 – Summary of studies reporting aldehyde concentrations in ENDS aerosols and their observations and conclusions. Studies are sorted according to the parameters related to aldehyde production: additives, battery settings, device types, flavorings, puffing regimes, and others.

Parameters	Study	Conclusions
Additives	(Duell et al., 2019)	Sucralose (a sweetener) increased the formation of aldehydes; it also promoted hemiacetal formation
Battery settings	(Kosmider et al., 2014)	Concentrations increased with power output; pure PG promoted aldehyde formation

Parameters	Study	Conclusions
	(Farsalinos et al., 2015d, 2017)	Users were asked to identify dry puffs while voltage settings were increased; comparison with laboratory analysis
	(Gillman et al., 2016)	Concentrations varied with device and power settings; some devices did not emit high aldehyde concentrations at high power outputs
	(Geiss et al., 2016)	Concentrations increased with power outputs; above 15 W, flavor was altered and the vapor was too hot for users
	(Sleiman et al., 2016)	Concentrations were higher for single-coil compared to double-coil devices; voltage also increased aldehyde formation
	(Jensen et al., 2017)	Identification of the compounds (including hemiacetals) resulting from thermal degradation of PG and VG at different power settings
	(Ogunwale et al., 2017)	Concentrations increased with power output; formation of formaldehyde-hemiacetal was observed (above 11.7 W)
	(Salamanca et al., 2017)	Formaldehyde and related hemiacetal concentrations increased with power output; hemiacetals concentrations were higher than free formaldehyde
	(Talih et al., 2017)	High powers do not necessarily produce high aldehyde concentrations; it correlated with the power per unit heating coil surface area
	(Farsalinos et al., 2018a)	Two power settings were tested; no difference was observed
	(Vreeke et al., 2018)	Triacetyl promoted the formation of aldehydes, including hemiacetals; increased also with power output
	(Cirillo et al., 2019)	E-liquid without nicotine increased aldehyde formation; concentrations were higher with lower resistance (the opposite for e-liquid with nicotine)
	(Stephens et al., 2019)	Concentrations increased with power output
	(Uchiyama et al., 2020)	Concentrations increased with the power output (significantly over 40 W); can be higher than cigarettes
Devices	(Blair et al., 2015)	Aldehydes were found in aerosol, supporting the degradation of VG; concentrations varied between devices

Parameters	Study	Conclusions
	(Talih et al., 2016)	Direct dripping was linked to higher temperatures, which promoted aldehyde emissions; results were higher than other ENDS
	(El-Hellani et al., 2018)	Concentrations had low variability across devices; they correlated with device brand and power output
	(Reilly et al., 2019)	Aldehyde emissions from Juul did not depend on flavorings and were lower than other ENDS devices
	(Talih et al., 2019)	Aldehyde emissions from Juul are similar to other ENDS; lower when nicotine normalized
	(Mallock et al., 2020)	European Juul version with lower nicotine had increased vaporization but did not emit higher aldehydes concentrations
	(Nicol et al., 2020)	A new-generation device with stainless steel mesh emitted less aldehydes than a nichrome wire surrounding by cotton wick
	(Rudd et al., 2020)	Aldehyde concentrations in aerosols from a pod device were below limits of quantification
	(Dusautoir et al., 2021)	Aldehydes were detected in ENDS aerosol and varied according to the power output; concentrations were lower than in HTPs or cigarettes
Flavors	(Khlystov and Samburova, 2016)	The presence of flavorings increased aldehyde formation by several orders of magnitude
	(Klager et al., 2017)	All tested devices emitted at least one aldehyde from the HPHC list; diacetyl was found in 60% of flavored e-liquid
	(Conklin et al., 2018)	Production of aldehydes depended on the PG:VG ratio and e-liquid flavors
	(Farsalinos and Voudris, 2018)	Flavorings might increase aldehyde concentrations, but they remain low
	(Qu et al., 2018)	Concentrations increased with flavoring content; fruity flavorings promoted aldehyde formation
	(Erythropel et al., 2019)	Flavorings can react with PG to form PG acetals; 50-80% of acetals were found in ENDS emissions, with unknown health effects
	(Gillman et al., 2020)	The variability of ENDS devices can be more important that the effects of flavorings on aldehyde emissions
Puffing regimes	(Uchiyama et al., 2016)	Concentrations increased with the number of puffs and the voltage; there were large discrepancies between devices

Parameters	Study	Conclusions
	(Sala et al., 2017)	Aldehydes concentrations were higher in aerosols than in e-liquid; longer puff durations promoted aldehyde formation
	(Korzun et al., 2018)	Concentrations were higher at a low flow rate (inducing higher temperature) at a fixed power
	(Kośmider et al., 2018)	Concentrations increased with the puff duration (using human topography data)
	(Beauval et al., 2019)	Concentrations varied by puffing regime (even while corrected by mass of vaporized e-liquid), but remained well below those in cigarette smoke
	(Bitzer et al., 2019)	Concentrations increased with puff volume and puff duration
	(Kosmider et al., 2020)	Concentrations were higher in low-nicotine than high-nicotine due to compensatory behavior
	(Son et al., 2020)	Concentrations were associated with the puffing regime, power settings, and flavored e-liquids
Others	(Goniewicz et al., 2014)	Concentrations were higher than nicotine inhalator, but at least 9 times lower than cigarette smoke
	(Papoušek et al., 2014)	Acrolein was detected in ENDS aerosols
	(Herrington and Myers, 2015)	Aldehydes were detected in aerosols but were absent in e-liquids; implication of the vaporization process
	(Jensen et al., 2015)	Hemiacetals can act as formaldehyde-releasing agents; formaldehyde concentrations in aerosols might be underestimated
	(Laugesen, 2015)	ENDS emitted 200 times less aldehydes than cigarette; also 73% less than 1st generation ENDS
	(Flora et al., 2016)	Aldehydes concentrations were below occupational exposure limits
	(Jo and Kim, 2016)	Aldehyde concentrations increased during vaporization; concentrations remained constant above 10 puffs
	(Margham et al., 2016)	Aldehydes were measured in aerosol; concentrations were lower than in cigarettes
	(Beauval et al., 2017)	Concentrations were lower than in cigarettes
	(Lee et al., 2017)	Acetaldehyde was under the limit of detection in ENDS aerosols
	(Lee et al., 2018a)	Aldehydes were detected both in e-liquids and in aerosols

Parameters	Study	Conclusions
	(Salamanca et al., 2018)	Formaldehyde-hemiacetal concentrations were higher than formaldehyde concentrations; above OSHA guidelines
	(Li et al., 2020)	Different compounds, including hydroxycarbonyls, were identified as significant components in aerosols

In most studies, aldehyde concentrations measured in ENDS aerosols were lower than those measured in cigarette smoke, except during dry puffs. Dry puffs are caused by an insufficient supply of e-liquid to the coil, resulting in local overheating of the coil and surrounding wicking material. High temperatures favor pyrolysis and thus emission of harmful products resulting from thermal degradation. In addition to aldehyde emission, formation of acetals and hemiacetals resulting from the reaction between aldehydes and glycols was also reported. The potential toxicity of these products is not known. Moreover, they may act as aldehyde-releasing agents in the body, implying that laboratory studies on ENDS aerosols underestimate actual aldehyde exposure.

Table 8 presents the observations and conclusions of studies on metals in ENDS aerosols. The reported concentrations varied greatly depending on the device and e-liquid tested. An increase of metal concentrations was observed with higher power output. While most metals were present in trace amounts, some were higher than in cigarette smoke. The most frequently identified metals were aluminum, iron, nickel, zinc, chromium, copper, and lead, and their concentrations were most often higher in aerosols compared to e-liquids.

Table 8 – Summary of studies reporting heavy metal and trace element concentrations in ENDS aerosol and their observations and conclusions. The studies were sorted according to the parameters that have an effect on metal concentrations: battery settings, device types, and others.

Parameters	Study	Conclusions
Battery settings	(Zhao et al., 2018)	B, Na, Al, Cu, and Zn were the main metals identified in aerosols
	(Zhao et al., 2019)	As, Cr, Cu, Fe, Mn, Ni, Pb, Sb, Sn, and Zn concentrations increased with power output; concentrations were higher in open systems than in closed systems
Devices	(Palazzolo et al., 2016)	Ni was found in ENDS aerosols; other metals were comparable to control and lower than cigarettes

Parameters	Study	Conclusions
	(Williams et al., 2017)	Ca, Na, Co, Mg, Sn, Pb, Zn, B, Al, Fe, Ge, Sn, Se, Ni, and Sb were found in most devices; Si was the most abundant one
	(Kim et al., 2018)	Five metals were quantified in aerosols of a sub-ohm device: Ca, Co, Fe, Mg, and Si
	(Olmedo et al., 2018)	Al, Cr, Ni, Pb, Zn, Mn, Fe, Cu, Sb, and Sn were detectable in most devices (n=56); Cd and Sn in most of them
	(Nicol et al., 2020)	Zn was detected in ENDS aerosols, but blank measures were fluctuating; comparison impossible with this analytical method
	(Ting et al., 2020)	Cr, Ni, Pb, and Cd were detected in ENDS aerosols; Cr exceeded PDE limit in 5% of ENDS
Others	(Goniewicz et al., 2014)	Cd, Ni, and Pb were detected in ENDS aerosols; most concentrations were comparable to Nicorette inhalator
	(Lerner et al., 2015)	Cu concentrations were 6.1 times higher than in cigarette smoke
	(Margham et al., 2016)	As, Ni, Zn, Fe, Co, and Cr were detected in ENDS aerosols; most of them close to blank values
	(Mikheev et al., 2016)	As, Cr, Ni, Co, Sb, Sn, and Zn varied across e-liquids (with or without nicotine)
	(Beauval et al., 2017)	Cd, Cr, and Sb were present in ENDS aerosols; Al, Co, Mn, Ni, and Pb were comparable to blanks
	(Lee et al., 2017)	Si, Cl, Ba, and In were detected in ENDS aerosols
	(Prokopowicz et al., 2019)	Cd and Pb were mostly under the LOQ in aerosols; concentrations were then lower than in cigarettes
	(Liu et al., 2020)	As was detected in ENDS aerosols; inorganic arsenic species were predominant and were higher in aerosols than in e-liquids

Table 9 presents the observations and conclusions of studies on PAHs in ENDS aerosols. Formation of PAHs occurs during pyrolysis or combustion processes of organic material at high temperatures. Detectable concentrations of light PAHs (2-4 rings) were observed at and above 400°C during the pyrolysis of cellulose (McGrath et al., 2003). The heating element temperature in ENDS is lower (250–350°C), which explains why PAHs were not quantified in most studies.

Table 9 – Summary of studies reporting polycyclic aromatic hydrocarbon (PAH) concentrations in ENDS aerosols and their observations and conclusions. The studies were sorted according to the parameters linked to PAH formation: device types and others.

Parameters	Study	Conclusions
Devices	(Wagner et al., 2018)	Benzo[a]pyrene was not detected in ENDS aerosols
	(Nicol et al., 2020)	Benz[j]aceanthrylene, benzo[a]anthracene, chrysene, naphthalene, benzo[b]fluoranthene, and benzo[k]fluoranthene were detected sporadically at trace levels; comparable to blank measurements
	(Rudd et al., 2020)	Benzo[a]pyrene was not detected in ENDS aerosols
	(Dusautoir et al., 2021)	22 PAHs were quantified in ENDS aerosols at very low concentrations (<10 pg/puff for most); concentrations in HTPs and cigarettes were higher
Others	(Flora et al., 2016)	Benzo[a]pyrene was not detected in ENDS aerosols
	(Margham et al., 2016)	On 16 PAHs, only naphthalene and chrysene were detected but at similar concentrations than blanks; concentrations were lower than cigarette
	(Beauval et al., 2017)	On the 16 PAHs, only naphthalene and acenaphthylene were detected in ENDS aerosols; concentrations were lower than in cigarette

Table 10 presents the observations and conclusion of studies on TSNAs in ENDS aerosols. TSNAs are impurities present in tobacco that can then be found in nicotine extracts following extraction from tobacco leaves. However, pharmaceutical grade nicotine is mainly used nowadays in e-liquids and the concentrations of TSNAs in nicotine are very low (in the ng/g range) (Moldoveanu et al., 2017). Moreover, some e-liquids contain synthetic nicotine in which these impurities are absent (Zettler et al., 2018).

Table 10 – Summary of studies reporting tobacco-specific nitrosamine (TSNA) concentrations in ENDS aerosols and their observations and conclusions.

Parameters	Study	Conclusions
Devices	(Nicol et al., 2020)	NNK was not detected in ENDS aerosols
	(Rudd et al., 2020)	NNN, NNK, NAB, and NAT were not detected in ENDS aerosols

Parameters	Study	Conclusions
Others	(Goniewicz et al., 2014)	NNN and NNK were quantified in ENDS aerosols; concentrations were higher than Nicorette inhalator
	(Farsalinos et al., 2015a)	NNN, NNK, NAB, and NAB were under limit of detection in ENDS aerosols; correlation between concentrations in spiked e-liquids and in aerosols
	(Flora et al., 2016)	NNN and NNK were below the limit of quantification in ENDS aerosols
	(Margham et al., 2016)	Traces of NNN were detected in ENDS aerosols; NNK, NAB, and NAT were not detected

Table 11 presents the observations and conclusions of studies on VOCs in ENDS aerosols. Concentrations of harmful VOCs were low, often undetected in most studies. Their concentrations are below what is found in cigarette smoke. However, it was observed that some VOCs (i.e., benzene, xylene and styrene) were formed during the vaporization process. Concentrations of benzene and toluene depended on device types and the power they delivered. Toxic flavorings were also identified in e-liquids and aerosols, such as diacetyl and acetyl propionyl. Diacetyl has been banned since 2016 in Europe (EU Tobacco Products Directive).

Table 11 – Summary of studies analyzing volatile organic compound (VOC) concentrations in ENDS emissions and their observations and conclusions. The studies were sorted according to the parameters associated to VOC formation: device types and others.

Parameters	Study	Conclusion
Devices	(Farsalinos et al., 2015c)	Diacetyl and acetyl propionyl were found in 72.3% of the tested samples; concentrations were similar in e-liquids and aerosols
	(Pankow et al., 2017)	Benzene was not detected in ENDS aerosols of Juul; benzene formation depended on the devices and their power outputs
	(Wagner et al., 2018)	1,3-butadiene, isoprene, acrylonitrile, benzene and toluene were not detected in ENDS aerosols
	(Zhao et al., 2018)	Benzene and toluene concentrations were dependent on brand, flavor, ENDS type, puffing regime, and voltage
	(Nicol et al., 2020)	Aromatic amines were below the limits of quantification

Parameters	Study	Conclusion
	(Rudd et al., 2020)	Vinyl chloride, 1,3-butadiene, ethylene oxide, isoprene, propylene oxide, acrylonitrile, benzene, toluene, ethylene benzene, styrene were not detected in ENDS aerosols
Others	(Goniewicz et al., 2014)	Out of 11 VOCs, only toluene and *m-/p*-xylene were quantified in ENDS aerosols; concentrations were higher than in Nicorette inhalator
	(Papoušek et al., 2014)	Acrylamide was not detected in ENDS aerosols
	(Herrington and Myers, 2015)	More than 150 VOCs can be analyzed with their method; presence of propylene oxide, benzene, toluene, xylene, styrene
	(Kim and Kim, 2015)	Benzene, xylene, styrene were detected in ENDS aerosols; they were formed during the vaporization process
	(Allen et al., 2016)	Diacetyl was detected in 39 of the 51 flavored e-liquids tested
	(Flora et al., 2016)	Acrylonitrile, benzene, 1,3-butadiene, isoprene and toluene were not detected in ENDS aerosols
	(Margham et al., 2016)	Styrene, 1,3-butadiene, isoprene, acrylonitrile, benzene, toluene, ethylbenzene, ethylene oxide, vinyl chloride, propylene oxide, acrylamide and aromatic amines were below the limits of detection
	(Lee et al., 2017)	Benzene and Toluene were detected in ENDS aerosols

Table 12 presents the observations and conclusions of studies on reactive oxygen species (ROS) and free radicals in ENDS aerosols. Free radicals are molecules that contain an unpaired electron in atomic orbital, which makes them very reactive due to their instability (Lobo et al., 2010). ROS are both oxygen-containing free radicals (e.g., hydroxyl radical) and molecules that can promote formation of free radicals (e.g., hydrogen peroxide). Mechanisms of formation of free radicals during ENDS use are not known. The temperature and oxygen supply, as well as the presence of catalytic metals (e.g., iron ions), might be involved in the mechanisms (Son et al., 2019). Concentrations of ROS and free radicals depended on the e-liquid composition and the device power output, but the concentrations varied greatly between the studies. Indeed, some reported that the quantities emitted were greater than conventional cigarettes, while others reported the opposite. Flavorings, higher PG content, higher power

output and increased puff number were associated with higher concentrations of free radicals in ENDS aerosols.

Table 12 – Summary of studies reporting reactive oxygen species (ROS) and free radical concentrations in ENDS aerosols and their observations and conclusions.

Study	Conclusions
(Goel et al., 2015)	Highly reactive free radicals are formed in ENDS aerosols
(Lerner et al., 2015)	ROS production was higher than in cigarette
(Bitzer et al., 2018a)	Free radical production was dependent on flavoring molecules; so was lipid peroxidation (MDA and 8-isoprostane formation)
(Bitzer et al., 2018b)	Free radical formation increased with PG content, power output, and temperature; lipid oxidation and 8-isoprostane formation increased with PG content and puff number
(Bitzer et al., 2019)	Free radicals formation were present in all ENDS aerosols, and was dependent on the device
(Cirillo et al., 2019)	ROS concentrations were increased in aerosols compared to control; sub-ohm resistance increased ROS generation
(Haddad et al., 2019)	ROS flux increased with the power output and PG content; it was also dependent on the device; flux can be similar to cigarette at high power
(Reilly et al., 2019)	Free radical formation was not influenced by flavors; free radical formation increased with PG content
(Shein and Jeschke, 2019)	Free radicals levels are of the order of air background; reduction of 99% or more compared to cigarette
(Son et al., 2019)	Hydroxyl radical formation increased with power output, ratio PG:VG, and air hole size; flavorings and puffing regime also had an effect
(Hasan et al., 2020)	Free radicals formation in ENDS was much lower than in cigarette; free radicals in ENDS seemed to be more potent

Previous studies on ENDS reported that most of the concentrations of harmful compounds were lower in ENDS aerosols than in cigarette smoke. However, the characteristics of the tested devices, as well as their parameters, lead to different emitted concentrations of harmful compounds, which makes it impossible to report typical concentration ranges. The presence of these harmful compounds in ENDS emissions suggests that these devices are not without risk to users' health.

1.8 Human exposure studies

Toxicological analysis of ENDS aerosols in laboratories showed results with wide variability, which complicates risk assessment and gives confusing messages to ENDS users. Human biomonitoring is an effective tool for assessing actual exposure to harmful compounds identified in ENDS aerosols. Upon inhalation, these compounds are distributed among body compartments, and they undergo chemical transformation (biotransformation) before being excreted. The final products resulting from the biotransformation processes, the so-called metabolites, are good candidates to be biomarkers. Ideally, a biomarker of exposure (BoE) is specific for the exposure of interest and is reliably detectable, using non-invasive sampling. Urine is a matrix of choice in biological biomonitoring as it considered as a non-invasive sampling method. The timing of sample collection should be chosen carefully, because biomarkers have different elimination half-lives ($t_{1/2}$), depending of the type and activity of the enzymes involved in the biotransformation (phenotype diversity).

The main advantage of human biomonitoring applied to ENDS exposure is that the measured biomarker concentrations directly reflect the total inhaled dose, independently of the type of ENDS and the participants' natural puffing regimes. However, biomonitoring takes into account all routes (i.e., inhalation, ingestion, and dermal absorption) and sources (e.g., air pollution, food) of exposure of the compounds of interest, which complicates the interpretation of the results.

Table 13 presents the urinary concentrations of BoE to tobacco alkaloids, heavy metals and trace elements, PAHs, TSNAs, and VOCs for ex-smokers, ENDS users, dual users (i.e., individuals both smoking and vaping), and smokers. Most ENDS users vape e-liquids containing nicotine, and therefore similar urinary concentrations of nicotine and its metabolites were observed in ENDS users compared to smokers. Lower concentrations in ENDS users were sometimes reported, which was closely related to nicotine delivery efficiency that was not optimal in the 1st or 2nd ENDS generations (Farsalinos et al., 2014; Rüther et al., 2018). Participants that used ENDS and cigarettes, referred to as dual users, had higher concentrations of nicotine compared to the smokers. Urinary concentrations of anabasine and anatabine, minor tobacco alkaloids, in ENDS users were similar to those in non-smokers. Therefore, they can be used to differentiate smokers and ENDS users.

Characterization of exposure to heavy metals and trace elements can be challenging. Many have long half-lives (in months or years) and can accumulate in different organs and tissues (bioaccumulation). Sources of metal exposure include food, air and water pollution, cigarette

smoking, and occupation. Most urinary metal concentration in ENDS users were not different from non-smokers. Only urinary concentrations of cadmium, chromium, lead, antimony, selenium, uranium, and zinc were statistically different between non-smokers and ENDS users, but not for all studies.

Urinary concentrations of PAHs were higher in smokers than in non-smokers as PAHs are formed during incomplete combustion of organic matter. Concentrations in ENDS users were similar to those in non-smokers, except for 1-OHP (pyrene metabolite), 2-hydroxyfluorene and 3-hydroxyfluorene (fluorene metabolites) in several studies.

TSNAs are impurities present in tobacco. Urinary concentrations of NNAL, NNN, NAT, and NAB were higher in smokers than in non-smokers. Although e-liquids should not contain these impurities, urinary concentrations of these biomarkers were slightly higher (significant difference) in ENDS users than in non-smokers. These concentrations were still very low compared to smokers, and most studies have shown no differences.

Of the 29 urinary BoE to VOCs, only CYMA, acrylonitrile metabolite, was almost systematically present in higher concentrations in ENDS users than in non-smokers. Similar results were observed for CYHA, another acrylonitrile metabolite, but only a few studies reported its urinary concentrations in ENDS users. Urinary concentrations of HEMA, metabolite of acrylonitrile, ethylene oxide, and vinyl chloride, were not different in non-smokers and in ENDS users in most studies. Other biomarkers for which at least two studies showed a significant difference between ENDS users and non-smokers were 3-HPMA (acrolein), CEMA (acrolein), AAMA (acrylamide), and 3-MHA + 4-MHA (xylene). For the overwhelming majority of biomarkers, urine concentrations were higher in smokers (and in dual users) than in non-smokers. Exposure to formaldehyde and acetaldehyde cannot be monitored by urine analysis. Formate and acetate, respectively, are the metabolites of formaldehyde and acetaldehyde, but they are not specific to these compounds and are therefore poor biomarkers.

Table 13 – Concentrations of urinary biomarkers of exposure (BoE) to tobacco alkaloids (part I), heavy metals and trace elements (part II), polycyclic aromatic hydrocarbons (part III), tobacco-specific nitrosamines (part IV), and volatile organic compounds (part V) reported in non-smokers, electronic nicotine delivery system (ENDS) users, dual users, and smokers. For each biomarker, its parent compound and the studies that have reported its urinary concentrations are listed.

Biomarker	Parent compound	Study	Non-smoker	ENDS users	Dual users	Smokers
Part I – Tobacco alkaloids						
Nicotine	-	(Goniewicz et al., 2017) *µg/g* [1]	-	584 (752)	-	1126 (821)
		(Shahab et al., 2017) *nmol/mg* [2]	0.8 (0.3-1.7)	2.5 (1.5-4.2)	4 (2.3-7.1)	1.9 (1.2-3.3)
		(Goniewicz et al., 2018) *ng/mg* [2]	31 (12.0, 800)	423.3 (306.7, 584.9)	1318.7 (1172.8, 1482.8)	1076.0 (967.7, 1195.2)
		(Lorkiewicz et al., 2018) *ng/mg* [1]	0.0 (0.0)	17.8 (30.9)	-	494.7 (1273.7)
		(Keith et al., 2019) *ng/mg* [1]	10.2 (15)	434.5 (769.5)	462.1 (639.2)	453.4 (771.7)
		(Rostron et al., 2020) *µg/g* [2]	-	1006.0 (801.1, 1263.3)	1244.2 (955.3, 1620.5)	-
		(Rudasingwa et al., 2021) *ng/mL* [3]	3.9 (3.9, 149.5)	339.1 (3.9, 4473.6)	-	1121.1 (42.3 4558.7)
Cotinine	Nicotine	(McRobbie et al., 2015) *ng/mg* [1]	-	889 (959)	1227 (679)	-
		(Goniewicz et al., 2017) *µg/g* [1]	-	1927 (1728)	-	2287 (1381)
		(Shahab et al., 2017) *nmol/mg* [2]	1.4 (0.6-3.5)	7.5 (4.5-12.4)	8.2 (4.6-14.8)	5.9 (3.8-9.3)
		(Czoli et al., 2018) *ng/mg* [2]	533.21 (326.59, 870.56)	733.67 (478.41, 1125.12)	1174.44 (859.41, 1604.72)	1282.04 (925.34, 1776.23)
		(Goniewicz et al., 2018) *ng/mg* [2]	0.42 (0.36, 0.49)	124.3 (68.9, 224.4)	2858.9 (2601.9, 3141.2)	1830.9 (1577.4, 2125.1)
		(Lorkiewicz et al., 2018) *ng/mg* [1]	0.1 (0.2)	147.3 (249.3)	-	69.5 (122.0)
		(Pulverset al., 2018) *ng/mg* [3]	-	361.45 (120.5, 710.5)	687.50 (247.3, 1193)	574.79 (99.53, 1417.02)
		(Keith et al., 2019) *ng/mg* [1]	2.6 (2.4)	855.8 (958.9)	851.6 (770.9)	910.9 (868.3)
		(Pulverset al., 2020) *ng/mL* [3]	-	928 (525, 1409)	699 (441, 1090)	1034 (836, 1502)
		(Rostron et al., 2020) *µg/g* [2]	-	2313.5 (1860.2, 2877.3)	2953.4 (2451.0, 3558.9)	-

Biomarker	Parent compound	Study	Non-smoker	ENDS users	Dual users	Smokers
		(Perez et al., 2021) *ng/mg* [2]	0.4 (0.4–0.5)	91.9 (34.7–243.2)	-	1,507.6 (1,067.5–2,129.3)
		(Rudasingwa et al., 2021) *ng/mL* [3]	0.9 (0.9, 0.9)	322.2 (0.9, 722.8)	-	729.5 (185.8, 1342.6)
Nornicotine	Nicotine	(Goniewicz et al., 2017) *µg/g* [1]	-	38 (38)	-	73 (39)
		(Shahab et al., 2017) *nmol/mg* [2]	0.1 (0.1-0.1)	0.2 (0.1-0.3)	0.3 (0.2-0.5)	0.2 (0.1-0.3)
		(Goniewicz et al., 2018) *ng/mg* [2]	3.4 (1.8, 6.2)	18.83 (14.72, 24.11)	67.94 (62.28, 74.11)	60.20 (56.04, 64.67)
		(Rostron et al., 2020) *µg/g* [2]	-	34.5 (29.2, 40.7)	50.7 (40.5, 63.6)	-
Nicotine-N-oxide	Nicotine	(Goniewicz et al., 2017) *µg/g* [1]	-	223 (232)	-	335 (231)
		(Shahab et al., 2017) *nmol/mg* [2]	0.2 (0.1-0.6)	0.9 (0.5-1.6)	1.3 (0.7-2.2)	0.7 (0.4-1.1)
		(Goniewicz et al., 2018) *ng/mg* [2]	11 (4.2, 31)	143.3 (105.3, 194.9)	387.4 (352.6, 425.6)	326.4 (302, 352.7)
		(Rostron et al., 2020) *µg/g* [2]	-	308.0 (244.8, 387.4)	371.8 (291.6, 474.2)	-
3-OH-cotinine	Nicotine	(Goniewicz et al., 2017) *µg/g* [1]	-	4686 (4409)	-	4765 (3163)
		(Shahab et al., 2017) *nmol/mg* [2]	2.8 (1.2-6.3)	11.4 (6.5-19.9)	10.9 (6-19.8)	8.5 (5.1-14.3)
		(Goniewicz et al., 2018) *ng/mg* [2]	0.69 (0.6, 0.8)	227.4 (128.9, 401.1)	4985.7 (4533.5, 5482.8)	3182.3 (2682.7, 3773.2)
		(Lorkiewicz et al., 2018) *ng/mg* [1]	0.0 (0.0)	498.2 (863.0)	-	535.6 (1105.0)
		(Keith et al., 2019) *ng/mg* [1]	6.4 (13.7)	3204.1 (2865.3)	2527.8 (2196.4)	2887.6 (2237)
		(Rostron et al., 2020) *µg/g* [2]	-	3776.9 (2886.2, 4942.3)	4469.2 (3657.6, 5460.9)	-
		(Perez et al., 2021) *ng/mg* [2]	0.7 (0.6–0.8)	181.5 (76.6–430.2)	-	2,609.5 (1,842.5–3,695.7)
		(Rudasingwa et al., 2021) *ng/mL* [3]	2.6 (2.6, 2.6)	820.3 (172.1, 2714.2)	-	2227.1 (500.3, 4802.3)
Norcotinine	Nicotine	(Goniewicz et al., 2017) *µg/g* [1]	-	108 (131)	-	136 (91)
		(Shahab et al., 2017) *nmol/mg* [2]	0.1 (0.1-0.1)	0.1 (0.1-0.2)	0.2 (0.1-0.4)	0.2 (0.1-0.2)
		(Goniewicz et al., 2018) *ng/mg* [2]	4.3 (2.3, 8.0)	31.93 (24.35, 41.87)	100.3 (93.4, 107.7)	90.51 (84.95, 96.43)

Biomarker	Parent compound	Study	Non-smoker	ENDS users	Dual users	Smokers
		(Rostron et al., 2020) *µg/g* [2]	-	3776.9 (2886.2, 4942.3)	4469.2 (3657.6, 5460.9)	-
Cotinine-N-oxide	Nicotine	(Goniewicz et al., 2017) *µg/g* [1]	-	349 (303)	-	392 (238)
		(Shahab et al., 2017) *nmol/mg* [2]	0.2 (0.1-0.4)	0.8 (0.5-1.3)	0.8 (0.5-1.4)	0.6 (0.4-1.0)
		(Goniewicz et al., 2018) *ng/mg* [2]	14 (6.8, 28)	117.5 (88.2, 156.5)	349.7 (326.5, 374.5)	297.4 (276.5, 319.9)
		(Rostron et al., 2020) *µg/g* [2]	-	244.0 (194.7, 305.7)	325.3 (266.0, 397.9)	-
Total Nicotine Equivalents (TNE)	TNE 2; cotinine, 3-OH-cotinine	(Goniewicz et al., 2018) *nmol/mg* [2]	0.006 (0.005 0.007)	2.000 (1.1, 3.5)	43.70 (39.8, 48.1)	27.90 (23.8, 32.7)
		(Rostron et al., 2020) *µmol/g* [2]	-	34.9 (28.1, 43.5)	41.6 (34.6, 50.0)	-
		(Smith et al., 2021a) *pmol/mg* [4]	0.007	21.55	48.57	41.72
	TNE 5; nicotine, cotinine, 3-OH-cotinine, nicotine-N-oxide, cotinine-N-oxide	(Goniewicz et al., 2017) *µmol/g* [1]	-	43 ± 40	-	50 ± 27
	TNE 6; nicotine, cotinine, 3-OH-cotinine, nicotine-N-oxide, cotinine-N-oxide, nornicotine	(Rostron et al., 2020) *µmol/g* [2]	-	49.2 (40.8, 59.3)	57.0 (47.2, 68.7)	-
	TNE 7; nicotine, cotinine, 3-OH-cotinine, nicotine-N-oxide, cotinine-N-oxide, norcotinine, nornicotine	(Shahab et al., 2017) *nmol/mg* [2]	6.3 (2.9-14.1)	25.0 (14.8-42.0)	28.8 (16.6-49.8)	21.1 (14.0-31.8)
Anabasine	-	(Shahab et al., 2017) *pmol/mg* [2]	5.5 (3.5-8.7)	6.2 (4.1-9.5)	25.5 (16.3-40.1)	17.0 (11.2-25.8)
		(Goniewicz et al., 2018) *ng/mg* [2]	0.605 (0.347 1.053)	1.144 (0.876 1.492)	8.231 (7.429 9.12)	7.799 (7.221 8.423)
		(Lorkiewicz et al., 2018) *ng/mg* [1]	0.0 (0.0)	0.0 (0.0)	-	1.6 (4.3)
		(Keith et al., 2019) *ng/mg* [1]	2.7 (2.7)	3.1 (3.0)	6.2 (7.7)	5.6 (5.6)
Anatabine	-	(Shahab et al., 2017) *pmol/mg* [2]	3.8 (2.4-6.2)	4.6 (2.8-7.6)	36.0 (22.0-59.1)	26.0 (16.3-41.4)
		(Goniewicz et al., 2018) *ng/mg* [2]	0.615 (0.306, 1.234)	0.886 (0.658, 1.192)	13.89 (12.35, 15.61)	13.02 (11.92, 14.22)
		(Lorkiewicz et al., 2018) *ng/mg* [1]	0.0 (0.0)	0.0 (0.0)	-	3.6 (9.3)
		(Keith et al., 2019) *ng/mg* [1]	0.9 (1.0)	2.4 (3.6)	4.7 (7.2)	5.3 (6.7)

Biomarker	Parent compound	Study	Non-smoker	ENDS users	Dual users	Smokers
Part II – Metals						
Al	-	(Olmedo et al., 2021) *µg/g* [3]	3.25 (2.56, 4.22)	3.94 (2.45, 7.39)	2.47 (1.95, 6.42)	-
As	-	(Olmedo et al., 2021) *µg/g* [3]	44.34 (21.36, 58.47)	15.93 (6.90, 41.65)	19.95 (7.54, 45.36)	-
		(Goniewicz et al., 2018) *ng/mg* [2]	0.054 (0.05, 0.057)	0.053 (0.048, 0.058)	0.047 (0.045, 0.05)	0.048 (0.046, 0.05)
Be	-	(Goniewicz et al., 2018) *ng/mg* [2]	0.011 (0.01 0.011)	0.012 (0.011 0.014)	0.013 (0.012 0.014)	0.012 (0.011 0.013)
Ba	-	(Prokopowicz et al., 2020) *µg/g* [3]	2.3 (1.1–3.2)	2.01 (1.2–4.4)	3.66 (2.04–4.45)	2.19 (1.24–3.06)
Cd	-	(Goniewicz et al., 2018) *ng/mg* [2]	0.149 (0.14 0.159)	0.193) 0.165 0.225)	0.28 (0.256 0.305)	0.277 (0.259 0.297)
		(Prokopowicz et al., 2020) *µg/g* [3]	0.35 (0.20–0.42)	0.29 (0.20–0.41)	0.26 (0.19–0.45)	0.28 (0.20–0.51)
		(Olmedo et al., 2021) *µg/g* [3]	0.08 (0.04, 0.14)	0.12 (0.05, 0.20)	0.10 (0.07, 0.22)	-
		(Perez et al., 2021) *ng/mg* [2]	0.1 (0.1–0.1)	0.2 (0.1–0.2)	-	0.2 (0.2–0.2)
		(Smith et al., 2021a) *ng/mg* [4]	0.152	0.22	0.26	0.26
Co	-	(Goniewicz et al., 2018) *ng/mg* [2]	0.564 (0.537 0.591)	0.579 (0.523 0.641)	0.6 (0.566 0.637)	0.542 (0.524 0.56)
		(Prokopowicz et al., 2020) *µg/g* [3]	0.51 (0.34–0.89)	0.46 (0.30–1.05)	0.44 (0.35–0.58)	0.61 (0.31–0.71)
		(Olmedo et al., 2021) *µg/g* [3]	0.16 (0.07, 0.30)	0.15 (0.09, 0.24)	0.17 (0.09, 0.20)	-
Cr	-	(Prokopowicz et al., 2020) *µg/g* [3]	0.14 (0.08–0.21)	0.06 (0.05–0.11)	0.12 (0.06–0.34)	0.09 (0.05–0.24)
		(Olmedo et al., 2021) *µg/g* [3]	0.20 (0.16, 0.30)	0.34 (0.23, 0.46)	0.28 (0.24, 0.38)	-
Cu	-	(Olmedo et al., 2021) *µg/g* [3]	1.46 (0.62, 2.56)	1.72 (0.64, 3.76)	2.36 (1.50, 4.46)	-
Fe	-	(Olmedo et al., 2021) *µg/g* [3]	3.47 (2.73, 4.64)	3.88 (2.41, 6.20)	3.32 (1.98, 5.38)	-
In	-	(Prokopowicz et al., 2020) *µg/g* [3]	0.004 (0.002–0.011)	0.005 (0.002–0.008)	0.006 (0.003–0.008)	0.004 (0.002–0.016)
Mn	-	(Goniewicz et al., 2018) *ng/mg* [2]	0.131 (0.124 0.138)	0.14 (0.124 0.158)	0.153 (0.143 0.165)	0.137 (0.13 0.143)
		(Prokopowicz et al., 2020) *µg/g* [3]	0.84 (0.59–1.60)	0.80 (0.60–1.15)	0.61 (0.51–1.43)	0.71 (0.49–0.92)

Biomarker	Parent compound	Study	Non-smoker	ENDS users	Dual users	Smokers
		(Olmedo et al., 2021) *µg/g* [3]	0.08 (0.06, 0.11)	0.08 (0.06, 0.17)	0.06 (0.05, 0.11)	-
Ni	-	(Prokopowicz et al., 2020) *µg/g* [3]	5.02 (3.1–7.2)	5.23 (2.41–6.72)	5.24 (3.03–6.55)	4.24 (2.74–6.98)
		(Olmedo et al., 2021) *µg/g* [3]	0.65 (0.33, 0.96)	0.65 (0.48, 0.99)	0.40 (0.24, 0.59)	-
Pb	-	(Goniewicz et al., 2018) *ng/mg* [2]	0.351 (0.33 0.373)	0.432 (0.382 0.488)	0.5 (0.475 0.526)	0.479 (0.462 0.496)
		(Prokopowicz et al., 2020) *µg/g* [3]	0.68 (<LOD–1.03)	0.66 (<LOD–1.14)	0.3 (<LOD–0.91)	0.98 (0.63–1.48)
		(Olmedo et al., 2021) *µg/g* [3]	0.22 (0.16, 0.35)	0.39 (0.26, 0.57)	0.44 (0.20, 0.64)	-
		(Perez et al., 2021) *ng/mg* [2]	0.3 (0.3–0.3)	0.4 (0.3–0.4)	-	0.4 (0.4–0.4)
		(Smith et al., 2021a) *ng/mg* [4]	0.364	0.43	0.46	0.45
Sb	-	(Prokopowicz et al., 2020) *µg/g* [3]	0.02 (<LOD–0.05)	0.04 (<LOD–0.05)	0.04 (0.03–0.13)	0.03 (<LOD–0.06)
		(Olmedo et al., 2021) *µg/g* [3]	0.12 (0.10, 0.18)	0.22 (0.16, 0.29)	0.14 (0.09, 0.20)	-
Se	-	(Sakamaki-Ching et al., 2020) *µg/g* [1]	41.8 (14.1)	54 (20.6)	-	39.7 (17.3)
Sn	-	(Olmedo et al., 2021) *µg/g* [3]	0.18 (0.08, 0.43)	0.26 (0.16, 0.56)	0.31 (0.21, 0.55)	-
Sr	-	(Goniewicz et al., 2018) *ng/mg* [2]	112.7 (106.8, 119)	118.9 (101, 140)	130.5 (121.3, 140.5)	113.7 (107.3, 120.6)
Tl	-	(Goniewicz et al., 2018) *ng/mg* [2]	0.172 (0.164, 0.18)	0.169 (0.153, 0.188)	0.163 (0.156, 0.17)	0.155 (0.15, 0.16)
U	-	(Goniewicz et al., 2018) *ng/mg* [2]	0.005 (0.005, 0.006)	0.007 (0.006, 0.008)	0.008 (0.007, 0.009)	0.007 (0.006, 0.008)
V	-	(Prokopowicz et al., 2020) *µg/g* [3]	7.0 (5.8–10.5)	6.9 (5.5–8.2)	7.8 (6.4–8.6)	8.5 (5.7–10.9)
Zn	-	(Sakamaki-Ching et al., 2020) *µg/g* [1]	413.6 (233.7)	584.5 (826.6)	-	470.7 (223.6)
		(Olmedo et al., 2021) *µg/g* [3]	259.24 (186.47, 398.05)	262.04 (199.69, 344.97)	189.67 (136.68, 341.01)	-
Part III – Polycyclic aromatic hydrocarbons (PAHs)						
1-Naphtol	Naphthalene	(Goniewicz et al., 2018) *ng/mg* [2]	1.3992 (1.28, 1.529)	1.550 (1.216, 1.975)	13.48 (11.91, 15.25)	11.11 (10.18, 12.12)

Biomarker	Parent compound	Study	Non-smoker	ENDS users	Dual users	Smokers
		(Wang et al., 2019) *µg/L* [2]	1.49 (1.35-1.64)	1.69 (1.39-2.06)	-	10.43 (9.73-11.18)
2-Napthol	Naphthalene	(Goniewicz et al., 2017) *ng/g* [1]	-	19 (14)	-	24 (13)
		(Goniewicz et al., 2018) *ng/mg* [2]	4.6311 (4.344, 4.937)	5.287 (4.693, 5.956)	14.79 (14.01, 15.61)	13.91 (13.21, 14.65)
		(Wang et al., 2019) *µg/L* [2]	4.94 (4.57-5.34)	5.43 (4.68-6.31)	-	12.93 (12.36-13.53)
		(Perez et al., 2021) *ng/mg* [2]	5.8 (5.3–6.4)	6.0 (5.4–6.7)	-	14.5 (13.0–16.0)
		(Smith et al., 2021a) *µg/mg* [4]	4.8	4.68	13.62	14.21
1-OHP	Pyrene	(Goniewicz et al., 2017) *ng/g* [1]	-	746 (627)	-	778 (338)
		(Czoli et al., 2018) *pg/mg* [2]	175.07 (134.28, 228.19)	141.06 (98.29, 202.49)	203.33 (153.85, 268.66)	249.23 (197.15, 315.14)
		(Goniewicz et al., 2018) *ng/mg* [2]	0.128 (0.121 0.136)	0.161 (0.143 0.181)	0.355 (0.339 0.373)	0.303 (0.287 0.321)
		(Wang et al., 2019) *ng/L* [2]	136 (129-144)	159 (146-174)	-	306 (295-318)
		(Perez et al., 2021) *ng/mg* [2]	0.1 (0.1–0.2)	0.2 (0.1–0.2)	-	0.3 (0.3–0.4)
		(Smith et al., 2021a) *ng/mg* [4]	136.95	151.87	329.13	302.07
1-Hydroxy-fluorene	Fluorene	(Goniewicz et al., 2017) *ng/g* [1]	-	592 (833)	-	1414 (864)
2-Hydroxy-fluorene	Fluorene	(Goniewicz et al., 2017) *ng/g* [1]	-	842 (495)	-	1029 (463)
		(Goniewicz et al., 2018) *ng/mg* [2]	0.167 (0.158, 0.177)	0.199 (0.178, 0.222)	1.141 (1.074, 1.212)	1.007 (0.947, 1.071)
		(Wang et al., 2019) *ng/L* [2]	177 (167-188)	220 (188-257)	-	951 (907-998)
3-Hydroxy-fluorene	Fluorene	(Goniewicz et al., 2017) *ng/g* [1]	-	451 (349)	-	679 (312)
		(Goniewicz et al., 2018) *ng/mg* [2]	0.064 (0.06, 0.068)	0.077 (0.068, 0.086)	0.630 (0.59, 0.673)	0.568 (0.53, 0.61)
		(Wang et al., 2019) *ng/L* [2]	67.9 (64-71.9)	86.7 (73.6-102)	-	537 (509-566)
1-hydroxy-phenanthrene	Phenanthrene	(Goniewicz et al., 2017) *ng/g* [1]	-	584 (415)	-	488 (211)
		(Goniewicz et al., 2018) *ng/mg* [2]	0.106 (0.101, 0.112)	0.107 (0.096, 0.12)	0.200 (0.191, 0.21)	0.178 (0.17, 0.186)

Biomarker	Parent compound	Study	Non-smoker	ENDS users	Dual users	Smokers
		(Wang et al., 2019) *ng/L* [2]	112 (106-119)	109 (97-121)	-	172 (166-178)
2-hydroxy-phenanthrene	Phenanthrene	(Goniewicz et al., 2017) *ng/g* [1]	-	968 (800)	-	655 (333)
		(Goniewicz et al., 2018) *ng/mg* [2,5]	0.129 (0.123, 0.135)	0.125 (0.113, 0.139)	0.316 (0.299, 0.333)	0.303 (0.289, 0.318)
		(Wang et al., 2019) *ng/L* [2]	136 (130-143)	128 (113-146)	-	286 (275-298)
3-hydroxy-phenanthrene	Phenanthrene	(Goniewicz et al., 2017) *ng/g* [1,6]	-	1410 (1262)	-	1314 (669)
4-hydroxy-phenanthrene	Phenanthrene	-	-	-	-	-
Part IV – Tobacco-specific nitrosamines (TSNAs)						
NNAL	NNK	(Goniewicz et al., 2017) *ng/g* [1]	-	80 (69)	-	225 (165)
		(Czoli et al., 2018) *pg/mg* [2]	19.76 (13.45, 29.03)	21.25 (14.34, 31.47)	30.26 (21.06, 43.48)	32.76 (23.89, 44.91)
		(Goniewicz et al., 2018) *pg/mg* [2]	0.921 (0.819 1.035)	4.887 (3.817 6.257)	262.6 (240 287.3)	203.5 (181.7 227.9)
		(Sakamaki-Ching et al., 2020) *pg/mg* [1]	2.8 (6.3)	13.3 (18.6)	-	105.7 (87.4)
		(Shahab et al., 2017) *pg/mg* [2]	4.83 (2.79-8.34)	1.47 (1.02-2.12)	44.5 (28.5-69.4)	53.4 (36.6-77.8)
		(Pulverset al., 2018) *pg/mg* [3]	-	22.15 (4.7, 119.3)	156.13 (52.5, 320.7)	102.75 (7.75, 291.17)
		(Rubinstein et al., 2018) *pg/mg* [3]	0 (0)	0.3 (0.7)	68.1 (68.7)	-
		(Pulverset al., 2020) *pg/mg* [3]	-	7 (3, 23)	47 (22, 103)	100 (70, 273)
		(Perez et al., 2021) *ng/mg* [2]	0.0009 (0.0008–0.001)	0.005 (0.004–0.007)	-	0.2 (0.1–0.2)
		(Rudasingwa et al., 2021) *pg/mL* [3]	4.9 (4.9, 4.9)	8.3 (4.9, 25.4)	-	32.0 (4.9, 69.8)
		(Smith et al., 2021a) *ng/mg* [4]	0.001	0.003	0.263	0.246
NNN	-	(Goniewicz et al., 2018) *pg/mg* [2]	1.923 (1.81, 2.043)	3.471 (3.033, 3.972)	11.78 (10.66, 13.01)	11.80 (10.84, 12.85)
NAT	-	(Shahab et al., 2017) *pg/mg* [2]	2.95 (1.81-4.81)	1.79 (1.21-2.67)	30.8 (18.5-51.1)	32.8 (20.5-52.5)

Biomarker	Parent compound	Study	Non-smoker	ENDS users	Dual users	Smokers
		(Goniewicz et al., 2018) *pg/mg* [2]	2.921 (2.739, 3.114)	3.909 (3.402, 4.493)	126.9 (111.7, 144.2)	96.06 (85.66, 107.7)
NAB	-	(Shahab et al., 2017) *pg/mg* [2]	1.52 (1.09-2.12)	1.07 (0.79-1.47)	6.02 (4.15-8.73)	6.17 (4.31-8.82)
		(Goniewicz et al., 2018) *pg/mg* [2]	1.067 (0.003, 1.135)	1.422 (1.256, 1.61)	20.85 (18.62, 23.34)	15.67 (14.12, 17.39)
Part V – Volatile organic hydrocarbons (VOCs)						
DHBMA	1,3-Butadiene	(Shahab et al., 2017) *ng/mg* [2]	204.2 (156.9-265.9)	156.3 (126.0-193.8)	294.9 (242.9-358.0)	202.7 (162.8-252.3)
		(Goniewicz et al., 2018) *ng/mg* [2]	359 (347.7 370.6)	360.2 (340.9 380.4)	532.7 (514.3 551.7)	499.8 (481.1 519.1)
		(Lorkiewicz et al., 2018) *ng/mg* [1]	368.6 (155.9)	359.1 (8.5)	-	381.8 (150.4)
		(Keith et al., 2019) *ng/mg* [1]	283.2 (104.5)	262.7 (107.7)	415.6 (209)	389.9 (194.4)
		(Frigerio et al., 2020) *µg/g* [3]	247.5 (163.6-348.5)	263.8 (177.3-298.7)	-	479.1 (273.2-925.6)
		(De Jesús et al., 2020) *µg/g* [7]	347 (6.17)	386 (11.5)	-	516 (6.27)
1-MHBMA	1,3-Butadiene	(Lorkiewicz et al., 2018) *ng/mg* [1]	0.1 (0.2)	0.0 (0.0)	-	0.4 (0.8)
		(Frigerio et al., 2020) *µg/g* [3,8]	0.27 (<LOQ-2.47)	0.55 (0.14-2.07)	-	4.07 (0.74-11.38)
2-MHBMA	1,3-Butadiene	(Goniewicz et al., 2017) *ng/g* [1]	-	305 (887)	-	1912 (1283)
		(Lorkiewicz et al., 2018) *ng/mg* [1]	0.2 (0.4)	1.3 (2.2)	-	0.1 (0.2)
3-MHBMA	1,3-Butadiene	(Shahab et al., 2017) *ng/mg* [2]	7.67 (5.08-11.6)	4.44 (3.42-5.78)	36.6 (25.4-52.6)	29.8 (19.9-44.8)
		(Goniewicz et al., 2018) *ng/mg* [2]	4.543 (4.348 4.745)	4.308 (3.843 4.829)	31.92 (29.64 34.38)	27.90 (26.04 29.89)
		(Lorkiewicz et al., 2018) *ng/mg* [1]	4.6 (3.7)	6.8 (11.7)	-	14.3 (19.3)
		(Rubinstein et al., 2018) *ng/mg* [3]	0 (0.5)	0 (0)	0 (0.1)	-
		(Keith et al., 2019) *ng/mg* [1]	3.6 (2.5)	6.8 (7.9)	18.7 (20.7)	19.5 (15.4)
		(De Jesús et al., 2020) *µg/g* [7]	4.43 (0.104)	4.58 (0.243)	-	32.6 (0.708)
BPMA	1-Bromopropane	(Lorkiewicz et al., 2018) *ng/mg* [1]	13.3 (9.2)	4.6 (7.9)	-	15.5 (10.9)

Biomarker	Parent compound	Study	Non-smoker	ENDS users	Dual users	Smokers
		(Keith et al., 2019) *ng/mg* [1]	13 (12.3)	6.0 (4.8)	17.5 (16.5)	20.6 (27.2)
3-HPMA	Acrolein	(McRobbie et al., 2015) ng/mg [1]	-	343 (178)	969 (807)	-
		(Goniewicz et al., 2017) *µg/g* [1]	-	410 (465)	-	937 (700)
		(Shahab et al., 2017) *ng/mg* [2]	236.1 (168.1-331.6)	175.3 (124-247.8)	574.5 (429.1-769.2)	488.4 (345.1-691.2)
		(Goniewicz et al., 2018) *ng/mg* [2]	272.4 (257 288.6)	314.8 (275.4 359.5)	1317.8 (1225 1417.7)	1143.5 (1064.3 1228.6)
		(Lorkiewicz et al., 2018) *ng/mg* [1]	294.3 (344.7)	332.5 (397.4)	-	544.7 (596.5)
		(Pulverset al., 2018) *ng/mg* [3]	-	370.34 (308.0, 518.2)	1014.69 (662.2, 3346.0)	818.90 (556.66, 818.90)
		(Rubinstein et al., 2018) *ng/mg* [3]	192.8 (261.6)	254.3 (191.4)	439.7 (224.1)	-
		(Keith et al., 2019) *ng/mg* [1]	223 (149.4)	338.6 (206.4)	569.5 (450.8)	724.4 (735.1)
		(Frigerio et al., 2020) *µg/g* [3]	160.6 (77.9-318.5)	222.1 (196.6-738.2)	-	1301.2 (328.9-3661.1)
		(De Jesús et al., 2020) *µg/g* [7]	262 (7.66)	354 (22.3)	-	1320 (33.0)
CEMA	Acrolein	(Shahab et al., 2017) *ng/mg* [2]	67.8 (49.3-93.2)	54.6 (41.7-71.4)	141.8 (106.7-188.4)	119.8 (88.2-162.9)
		(Goniewicz et al., 2018) *ng/mg* [2]	98.14 (93.89, 102.6)	108.0 (95.93, 121.6)	302.0 (283.3, 321.8)	271.5 (255.1, 289)
		(Lorkiewicz et al., 2018) *ng/mg* [1]	51.5 (26.9)	18.4 (16.1)	-	110.2 (118.5)
		(Keith et al., 2019) *ng/mg* [1]	79 (62.5)	120.8 (90.3)	188.1 (160)	180.1 (134.8)
		(Frigerio et al., 2020) *µg/g* [3]	0.9 (<LOQ-2.1)	2.7 (0.9-36.5)	-	163.1 (45.8-358.4)
		(De Jesús et al., 2020) *µg/g* [7]	93.7 (2.26)	115 (7.01)	-	299 (6.31)
		(Perez et al., 2021) *ng/mg* [2]	87.0 (81.1–93.3)	98.7 (84.2–115.7)	-	235.0 (208.6–264.8)
		(Rudasingwa et al., 2021) *ng/mL* [3]	17.5 (10.0, 95.6)	11.9 (10.0, 92.7)	-	166.1 (25.3, 532.1)
		(Smith et al., 2021a) *ng/mg* [4]	98.96	107.77	292.45	270.85
AAMA	Acrylamide	(Goniewicz et al., 2017) *µg/g* [1]	-	110 (97)	-	254 (148)

Biomarker	Parent compound	Study	Non-smoker	ENDS users	Dual users	Smokers
		(Shahab et al., 2017) *ng/mg* [2]	33.6 (25.8-43.7)	29.3 (22.3-38.3)	82.4 (66.1-102.8)	65.6 (50.6-85.1)
		(Goniewicz et al., 2018) *ng/mg* [2]	47.28 (45.03 49.65)	56.05 (51.07 61.5)	144.0 (136.4 151.9)	136.4 (129.3 143.8)
		(Lorkiewicz et al., 2018) *ng/mg* [1]	59.1 (57.8)	136.2 (185.3)	-	84.4 (57.3)
		(Pulvers et al., 2018) *ng/mg* [3]	-	96.52 (82.3, 157.3)	268.46 (168.6, 394.6)	192.28 (100.93, 294.92)
		(Rubinstein et al., 2018) *ng/mg* [3]	34.5 (41.6)	67.3 (69)	235.6 (239.8)	-
		(Keith et al., 2019) *ng/mg* [1]	67.8 (60.5)	88.5 (43.6)	181.8 (157.4)	191.9 (136.1)
		(Frigerio et al., 2020) *µg/g* [3]	47.9 (24.2-95.4)	55.8 (34.4-65.5)	-	114.6 (55.1-223.9)
		(De Jesús et al., 2020) *µg/g* [7]	45.0 (1.16)	61.7 (4.18)	-	152 (2.55)
		(Perez et al., 2021) *ng/mg* [2]	44.9 (41.8–48.1)	58.8 (51.2–67.6)	-	135.1 (122.9–148.4)
		(Smith et al., 2021a) *ng/mg* [4]	48.75	59.03	134.83	134.67
GAMA	Acrylamide	(Shahab et al., 2017) *ng/mg* [2]	12.1 (9.5-15.5)	10.0 (7.6-13.2)	24.3 (19.6-30.2)	18.5 (14.7-23.3)
		(Goniewicz et al., 2018) *ng/mg* [2]	9.022 (8.584 9.482)	9.924 (9.076 10.85)	18.52 (17.57 19.52)	17.33 (16.49 18.21)
		(Lorkiewicz et al., 2018) *ng/mg* [1]	82.8 (69.3)	21.0 (36.4)	-	146.7 (218.1)
		(Keith et al., 2019) *ng/mg* [1]	25.4 (21.2)	36.5 (24.7)	39 (30.8)	43.6 (34.8)
		(Frigerio et al., 2020) *µg/g* [3]	2.5 (<LOQ-7.1)	3.9 (1.4-6.7)	-	5.3 (1.7-30.4)
		(De Jesús et al., 2020) *µg/g* [7]	8.57 (0.253)	11.4 (0.743)	-	18.8 (0.255)
CYHA	Acrylonitrile	(Goniewicz et al., 2018) *ng/mg* [2]	1.870 (1.766, 1.981)	2.431 (2.114, 2.794)	25.09 (22.82, 27.58)	21.80 (20.05, 23.69)
CYMA	Acrylonitrile	(Goniewicz et al., 2017) *µg/g* [1]	-	45 (66)	-	212 (178)
		(Shahab et al., 2017) *ng/mg* [2]	3.7 (2.1-6.5)	1.4 (1.1-1.9)	51.6 (33.6-79.2)	49.2 (32.9-73.6)
		(Goniewicz et al., 2018) *ng/mg* [2]	1.315 (1.23 1.406)	3.959 (3.002 5.219)	146.2 (133.8 159.8)	123.9 (109.9 139.7)
		(Lorkiewicz et al., 2018) *ng/mg* [1]	4.7 (15.6)	43.5 (75.3)	-	33.0 (51.2)

Biomarker	Parent compound	Study	Non-smoker	ENDS users	Dual users	Smokers
		(Pulverset al., 2018) *ng/mg* [3]	-	20.26 (8.4, 32.7)	120.23 (51.0, 422.4)	89.56 (33.69, 276.35)
		(Rubinstein et al., 2018) *ng/mg* [3]	0 (1.1)	1.3 (3.2)	59.4 (81.3)	-
		(Keith et al., 2019) *ng/mg* [1]	3.0 (12.3)	29.3 (31.3)	97 (78.6)	129.8 (126)
		(De Jesús et al., 2020) *µg/g* [7]	1.27 (0.043)	4.51 (0.560)	-	172 (4.77)
		(Perez et al., 2021) *ng/mg*	1.2 (1.1–1.3)	3.8 (2.8–5.1)	-	97.1 (76.3–123.7)
		(Rudasingwa et al., 2021) *ng/mL* [3]	0.4 (0.4, 304.7)	0.4 (0.4, 257.3)	-	179.9 (0.4, 592.4)
		(Smith et al., 2021a) *ng/mg* [4]	1.42	2.98	153.92	147.59
HEMA	Acrylonitrile, ethylene oxide, vinyl chloride	(Goniewicz et al., 2017) *ng/g* [1]	-	1480 (1573)	-	3821 (3120)
		(Shahab et al., 2017) *ng/mg* [2]	0.64 (0.48-0.84)	0.42 (0.32-0.55)	1.15 (0.84-1.57)	0.81 (0.61-1.07)
		(Goniewicz et al., 2018) *ng/mg* [2]	0.955 (0.893 1.02)	1.076 (0.945 1.224)	3.194 (2.936 3.475)	2.744 (2.545 2.958)
		(Lorkiewicz et al., 2018) *ng/mg* [1]	1.1 (1.4)	0.0 (0.0)	-	1.2 (1.1)
		(Pulverset al., 2018) *ng/mg* [3]	-	0.78 (0.8, 1.9)	3.00 (1.5, 7.4)	2.15 (0.77, 6.39)
		(Rubinstein et al., 2018) *ng/mg* [3]	1.3 (2.3)	0.5 (1.1)	1.0 (1.4)	-
		(Keith et al., 2019) *ng/mg* [1]	1.7 (1.3)	1.1 (0.9)	4.5 (5.4)	4.2 (5.2)
		(Frigerio et al., 2020) *µg/g* [3]	1.3 (0.1-4.1)	2.0 (1.3-2.2)	-	3.2 (1.0-26.7)
		(De Jesús et al., 2020) *µg/g* [7]	0.965 (0.037)	1.09 (0.076)	-	3.11 (0.084)
SPMA	Benzene	(Goniewicz et al., 2017) *ng/g* [1]	-	188 (481)	-	792 (674)
		(Shahab et al., 2017) *ng/mg* [2]	0.52 (0.37-0.71)	0.74 (0.55-0.98)	1.43 (1.11-1.83)	0.64 (0.48-0.84)
		(Goniewicz et al., 2018) *ng/mg* [2]	1.038 (0.967 1.114)	1.007 (0.9 1.125)	1.071 (1.017 1.127)	1.090 (1.035 1.147)
		(Pulverset al., 2018) *ng/mg* [3]	-	0.09 (0.07, 0.6)	1.06 (0.6, 2.5)	0.71 (0.19, 2.24)
		(Rubinstein et al., 2018) *ng/mg* [3]	0 (0)	0.3 (0.7)	68.1 (68.7)	-

Biomarker	Parent compound	Study	Non-smoker	ENDS users	Dual users	Smokers
		(Frigerio et al., 2020) *µg/g* [3]	0.06 (<LOQ-0.23)	0.16 (0.03-0.34)	-	0.48 (0.08-1.45)
		(De Jesús et al., 2020) *µg/g* [7]	1.01 (0.039)	1.04 (0.070)	-	1.06 (0.0210)
tt-MA	Benzene	(Shahab et al., 2017) *ng/mg* [2]	131.8 (94.1-184.5)	55.2 (42.3-71.9)	135.0 (102.3-178.1)	78.6 (58.2-106.2)
		(Lorkiewicz et al., 2018) *ng/mg* [1]	144.0 (80.4)	317.5 (92.7)	-	186.9 (74.9)
		(Keith et al., 2019) *ng/mg* [1]	138.8 (92.3)	211 (179.3)	156.2 (147.6)	132.4 (102.7)
TTCA	Carbon disulfide	(Shahab et al., 2017) *ng/mg* [2]	13.4 (9.07-19.7)	6.84 (4.33-10.8)	9.95 (6.85-14.5)	6.03 (4.40-8.27)
		(Goniewicz et al., 2018) *ng/mg* [2]	21.61 (19.51, 23.93)	19.36 (15.99, 23.43)	22.89 (20.98, 24.99)	21.91 (20.52, 23.38)
		(Lorkiewicz et al., 2018) *ng/mg* [1]	19.4 (19.6)	6.4 (11.0)	-	96.1 (147.9)
		(De Jesús et al., 2020) *µg/g* [7]	20.6 (1.27)	19.8 (2.12)	-	21.5 (0.556)
HPMMA	Crotonaldehyde	(Goniewicz et al., 2017) *µg/g* [1]	-	616 (575)	-	1857 (1379)
		(Shahab et al., 2017) *ng/mg* [2]	366.3 (266.0-504.5)	235.9 (179.1-310.7)	1199.5 (881.9-1631.6)	804.2 (563.8-1147.1)
		(Goniewicz et al., 2018) *ng/mg* [2]	457.7 (433.4 483.3)	442.8 (387.6 505.8)	2707.7 (2515.8 2914.3)	2359.3 (2188.2 2543.8)
		(Lorkiewicz et al., 2018) *ng/mg* [1]	157.6 (48.0)	275.9 (245.0)	-	358.1 (491.0)
		(Pulvers et al., 2018) *ng/mg* [3]	-	251.63 (157.8, 765.9)	305.74 (228.6, 918.7)	303.35 (193.91, 480.53)
		(Rubinstein et al., 2018) *ng/mg* [3]	100.4 (129.9)	148.7 (99)	185.4 (156.6)	-
		(Keith et al., 2019) *ng/mg* [1]	138.7 (49.5)	179.1 (112.4)	433.5 (399.8)	462.4 (398.6)
		(Frigerio et al., 2020) *µg/g* [3]	48 (15-265)	38 (19-133)	-	268 (96-580)
		(De Jesús et al., 2020) *µg/g* [7]	442 (13.1)	432 (36.1)	-	2740 (68.4)
CMEMA	Crotonaldehyde	(Frigerio et al., 2020) *µg/g* [3]	273 (122-603)	233 (154-542)	-	400 (220-774)
		(De Jesús et al., 2020) *µg/g* [7]	1.82 (0.057)	2.75 (0.227)	-	28.2 (0.734)
ATCA	Cyanide	(Shahab et al., 2017) *ng/mg* [2]	102.0 (72.6-143.4)	55.3 (41.0-74.5)	132.3 (97.8-179.0)	91.2 (69.6-119.5)

Biomarker	Parent compound	Study	Non-smoker	ENDS users	Dual users	Smokers
		(Lorkiewicz et al., 2018) *ng/mg* [1]	115.5 (77.1)	439.7 (257.8)	-	343.2 (444.5)
AMCC	N,N-Dimethyl-formamide	(Shahab et al., 2017) *ng/mg* [2]	100.2 (72.4-138.7)	60.8 (44.4-83.3)	176.3 (129.1-240.5)	162.2 (120.6-218.1)
		(Goniewicz et al., 2018) *ng/mg* [2]	105.1 (92.26, 112.3)	153.8 (136.1, 173.8)	550.3 (519.2, 583.2)	482.9 (454, 513.7)
		(Lorkiewicz et al., 2018) *ng/mg* [1]	113.9 (83.7)	201.8 (85.1)	-	237.2 (135.7)
		(Keith et al., 2019) *ng/mg* [1]	127.1 (90.5)	169.7 (105.4)	354 (251.6)	327.7 (226.3)
		(Frigerio et al., 2020) *µg/g* [3]	142 (55-434)	243 (60.519)	-	405 (90-844)
		(De Jesús et al., 2020) *µg/g* [7]	104 (3.08)	203 (12.0)	-	540 (11.0)
PGA	Ethylbenzene, styrene	(Shahab et al., 2017) *ng/mg* [2]	88.1 (60.6-128.2)	71.1 (53.7-94.1)	124.5 (91.1-170.0)	88.0 (62.6-123.8)
		(Goniewicz et al., 2018) *ng/mg* [2]	203.3 (196.5, 210.2)	223.7 (197.4, 253.5)	416.5 (399, 434.8)	375.8 (360.3, 391.8)
		(Lorkiewicz et al., 2018) *ng/mg* [1]	205.2 (75.4)	324.5 (75.5)	-	216.6 (77.7)
		(Keith et al., 2019) *ng/mg* [1]	186.2 (66.9)	191.2 (52.3)	286.3 (139.3)	330.2 (177.3)
		(De Jesús et al., 2020) *µg/g* [7]	202 (4.26)	220 (9.80)	-	398 (5.73)
IPMA3	Isoprene	(Goniewicz et al., 2018) *ng/mg* [2]	3.378 (3.155, 3.617)	3.747 (3.247, 4.323)	39.79 (36.33, 43.56)	33.50 (30.69, 35.56)
		(De Jesús et al., 2020) *µg/g* [7]	3.23 (0.103)	3.60 (0.263)	-	42.1 (1.18)
2-HPMA	Propylene oxide	(Goniewicz et al., 2017) *µg/g* [1]	-	21 (15)	-	45 (24)
		(Shahab et al., 2017) *ng/mg* [2]	37.4 (28.7-48.9)	29.3 (21.9-39.3)	68.9 (52.6-90.4)	41.1 (30.4-55.6)
		(Goniewicz et al., 2018) *ng/mg* [2]	33.79 (30.63 37.26)	34.45 (30.12 39.4)	84.13 (78.12 90.6)	71.10 (67.59 74.8)
		(Lorkiewicz et al., 2018) *ng/mg* [1]	84.0 (133.9)	37.0 (33.6)	-	89.2 (103.4)
		(Pulverset al., 2018) *ng/mg* [3]	-	38.03 (29.2, 133.3)	105.08 (62.0, 175.3)	68.39 (32.35, 29.45)
		(Rubinstein et al., 2018) *ng/mg* [3]	15.2 (14.4)	28.8 (25)	40.2 (27.9)	-
		(Keith et al., 2019) *ng/mg* [1]	84.4 (214.7)	34.8 (27.6)	48.6 (33.6)	60.1 (57.1)

Biomarker	Parent compound	Study	Non-smoker	ENDS users	Dual users	Smokers
		(Frigerio et al., 2020) *µg/g* [3]	8.8 (4.2-16.4)	9.8 (6.7-17.4)	-	28.4 (9.4-70.9)
		(De Jesús et al., 2020) *µg/g* [7]	31.6 (1.60)	39.0 (2.71)	-	79.8 (1.62)
MA	Styrene	(Shahab et al., 2017) *ng/mg* [2]	173.0 (127.3-235.3)	100.8 (78.2-129.9)	227.2 (181.1-284.9)	188.6 (147.4-241.2)
		(Goniewicz et al., 2018) *ng/mg* [2]	131.7 (126.6, 136.9)	198 (138.5, 119.5)	287.8 (274.4, 301.9)	279.3 (268.1, 290.8)
		(Lorkiewicz et al., 2018) *ng/mg* [1]	132.0 (41.0)	197.2 (35.9)	-	187.9 (61.9)
		(Keith et al., 2019) *ng/mg* [1]	126.8 (52.2)	146.8 (70.5)	244.6 (110.7)	284.9 (251.8)
		(De Jesús et al., 2020) *µg/g* [7]	128 (2.97)	143 (6.52)	-	300 (4.14)
PHEMA	Styrene	(Shahab et al., 2017) *ng/mg* [2]	0.75 (0.55-1.00)	0.48 (0.36-0.63)	1.09 (0.8-1.48)	0.75 (0.57-0.98)
		(Lorkiewicz et al., 2018) *ng/mg* [1]	0.4 (1.0)	0.4 (0.7)	-	0.8 (1.3)
		(Keith et al., 2019) *ng/mg* [1]	0.9 (0.9)	1.0 (0.7)	1.5 (1.1)	2.3 (2.5)
		(Frigerio et al., 2020) *µg/g* [3]	0.53 (0.09-1.36)	0.68 (0.17-1.29)	-	1.05 (0.39-2.55)
BMA	Toluene	(Lorkiewicz et al., 2018) *ng/mg* [1]	6.7 5.6)	1.5 (1.3)	-	6.6 (4.6)
		(Goniewicz et al., 2018) *ng/mg* [2]	6.314 (5.965, 6.683)	6.985 (6.088, 8.015)	7.394 (6.836, 7.998)	6.696 (6.238, 7.188)
		(Keith et al., 2019) *ng/mg* [1]	10.6 (14.0)	5.0 (2.5)	8.3 (6.3)	12.8 (29.5)
		(Frigerio et al., 2020) *µg/g* [3]	2.22 (0.55-12.74)	1.42 (0.40-4.28)	-	1.47 (0.53-2.96)
		(De Jesús et al., 2020) *µg/g* [7]	6.10 (0.189)	7.45 (0.659)	-	6.58 (0.179)
1,2-DCVMA	Trichloroethylene	(Lorkiewicz et al., 2018) *ng/mg* [1]	2.7 (4.4)	1.6 (2.7)	-	6.7 (17.8)
2,2-DCVMA	Trichloroethylene	(Lorkiewicz et al., 2018) *ng/mg* [1]	0.0 (0.0)	0.0 (0.0)	-	1.0 (2.5)
2-MHA	Xylene	(Shahab et al., 2017) *ng/mg* [2]	19.6 (13-29.7)	10.5 (7.80-14.2)	56.9 (41.8-77.4)	41.9 (30.1-58.4)
		(Goniewicz et al., 2018) *ng/mg* [2]	20.99 (19.35, 22.77)	27.77 (23.6, 32.67)	109.3 (101.6, 117.6)	98.769 (91.78, 106.1)
		(Lorkiewicz et al., 2018) *ng/mg* [1]	10.4 (7.9)	20.8 (27.9)	-	59.4 (79.1)

Biomarker	Parent compound	Study	Non-smoker	ENDS users	Dual users	Smokers
		(Keith et al., 2019) *ng/mg* [1]	8.6 (14.6)	17.1 (12.7)	23.8 (21.7)	33.3 (35.2)
		(De Jesús et al., 2020) *µg/g* [7]	22.2 (0.857)	32.1 (2.42)	-	116 (2.78)
3-MHA + 4-MHA	Xylene	(Shahab et al., 2017) *ng/mg* [2]	76.3 (48.8-119.4)	51.4 (38.5-68.6)	273.2 (201.1-371.0)	266.5 (182.1-390.1)
		(Goniewicz et al., 2018) *ng/mg* [2]	154.9 (145.8, 164.5)	185.1 (159, 215.6)	758.4 (710.4, 809.6)	678.4 (633.6, 726.2)
		(Lorkiewicz et al., 2018) *ng/mg* [1]	71.9 (29.6)	316.3 (349.1)	-	197.9 (207.8)
		(Keith et al., 2019) *ng/mg* [1]	115.9 (129.3)	165.3 (104.7)	289.1 (213.5)	365.7 (305.8)
		(De Jesús et al., 2020) *µg/g* [7]	151 (4.48)	195 (13.4)	-	781 (17.9)

[1]Mean (standard deviation; SD); [2]geometric mean (95% confidence interval; CI); [3]median (1st–3rd quartiles; interquartile range IQR); [4]geometric mean retrieved from figure; [5]2- and 3-hydroxyphenanthrene; [6]3- and 4-hydroxyphenanthrene; [7]geometric means [standard error; SE]; [8]1- and 2-MHBMA.

1,2-DCVMA N-acetyl-S-(1,2-dichloroethenyl)-L-cysteine; 1-MHBMA N-acetyl-S-(1-hydroxymethyl-2-propenyl)-L-cysteine; 1-OHP 1-Hydroxypyrene; 2,2-DCVMA N-acetyl-S-(2,2-dichloroethenyl)-L-cysteine; 2-HPMA N-acetyl-S-(2-hydroxypropyl)-L-cysteine; 2-MHA 2-Methylhippuric acid; 2-MHBMA N-acetyl-S-(2-hydroxy-3-butenyl)-L-cysteine; 3-HPMA N-acetyl-S-(3-hydroxypropyl)-L-cysteine; 3-MHA 3-Methylhippuric acid; 3-MHBMA N-acetyl-S-(4-hydroxy-2-buten-1-yl)-L-cysteine; 3-OH-cotinine Trans-3'-hydroxycotinine; 4-MHA 4-Methylhippuric acid; AAMA N-acetyl-S-(2-carbamoylethyl)-L-cysteine; AMCC N-acetyl-S-(N-methylcarbamoyl)cysteine; ATCA 2-Aminothiazoline-4-carboxylic acid; BMA Benzylmercapturic acid; BPMA N-Acetyl-S-propyl-L-cysteine; CEMA N-acetyl-S-(2-carboxyethyl)-L-cysteine; CMEMA N-acetyl-S-(3-carboxy-2-propyl)-L-cysteine; CYHA N-acetyl-S-(1-cyano-2-hydroxyethyl)-L-cysteine; CYMA N-acetyl-S-(2-cyanoethyl)-L-cysteine; DHBMA N-acetyl-S-(3,4-dihydroxybutyl)-L-cysteine, GAMA N-acetyl-S-(2-carbamoyl-2-hydroxyethyl)-L-cysteine; HEMA N-acetyl-S-(2-hydroxyethyl)-L-cysteine; HPMMA N-acetyl-S-(3-hydroxypropyl-1-methyl)-L-cysteine; IPMA3 N-acetyl-S-(4-hydroxy-2-methyl-2-trans-buten-1-yl)-L-cysteine; MA Mandelic acid; NAB N-nitrosoanabasine; NAT N-nitrosoanatabine; NNAL 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol; NNN N-nitrosonornicotine; NNK 4-(N-nitrosomethylamino)-1-(3-pyridyl)-1-butanone; PHEMA N-acetyl-S-(2-hydroxy-1-phenylethyl)-L-cysteine + N-acetyl-S-(2-hydroxy-2-phenylethyl)-L-cysteine; PGA Phenylglyoxylic acid; SPMA N-acetyl-S-(phenyl)-L-cysteine; TTCA 2-thiothiazolidine-4-carboxylic acid; tt-MA Trans, trans-muconic acid

In addition to BoE, other biomarkers used in human biomonitoring of exposures to tobacco products and alternatives are biomarkers of potential harm (BoPH). While the BoE reveal the concentration of a xenobiotic substance or its metabolites in biological matrices, BoPH are defined as “measurement of an effect due to exposure; these include early biological effects, alterations in morphology, structure, or function, and clinical symptoms consistent with harm; also includes preclinical changes” (Institute of Medicine (US) Committee to Assess the Science Base for Tobacco Harm Reduction, 2001). From decades of cigarette research, we know that

clinical outcomes associated with smoking, such as cancer, cardiovascular diseases and chronic obstructive pulmonary diseases occur after many years and are therefore not appropriate for judging the safety of new products. BoPH have the potential to assess the potential health risks of smoked and smokeless tobacco products and other means of nicotine delivery, because they are predictive of future disease development (Chang et al., 2019). Several BoPH were selected for tobacco product evaluation, but only a few were validated as specific, predictive indicator of disease development. A type of BoPH are biomarkers of oxidative stress.

Oxidative stress is defined as the imbalance between the generation and elimination of reactive oxygen species (ROS). ROS include free radicals (e.g., superoxide anion radical ($^{\bullet}O_2^-$) and hydroxyl radical ($^{\bullet}OH$)) and peroxides (e.g., hydrogen peroxide (H_2O_2)). Most ROS are generated in the mitochondria where electrons can escape the electron transport chain and react with molecular oxygen. Other sources involve some oxidases, such as cytochromes P450, xanthine oxidase, and NADPH oxidase complex. Elimination of ROS is carried out by the antioxidant defenses of the body, which are composed of low and high molecular mass antioxidants. The low molecular mass antioxidants include molecules that come from the diet (e.g., vitamins C and E) or that are synthesized in the body (e.g., glutathione and melatonin). The high molecular mass antioxidants are enzymes like superoxide dismutase, catalases or peroxidases (Lushchak, 2014).

Although ROS were identified as toxic by-products of aerobic metabolism, they are also active participants in signaling function and are involved in inflammatory processes. When ROS generation exceeds elimination, ROS can attack cellular components (e.g., DNA, proteins, and lipids) as they are highly reactive, and they can disturb the cellular metabolism (Pizzino et al., 2017). Many factors can alter ROS homeostasis such as increased concentrations of endogenous or exogenous substances that interfere with the ROS generation and decreased concentrations of antioxidant molecules and enzymes. Several diseases were associated to oxidative stress, including cancers, cardiovascular diseases and diabetes (Sharifi-Rad et al., 2020). Biomarkers of oxidative stress are currently being studied as possible BoPH, although they have no predictive validity to date.

Human biomonitoring of oxidative stress can be achieved by measuring the products that are formed by the reactions between ROS and the cellular components. ROS are unstable and short-lived, which complicates their measurement in biological media. Compounds resulting from the ROS attack on DNA or lipids, for example, are much more stable, reflect excess ROS, and can be measured in non-invasive matrices, such as urine or exhaled breath condensate.

Two well-known biomarkers of oxidative stress are 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG), a marker of DNA damage, and 8-iso-prostaglandin $F_{2\alpha}$ (8-isoprostane), a marker of lipoperoxidation (Cooke et al., 2000; Milne et al., 2005).

Very few studies have investigated the effect of electronic cigarettes on oxidative stress via the measurement of urinary biomarker of oxidative stress (see Table 14). Urinary concentrations of 8-isoprostane and 8-oxodG were reported to be higher in smokers and ENDS users than in non-smokers. However, further studies are needed to verify these findings.

Table 14 – Concentrations of urinary biomarkers of oxidative stress reported in non-smokers, electronic nicotine delivery system (ENDS) users, dual users, and smokers.

Biomarker	Study	Non-smoker	ENDS users	Dual users	Smokers
8-Isoprostane	(Sakamaki-Ching et al., 2020) *pg/mg* [1]	411.2 (287.4)	750.8 (433)	-	784.2 (546.1)
	(Perez et al., 2021) *ng/mL* [2]	0.4 (0.4-0.4)	0.5 (0.4-0.5)	-	0.6 (0.5-0.6)
8-OxodG	(Sakamaki-Ching et al., 2020) *ng/mg* [1]	221.6 (157.8)	442.8 (300.7)	-	388 (235)

[1]Mean (standard deviation; SD); [2]geometric mean (95% confidence interval; CI).

The association between smoking and urinary concentrations of 8-oxodG and 8-isoprostane was not reported consistently. This might be due to the lack of characterization of exposure, small cohorts, and inconsistent use of measurement methods. Moreover, the relationships between BoEs resulting from tobacco smoke exposures and 8-ododG and 8-isoprostane have never been studied among smokers in a large cohort. If such an association exists, the likelihood of finding it will be in a large cohort of smokers where the biomarkers are measured using the same analytical method.

1.9 The debates on ENDS

ENDS were promising devices to substitute the conventional cigarette and thus help smokers quit smoking. However, the growing popularity of these devices quickly led public health organizations to fear that a "new cigarette" was being marketed. After years of studies in various fields and thousands of articles published on ENDS, both public health organizations and the scientific community remain torn on this subject. The main topics still open to

discussion will be presented in this sub-chapter (Wagener, 2018; Abbott, 2019; Fairchild et al., 2019a, 2019b; The debate over e-cigarettes demands stronger evidence of their value, 2019; E-cigarettes and vaping: the bad, the good, and the unknown, 2020; Boseley, 2020; Michalopoulos, 2021; Smith et al., 2021b).

Here are some of main issues related to the debate surrounding ENDS:

1) Are ENDS a good tool for smoking cessation?
2) Do ENDS increase the risk of smoking?
    a. Should we be concerned about the increase in ENDS use by young people?
    b. Do ENDS increase the risk of trivializing nicotine addiction?
3) Are ENDS safe?
    a. Will ENDS have long-term effects?
    b. Should we be concerned about the administration of other drugs (e.g., cannabinoids) with ENDS?
4) Are ENDS, and especially their waste, harmful to our environment?
5) How ENDS should be regulated?
    a. Should ENDS be banned?
    b. If not, should it be regulated as a tobacco product or a pharmaceutical drug?
    c. Should tobacco companies be excluded from the discussion on ENDS regulation?

Together with the growing number of studies related to ENDS, two opposing camps have emerged: the precautionary principle approach and the risk reduction approach with two diametrically opposed objectives. The first camp aimed at the suppression and denormalization of smoking, including restrictions on tobacco products and ENDS. The second camp focused on reducing the harms associated with tobacco use and considered ENDS, as well as NRT, as useful tools to achieve this goal. However, not all opinions are so clear-cut, and a significant part of the actors involved in the debate are somewhere between these two opposite visions.

The field of public health strives to base decision-making on scientific evidences. However, preliminary evidence or lack of evidence in debated topics do not always help to resolve policy controversies because they can be interpreted in completely different ways. The purpose of this thesis is not to answer all the questions mentioned, but elements of an answer for question 4, “Are ENDS safe?” will be presented. The thesis results will help fill the evidence gap.

Chapter 2 – Framework of the thesis

Chapter 1 summarized the current knowledge of harmful compounds in ENDS aerosols and the urinary concentrations of BoE to harmful compounds resulting from ENDS use. Biomarkers of oxidative stress were also introduced, but only a few studies have analyzed them in ENDS users' urine samples. Overall, there is a lack of scientific evidence on ENDS toxicity, particularly data from rigorous randomized controlled trials.

This chapter starts with a brief description of the ESTxENDS clinical study (Subchapter 2.1). The following subchapters present the objectives of the thesis: the characterization of ENDS aerosols (Subchapter 2.2), the analysis of BoE to harmful compounds in urine (Subchapter 2.3), and the analysis of biomarkers of oxidative stress in urine (Subchapter 2.4). All objectives are then summarized in Subchapter 2.5. Figure 1 presents the different parts of the thesis that are the subject of the next chapters (chapter 3 to 6).

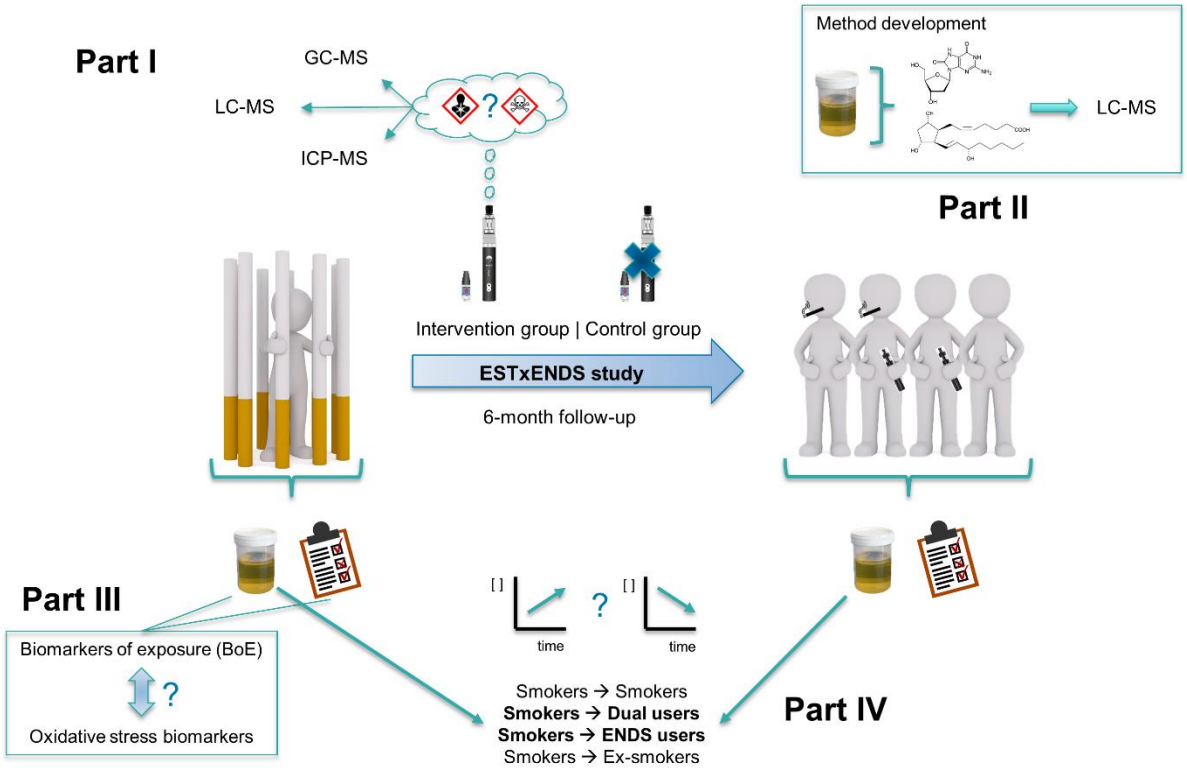


Figure 1– Explanatory scheme of the thesis objectives: analyses of ENDS emissions (part I), development of a method of analysis of urinary biomarkers of oxidative stress (part II), evaluation of the association between biomarkers of exposure to tobacco smoke and biomarkers of oxidative stress (part III), and effects of smoking cessation on urinary biomarker concentrations after 6 months.

2.1 The ESTxENDS study

This thesis work was part of the clinical study entitled “Efficacy, Safety and Toxicology of Electronic Nicotine Delivery Systems as an aid for smoking cessation: The ESTxENDS multicenter randomized controlled trial” and funded by the Swiss National Science Foundation. This study was approved by the ethics committees of Bern, Geneva, and Lausanne (Project-ID: 2017-02332). It was a multicenter (Bern, Geneva, Lausanne, St. Gallen, and Zurich) clinical trial in which smokers (n=1,172) try to stop smoking over a 6-month period with professional smoking cessation counselling (SCC) and with or without ENDS (intervention and control groups, respectively). Classical nicotine replacement therapies (NRT) could be used as needed (at participants’ own expense). The participants in the intervention group received a free ENDS device and e-liquids for 6-month use. Clinical visits were planned at baseline (t=0) and at follow-up (t=6-month) during which questionnaires were administered and clinical tests were performed. Participants also collected their morning urine on the days of the clinical visits. The ESTxENDS study objectives were: 1) to determine the efficacy of ENDS to reduce or stop smoking, 2) to evaluate the safety of ENDS in terms of adverse events, 3) to evaluate the reduction of the exposure to inhaled toxic compounds between cigarettes smokers and ENDS users, and 4) to evaluate the effects of ENDS on health. The thesis work focused on the third objective.

2.2 Aerosols generated by ENDS

A crucial point of the ESTxENDS clinical study was the selection of the ENDS model and e-liquid brand. Ideally, the ENDS had to be easy to use and suitable for ENDS beginners. The choice was made based on the models available in 2017 and on advices of associations of ENDS users and tenants of vape shops. The selection criteria of the e-liquid brand was to know the origin of the products and to have information on the e-liquid composition. The device selected was the Innokin Endura T20-S starter kit (China) and the e-liquids were “Alfaliquids” (Gaïatrend, France) with six different flavors. Neither the ENDS nor the e-liquids have been previously included in human exposure studies of harmful compounds.

The research question was “does the selected ENDS emit less toxic compounds than cigarettes?”. This analytical step was necessary in the good conduct of the ESTxENDS study to make sure that our approach was in line with risk reduction when proposing to our participants to use an ENDS to stop smoking cigarettes.

- The objective was thus to measure the concentrations of harmful compounds both in e-liquids and in aerosols generated by the selected ENDS. Based on previous studies (Subchapters 1.5 and 1.7), the following chemical families were selected: aldehydes, heavy metals and trace elements, TSNAs, and VOCs.

The complete list of compounds is given below:

Aldehydes: formaldehyde, acetaldehyde, acrolein, propanal, crotonaldehyde, butyraldehyde, benzaldehyde, isovaleraldehyde, valeraldehyde, *o*-tolualdehyde, *m-/p*-tolualdehyde, hexanal, and 2,5-dimethylbenzaldehyde.

Metals: beryllium (Be), aluminum (Al), vanadium (V), chromium (Cr), manganese(Mn), iron (Fe), cobalt (Co), nickel (Ni), copper (Cu), zinc (Zn), arsenic (As), selenium (Se), molybdenum (Mo), palladium (Pd), silver (Ag), cadmium (Cd), tin (Sn), antimony (Sb), platinum (Pt), and lead (Pb).

TSNAs: N-nitrosonornicotine (NNN), 4-(methylnitrosamino)-1-(3-bipyridyl)-1-butanone (NNK), N-nitrosoanatabine (NAT), and N-nitrosoanabasine (NAB).

VOCs: 1,3-butadiene, isoprene, acrylonitrile, benzene, toluene, acrylamide, and naphthalene.

2.3 Biomonitoring of inhaled harmful compounds

Characterization of ENDS emissions provides a good overview of the harmful compounds that participants are likely to inhale during ENDS use. However, the inhaled doses will depend heavily on the ENDS use, both in terms of e-liquid consumption and puffing topography (Subchapter 1.6). Indeed, exposure assessment is complicated because participants may be exposed to different concentrations of harmful compounds and at varying frequencies and durations. It would theoretically be possible to record vaping sessions during the day with a puff monitor. However, the puffing regime would still need to be replicated in the laboratory to quantify harmful compounds in ENDS emission, which is tedious. Therefore, this option was abandoned. Instead, human biomonitoring was preferred, as it is an efficient tool to assess exposure to harmful compounds (Subchapter 1.8).

The research question was “are the urinary biomarkers of exposure to the selected harmful compounds reduced six month after smoking cessation when using ENDS?”. Exposure biomarkers also allow the comparison between urinary concentrations observed in ENDS

users and abstainers (i.e., no smoking, no vaping), which would correspond to the unexposed group.

The objectives were to:

- quantify urinary concentrations of BoE to tobacco smoke listed below both at baseline and at 6-month follow-up.
- compare the urinary concentrations of BoE between ex-smokers, ENDS users, dual users, and smokers after 6 months follow-up.

The list of BoE is given below:

Alkaloids: Nicotine, cotinine, norcotinine, trans-3'-hydroxycotinine (3-OH-cotinine), anabasine

Metals: Be, Al, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Se, Mo, Pd, Ag, Cd, Sn, Sb, Pt, and Pb.

PAHs: 1-naphthol, 2-naphthol, 1-hydroxypyrene (1-OHP)

TSNAs: NNAL

VOCs: N-acetyl-S-(2-carbamoylethyl)-L-cysteine (AAMA), N-acetyl-S-(2-cyanoethyl)-L-cysteine (CYMA), N-acetyl-S-(3,4-dihydroxybutyl)-L-cysteine (DHBMA), N-acetyl-S-(2-carbamoyl-2-hydroxyethyl)-L-cysteine (GAMA), N-acetyl-S-(2-hydroxyethyl)-L-cysteine (HEMA), N-acetyl-S-(2-hydroxypropyl)-L-cysteine (2-HPMA), N-acetyl-S-(3-hydroxypropyl)-L-cysteine (3-HPMA), N-acetyl-S-(3-hydroxypropyl-1-methyl)-L-cysteine (HPMMA), N-acetyl-S-(1-hydroxymethyl-2-propenyl)-L-cysteine + N-acetyl-S-(2-hydroxy-3-butenyl)-L-cysteine (1-MHBMA + 2-MHBMA), N-acetyl-S-(4-hydroxy-2-buten-1-yl)-L-cysteine (3-MHBMA), and N-acetyl-S-(phenyl)-L-cysteine (SPMA).

2.4 Vaping and oxidative stress level

Oxidative stress levels are increased by environmental factors, including exposure to exogenous harmful compounds. While BoE are extremely effective in assessing exposure to specific harmful compounds, they provide little information about the effects that these compounds cause in the body. Biomarkers of oxidative stress have the potential to show the effects of exposure to ENDS emissions, taking into account all compounds, identified or not, present in aerosols. As oxidative stress has been associated with the development of several diseases, as well as with inflammatory processes, this approach can provide important

information on the short-term effects and, in the case of the induction of chronic oxidative stress, predict long-term diseases.

The use of the two selected biomarkers of oxidative stress, 8-oxodG and 8-isoprostane, have clinical utility that is currently limited to research. Indeed, the perturbations of ROS homeostasis and the subsequent oxidative stress result from complex mechanisms. Many factors (e.g., physiological, behavioral, and environmental) can influence oxidative stress level, which induces a large inter-individual variability. Only a very limited number of studies have reported urinary concentrations of 8-oxodG and 8-isoprostane in the urine of ENDS users.

To date, no study has attempted to understand the impact of internal doses of different families of harmful compounds (exposure to chemical mixtures) from cigarette smoking on oxidative stress biomarkers in a large cohort of smokers. Associations between BoE and biomarkers of oxidative stress give an indication of which harmful compounds are related to oxidative stress in smokers as well as an overall assessment of exposure to this mixture. The main advantage of the study design is that it is includes more than 250 participants, both oxidative stress biomarkers and BOEs are measured in the same individual in the same sample by the same laboratory. Furthermore, the same individuals are followed over time, and so other environmental factors that contribute to oxidative stress (e.g., place of residence, diet, occupation) are similar between the two clinical visits.

The research questions were “are the biomarkers of oxidative stress associated with biomarkers of exposure to tobacco smoke?” and “are the urinary biomarkers of oxidative stress reduced six months after smoking cessation when using ENDS?”. The selected biomarkers of oxidative stress could provide valuable information on possible health effects in the absence of the hindsight needed to conduct epidemiological studies to investigate the link between vaping and disease development. If these biomarkers were indeed associated with harmful compounds found in cigarette smoke, one would expect a reduction in their urinary concentration in ENDS users.

Therefore, the objectives were to:

- develop a new LC-MS/MS analytical method for the simultaneous quantification of 8-oxodG and 8-isoprostane in urine.
- assess the associations between BoPH and the selected BoE in smokers and determine which BoE are related to oxidative stress.

- assess the changes in urinary concentrations of BoPH after 6-month follow-up and compare the concentrations between ex-smokers, ENDS users, dual users, and smokers.

2.5 Summary of objectives

In summary, the dissertation has two main objectives: 1) to assess exposures to harmful compounds resulting from vaping in the context of smoking cessation, and 2) to evaluate the use of oxidative stress biomarkers as biomarkers of potential harm. The results are separated in four chapters, as follows:

- Objective 1 – To quantify harmful compounds present in the aerosols of the selected ENDS during a series of laboratory experiments where ENDS aerosols were generated using an in-house built vaping machine and measured in emissions using air sampling methods and chemical analytical instruments for quantification.

- Objective 2 – To develop and validate a new, robust, and sensitive LC-MS/MS method for the simultaneous determination of two urinary oxidative stress biomarkers, 8-oxodG and 8-isoprostane.

- Objective 3 – To determine possible associations between urinary oxidative stress biomarkers and BoE to tobacco smoke in a cohort of smokers.

- Objective 4 – To quantify the changes of urinary concentrations of BoE and oxidative stress biomarkers between baseline and follow-up as a function of participants' final smoking status.

Chapter 3 – Characterization of ENDS emissions

Results from the analysis of e-liquids and ENDS aerosols were subject of a manuscript. The manuscript has not yet been submitted to a peer-reviewed journal as it is currently still under review by the co-authors. It should be submitted in 2022.

Author contributions: Reto Auer (R.A.) and Aurélie Berthet (A.B.) managed funding acquisition; Nicolas Sambiagio (**N.S.**) planned and carried out the analysis of e-liquids and aerosols; Nicolas Concha-Lozano (N.C.-L.) and **N.S.** performed the elemental analysis on the metallic components of a dissected coil; A.B. validated the analysis plan and supervised the analysis progress; **N.S.** wrote the manuscript, which was further amended by all authors.

3.1 Introduction

In the ESTxENDS study, smokers in the intervention group tried to quit smoking with an ENDS that were provided to them. This ENDS was easy to use (i.e., battery parameters were not modifiable) and was therefore suitable for new ENDS users. Participants chose one or more flavored e-liquids from six different flavors and four nicotine concentrations, and they received free supply during six months. Characterization of ENDS aerosols was important to inform participants of what they are exposed to when using this device compared to conventional cigarette.

An in-house build vaping machine was used to generate ENDS aerosols according to standard conditions set by CORESTA (recommended method n°81). Forty-four harmful compounds, including aldehydes, metals, TSNAs, and VOCs, were analyzed both in e-liquids and in ENDS aerosols. An elemental analysis of a dissected coil was also performed by SEM-EDX to identify the composition of the metallic surfaces that were in contact with e-liquid.

Concentrations of harmful compounds measured in aerosol were compared with those in cigarette smoke previously reported in the literature. To facilitate the understanding of these results, measured concentrations were also compared to occupational exposure limits, regulatory limits, and environmental concentrations.

3.2 Overview of results and discussion

Of the four families of harmful compounds analyzed, only concentrations of aldehydes and metals could be quantified in ENDS aerosols. The seven VOCs and the four TSNAs were below the limits of quantification (LOQs).

Thermal degradation of e-liquids was observed with the emission of formaldehyde and acetaldehyde (<10 µg/g e-liq in average). An effect of flavorings on aldehyde formation was observed, although the concentrations remained very low (<15 µg/g e-liq). This effect had previously been shown (Table 7 – Subchapter 1.7). Estimated exposures to aldehydes from ENDS use were compared to 1) exposures from tobacco smoke, 2) environmental exposures (indoor air), and 3) the short-term exposure limits (STEL; acceptable average exposure in 15 minutes) in Switzerland. Formaldehyde and acetaldehyde exposures resulting from a day of vaping were at least 7 times and 200 times lower than one day of cigarette smoking, respectively, and approximatively half as much as spending 8 hours in a house in Europe (in average – without ventilation). Furthermore, one would have to vape around 200 puffs in 15 minutes (one puff every 4.5 s) to reach the STEL value of formaldehyde, whereas it would be impossible to reach the STEL value of acetaldehyde only by vaping.

The e-liquids used in the ESTxENDS study, “Alfaliquids”, were certified by AFNOR (norm XP-D90-300.2), which means that they do not contain heavy metals (Pb, As, Cd, and Sb). The maximal concentrations limits are defined as follows: Pb <10 mg/L, As <3 mg/L, Cd <1 mg/L, and Sb <5 mg/L. Concentrations of these elements measured in e-liquids were three orders of magnitude below these limits (in ng/g e-liq or ng/mL). For the other elements, the concentrations were also present in trace amounts (<20 ng/g e-liq). Nineteen metals were quantified in ENDS aerosols, most at low concentrations (<10 ng/g e-liq). However, Al, Fe, Ni, Cu, Zn, and Pb concentrations were above 100 ng/g e-liq, which suggested that these elements leached from the ENDS metallic part. An elemental analysis of the coil identified two alloys (nichrome and stainless steel) and one element (Ni), but failed to show the presence of Al, Cu, and Pb. Therefore, an ENDS coil and an ENDS clearomizer were placed separately in an acid solution to dissolve surface metals, and we identified the same six metals at high concentrations (>1000 µg/samples – qualitative experiment). The concentrations of metals in ENDS aerosols were below the minimal risk levels (MRLs) defined by the Agency for Toxic Substances and Disease Registry (ATSDR), the permitted daily exposures (PDE) issued by the European Medicines Agency (EMA) and the recommended exposure limits (RELs) fixed by the National Institute for Occupational Safety and Health (NIOSH). However, the quality of

materials used for the manufacturing of these devices should be better controlled to ensure the lowest toxicant emission possible.

In conclusion, analysis of ENDS aerosols showed that these new products emit the selected harmful compounds at lower concentrations compared to cigarettes. That means that participants would strongly reduce their exposure by switching from smoking to vaping. However, the presence of aldehydes and metals in the emissions indicated that these ENDS could not be considered as “healthy” or “risk-free”. While the measured concentrations were below certain guidelines (e.g., PDE) and occupational limits (e.g., REL), it should be kept in mind that there are other sources of exposures to these compounds (e.g., air pollution or food) and that the exposures are cumulative. In addition, the long-term effects of repeated exposure to a mixture of harmful compounds at low concentrations are not known. The recommendation is therefore to promote ENDS, but only with the objective of replacing conventional cigarettes.

These results will be used as a basis for interpretation of the metabolite concentrations in urine (see Chapter 6).

3.3 Manuscript 1

See next page.

Assessment of toxicant and carcinogen concentrations in electronic cigarette liquid and aerosol

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Keywords: E-cigarette; Electronic Nicotine Delivery Systems (ENDS); Toxicology

Electronic nicotine delivery systems (ENDS or e-cigarettes) have gained popularity in recent years because they have been marketed as safer than conventional cigarettes. However, whether the safety risks of ENDS are definitively lower than conventional cigarettes needs to be confirmed by additional scientific evidences. This is particularly important when it comes to the use of ENDS being recommended by health professionals as an aid for smoking cessation.

We analyzed several toxicants and carcinogens, including aldehydes, volatile organic compounds (VOCs), metals, and tobacco specific nitrosamines (TSNAs) in six flavored e-liquids and their aerosols. We selected a commercially available ENDS suitable for beginners, and we used a machine for ENDS aerosol generation following the CORESTA standard conditions.

We detected several aldehydes and metals in ENDS emissions, but most of them were present in lower concentrations than those reported in cigarette smoke. Concentrations of metals in e-liquids were lower than in aerosols, indicating that they originated from the device itself. None of the selected VOCs was detected in ENDS emissions. Concentrations of TSNAs were also under the limits of quantification in both e-liquids and aerosols.

Comparison of concentrations found in ENDS aerosols with cigarette smoke indicated that use of ENDS greatly reduces exposure to the selected toxicants and carcinogens compared to conventional cigarettes. However, the quality of ENDS and e-liquids is essential to ensure the lowest possible toxicant emissions.

1. Introduction

Electronic nicotine delivery systems (ENDS or e-cigarettes) are relatively new products whose use has increased significantly over the past decade. This new way of administering nicotine has seduced many users with a wide variety of devices and flavored e-liquids. However, the scientific community is particularly concerned about the possible health effects of ENDS, especially regarding potential long-term effects resulting of chronic exposure to ENDS emissions. Public health experts are divided on whether or not to support ENDS, because there is no consensus on the safety and efficacy of these products for smoking cessation.

ENDS consist of a battery part, a liquid storage tank, and an atomizer. They are filled with a so-called e-liquid, which is heated to generate an aerosol. E-liquids are mainly composed of propylene glycol and glycerol in different proportions (more than 90% of the total volume), to which flavorings, water, ethanol, and nicotine may be added. Aerosolization process takes places at temperature between 150 to 250°C, which is much lower than the combustion process of conventional cigarettes where temperatures of 800 to 900°C have been reported[1,2]. Consequently, ENDS deliver nicotine from a completely different way compared to conventional cigarettes.

The absence of combustion in ENDS may indicate that many toxicants and carcinogens are not likely to be emitted from these devices. Several research groups have used the harmful and potentially harmful constituents (HPHCs) in tobacco products and tobacco smoke issued by the U.S. Food and Drug Administration (FDA) to compare emissions generated by ENDS and cigarette smoke[3–6]. These chemicals were selected based on their potential association with one or more of the five health effects caused by smoking: cancer, cardiovascular diseases, respiratory effects, reproductive problems, and addiction. This list includes some volatile organic compounds (VOCs) including aldehydes, some metals, tobacco-specific nitrosamines (TSNAs), and some polycyclic aromatic hydrocarbons (PAHs). Table 1 shows the HPHCs analyzed in this study.

Goniewicz et al. (2014) reported that the aerosol concentrations of a selection of HPHCs were 9 to 450 times lower than in cigarette smoke7. Other studies have since supported these observations[5,6,8,9]. This is why part of the scientific community has proposed ENDS as a safer alternative to smoking. However, this reduction in concentrations does not mean that these products are safe for the health of users. In particular, aldehyde and metal emissions are of

concern. The use of food flavorings that have not been tested for inhalation exposure as well, but this topic is not covered in our study

Table 1 – Compounds from the list of harmful and potentially harmful constituents (HPHCs) in tobacco products and tobacco smoke issued by the U.S. Food and Drug Administration (FDA) analyzed in this study and their properties such as chemical family (aldehydes, volatile organic compounds (VOCs), metals, and tobacco-specific nitrosamines (TSNAs)) and potential health effects (addictive (AD), carcinogen (CA), cardiovascular toxicant (CT), reproductive or developmental toxicant (RDT), and respiratory toxicant (RT)).

Chemical family	Compounds	Potential health effects
Aldehydes	Acetaldehyde	CA, RT, AD
	Acrolein	RT, CT
	Crotonaldehyde	CA
	Formaldehyde	CA, RT
	Propanal	RT, CT
VOCs	Acrylamide	CA
	Acrylonitrile	CA, RT
	Benzene	CA, CT, RDT
	1,3-Butadiene	CA, RT, RDT
	Isoprene	CA
	Naphthalene [1]	CA, RT
	Toluene	RT, RDT
Metals	Arsenic	CA, CR, RDT
	Beryllium	CA
	Cadmium	CA, RT, RDT
	Chromium	CA, RT, RDT
	Cobalt	CA, CT
	Lead	CA, CT, RDT
	Nickel	CA, RT
	Selenium	RT
TSNAs	NNK [2]	CA
	NNN [2]	CA

[1]Naphthalene is both a VOC and the simplest polycyclic aromatic hydrocarbon (PAH); [2]4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), N-Nitrosonornicotine (NNN)

Aldehyde emissions can be caused by the thermal degradation of the main constituents of e-liquids, namely propylene glycol and glycerol[10]. This can occur in case of lack of liquid in the tank ("dry puff") or insufficient liquid supply to the coil, both inducing excess heat[11,12].
The extent of aldehyde generation varies depending on the device, the e-liquid, and the puffing regime[13]. The main aldehydes emitted by ENDS include formaldehyde (carcinogen group 1, International Agency for Research on Cancer – IARC), acetaldehyde (carcinogen group 2B, IARC) and acrolein (carcinogen group 2B, IARC). The design and use of ENDS are therefore important factors in reducing exposure to these carcinogens.

In recent years, several scientific studies have shown that ENDS emit varying amounts of heavy metals depending on the type of device[14]. Although some e-liquids contained metal contamination, the highest concentrations have been found in aerosols, indicating that they leach from the metal parts of the ENDS. The presence of metals in ENDS aerosols has raised concerns as they are linked to serious health effects such as cancers, neurotoxicity, cardiovascular diseases, and kidney toxicity[15]. The role of ENDS in the daily intake of heavy metals is not yet quantified.

Characterization of ENDS emissions is therefore important to assess the potential health risks of these devices. As they are rapidly evolving, additional scientific evidence is needed to ensure that changes are not made to the detriment of the users and to inform users of the nature of the toxicants and carcinogens to which they may be exposed when using ENDS. For this study, we selected a commercially available ENDS recommended for beginners (Innokin T20S, China), which was used in an on-going clinical trial on smoking cessation. We aimed to determine the concentrations of several HPHCs (aldehydes, VOCs, metals, and TSNAs) in emissions of the selected ENDS using six different flavored e-liquids. We also investigated the impact of the coil and the ENDS device metal composition on metal concentrations in emissions.

2. Methods

2.1 ENDS device and e-liquids

We conducted all experiments on aerosol generation using the Innokin Endura T20-S (Innokin, China). This device is composed of a 1-cell Li-Po battery, with a capacity of 1500 mAh and a Prism-S tank and coil system (clearomizer). Technical specifications included a maximal output wattage of 18 W, a maximum output current of 6 A, and the use of resistance of minimum 0.6 Ω. The Prism-S tank has a capacity of 2 mL. Innokin Prism-S coils (Innokin, China) are sold with two different resistance: 0.8 Ω and 1.5 Ω. We used the device with Innokin Prism-S coils of 0.8 Ω to increase the vapor production.

We tested six “Alfaliquids” (e-liquids from Gaïatrend, France) with different flavors: FR-M (tobacco, red fruit), FR4 (tobacco, caramel), Fresh Mint (FM; mint, candy), Red Fruits (RF; strawberry, raspberry, blackberry, blueberry), Raspberry #2 (R2; raspberry), and Green Apple (GA; Granny Smith apple). These flavors represented some of the most sold flavors in the Netherlands in 2017: fruits (40%), tobacco (16%), and menthol (8%)[16]. They should therefore reflect the most popular flavors among ENDS users. Formation of toxicants during aerosolization process may depend on flavoring molecules, so we have selected several flavors[17]. Propylene glycol and glycerol ratio (PG:VG ratio) was 76:24 and nicotine concentrations was 19.6 mg/mL for all the tested flavored e-liquids. Gaïatrend is certified by AFNOR (XP D90-300-2 norm, March 2015) for the fabrication of e-liquid.

Both ENDS and e-liquids are authorized for sale in Switzerland. The selected device and e-liquids were used in an on-going randomized controlled trial on smoking cessation: “Efficacy, Safety and Toxicology of Electronic Nicotine Delivery Systems as an aid for smoking cessation: the ESTxENDS multicenter randomized controlled trial” (ClinicalTrials.gov Identifiers: NCT03589989).

2.2 Vaping machine

We used a vaping machine that was developed in our laboratory according to the CORESTA standard conditions[18]. It included three ports (510 connectors) for ENDS clearomizers, a piston syringe, a stabilized power supply, a pinch-valve system and silicon tubing.

The batteries of ENDS were replaced by a controlled power supply in order to monitor and provide a steady state power of 12 W along all aerosol generation experiments. This was done to avoid including the battery charge state as an additional variable parameter and to control precisely the timing of puff duration. The ENDS clearomizers were placed on a tilting support to set a realistic orientation of the clearomizers of 45°. The pinch valve system in combination with the piston syringe allowed the connection of three sampling supports in parallel. The last valve was used to connect the outlet hose of the system. Figure 1 presents the design of the vaping machine.

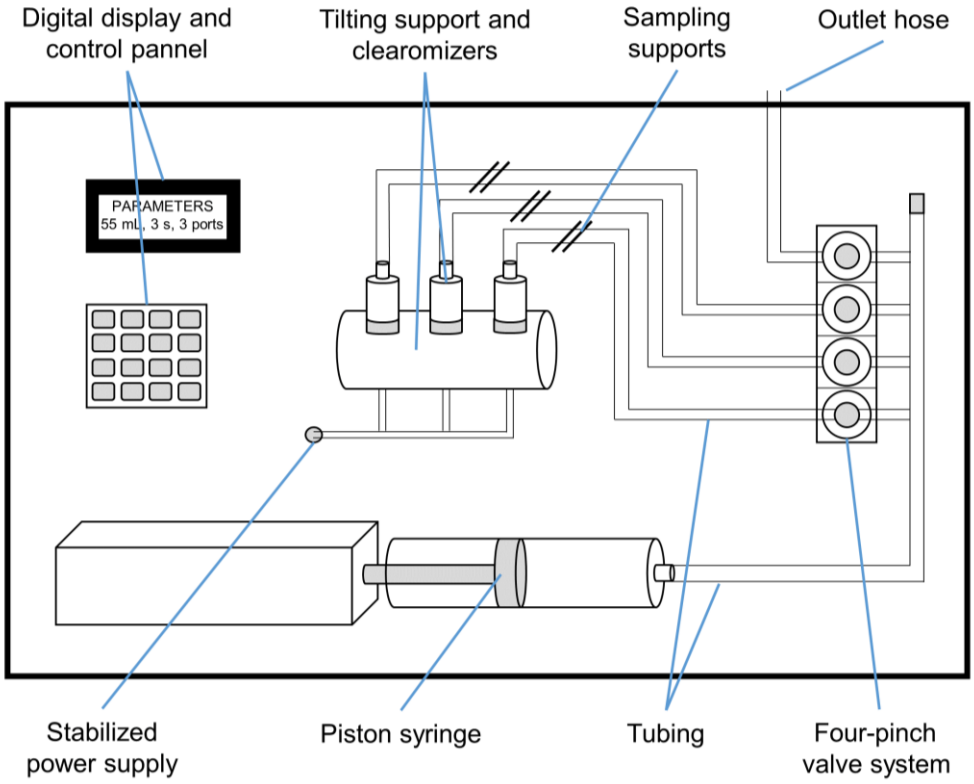


Figure 1 – Schema of the vaping machine design. The sampling supports are represented by two slashes as they can vary depending on the method of collection.

An integrated circuit allowed selecting the puffing regime parameters and monitoring the puff count, voltage and current through the coil in real time. The puffing regime parameters were chosen according to the CORESTA recommended method (CRM) n°81: 3 s puff duration, 55 mL puff volume, and 30 s puff frequency[18]. Number of puff cycles was adjusted according to the family of compounds measured. Before each experiment, clearomizers were filled with e-liquid, weighted, and screwed to the tilting support. A waiting period of 10 min was respected in order to ensure the cotton wick was fully impregnated. Aerosols were then sampled by connecting plastic or glass impingers to clearomizers using the shortest length of tubing to limit concentration underestimation due to aerosol deposition on the connecting tubes.

2.3 Aldehydes analysis

Thirteen aldehydes were analyzed (five from the HPHC list; Table 1): formaldehyde, acetaldehyde, acrolein, propanal, crotonaldehyde, butyraldehyde, benzaldehyde, isovaleraldehyde, valeraldehyde, o-tolualdehyde, m+p-tolualdehyde, hexanal, and 2,5-dimethylbenzaldehyde. The collection of aldehydes was based on the method of Gillman et al. (2016) and the quantification was performed by a routine high-performance liquid chromatography-ultraviolet (HPLC-UV) method[12]. The six e-liquids were tested in three replicates for each clearomizer (n = 54 samples). For this method, only 30 puffs were collected to avoid overheating of the device, which could generate higher concentrations of aldehydes. The aerosol generated by the ENDS was passed through two successive glass impingers with fritted nozzle containing a solution of a 2,4-dinitrophenylhydrazine (DNPH) derivatization solution (30 mL each; 1.3 mM DNPH; Merck, Schaffhausen, Switzerland), with 10 mM phosphoric acid (85%; Merck, Schaffhausen, Switzerland) in H_2O/ACN (v/v, 50:50). One sample (1 mL) of each impinger was collected and directly analyzed by HPLC (Dionex Ultimate 3000 system, Thermo Scientific, Reinach, Switzerland) equipped with a UV Detector (DAD-3000 RS rapid separation diode array detector, Thermo Scientific, Reinach, Switzerland). Limit of quantification (LOQ) was 0.05 µg/mL (calibration ranges from 0.05 µg/mL to 5 µg/mL for each compound). Details of the HPLC-UV method can be found in the supplementary data. We calculated intra-coil variations (i.e., the differences between the measured concentrations from the three replicates of a same coil for a same flavor) and inter-coil variations (i.e., the differences between the measured concentrations from the three coils for a same flavor) for both formaldehyde and acetaldehyde, and expressed as coefficient of variations (CV).

2.4 VOC analysis

The following seven VOCs were quantified (all from the HPHC list; Table 1): 1,3-butadiene, isoprene, acrylonitrile, benzene, toluene, acrylamide, and naphthalene. Naphthalene belongs also to the polycyclic aromatic hydrocarbon (PAH) family, and it was quantified by the VOC method as it was technically possible. VOCs were only quantified in aerosols. Aerosols were generated by simulating 100 puffs for three replicates of each flavored e-liquid (n = 18 samples overall). Aerosols were collected in two glass impingers with fritted nozzle containing methanol (30 mL). Both impingers were placed in cooling baths (1st one in isopropanol/dry ice at -78°C, and second one in ice with salt at 0°C.). One sample of each impinger (1 mL) was analyzed by gas chromatography mass spectrometry (GC-MS; Thermo Trace 1310 GC with Triplus RSH autosampler + Thermo ISQ LT, Thermo Scientific, Reinach, Switzerland). Limit of

quantification were 40 ng/mL for each compound (calibration ranges from 10 ng/mL to 1 µg/mL for each compound). Internal standard was benzene-d6 (Merck, Schaffhausen, Switzerland). Details of the GC-MS method and detection parameters can be found in supplementary data.

2.5 Metal analysis

The concentrations of the following 20 metals were quantified (eight from the HPHC list; Table 1): beryllium (Be), aluminum (Al), vanadium (V), chromium (Cr), manganese(Mn), iron (Fe), cobalt (Co), nickel (Ni), copper (Cu), zinc (Zn), arsenic (As), selenium (Se), molybdenum (Mo), palladium (Pd), silver (Ag), cadmium (Cd), tin (Sn), antimony (Sb), platinum (Pt), and lead (Pb). The metal quantification was performed using inductively coupled plasma mass spectrometry (ICP-MS; iCAP TQ, Thermo Scientific, Reinach, Switzerland). The metal content was determined for the six flavored e-liquids (n = 6 samples), but in the emission of only one flavored e-liquid: FM, 19.6 mg/mL (n = 9 samples), based on the results of e-liquid quantification. This flavored e-liquid was representative for the five other flavors. Concentrations of metal in e-liquids were quantified by dissolving e-liquid (1 g) in a solution of 0.5% nitric acid (HNO_3, 5 mL; 69% solution from SCP Science, Marktoberdorf, Germany). MilliQ water was prepared in the laboratory with a water purification system (MilliQ Advantage, Merck, Schaffhausen, Switzerland). For emission, 100 puffs were generated and condensed in two empty 50 mL plastic centrifuge tubes (DECCS 14 2TDS, Medivac, Parma, Italy) placed in a cooling bath (isopropanol/dry ice at -78°C). Condensed aerosol in both tubes was diluted and mixed in 0.5% HNO_3 (5 mL). Standard solutions of metals for calibration curves were bought from Labkings (Hilversum, Netherlands), except Fe from SCP Science (Marktoberdorf, Germany). LOQs and calibration ranges are presented in supplementary data as they are different for each metal. Yttrium (Y) was used as internal standard. Details of the ICP-MS method can be found in supplementary data.

2.6 TSNA analysis

Concentrations of the four main tobacco-specific nitrosamines were measured: N-nitrosonornicotine (NNN), 4-(methylnitrosamino)-1-(3-bipyridyl)-1-butanone (NNK), N-nitrosoanatabine (NAT), and N-nitrosoanabasine (NAB). Concentrations were determined in the six flavored e-liquids (n = 6 samples) and in the emission of only one flavored e-liquid (FM; n = 9 samples). This was based on the results of the quantification of TSNAs in e-liquids, and we considered that this flavored e-liquid was representative of the other flavors. In e-liquids,

the TSNAs were quantified by diluting e-liquid (20 mg) in 50 mM ammonium acetate in water (1 mL; Merck, Schaffhausen, Switzerland). For emission, 100 puffs were generated and collected in two empty 50 mL plastic centrifuge tubes (DECCS 14 2TDS, Medivac, Parma, Italy) placed in a cooling bath (isopropanol/dry ice, -78°C). Condensed aerosol in both tubes was recovered and mixed in 0.5% HNO_3 (5 mL).

A sample of this solution (100 µL) was further diluted in ammonium acetate in water (50 mM, 900 µL) and analyzed by liquid chromatography – tandem mass spectrometry (LC-MS/MS; Dionex Ultimate 3000 system + TSQ Quantiva, Thermo Scientific, Reinach, Switzerland). LOQs were 0.05 µg/mL for each compounds (calibration ranges from 10 ng/mL to 10 µg/mL). Internal standards were NNN-d4, NNK-d4, NAB-d4 and NAT-d4. Details of the LC-MS/MS method can be found in supplementary data.

2.7 Elemental analysis

Innokin Prism-S coil was carefully dissected to isolate different components: coil wire, internal metallic part, wick, and grid (Figure 2 and 3). They were then analyzed by scanning electron microscope equipped with an energy dispersive X-ray spectrometer (SEM-EDX Phenom XL, Thermo Scientific, Reinach, Switzerland) to analyze their morphology and elemental composition (BSD detector and 15 kV beam). The EDX count time was set to reach a LOD of about 0.1 wt%.

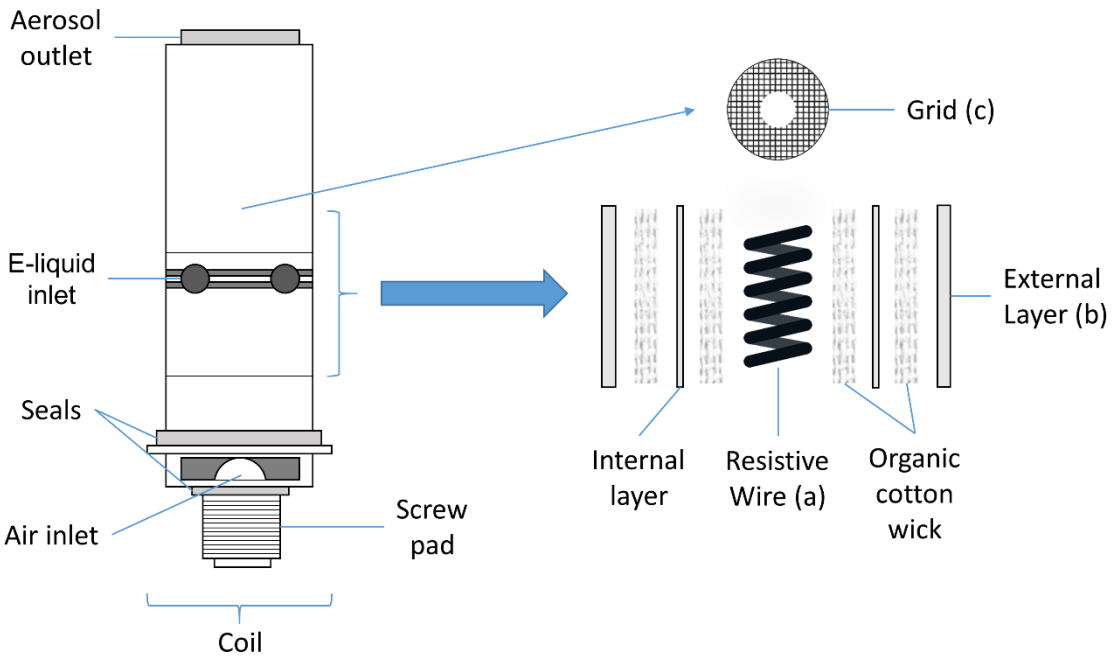


Figure 2 – Description of the composition of the Innokin Prism-S coil (Innokin, China).

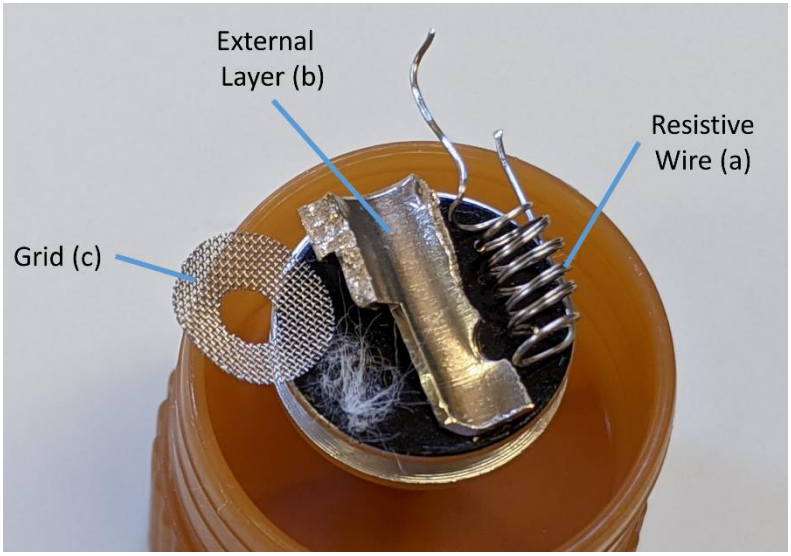


Figure 3 – Picture of the three components of the Innokin Prism-S coil (Innokin, China) used for the elemental analysis by scanning electron microscope equipped with an energy dispersive X-ray spectrometer (SEM-EDX): resistive wire (a), external layer (inner side; b), and grid (c).

2.8 Interpretation of toxicant and carcinogen concentrations in ENDS emissions

Concentrations of toxicants and carcinogens in ENDS emissions may be of concern. However, it is important to put them in perspective with other sources of exposure, such as cigarette smoking or environmental sources, or with toxicological limits set by public health or occupational health agencies. This subchapter provides the basis for the calculations and approximations used to make these comparisons that are presented in the discussion section.

2.8.1 Aldehyde – comparison with cigarette smoke

Exposure to formaldehyde and acetaldehyde were compared between smokers and ENDS users. We defined a light smoker as a person smoking 5 cigarettes per day (cig/day) and a heavy smoker as a person smoking 20 cig/day[19]. The reported mainstream formaldehyde and acetaldehyde amounts of most commercially available cigarettes are in the ranges of 30–60 micrograms per cigarette (µg/cig) and 500–1000 µg/cig, respectively[20]. The daily dose of formaldehyde to which light and heavy smokers are exposed would then be (5 cig/day x (30–60 µg/cig) =) 150–300 µg/day and 600–1200 µg/day, respectively. Similarly, the daily dose of acetaldehyde would be 2500–5000 µg/day and 10–20 milligrams per day (mg/day) for light and heavy smokers, respectively.

For ENDS users, we approximated the daily consumption of e-liquid to 3 mL per day (mL e-liq/day) based on observations we made during the ESTxENDS clinical study. Density of e-liquid was estimated to 1 g/mL to facilitate the calculations (3 mL e-liq/day = 3 g e-liq/day). Therefore, an estimation of the daily exposure dose to formaldehyde and acetaldehyde can be calculated by multiplying the concentrations in aerosol (expressed as micrograms by gram of vaporized e-liquid; µg/g e-liq) with the quantity of vaporized e-liquid inhaled per day (3 g e-liq/day). Daily doses, expressed in µg/day, from cigarette smoke and ENDS aerosol could directly be compared.

2.8.2 Aldehyde – comparison with indoor air

Environmental sources of aldehydes include air pollution, traffic, and industrial waste [21]. Indoor air can also be contaminated by these substances. Indeed, the average concentration of formaldehyde and acetaldehyde in homes in Europe randomly selected has been measured at about 20–30 µg/m^3 and 10–25 µg/m^3, respectively[22,23]. It varies according to the building materials, the presence of new materials and products, and the degree of ventilation (air exchange rate). We decided to compare the cumulative dose of formaldehyde and acetaldehyde inhaled during an 8-hour period in this environment with the daily dose resulting from ENDS use.

We considered an average adult resting at home for 8 hours who would inhale 6 liters of air per minute (L/min; frequency of 12 inhalations per minute with a tidal volume of 500 mL) [24,25]. We also considered that there would be no ventilation (i.e., closed windows) and therefore that the concentrations of formaldehyde and acetaldehydes do not vary during the exposure period. The total dose of formaldehyde inhaled over an 8-hour period would thus be 20–30 µg/m^3 x (6 L/min x 0.001) x (8 h x 60) = 58–86 µg. Similarly, the total dose of acetaldehyde would be 29–72 µg.

For the ENDS user, we use the same approximations made in the previous paragraph (an e-liquid consumption of 3 g e-liq/day). The total doses of aldehydes resulting from an exposition to indoor air during an 8-hour period were compared to the daily doses resulting from vaping.

2.8.3 Aldehyde – comparison with occupational exposure limits

ENDS users are exposed to aldehydes only during a short period of time. Indeed, a daily consumption of 3 g e-liq/day would correspond to ~325 puffs (based on our experimental data with the CRM n°81 puffing regime), which is 975 s or less than 17 min of use per day.

Such short exposure can be compared to occupational exposure, and particularly to short-term exposure limit (STEL; acceptable time-weighted average concentration to which workers can be exposed continuously for a short period of time).

The Swiss STEL for formaldehyde and acetaldehyde are 0.6 ppm or 0.74 mg/m^3 and 50 ppm or 90 mg/m^3, respectively, over a period of 15 minutes. We can determine aerosol exposure time to reach this limit since STEL is time-weighted average. We can use Equation 1 by considering the concentration in air (i.e., air inhaled when not using ENDS) to be negligible.

Equation 1:

$$STEL = \frac{t_a * c_a + t_r * c_r}{15\ min}$$

With t_a the aerosol exposure time and t_r the rest of the time ($t_a + t_r = 15$ min), and c_a the aldehyde concentration in ENDS aerosol and c_r the aldehyde concentration in ambient air.

As mentioned above, c_r is assumed negligible and the equation can be rearranged to calculate the aerosol exposure time t_a. It gives: t_a (min) = STEL (mg/m^3) x 15 (min) / c_a (mg/m^3). Aerosol concentration should be expressed in mg/m^3, which can be done by transforming results expressed in ng/puff into ng/mL (= mg/m^3), knowing that the puff volume was 55 mL. The number of puff can then be calculated considering a puff duration of 3 s: puff number = t_a (min) x 60 / 3 (s). Therefore, this puff number gives an indication on the frequency of ENDS use required in 15 minutes to approach the STEL values.

2.8.4 Metals – minimal risk levels (MRLs)

Minimal risk levels (MRLs) were issued by the Agency for Toxic Substances and Disease Registry (ATSDR) and are defined as the daily dose of a chemical a person can be exposed to by ingestion, inhalation, or external radiation without a detectable risk to health (other than cancer)[26]. MRLs exist for 8 of the 19 metals analyzed (V, Cr, Mn, Co, Ni, Mo, Cd, and Sb). MRLs for chronic inhalation exposure were selected, and the units were converted to ng/m^3. These values were multiplied by the total volume of air inhaled per day (m^3/day) to calculate the acceptable daily exposure dose (ng/day). We calculated total volume of air inhaled per day based on the volume of breath (tidal volume = 500 mL) multiplied by the frequency of inspiration per minute (12 x/min) and the number of minutes in 24 h (n= 1440 min). Total inhaled volume was estimated to be 8.64 m^3 (for an average adult resting for 24 h).

Metal concentrations in aerosol (expressed as nanograms by gram of vaporized e-liquid; ng/g e-liq) were transformed to daily dose (ng/day) using the daily consumption of e-liquid (on average 3 mL e-liq/day or 3 g e-liq/day considering a density of 1 mg/mL). MRLs and metal concentration transformed and expressed in ng/day could be directly compared.

2.8.5 Metals – permitted daily exposures (PDEs)

Permitted daily exposure (PDEs) were obtained from the ICH Q3D guideline for elemental impurities defined by the European Medicines Agency (EMA)[27]. PDEs are defined as the maximum permitted amounts of metals resulting from the daily intake of a drug product (oral, parental or inhalation routes). PDEs exist for 14 of the 19 metals analyzed in this study (V, Cr, Co, Ni, Cu As, Mo, Pd, Ag, Cd, Sn, Sb, Pt, and Pb). PDEs are expressed in µg/day or can be transformed in µg/g by multiplying with the daily dose of the product of interest (g/day). PDEs were thus multiplied by 3 by estimating an average daily consumption of 3 g of e-liquid per day. PDEs were transformed in ng/g to allow direct comparison with metal concentration expressed as ng/g e-liq.

2.8.6 Metals – recommended exposure limits (RELs)

Recommended exposure limits (RELs) were proposed by the National Institute for Occupational Safety and Health (NIOSH)[28]. RELs are maximum concentrations of a chemical that are permitted in occupational settings and have three subcategories: time weighted average (TWA; for an 8-hour period), ceiling value (CV; at any time), and short-term exposure limit (STEL; for a 15-minute period). RELs are available for 17 of the 19 metals analyzed (all except Pd and Cd). Missing RELs were replaced by permissible exposure limits (PELs) issued by the Occupational Safety and Health Administration (OSHA). RELs and PELs were transformed in µg/m^3. All were TWA, except two that were CV (for Be and As).

Metal concentration in aerosol (µg/m^3) could directly be compared to CV. This was not the case for TWA: ENDS users are not exposed to ENDS emissions over an 8-hour period (480 min), but only during ~340 puffs (for 3 g e-liq/day; based on experimental data). Thus, daily time of exposure would be 1020 s or 17 min (1 puff = 3 s). Assuming no exposure to metals outside of vaping, metal concentrations in ENDS emissions (µg/m^3) must be corrected by a 17:480 factor and could then be compared to TWA.

3. Results

3.1 Aldehydes

No aldehydes were detected in ENDS emissions, except formaldehyde, acetaldehyde and acrolein. Only formaldehyde and acetaldehyde were above the LOQ (0.05 µg/mL), but not in all replicates. Table 2 presents the concentrations in aerosols of the six flavored e-liquids. On average, the concentrations of formaldehyde and acetaldehyde generated per gram of aerosolized e-liquid were 7 ± 4 µg/g e-liq and 4 ± 3 µg/g e-liq, respectively. Intra-coil variations were under 20% for formaldehyde and under 25% for acetaldehyde. Inter-coil variations were under 25% for formaldehyde and under 40% for acetaldehyde.

Table 2 – Concentrations of formaldehyde, acetaldehyde and acrolein in electronic nicotine delivery system (ENDS) aerosols of six flavored e-liquids, expressed in micrograms per gram of vaporized e-liquid (µg/g e-liq) as mean with standard deviation. Nine replicates were analyzed for each flavored e-liquid.

Flavors	**Formaldehyde** (µg/g e-liq)	**Acetaldehyde** (µg/g e-liq)	**Acrolein** (µg/g e-liq)
FR-M	9.2 (2.3)	6.5 (1.4) [1]	ND
FR4	3.6 (0.5) [1]	<LOQ	ND
FM	3.1 (1.2) [1]	<LOQ	ND
RF	8.2 (3.8) [1]	<LOQ	ND
R2	<LOQ	<LOQ	<LOQ
GA	12.1 (2.1)	6.9 (1.3)	ND

[1]One or more replicates were below limit of quantification (LOQ).

ND means non-detected and <LOQ means concentrations in impinger <0.05 µg/mL (corresponding to ≈ 5 µg/g e-liq). Flavors: FR-M (tobacco, red fruit), FR4 (tobacco, caramel), Fresh Mint (FM; mint, candy), Red Fruits (RF; strawberry, raspberry, blackberry, blueberry), Raspberry #2 (R2; raspberry), and Green Apple (GA; Granny Smith apple). Results expressed in ng/puff can be calculated considering that one puff consumed about 9.22 ± 0.66 mg of e-liquid and that puff cycles of 30 puffs were used for each aerosol generation.

3.2 VOCs

None of the seven VOCs analyzed (including naphthalene; a semi-VOC) was detected in the ENDS emissions (n = 18 samples). Limits of detection (LODs) were 10 ng/mL for each compound, except for acrylonitrile (20 ng/mL).

3.3 Metals

Table 3 presents the concentrations of metals in e-liquids and aerosols. Overall, the concentrations found in aerosols were higher than in e-liquids. Six metals had aerosol concentrations above 100 ng/g e-liq: Al, Fe, Ni, Cu, Zn, and Pb.

Table 3 – Metal concentrations in e-liquids and electronic nicotine delivery system (ENDS) aerosols, expressed in nanograms per gram vaporized e-liquid (ng/g e-liq) as mean with standard deviation. We analyzed six flavored e-liquids and nine replicates of one flavor only for aerosols (fresh mint; FM). Metals are classified by increasing atomic number.

Metals	E-liquid concentration (ng/g e-liq)	Aerosol concentration (ng/g e-liq)
Be	<LOQ	0.04 (0.04) [1]
Al	<LOQ	191 (78.7)
V	0.12 (0.11) [1]	0.31 (0.15)
Cr	1.38 (0.30) [1]	4.79 (1.60)
Mn	1.61 (2.08) [2]	7.42 (3.67)
Fe	8.78 (10.5)	161 (89.0)
Co	<LOQ	0.31 (0.61) [1]
Ni	2.72 (2.10)	166 (44.3)
Cu	7.66 (6.58)	640 (348)
Zn	15.1 (16.9) [1]	797 (272)
As	2.12 (0.69)	2.51 (0.82)
Se	<LOQ	<LOQ
Mo	0.60 (0.39)	0.41 (0.36)
Pd	0.45 (0.28)	1.90 (0.38)
Ag	0.01 (0.01) [1]	0.17 (0.05)
Cd	0.02 (0.04) [1]	3.98 (5.45)
Sn	0.39 (0.30)	6.36 (5.53)
Sb	0.26 (0.15)	2.48 (1.26)
Pt	0.92 (1.58)	0.03 (0.01)
Pb	1.06 (1.32)	265 (99.0)

[1]One or more replicates were below the limit of quantification (LOQ). LOQs in e-liquids were 0.05 ng/mL for Be, 5 ng/mL for Al, 0.02 ng/mL for V, 0.05 ng/mL for Cr, 0.1 ng/mL for Co, 1 ng/mL for Zn, 0.5 ng/mL for Se, 0.002 ng/mL for Ag, and 0.005 ng/mL for Cd. LOQs in aerosols were 0.005 ng/mL for Be, 0.1 ng/mL for Co, and 0.5 ng/mL for Se; [2]One e-liquid was removed. Results expressed in ng/puff can be calculated considering that one puff consumed about 8.87 ± 0.09 mg of e-liquid that puff cycles of 100 puffs were used for each aerosol generation.

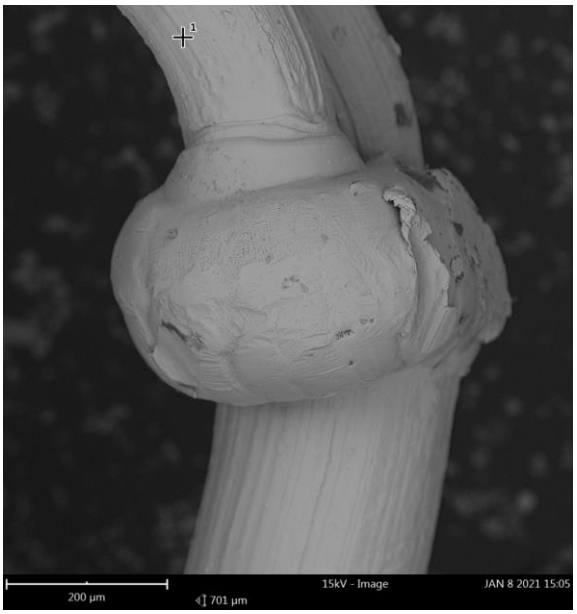
The cold trap sampling system allowed a high level of recovery: on average, 97 ± 5% of the aerosolized e-liquid mass was collected in the two collection tubes. Of the 20 metals analyzed, only Be, Al, and Co could not be quantified in e-liquids, and Se both in e-liquids and aerosols.

3.4 TSNAs

The four nitrosamines, NNN, NNK, NAB, and NAT, were not detected in flavored e-liquids (n = 6 samples) neither in ENDS emissions (FM flavor only; n = 9 samples). LODs were between 10 and 20 ng/mL.

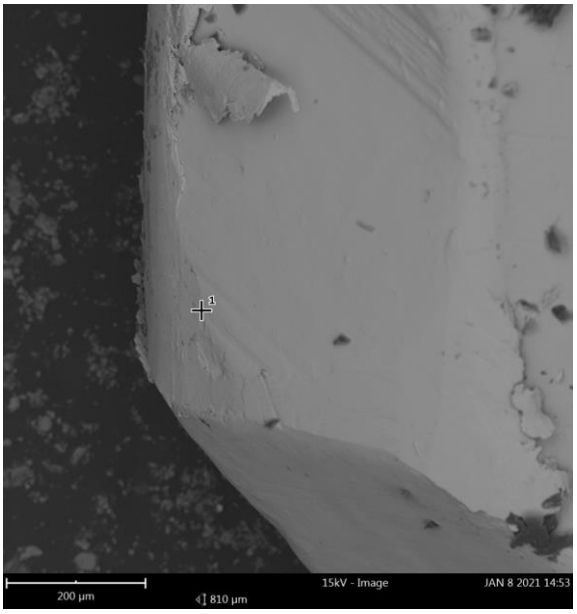
3.5 Elemental composition

We performed an elemental analysis of different components of the coil to investigate the sources of the metals detected in ENDS emissions (Figure 2 and 3). ENDS coil was composed of a resistive wire that heated the e-liquids to generate the aerosol inhaled by the user. The resistive wire consisted of two parts (Figure 4): the internal coil was a double nichrome wire (allow of nickel and chrome; 2x 50 mm, Ø170µm) welded to two nickel legs (2x 20 mm, Ø250µm). The organic cotton wick, whose function was to carry the e-liquid to the resistive wire, was held in place by internal layers. We analyzed the inner side of the external layer, which was in contact with e-liquid, and we observed that it was composed of nickel (Figure 5). A grid was located above the cotton to prevent probably the fibers from passing. It was composed of stainless steel (alloy of iron, chrome, and nickel; Figure 6).



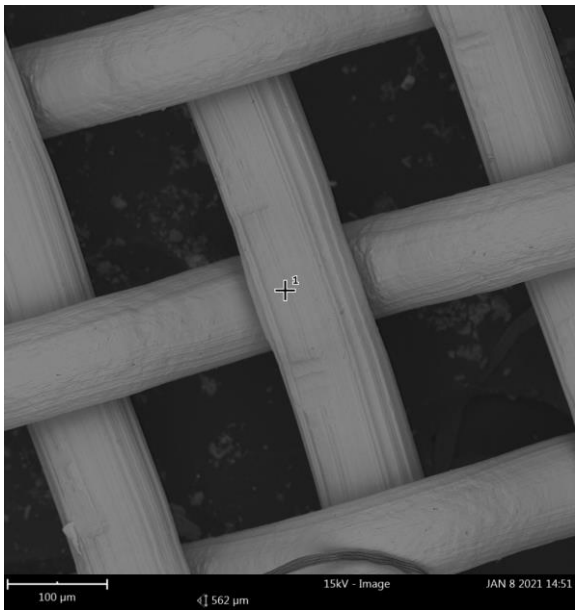
Element Symbol	Atomic percentage	Weight percentage
Ni	70.08	74.28
Cr	24.54	23.04

Figure 4 – Backscattered scanning electron microscope image (BSD-SEM) of the resistive wire (a) and table of the elemental composition obtained from the energy dispersive X-ray analysis (SEM-EDX).



Element Symbol	Atomic percentage	Weight percentage
Ni	100	100

Figure 5 – Backscattered scanning electron microscope image (BSD-SEM) of the external layer (b) and table of the elemental composition obtained from the energy dispersive X-ray analysis (SEM-EDX).



Element Symbol	Atomic percentage	Weight percentage
Fe	71.58	72.69
Cr	19.12	18.07
Ni	8.05	8.59

Figure 6 – Backscattered scanning electron microscope image (BSD-SEM) of the grid (c) and table of the elemental composition obtained from the energy dispersive X-ray analysis (SEM-EDX).

4. Discussion

We analyzed aerosols generated by one ENDS with six flavored e-liquids for the presence of several compounds from the Harmful and Potentially Harmful Constituents (HPHC) list. We also investigated the coil composition to identify the metals that are in contact with e-liquids as the coil might be the primary source of metals detected in ENDS emissions. The choice of was motivated by its ease to use, especially for beginner ENDS users. This was one of the most important criteria for the device selection in the on-going clinical study on smoking cessation "Efficacy, Safety and Toxicology of Electronic Nicotine Delivery Systems as an aid for smoking cessation: the ESTxENDS multicenter randomized controlled trial".

Puff parameters are factors with a wide inter-individual variation and some research groups recommended to use laboratory test results with caution to assess the real exposure to toxicants among vapers[29,30]. Thus, concentrations of the measured compounds should be expressed per gram of vaporized e-liquid to reduce the influence of different puffing regimes and the variability between ENDS devices.

Several studies have reported varying levels of aldehydes in ENDS emissions over the past 10 years[31]. Aldehydes can be formed during the thermal degradation of the main components of e-liquids in overheating conditions. This might happen when the e-liquid supply to the coil is insufficient, either because the device has been poorly designed (e.g., some devices of older generations) or because the e-liquid tank is empty. For example, Hutzler et al. (2014) collected puff blocks until no aerosol was produced by the ENDS[32]. The resulting high aldehydes concentrations (similar to conventional cigarettes) observed during the last puffs could be attributed to insufficient e-liquid supply to the heat source. Talih et al. (2016) tested an outdated ENDS device that needed to be filled regularly to keep the wick wet[33]. The high aldehyde concentrations were therefore not representative of what is achieved by the ENDS nowadays. Formation of significant amount of aldehydes during overheating (or dry puff) cannot be detected when testing with a vaping machine in the laboratory before analyzing the collected samples. However, ENDS users will immediately recognize the problem as the aerosol will have an unpleasant burnt taste. Informed ENDS users know that they should change the resistance of their device periodically. It is therefore important to use a resistance compatible with the device power and e-liquid with suitable PG:VG ratio when performing laboratory testing, as glycerol is more viscous than propylene glycol and wets the absorbent material more slowly.

The ENDS we tested emitted concentrations of formaldehyde and acetaldehyde under 20 µg/g e-liq, which was coherent with the results of other studies on devices with similar tank design[34–36].

The intra- and inter-coil variations indicated that the collection and analysis method was efficient, but that the coils emitted slightly different concentrations of formaldehyde and acetaldehyde, possibly due to slight differences in manufacturing. However, intra-coil and inter-coil variation were higher for acetaldehyde. This can be explained by the measured concentrations were close to the LOQ (0.05 µg/mL), where the uncertainty is higher (CV < 30% for the lowest limit of quantification (LLOQ) is acceptable).

We observed differences in aldehyde concentrations between the flavored e-liquids and the variability was higher than the intra- and inter-coil variations (CV ≈ 60%). It indicated that formaldehyde and acetaldehyde formation was dependent on the flavoring molecules present in e-liquid as the PG:VG ratio and the nicotine concentration were the same for the six flavored e-liquid. This was previously demonstrated by Khlystov and Samburova (2016), although the measured concentrations were much higher (176–5570 µg/g e-liq and 58.4–2670 µg/g e-liq for formaldehyde and acetaldehyde, respectively) than those measured in our study[37]. However, we could not identify a group of flavors that would promote aldehyde formation. Indeed, one tobacco flavor out of two promotes a higher aldehyde formation (e.g., for formaldehyde: FR-M vs FR4: 9.2 µg/g e-liq vs 3.6 µg/g e-liq) and two fruity flavors out of three (e.g., for formaldehyde: GA and RF vs R2: 12.1 and 8.2 µg/g e-liq vs < LOQ). It would therefore be necessary to look at the precise composition of each flavor to identify common molecules and/or to identify other factors (e.g., effects of acidic or basic compounds) that may explain these results. This was however outside the scope of this study.

Detection of formaldehyde and acetaldehyde in ENDS emissions may be of concern, as both compounds are carcinogens. It is important to put the measured concentration in perspective with other sources of exposure or with limits set by public or occupational health agencies.
We compared the concentrations of formaldehyde and acetaldehyde with the ones reported in conventional cigarette smoke (a), the ones resulting from an environmental exposure (indoor air; b), and the ones corresponding to occupational exposure limits (c):

(a) Compared to the daily dose of formaldehyde resulting from cigarette smoking for light and heavy smokers, an ENDS user (3 mL e-liq/day) would be exposed to an amount at least 7x and 28x lower, respectively. Similarly, they would be exposed to a daily amount of acetaldehyde at least 200x and 800x lower compared to a light and heavy smoker, respectively.

(b) Indoor air might be contaminated with aldehydes. We calculated that an adult resting at home during 8 hours would be exposed to a dose of formaldehyde and acetaldehyde of 58–86 µg and 29–72 µg/m^3, respectively. This is more than twice the inhaled daily doses of ENDS users (3 mL e-liq/day): 21 ± 12 µg for formaldehyde and 12 ± 9 µg for acetaldehyde. However, an important distinction in our example is that the exposure for the adult resting at home is continuous, whereas the ENDS user is exposed discontinuously, over a much smaller volume. Therefore, the effective concentrations to which they are exposed are higher, but for a shorter time. In addition, the fact that the measured concentrations in ENDS emissions are lower than those from one environmental source does not mean that these devices are safe, because these exposures accumulate.

(c) ENDS users are exposed to high concentrations of formaldehyde (1.15 mg/m^3) and acetaldehyde (0.64 mg/m^3) over a short period of time (~17 min per day). We estimated the number of puffs required in 15 min to reach the STEL limit. For formaldehyde, ENDS users would have to puff during 9.7 min or take 194 puffs (of 3 s) in 15 min to reach the STEL. That corresponds to 1.7 g of e-liquid, or almost a complete tank refill. It is unlikely that a user would consume this amount in 15 minutes. For acetaldehyde, the concentration in aerosol (0.64 mg/m^3) is lower than the STEL (90 mg/m^3), so ENDS users will never reach this limit even if their vape non-stop for 15 minutes.

In conclusion, the tested ENDS does emit aldehydes, mainly formaldehyde and acetaldehyde, but the concentrations are lower than the ones reported in cigarette smoke and lower than occupational exposure limits. Moreover, we showed that environmental exposure could be more important that the one resulting from vaping.

Emissions of metals by ENDS have been less studied than emission of aldehydes. The concentrations found in aerosols present a high variability between studies[14,15]. It can be explained by different generations of ENDS (cig-a-likes, tanks, pods; open or closed systems), power settings, collection methods and puffing regimes[38–40].

Metals with the highest reported concentrations are similar to the ones identified in this study: Al, Fe, Ni, Cu, Zn, and Pb, although they were not detected in emissions of all devices.

There is no standardized method to collect metals from ENDS aerosols. We tested two collection methods: filter and cold trap. Similarly to Palazzo et al. (2016), we tested metal collection on a mixed cellulose ester (MCE) membrane (0.8 µm, 25 mm, SKC, USA)[41]. However, the filters were saturated after ≈ 10 puffs, and they became airtight, which stopped the experiment. The pore size (0.8 µm) was probably too small. We then tried a cold trap system. We selected plastic centrifuge tubes to avoid using glass impingers that may contain

traces of metals. Tubes are normally intended for exhaled breath condensate (EBC) collection in a cooling device (Medivac system). They were previously analyzed to ensure they did not contain metal contamination (data not shown). We tested first the collection in an ice bath (0 °C) and observed gravimetrically that only about 57% of the aerosol was collected. This percentage increased to more than 92% when isopropanol/dry ice bath was used (-70°C). This meant that almost all the aerosol generated was collected in the two tubes. Other groups also described new approaches of aerosol condensation using pipette tips and plastic tubes or fluoropolymer trap with high recovery (70 to 95%)[38,42]. Our approach has the advantage of being able to determine easily the recovery level gravimetrically, which is not the case when using filters or impingers.

However, one disadvantage of our strategy, such as those using condensation in tubing, is that it is difficult to measure a blank sample. We made sure that the tubes did not contain traces of metals, we used the same solvent to dilute condensed aerosol and prepare the calibration standard solutions, but we did not perform any measurement in the air of the laboratory. Thus, we cannot exclude that a fraction of the measured metal concentrations comes from the ambient air. However, this contribution would be very small due to the limited volume of air needed to generate 100 puffs (5.5 L or 0.0055 m^3). Based on the data from the German Environment Agency (UBA), the values obtained in our study for Cu, Ni, Pb, and Zn are two to three orders of magnitude higher than metal concentrations measured in air by industrial stations (i.e., air measuring stations near industries) in Germany[43]. It was therefore unlikely that laboratory air was source of the levels of contamination we measured. Yet, Zhao et al. (2020) recommended reporting blank or control-corrected metal concentrations[14]. Therefore, we would suggest performing air sampling the same day of the experiment to obtain metal concentrations in laboratory air that would serve as blank values.

Concentrations of metals in ENDS emissions were greater than in e-liquids (most of them < 5 ng/g e-liq), indicating that metal contamination of e-liquid was not the primary source of the selected metals. SEM-EDX did not allow identifying trace elements (roughly < 0.1 wt%). Therefore, we immersed a coil and an ENDS head separately in acidified water (HNO_3 6.5% in H_2O, heated at 95°C for 40 min) to dissolve the metals on the surface. The six metals (Al, Fe, Ni, Cu, Zn, and Pb) were found in high concentrations in both solutions from the coil and the head (> 1000 µg/sample for each metal – data not shown). This was not a quantitative experiment, but it was an additional indication that the metals in aerosols originated from the metal parts of ENDS in contact with e-liquid.

Although the concentrations of several metals in ENDS emissions seemed elevated, we compared these values with three existing toxicity limits. Metal emissions from ENDS were lower than the MRLs issued by the ATSDR, the PDEs from the ICH Q3D guideline for elemental impurities defined by the EMA, and the RELs proposed by NIOSH[26–28]. Table 4 summarizes the comparisons between metal concentrations in aerosol and safety limits.

No metal concentrations in aerosols exceeded MRLs, PDEs or RELs. Even the six metals found in elevated concentrations (100-1000 ng/g e-liq), namely Al, Fe, Ni, Co, Zn, and Pb, did not approach the limit values. Comparisons with MRLs may indicate that ENDS users would not increase the risk for their health by vaping (considering exposure to metals only, and without considering cancer risk). Comparisons with PDEs may indicate that, if the selected ENDS device were a pharmaceutical product, it would respect the guidelines on metal impurities. However, this also evidences that quality of materials used in the manufacture of ENDS should be better controlled, even if the levels emitted by the tested device remain low.

The rest of the compounds (VOCs and TSNAs) were not detected in e-liquids or ENDS aerosols. PAHs are formed primary by combustion processes that are unlikely to happen in ENDS. Therefore, we decided not to analyze this family. Nonetheless, we quantified naphthalene, which is both a VOC and the simplest compound from the PAH family. We used the VOC method to quantify it, as it was technically possible. Naphthalene was previously detected in ENDS aerosol only in very low concentrations (in pg/mL puff)[9]. Concerning the VOCs, they were previously shown to be mostly undetectable in ENDS aerosol or quantified in low concentrations[44]. TSNAs are impurities present in tobacco leaves. Low levels were previously detected in e-liquids and aerosols, comparable to pharmaceutical nicotine products[45].

Table 4 – Comparison of metal concentrations in aerosols with minimal risk levels (MRLs), permitted daily exposure (PDEs), and recommended exposure limits (RELs). Metal concentrations and safety limits have been transformed into the same units to allow direct comparison. Missing limits are indicated by a dash.

Metal	**Metal concentration** (ng/day)	**MRL** (ng/day)	**Metal concentration** (ng/g)	**PDE** (ng/g)	**Metal concentration** (µg/m³)	**REL** (µg/m³)
Be	<3	-	<1	-	<0.01	0.5 [1]
Al	573	-	191	-	1.08	5,000
V	<3	864	<1	3,000	<0.01	1,000
Cr	14	364	5	9,000	0.03	500

Metal	Metal concentration (ng/day)	MRL (ng/day)	Metal concentration (ng/g)	PDE (ng/g)	Metal concentration (µg/m³)	REL (µg/m³)
Mn	22	2,592	7	-	0.04	1,000
Fe	483	-	161	-	0.91	5,000
Co	<3	864	<1	9,000	<0.01	50
Ni	498	778	166	15,000	0.95	15
Cu	1,920	-	640	90,000	3.74	1,000
Zn	2,391	-	797	-	4.50	5,000
As	8	-	3	6,000	0.01	2 [1]
Se	ND	-	ND	-	ND	200
Mo	1.2	17,280	<1	30,000	<0.01	15,000
Pd	6	-	2	3,000	0.01	5,000 [2]
Ag	<3	-	<1	21,000	<0.01	10
Cd	12	86	4	9,000	0.02	5 [2]
Sn	19	-	7	180,000	0.04	500
Sb	7	2,592	2	60,000	0.01	2,000
Pt	<3	-	<1	3,000	<0.01	1,000
Pb	795	-	265	15,000	1.50	50

[1]Ceiling values (CV); [2]Permissible exposure limits (PELs)

ND means non-detected. Metal concentrations under 1 ng/g e-liq are reported as <1 ng/e-liq (or <3 ng/day or <0.01 µg/m³ over a 8-hour period).

Characterization of ENDS emissions carried out during our study supports the fact that smokers would greatly reduce their exposure to the selected toxicants and carcinogens present in cigarette smoke, and therefore the risks to their health related to those compounds, by becoming exclusively ENDS users.

However, we cannot exclude that ENDS users could be exposed to other compounds that we have not measured and that could have adverse health effects. Further investigations must be conducted on these other potential compounds. Concerning our study, metals may be found at higher concentrations in ENDS aerosols compared to cigarette smoke, especially Co, Ni, and Pb. Concentrations measured in ENDS emissions should probably not increase a risk for ENDS users' health as exposure estimates were lower than the toxicological values MRLs, PDEs, and RELs. However, it should be mentioned that ENDS is not the only source of these metals and that these daily doses come in addition to those from food and pollution.

In conclusion, the overall exposure to the selected HPHC is undoubtedly lower for ENDS users compared to smokers. Current concerns about ENDS focus primarily on thermal degradation of the e-liquid, flavorings, and metal leakage. Fortunately, we currently have the technical means to limit the presence or generation of toxicants by ensuring good quality ingredients and material used. ENDS can be proposed as an aid for smoking cessation because overall exposure to toxicants and carcinogen is reduced in vaping compared to tobacco smoking.

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Chapter 4 – Oxidative stress biomarker method

The development, validation and application of the method of oxidative stress biomarkers were subject of an article titled “Rapid Liquid Chromatography – Tandem Mass Spectrometry Analysis of Two Urinary Oxidative Stress Biomarkers: 8-oxodG and 8-isoprostane” by N. Sambiagio, J.-J. Sauvain, A. Berthet, R. Auer, A. Schoeni, and N.B. Hopf. The article was published in the journal Antioxidants (international, peer-reviewed, and open access journal published by MDPI) on December 31, 2020 (doi: 10.3390/antiox10010038).
Author contributions (from the article): Reto Auer (R.A)., Nancy B. Hopf (N.B.H.), Aurélie Berthet (A.B.), and Jean-Jacques Sauvain (J.-J.S.) conceived the project and managed funding acquisition; Anna Schoeni (A.S.) and R.A. managed the project coordination; Nicolas Sambiagio (**N.S.**) and J.-J.S. carried out the method development, **N.S.** validated the method; **N.S.** and A.S. selected the participants; **N.S.** applied the method on participants’ urine; A.B., J.-J.S., and N.B.H. supervised the method elaboration processes; **N.S.** wrote the manuscript, which was further amended by all authors. All authors have read and agreed to the published version of the manuscript.

4.1 Introduction

The analysis of the two selected urinary oxidative stress biomarkers, 8-oxodG and 8-isoprostane, required the implementation of a new method. Several chemical analyses had been previously developed to quantify 8-oxodG and 8-isoprostane in urine, based on liquid chromatography – mass spectrometry (LC-MS) or on enzyme-linked immunosorbent assay (ELISA). However, few quantified both biomarkers simultaneously, saving time and money.

A LC-MS method was developed and validated. Briefly, urines samples were spiked with internal standards, passed through solid-phase extraction (SPE) cartridges, and the extracts were evaporated and reconstituted in the injection solvent for subsequent analysis by LC-MS (see Figure 2). The validation parameters included limits of detection (LODs), limits of quantification (LOQs), linearity, intra- and inter-day precision and accuracy, recovery, and matrix effects. During the method development, emphasis was placed on matrix effects in order to find strategies to reduce them. Three different urine samples with different creatinine contents (representing different hydration status) were used to perform the method validation.

The method was finally applied to ex-smokers to verify that the measured concentration ranges matched the ones previously reported in the literature.

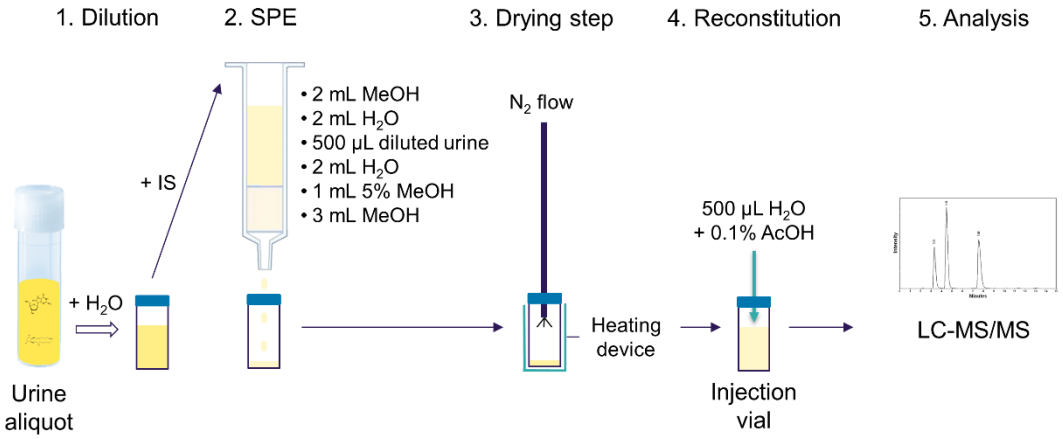

Figure 2 – Overview of the LC-MS method developed for the simultaneous analysis of 8-oxodG and 8-isoprostane in urine.

4.2 Overview of results and discussion

In urine samples, LOQs were 0.5 ng/mL for 8-oxodG and 0.1 ng/mL for 8-isoprostane. Linearity from 0.5 to 20 ng/mL for 8-oxodG and from 0.1 to 5 ng/mL for 8-isoprostane was observed (R^2>0.999). The accuracy and precision were determined by spiking two different urine samples at three different concentrations (0.5, 1 and 10 ng/mL for 8-oxodG and 0.1, 0.2, and 0.5 ng/mL for 8-isoprostane). Intra-day accuracy and precision ranged from 92 to 114% with a coefficient of variation (CV) lower than 5.7% and 97 to 114% with a CV lower than 7% for 8-oxodG and 8-isoprostane, respectively. The measurements were repeated for three days to determine inter-day accuracy and precision: 92 to 103% with a CV lower than 10% and 97 to 114% with a CV lower than 8.1% for 8-oxodG and 8-isoprostane, respectively. Extraction recoveries of 8-oxodG and 8-isoprostane were 97% and 91%. Matrix effects were urine-dependent and varied from 4% to 67% for 8-oxodG and from 25% to 83% for 8-isoprostane. Urine samples with high creatinine content (i.e., participant with a poor hydration status) were diluted before SPE to avoid signal suppression during the analysis of urine samples by LC-MS due to co-eluting impurities.

Fifty-six morning urine samples of ex-smokers (6-month abstinence) were analyzed. The concentrations of 8-oxodG and 8-isoprostane were 4.04 (3.42–5.37) ng/mg creatinine and 0.23 (0.14–0.28) ng/mg creatinine, respectively (median with IQR). These concentrations were

similar to the values for healthy adults reported in two recent systematic reviews (Graille et al., 2020a, 2020b).

The method was used to assess the associations of oxidative stress biomarkers and BoE to tobacco smoke and to monitor the change of oxidative stress during smoking cessation (Chapter 5 and 6).

4.3 Manuscript 2 (published)

See next page.

Rapid Liquid Chromatography—Tandem Mass Spectrometry Analysis of Two Urinary Oxidative Stress Biomarkers: 8-oxodG and 8-isoprostane

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Abstract: Human biomonitoring of oxidative stress relies on urinary effect biomarkers such as 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG), and 8-iso-prostaglandin $F_{2\alpha}$ (8-isoprostane); however, their levels reported for similar populations are inconsistent in the scientific literature. One of the reasons is the multitude of analytical methods with varying degrees of selectivity used to quantify these biomarkers. Single-analyte methods are often used, requiring multiple injections that increase both time and cost. We developed a rapid ultra-high-performance liquid chromatography–tandem mass spectrometry (UPLC-MS/MS) method to quantify both urinary biomarkers simultaneously. A reversed-phase column using a gradient consisting of 0.1% acetic acid in water and 0.1% acetic acid in methanol/acetonitrile (70:30) was used for separation. The MS detection was by positive (8-oxodG) and negative (8-isoprostane) ion-mode by multiple reaction monitoring. Very low limit of detection (<20 pg/mL), excellent linearity ($R^2 > 0.999$), accuracy (near 100%), and precision (CV < 10%) both for intra-day and inter-day experiments were achieved, as well as high recovery rates (>91%). Matrix effects were observed but were compensated by using internal standards. Our newly developed method is applicable for biomonitoring studies as well as large epidemiological studies investigating the effect of oxidative damage, as it requires only minimal clean up using solid phase extraction.

Keywords: oxidative stress; biomarker; 8-oxodG; 8-isoprostane; biomonitoring; liquid chromatography; mass spectrometry

1. Introduction

Oxidative stress is a major contributor to the pathophysiology of a variety of diseases [1]. It represents an unbalanced biological state where the natural antioxidant defenses are exceeded due to the presence of reactive oxygen species (ROS). This antioxidant mechanism regulates oxidative stress in the human body against environmental factors such as exposures to UV and pollution, and behavioral habits, such as smoking, diet, drinking, and excessive physical activity as well as ageing and body mass [2]. Excess ROS can cause cellular damage by reacting with cellular components such as proteins, lipids, or DNA [3]. In the human body, oxidative stress plays a crucial role in the onset of several diseases including cancer, diabetes, cardiovascular and respiratory diseases [4,5]. Oxidative stress can both be a cause and a consequence of inflammation. Inflammatory cells such as macrophages and neutrophils are activated upon infection or injury. While fighting off invading pathogens, inflammatory cells produce oxidative stress to an excessive extent, which in turn damages healthy cells, leading to inflammation. Under normal conditions, inflammation decreases after the infection is eliminated or the injury is repaired. Yet, oxidative stress can also trigger the inflammatory response, which generates more oxidative stress, creating a vicious cycle.

ROS concentrations in body fluids cannot be easily quantified as they are highly reactive and have short half-lives. However, biomonitoring of oxidative stress can be achieved by quantifying excreted and stable oxidation products [6]. Several oxidative stress biomarkers in body fluids exist, such as 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) and 8-iso-prostaglandin $F_{2\alpha}$ (8-isoprostane). 8-oxodG is one of the major compounds resulting from oxidative damage to DNA [7]. Another name for this biomarker is 8-hydroxy-2′-deoxyguanosine (8-OHdG), a chemically less stable tautomer (Figure 1). The scientific community uses both naming conventions interchangeably [8,9]. The oxidized nucleosides, which are a result of the oxidation of DNA by ROS, are excreted into the urine. Their measurement therefore represents the cumulative total body oxidative stress [10]. In clinical settings, 8-oxodG has been proven to be a predictive factor for the development of diseases. High oxidation of DNA, which is associated with high excretion of urinary 8-oxodG, is predictive for lung and breast cancer risks [11,12].

8-isoprostane is part of the F_2-isoprostane family. It is formed after oxidation of arachidonic acid, which is present in the membrane phospholipids of the body's cells [13]. There are 64 F_2-isoprostane isomers and the most predominant one is 8-isoprostane (also abbreviated as 15-F_{2t}-IsoP, 8-iso-$PGF_{2\alpha}$, 8-epi-$PGF_{2\alpha}$, or $iPF_{2\alpha}$-III) (Figure 2) [14].

F_2-isoprostanes are frequently viewed as the most reliable biomarkers for monitoring oxidative stress in vivo [15,16]. In clinical settings, for example, elevated urinary concentrations of F_2-isoprostane are found in cardiovascular disease, correlating with severity of disease, and predicting clinical outcomes [17].

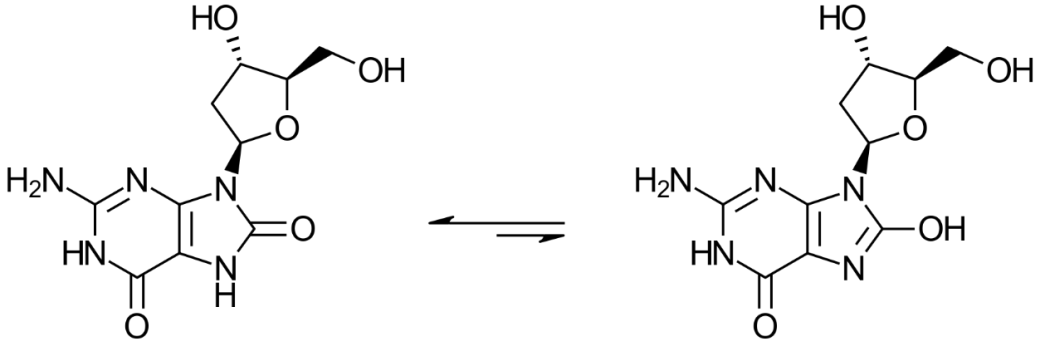


Figure 1. Structure of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) and its tautomer 8-OHdG.

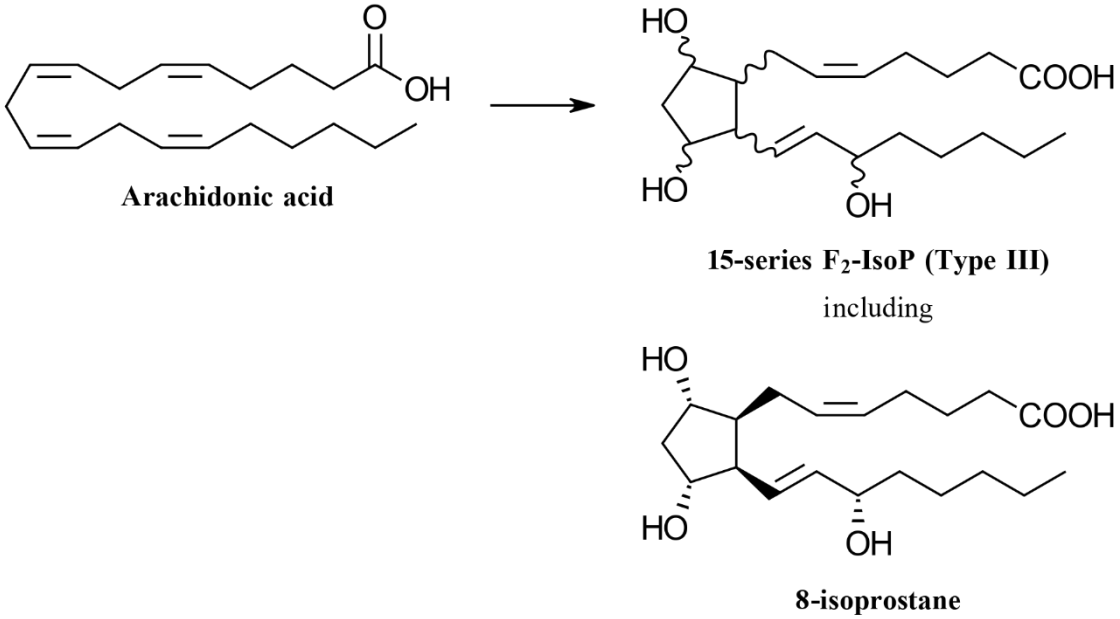


Figure 2. 8-Isoprostane is one of the 64 isomers formed by the oxidation of arachidonic acid. It is part of the 15-series F_2-isoprostanes.

Several analytical methods have been developed to quantify 8-oxodG and 8-isoprostane in different biological matrices, including blood, saliva, urine, and exhaled air condensate (EBC). Urine is the preferred matrix in biological monitoring because its collection involves a simple, non-invasive sampling method. Both biomarkers can be quantified by two principal analytical approaches: liquid (LC) or gas (GC) chromatography coupled with mass spectrometry (MS) or enzyme-linked immunosorbent assay (ELISA) [18–20]. LC-MS/MS is usually preferred to GC-MS/MS as the latter requires a derivatization step, which introduces possible losses of biomarkers and increases the overall time needed to conduct the analyses.

Immunological methods are less sensitive and lack specificity compared to mass-based methods [21]. However, they are still used, as they are faster and do not require expensive analytical instruments.

Four studies have reported concurrent quantification of 8-oxodG and 8-isoprostane in urine by LC-MS/MS. Wu et al. (2016) reported the simultaneous analysis of 8-oxodG, 8-nitroguanine (8-NO_2Gua), 8-isoprostane, and N-acetyl-S-(tetrahydro-5-hydroxy-2-pentyl-3-furanyl)-L-cysteine (HNE-MA) with solid-phase extraction [22]. Zhao et al. (2017) reported the determination of 8-oxoguanosine, 8-oxodG, and 8-isoprostane with solid-phase extraction [23]. Saito et al. (2018) described the concurrent analysis of 8-isoprostane, 8-oxodG, and 3-nitro-L-tyrosine by online solid-phase microextraction [24]. Martinez and Kannan (2018) reported the determination of 8-oxodG, o-o'-dityrosine (DiY), malondialdehyde (MDA), and four F_2-isoprostane isomers (including 8-isoprostane) after 2,4-dinitrophenylhydrazine (DNPH) derivatization and solid-phase extraction [25]. Table 1 summarizes the method validation parameters for the different analytical methods. These parameters include limit of detection (LOD), limit of quantification (LOQ), linearity, intra- and inter-day precision and accuracy, recovery, and matrix effects.

Sensitivity of LC-MS methods is dependent on matrix effects when analyzing biological fluids. These effects can be manifested by either a decrease in MS response (signal suppression) or an increase in MS response (signal enhancement) [26]. During method validation, it is important to determine the influence of these matrix effects on MS responses and to find strategies to minimize their impact. It is also advisable to use several sources of biological fluids in this step as matrix effects can vary greatly between urine samples [27]. During our method development, we selected three urine samples with different creatinine concentrations, which represent the different hydration status of the donor. Urine samples with high creatinine concentrations contain more matrix components that can affect the analysis of the biomarkers of interest. We propose several recommendations to reduce or control matrix effects.

This study aimed to optimize the simultaneous analysis of 8-oxodG and 8-isoprostane in urine by LC-MS/MS and to validate a new method following the US Food and Drug Administration (FDA) guidelines for bioanalytical method validation. Our method included the development of a sample preparation procedure (solid-phase extraction) and the optimization of the LC-MS parameters. We applied the method to urine samples of ex-smokers known to have low concentrations of these biomarkers. We confirmed the non-smoking status of the participants by analysis of nicotine and its metabolites in their urine (total nicotine equivalent <2 nmol/mg creatinine).

The ranges of creatinine-adjusted 8-oxodG and 8-isoprostane concentrations were in agreement with the reference values reported in the general population. Therefore, non-smokers can be used as controls in oxidative stress research.

Table 1. Descriptions of the multi-analyte analytical methods previously developed and our method.

	Wu et al. (2016) [22]	Zhao et al. (2017) [23]	Saito et al. (2018) [24]	Martinez and Kannan (2018) [25]	Our Study
Parameters					
LOD					
8-oxodG	20 pg/mL	170 pg/mL	12.6 pg/mL	30 pg/mL	10 pg/mL
8-isoprostane	8 pg/mL	40 pg/mL	3.4 pg/mL	10 pg/mL	20 pg/mL
LOQ					
8-oxodG	50 pg/mL	570 pg/mL	20 pg/mL	100 pg/mL	30 pg/mL
8-isoprostane	30 pg/mL	130 pg/mL	29 pg/mL	20 pg/mL	50 pg/mL
Linearity					
8-oxodG	$R^2 > 0.998$	$R^2 > 0.999$	$R^2 > 0.999$	$R^2 > 0.999$	$R^2 > 0.999$
8-isoprostane	$R^2 > 0.998$	$R^2 > 0.999$	$R^2 > 0.999$	$R^2 > 0.999$	$R^2 > 0.999$
Intra-/inter-day accuracy					
8-oxodG	98.8–102.2%/ 98.5–101.6%	n.a./ n.a.	91.1–97%/ n.a.	92–101%/ n.a.	92–114%/ 92–103%
8-isoprostane	98.5–101.7%/ 99–102.1%	n.a./ n.a.	95.7–100%/ n.a.	93–103%/ n.a.	97–114%/ 97–114%
Intra-/inter-day precision					
8-oxodG	<8.1%/<8.5%	<1.9%/<3.9%	<5%/<6.1%	<9%/n.a.	<5.7%/<10%
8-isoprostane	<4.6%/<5.1%	<2.3%/<5.3%	<2.1%/<4.5%	<9%/n.a.	<7.0%/<8.1%
SPE recovery					
8-oxodG	90.1–90.7%	90.1–100%	n.a.	n.a.	97%
8-isoprostane	94.3–95%	89.2–108%	n.a.	n.a.	91%
Matrix effects [1]					
8-oxodG	89.2%	n.a.	n.a.	n.a.	20%
8-isoprostane	96.6%	n.a.	n.a.	n.a.	70%

[1] Absolute matrix effects.

2. Materials and Methods

2.1. Standards, Chemicals, and Material

8-OxodG (≥98% (TLC), CAS Number 88847-89-6) was obtained from Merck KGaA (Buchs, St. Gallen, Switzerland). The isotopically labelled [$^{15}N_5$]-8-oxodG (CAS Number 569649-11-2) was used as the internal standard (IS) and bought from Cambridge Isotope Laboratories, Inc. (Tewksbury, MA, USA). 8-Isoprostane ((5Z,8β,9α,11α,13E,15S)-9,11,15-trihydroxyprosta-5,13-dien-1-oic acid; ≥95%, CAS Number 27415-26-5) and its deuterated isomer (IS) 8-isoprostane-d_4 ((5Z,8β,9α,11α,13E,15S)-9,11,15-trihydroxyprosta-5,13-dien-1-oic-3,3,4,4-d4 acid; CAS Number 211105-40-7), were obtained from Cayman Chemical (Ann Arbor, MI, USA). LC-MS grade solvents, water, methanol, and acetonitrile, were obtained from Carlo Erba Reagents (Chaussée du Vexin, Val de Reuil, France). LC-MS grade acetic acid was obtained from Honeywell (Seelze, Germany). MilliQ water was produced in the laboratory with a water purification system (MilliQ Advantage) from Merck (Schaffhausen, Switzerland). Solid phase extraction (SPE) cartridges (Chromabond C18 ec SPE 500 mg 3 mL) were purchased from Macherey-Nagel (Oensingen, Switzerland).

2.2. Urine Samples for Method Validation

Three urine samples collected from healthy, consenting adults were aliquoted in tubes (8 mL) before storing at −20 °C. We chose to focus our study on volunteer hydration to investigate its consequences on matrix effects; thus, we chose urine samples by color and creatinine concentration. Indeed, even if urine is a relatively clean matrix, it contains many compounds that can interfere with the analysis [28]. This is especially important with high creatinine urine samples (“dark urine”, indicative of poor hydration). Two samples were chosen to reflect extreme cases: light colored urine or “light urine” with a creatinine concentration of 0.2 mg/mL, and dark colored urine or “dark urine” with a creatinine concentration of 3.58 mg/mL. Both samples were used for method validation. A third urine sample was an intermediate colored urine or “medium urine” with a creatinine concentration of 1.65 mg/mL. The latter urine sample was used to prepare the calibration standards during the method development and validation. Thus, we conducted the method development on three urine samples with different creatinine concentrations.

2.3. Calibration Curve and Quality Controls (QC)

Calibration curves were prepared by spiking “medium urine” with six different concentrations of 8-oxodG and 8-isoprostane and a constant concentration of IS (standard stock solution description in Supplementary Information). The six concentration levels were 0.5, 1, 2.5, 5, 10, and 20 ng/mL for 8-oxodG and 0.1, 0.2, 0.5, 1, 2.5, and 5 ng/mL for 8-isoprostane.

The “light urine” was used to prepare low QC concentrations: 0.63 ng/mL of 8-oxodG and 0.10 ng/mL of 8-isoprostane. The “medium urine” was used to prepare the high QC concentrations: 3.30 ng/mL of 8-OHdG and 0.45 ng/mL of 8-isoprostane.

2.4. Solid-Phase Extraction (SPE)

Urine samples were thawed at room temperature and vortexed. Urine amounts for analysis were adjusted according to the creatinine concentration: 500 µL of urine for 1 mg/mL of creatinine (in other words, we adjusted the urine volume to load 0.5 mg of creatinine). Water (400 µL), IS (100 µL), and 10% formic acid (100 µL) were added to form the SPE loading solution. SPE cartridges were first conditioned with methanol (2 mL) and water (2 mL). Urine samples were loaded onto the SPE, washed with water (2 mL) and then 5% methanol (2 mL), dried with air (PRESSURE+ from Biotage, Uppsala, Sweden), and eluted with methanol (3 mL). The extract was filtered (0.45 µm), evaporated under a nitrogen flow with a Pierce Reacti-Therm III evaporator (Thermo Scientific, Reinach, Switzerland Switzerland), and reconstituted in the injection solvent (500 µL 0.1% acetic acid in water). Calibration standards and QC were treated identically to the samples.

2.5. LC-MS/MS Analysis

Analysis of 8-oxodG and 8-isoprostane was performed using a UPLC (Dionex Ultimate 3000 system, Thermo Scientific, Reinach, Switzerland) coupled with a triple-stage quadrupole mass spectrometer (TSQ Quantiva, Thermo Scientific, Reinach, Switzerland) equipped with a heated electrospray ionization source (ESI) operated in positive ion mode for 8-oxodG and in negative ion mode for 8-isoprostane.

The compounds were separated using a C18 column (2.1 × 100 mm, 1.8 µm; Zorbax Eclipse Plus, Agilent, Morges, Switzerland). The column temperature was maintained at 30 °C. The mobile phase consisted of: eluent A composed of 0.1% acetic acid in water, and eluent B of 0.1% acetic acid in methanol/acetonitrile (7:3, v/v). The solvent gradient program was: t = 0 min: 0% B, t = 1.1 min: 55% B, t = 12 min: 65% B, t = 12.5 min: 90% B, t = 14.5 min: 90% B, t = 15.5 min: 0% B, t = 22 min: 0% B, at a flow rate of 250 µL/min. Using methanol and acetonitrile mixture as the mobile phase (B) was based on previous work from Prasain et al. (2013) reporting that F_2-isoprostane isomers’ separation was not achieved with a mobile phase of 100% methanol [29]. Multi-reaction monitoring (MRM) transitions and ESI parameters can be found in Supplementary Information. All data acquisition and processing were accomplished using the Thermo Scientific Chromeleon software (version 7.2.10).

2.6. Application to Urine Samples Obtained from Healthy Participants

For method application, urine samples were collected from an on-going randomized controlled trial on smoking cessation: "Efficacy, Safety, and Toxicology of Electronic Nicotine Delivery Systems as an aid for smoking cessation: the ESTxENDS multicenter randomized controlled trial" (ClinicalTrials.gov Identifiers: NCT03589989) approved by the Ethics committees of Bern, Geneva, and Lausanne (Project-ID: 2017-02332), Switzerland. The study was conducted in accordance with the ethical principles of the World Medical Association Declaration of Helsinki and the International Committee on Harmonization for Good Clinical Practice and Swiss law. All participants provided a written informed consent and the following information: age, gender, and anthropometric data (height, weight).

For this study, we selected participants who reported that they were cigarette abstinent for more than four months, were not using any other nicotine delivery systems (e-cigarettes, nicotine replacement therapy or any other nicotine containing device) and had donated their first-void urine sample (first morning urine sample). We validated the smoking abstinence by assessing total urinary nicotine equivalent (<2 nmol/mg creatinine). The urine samples were first stored at 4 °C (for 1 to 7 days), and urine aliquots were then stored at −20 °C until analysis.

2.7. Participant Description

Fifty-six participants provided first-void urine samples for the quantification of 8-oxodG and 8-isoprostane. Mean age of the participants was 43.5 years old with a BMI mean of 26. Twenty-six participants were women (46%) and 30 participants were men (54%). Participant demographics are described in Table 2.

Table 2. Summary of participant demographics and verification of smoking abstinence.

Characteristic	Non-Smokers (*n* = 56)
Age (years)	43.5 (35.5–54.25) *
Sex	
Men	30 (54) **
Women	26 (46)
BMI (kg/m^2)	26 (23–28)
≤25	22 (39)
>25	34 (61)
TNE (nmol/mg creatinine)	0.01 (0.00–0.02)

* Median (IQR: 25–75%); ** Number (% of total).

2.8. Other Bioanalytical Methods

Urinary concentrations of 8-oxodG and 8-isoprostane were adjusted with creatinine concentration to account for the hydration status of the participants and allow inter-individual comparison. There is an acceptable correlation between creatinine corrected spot-urine and 24 h urine [30–33]. Creatinine was quantified at the Unit of Forensic Toxicology and Chemistry, University Center of Legal Medicine (Lausanne—Geneva, Switzerland) with a routine clinical method based on Jaffe (1886) [34].

Total nicotine equivalent (TNE) is considered as the gold standard biomarker of daily nicotine intake [35]. In most studies, TNE is based on six metabolites (nicotine, cotinine, trans-3′-hydroxycotinine, cotinine-*N*-glucoronide, nicotine-*N*-glucoronide, and trans-3-hydroxycotinine-*O*-glucoronide). In this study, only four metabolites were included (TNE 4) as it was sufficient for smoking status verification. Nicotine, cotinine, trans-3′-hydroxycotinine, and norcotinine were analyzed at the Unit of Forensic Toxicology and Chemistry, University Center of Legal Medicine (Lausanne—Geneva, Switzerland) by LC-MS/MS with a routine method based on an application note of Thermo Fisher Scientific (n°20709, 2013). TNE was calculated as TNE = (nicotine/162.23 + cotinine/176.22 + trans-3′-hydroxycotinine/192.22 + norcotinine/162.19)/creatinine, expressed in nmol/mg creatinine).

2.9. Data Presentation and Statistical Analysis

Method validation parameters were calculated based on the peak areas that were integrated by the UPLC-MS/MS software. Description of these parameters can be found in Supplementary Information. Total nicotine equivalent was calculated as the molar sum of nicotine, cotinine, trans-3′-hydroxycotinine, and norcotinine (corrected by the creatinine concentration). Oxidative stress biomarkers were creatinine-corrected and were presented as median with the 1st and 3rd quartile. All calculations were performed with the R program (R version 3.6.2 (12 December 2019)—“Dark and Stormy Night”).

3. Results

3.1. LC-MS/MS Analysis

After the SPE on C18 cartridge (optimization description in Supplementary Information), the samples were analyzed by LC-MS. LC separation was performed on a C18 column with a gradient of 0.1% acetic acid in water (A) and 0.1% acetic acid in methanol/acetonitrile 70:30 (%, v/v; B) at a flow rate of 250 µL/min. Retention times were 4.7 min for 8-oxodG and 10.2 min for 8-isoprostane (Figure 3). Internal standards’ retention times were similar.

Elution of 8-oxodG and its internal standard occurred after the solvent front, indicating that the column did retain the compound. This also helped to reduce the signal suppression during the mass spectrometry process.

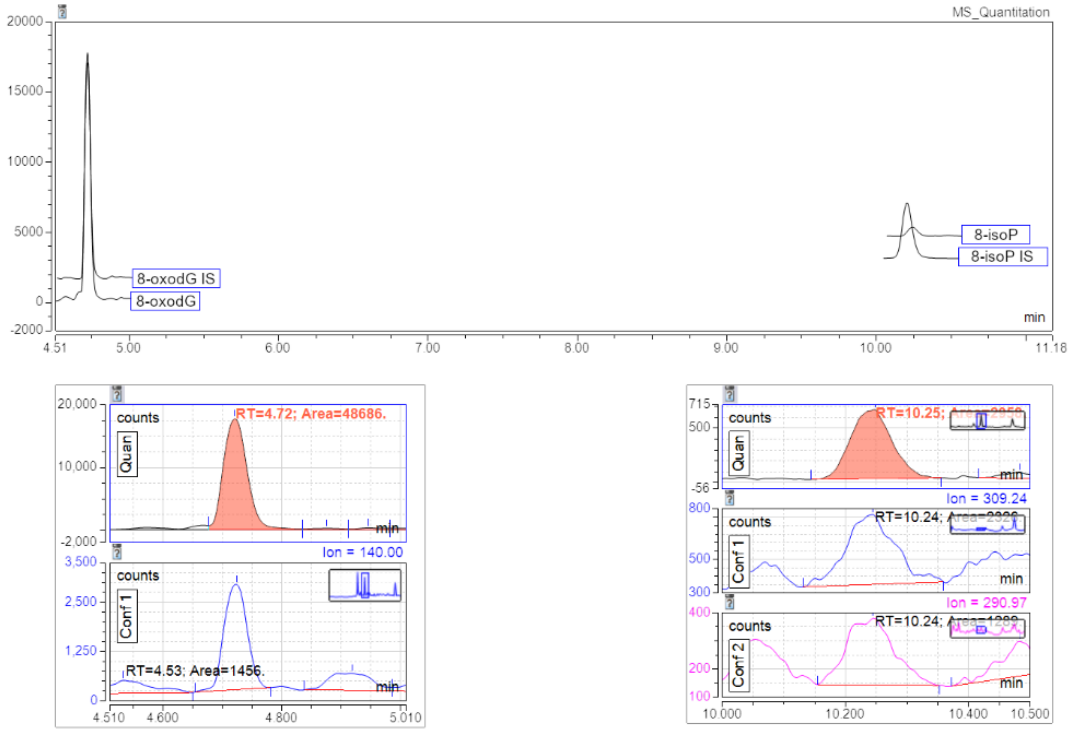
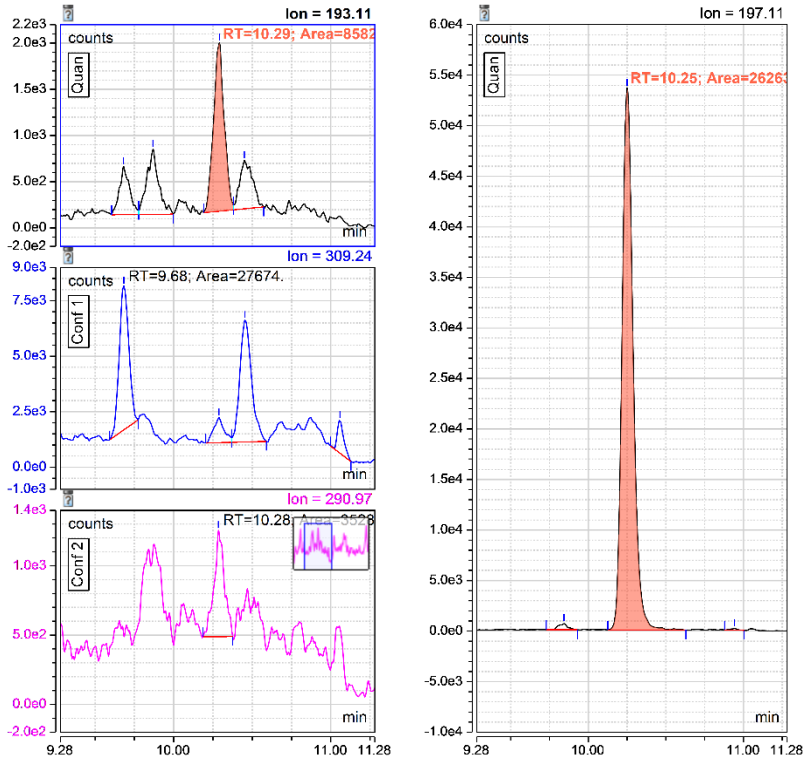


Figure 3. Chromatogram of spiked "light urine"; chromatogram with retention times and multi-reaction monitoring (MRM) transitions for 8-oxodG (left) and 8-isoprostane (right).

ESI mode interface was operated in positive ion mode for the first segment of the run (0.5–8 min) and in negative ion mode for the second segment (8.5–14 min) to optimize the detection of both analytes. Ion source parameters, as well as m/z transitions for the multiple reaction monitoring, were determined by infusion of aqueous standard of 8-oxodG (5 µg/mL) and 8-isoprostane (5 µg/mL). Mass transitions, collision energy, and RF lens are shown in Supplementary Information (Table S1).

MRM transitions for 8-isoprostane showed the probable presence of other F_2-isoprostane isomers in urine (Figure 4). Separation gradient was optimized to allow the peak separation of 8-isoprostane with other potential isomers in urine samples.

We tested a lower concentration of acetic acid in the mobile phase (0.01%), but it did not increase 8-isoprostane signal and decreased 8-oxodG signal. Use of formic acid (0.1%) reduced the signals of both analytes.



(**a**) (**b**)

Figure 4. (**a**) MRM transitions for 8-isoprostane in "light urine"; presence of potential other F_2-isoprostane isomers; (**b**) corresponding 8-isoprostane-d_4 chromatogram.

3.2. Sensitivity, Linearity, Accuracy, and Precision

We determined LODs at 10 pg/mL for 8-oxodG and 20 pg/mL for 8-isoprostane (S/N ≥ 3) and the LOQs at 30 pg/mL for 8-oxodG and 50 pg/mL for 8-isoprostane (S/N ≥ 10 and coefficient of variation <20%) in aqueous solution. In urine, our lowest calibration standard was 0.5 ng/mL for 8-oxodG and 0.1 ng/mL for 8-isoprostane. These concentrations were low enough to quantify these biomarkers in participants' urine samples and were in accordance with previous published methods. Therefore, calibration curves were constructed with six levels from 0.5 to 20 ng/mL for 8-oxodG and 0.1 to 5 ng/mL for 8-isoprostane in urine. Linear regression with 1/x weighting was performed on analyte/IS peak area ratio versus standard concentrations. Linearity of the working ranges was observed with a regression coefficient of $R^2 > 0.999$. Slopes of the calibration curves were similar for urine and water: 2 ± 7% for 8-oxodG and 3 ± 6% for 8-isoprostane.

Intra-day precision and accuracy were determined by analyzing three replicates of two urine samples spiked at three concentrations: 0.5, 1, and 10 ng/mL for 8-oxodG and 0.1, 0.2, and 0.5 ng/mL for 8-isoprostane.

Intra-day accuracy ranged from 92% to 114% with a coefficient of variation lower than 5.7% for 8-oxodG, and from 97% to 114% with a coefficient of variation lower than 7% for 8-isoprostane. Injections were performed for three days to determine the inter-day precision and accuracy. The inter-day accuracy for 8-oxodG ranged from 92% to 103% with a coefficient of variation lower than 10%, and from 97% to 114% with a coefficient of variation lower than 8.1% for 8-isoprostane. Accuracy and precision details are shown in Table 3.

Table 3. Accuracy and precision for 8-oxodG and 8-isoprostane at three concentrations in two different urine samples.

Urine	Compound	Concentration [1]	Intra-Day Accuracy [2] and Precision [3]		Inter-Day Accuracy and Precision	
"Light urine"	8-oxodG	0.5	94	0.9	94	2.5
		1	92	1.9	92	2.7
		10	99	0.5	99	2.4
	8-isoprostane	0.1	97	5.9	97	8.1
		0.2	99	4.4	99	3.4
		0.5	100	3.3	100	2.7
"Dark urine"	8-oxodG	0.5	113	5.4	103	10
		1	107	5.7	100	8.4
		10	98	0.8	99	4.4
	8-isoprostane	0.1	114	7.0	114	2.1
		0.2	100	5.5	100	4.9
		0.5	102	2.0	102	2.5

[1] [ng/mL]; [2] %; [3] coefficient of variation [%].

3.3. Extraction Recovery and Matrix Effects

During the method development, extraction recoveries were calculated for each concentration used in the calibration curve. We observed stable extraction recoveries. The extraction recovery and the matrix effects for the concentrations corresponding to the highest calibration curve levels, 20 ng/mL for 8-oxodG and 5 ng/mL for 8-isoprostane, are presented. To determine extraction recovery, three replicates in urine spiked before SPE with 8-oxodG and 8-isoprostane and three replicates in urine spiked after SPE with the same solution were analyzed. Extraction recovery was 97% for 8-oxodG and 91% for 8-isoprostane. To determine absolute matrix effects, three replicates in water spiked with 8-oxodG and 8-isoprostane (without SPE) were compared to three replicates in urine spiked after SPE with the same solution. Matrix effects were found to be urine-dependent, and we observed matrix effects up to 20% for 8-oxodG and 70% for 8-isoprostane for "medium urine" (100% corresponds to no matrix effects). Variation of the analyte to IS ratio was lower than 4% indicating that the observed signal reduction was compensated by using a stable isotopic internal standard.

Matrix effects were also observed for “light urine” (67% for 8-oxo-dG and 83% for 8-isoprostane) and “dark urine” (4% for 8-oxodG and 25% for 8-isoprostane), estimated by the IS variation. A simple dilution by a factor of two of the “dark urine” reduced matrix effects to 19% for 8-oxodG and 58% for 8-isoprostane (more information in the Supplementary Information). This indicated that signal suppression can be reduced by diluting urine samples prior to analysis or by taking a lower volume of urine for analysis.

Relative matrix effects were estimated by comparing the slopes of calibration curves in three different urine samples. Coefficient of variation for the slopes of both 8-oxodG and 8-isoprostane were <5%, which emphasizes the importance of the stable isotopic internal standard for matrix effect correction.

3.4. Stability

We evaluated stability of 8-oxodG and 8-isoprostane in urine at −20 °C by monitoring the QC (low and high) over a 6-month period. 8-oxodG concentration was 8–9% higher after 6 months for both low (0.65 ng/mL) and high QC (3.42 ng/mL). The variation of the concentration over the whole period (65 injections) was less than 5% for both. 8-isoprostane concentration was 15% lower after 6 months for low QC (0.09 ng/mL) and 7% higher for high QC (0.46 ng/mL). The variation of the concentration over the whole period (65 injections) was 13% and 7% for low and high QC, respectively.

Stability of the analytes in processed urine at room temperature was also monitored by the QC (low and high). QCs were injected three times in an injection sequence (at the beginning, in the middle, and at the end), seven hours apart. The average of the intra-sequence variation of 8-oxodG was 1.42% and 1.34% for low and high QC, respectively. The average of the intra-sequence variation of 8-isoprostane was 8.9% and 6.1% for low and high QC, respectively. There was no tendency for signals to increase or decrease between the 1st and the 3rd injection (i.e., after about 14 h), meaning that the analytes were stable in processed urine during this period.

3.5. Oxidative Stress Biomarkers’ Concentrations in Healthy Participants

Oxidative stress biomarkers’ concentrations were determined in 56 morning urine samples obtained from the participants. The two analytes were quantified in all samples. After creatinine correction, the median of 8-oxodG concentration was 4.04 ng/mg creatinine (1st quartile–3rd quartile: 3.42–5.37 ng/mg creatinine).

The median of 8-isoprostane concentration was 0.23 ng/mg creatinine (1st quartile–3rd quartile: 0.14–0.28 ng/mg creatinine). Details are shown in Table 4.

Table 4. 8-oxodG and 8-isoprostane concentrations in participants' urine.

8-oxodG			8-isoprostane		
All Participants	**BMI**		**All Participants**	**BMI**	
4.04 * (3.42–5.37)	BMI ≤ 25 (*n* = 22)	4.28 (3.62–6.11)	0.20 (0.14–0.28)	BMI ≤ 25 (*n* = 22)	0.19 (0.14–0.30)
	BMI > 25 (*n* = 34)	3.96 (2.81–4.97)		BMI > 25 (*n* = 34)	0.21 (0.14–0.27)

* Median (IQR: 25–75%), expressed in ng/mg creatinine.

4. Discussion

We successfully optimized the simultaneous quantification of urinary 8-oxodG and 8-isoprostane by LC-MS/MS and efficiently applied the method to 56 urine samples from participants (non-smoking status confirmed by total nicotine equivalent <2 nmol/mg creatinine). The creatinine-adjusted concentrations ranges of 8-oxodG and 8-isoprostane were in agreement with the reference values of the population [36,37]. This is interesting, because this would allow non-smokers to be used as controls in studies investigating the effects of a particular exposure (e.g., air pollution, UV) or behavioral habit (e.g., smoking, intense activity) on the oxidative stress level.

Matrix effects are commonly observed in analysis of biological fluids, and they can obscure the signal in an otherwise selective and sensitive LC-MS method. The matrix effects' mechanisms are not fully understood, but they involve co-elution of matrix components that induce a loss of response (signal suppression) or an increase of response (signal enhancement). As all urine samples have different compositions, Matuzewski et al. (2003) recommended performing method validation in five different sources instead of a single one [27].

We hypothesized that "dark urine" samples (from individuals with a low hydration status) cause greater matrix effects than "light urine" samples (from individuals with a high hydration status). We selected urine samples according to the aspect (color) and urinary creatinine concentration. The latter is dependent on hydration status, as an increased amount of water in urine will lower the creatinine concentration. We demonstrated that matrix effects were proportional to urinary creatinine concentrations.

This finding is of primary importance, because even if the matrix effect is compensated by the use of internal standards, the sensitivity of the method is decreased due to signal suppression (comments on matrix effects and method performance in Supplementary Information).

Therefore, it is highly recommended to construct calibration curves in the same biological fluid as the samples. It is also important to assess relative matrix effects by comparing calibration curve slopes constructed in different urine samples. Similar slopes indicate that sample matrix and recovery differences do not alter precision and accuracy. This is an additional argument for the use of multiple urine sources during method development and validation. In order to have comparable MS response intensities for urine samples, we adjusted the urine volume according to the creatinine concentration.

As oxidative stress biomarkers are usually corrected by creatinine concentrations for spot urine samples, it is therefore reasonable to use these known concentrations during sample preparation. It would also be possible to adjust the volume of urine used for analysis by the density or the total urine volume. We chose 500 µL of urine for 1 mg/mL creatinine because 1 mg/mL is close to the average creatinine concentration in spot urine samples (1.3 mg/mL [38]). Furthermore, we obtained good precision and accuracy for low concentrations (0.5 ng/mL for 8-oxodG and 0.1 ng/mL for 8-isoprostane). This allows us to control for matrix effects.

We planned initially to include malondialdehyde (MDA), with 8-oxodG and 8-isoprostane, in the method. Simultaneous analysis of different biomarkers presents many advantages such as saving time and money. It can be challenging if the analytes have different physicochemical properties. This is the case for 8-oxodG, 8-isoprostane, and MDA. 8-oxodG is composed of a purine and is a polar molecule due to the presence of polar functional groups (amides, hydroxyls, and amine). Moreover, it is uncharged under low and neutral pH and forms anions and dianions at higher pH values (pKa values at 8.6 and 11.7) [39,40]. 8-isoprostane is mostly non-polar due to its alkane chains. Nevertheless, solubility in water is possible due to the presence of polar functional groups (hydroxyls and carboxyl). The molecule is neutral in acidic conditions and forms an anion under neutral and alkaline conditions (pK_a value at pH ~5) [41]. MDA is a small, reactive molecule that undergoes keto-enol tautomerism. Most of the analytical methods involve a derivatization step [42].

We used dinitrophenylhydrazine (DNPH) solution (5 mM) in water/acetonitrile/acetic acid (6.5:1:2.5) for the derivatization as described by Martinez and Kannan (2018) [25]. However, we were not able to reproduce the results because 8-oxodG was not completely retained on the SPE cartridge during the loading and washing steps.

This could be due to the presence of acetonitrile from the MDA derivatization step prior to SPE. We performed the tests with the four non-polar cartridges listed in Supplementary Information (Table S2) without success. We therefore decided to exclude MDA from the method to improve the retention of 8-oxodG. We decided to keep 8-oxodG as it is an important biomarker of DNA damage, and not include MDA, as MDA and 8-isoprostane are both biomarkers of lipid peroxidation. Moreover, the physiological validity of MDA as a biomarker of oxidative stress is rather poor. The main reasons are that MDA formation is not specific to lipid peroxidation, there is a lack of association between MDA and oxidative stress in humans, and urinary MDA concentrations are potentially also modified by diet [43].

We were able to quantify low concentrations of oxidative stress biomarkers (0.5 ng/mL for 8-oxodG and 0.1 ng/mL for 8-isoprostane) in 56 morning urine samples from non-smoking healthy participants. Matrix effects were observed during the analysis of urine samples and their magnitude was directly linked to the urinary creatinine concentration, a measure of hydration level of the individual. This also meant that the sample clean-up was not complete as matrix components induce MS signal suppression effects. Solid-phase extraction with a reversed-phase cartridge was chosen because it retained both analytes well. However, 8-oxodG is more polar than 8-isoprostane and eluted with low percentage of methanol (10%). Therefore, the SPE washing step could not be optimized further to remove more matrix components without losing 8-oxodG. Both 8-oxodG and 8-isoprostane are negatively charged at high pH. Nevertheless, 8-isoprostane was not recovered during the elution step for the two anionic SPE cartridges we tested. Due to the different physicochemical properties of the two analytes, the reversed-phase was a good compromise.

The various existing F_2-isoprostane isomers can complicate the 8-isoprostane quantification due to possible co-elution [29]. As we observed several peaks on the UPLC chromatograms close to the retention time of 8-isoprostane, we adjusted the separation gradient to isolate the 8-isoprostane peak. This was the main reason why the total duration of the analytical run could not be shortened. Other peaks might be other F_2-isoprostane isomers but this was not explored further.

The obtained concentration medians for 8-oxodG (4.04 ng/mg creatinine) and 8-isoprostane (0.23 ng/mg creatinine) in our participants were comparable to the values in healthy adults reported in two systematic reviews by Graille et al. (2020 a, 2020 b) [36,37]. For 8-oxodG, the authors reported a median value obtained by chromatographic analytical techniques in healthy adults of 3.9 ng/mg creatinine (3–5.5 ng/mg creatinine) with a BMI ≤ 25 and 2.8 ng/mg creatinine (2.4–3.5 ng/mg creatinine) with a BMI > 25.

For 8-isoprostane, they reported a median value of 0.249 ng/mg creatinine (0.186–0.407 ng/mg creatinine) for healthy adults with a BMI ≤ 25 and 0.508 ng/mg creatinine (0.180–0.553 ng/mg creatinine) for healthy adults with a BMI > 25. Therefore, our method is sufficiently sensitive in quantifying background population concentrations of these oxidative stress biomarkers, and it could be applied in clinical or epidemiological studies.

Oxidative stress biomarkers and inflammation markers have been analyzed together in several studies. Helmersson et al. (2004) and Tatsch et al. (2015) showed respectively that type 2 diabetes led to chronic inflammation followed by oxidative damage and that patients with higher 8-oxodG concentrations had higher degrees of inflammation and higher insulin resistance [44,45]. Altemose et al. (2017) and Squillacioti et al. (2020) reported that exposure to air pollution (including PAHs and aldehydes) contributed to the induction of oxidative stress and airways inflammation [46,47]. Ochoa et al. (2011), Mrakic-Sposta et al. (2015), and Larsen et al. (2020) investigated the effect of intense exercise on elevation of oxidative stress and inflammation markers [48–50]. Several researches have been conducted on the effect of diet on these markers, including those of Helmersson et al. (2008) and Holt et al. (2009), which showed the importance of a healthy diet and highlighted the beneficial effect of fruits and vegetables [51,52].

Of these nine studies, only one used LC-MS as an analytical technique and only two studies quantified both analytes with two separate analyses. The method we propose would provide a simultaneous quantification of 8-oxodG and 8-isoprostane. By addressing both analytes in one run, this method saves time and consequently money, and it can thus be used in larger epidemiological studies. This method can help in gaining a better understanding of the relationship between oxidative stress and inflammation, and to understand the underlying mechanisms, as currently these biomarkers are not completely understood. We would also highlight that the measurement variability of our method is lower than the intra-individual variabilities for both 8-oxodG and 8-isoprostane, which renders them excellent in molecular epidemiological studies [30,53].

From a clinical perspective, 8-isoprostane and 8-oxodG are important oxidative stress biomarkers, which have a diagnostic and prognostic value and correlate with disease degree. Therefore, screening for 8-isoprostane and 8-oxodG in a fast and cost-effective way could help to identify people at risk and monitor the potential effect of interventions.

5. Conclusions

Our concurrent analysis of urinary 8-oxodG and 8-isoprostane method is rapid, stable, and robust. We recommend using a stable isotopic internal standard to compensate for matrix effects. The matrix effect was related to creatinine content; consequently, we suggest diluting "dark urine" (high creatinine concentration) to reduce ion suppression effects and increase the loading volume of "light urine" (low creatinine concentration) to allow quantification. We successfully analyzed 56 urine samples from healthy non-smoking participants and were able to quantify background levels of oxidative stress biomarkers. Our method is suitable for large epidemiological or biomonitoring studies.

Supplementary Materials: The following are available online at https://www.mdpi.com/2076-3921/10/1/38/s1, Table S1:Multi-reaction monitoring parameters, Table S2: Summary of tested SPE cartridge during method development.

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Data Availability Statement: The data presented in this study are available on request from the corresponding author. The data are not publicly available because the ESTxENDS trial is ongoing. Additional data are provided in supplementary material.

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Chapter 5 – Oxidative stress in smokers

The analysis of the associations between oxidative stress biomarkers and BoE to tobacco smoke was subject of a manuscript. The manuscript has not yet been submitted to a peer-reviewed journal, but was submitted to the publication committee (PC) of the ESTxENDS study and has been reviewed by an external, independent reviewer. Recommendations of this reviewer included several modifications and shortening of the manuscript. The manuscript will be submitted to a peer-reviewed journal in 2022.

Author contributions: Reto Auer (R.A.), Nicolas Rodondi (N.R.), Jean-Paul Humair (J.-P.H.), Ivan Berlin (I.B.), Nancy B. Hopf (N.B.H.), Aurélie Berthet (A.B.), and Jean-Jacques Sauvain (J.-J.S.) conceived the project and managed funding acquisition; Anna Schoeni (A.S.) and R.A. managed the project coordination; N.R., J.-P.H., I.B. were responsible for the study centers in Bern, Geneva, and Lausanne, respectively; Nicolas Sambiagio (**N.S**)**.** and A.B. organized the biological sample logistics; **N.S.** and A.S. selected the participants; **N.S.** and A.B. organized the analysis; **N.S.** carried out the analysis of the biomarkers of oxidative stress; Florian Breider (F.B.), Dominique Grandjean (D.G.) and **N.S.** performed the TSNA analysis; **N.S.** and A.S. prepared the database; Pascal Wild (P.W.) and **N.S.** performed the statistical analysis; A.B. and N.B.H. supervised the study progress; **N.S.** wrote the manuscript, which was further amended by all authors.

5.1 Introduction

Oxidative stress biomarkers include both molecules that have been altered due to a disruption of ROS homeostasis and molecules of the antioxidant system that changed in response to high ROS concentrations. The advantage of using oxidative stress biomarkers is that they can provide valuable information of the effects of exposures, especially chemical mixtures, for which there are no BoE or for which the compounds involved are not identified. Concerning ENDS, this approach allows taking into account exposure to all compounds present in emissions, including those not identified or not quantified. However, a significant drawback with the use of oxidative stress biomarkers is their lack of specificity. Indeed, they are not linked to a specific exposure, and they depend on many factors such as environmental or behavioral factors. This complicates the interpretation of the relationship between the measured concentrations and the exposure(s) or condition(s) of interest.

Although smoking causes a depletion of antioxidant defenses, significantly different concentrations of oxidative stress biomarkers, 8-oxodG and 8-isoprostane, between smokers and non-smokers have not been consistently reported in all studies (Faux et al., 2009; Graille et al., 2020b, 2020a). Thus, the aim was to investigate the factors that are associated with oxidative stress levels, with a focus on PAH and VOC exposures. No study investigated the associations between the selected oxidative stress biomarkers and BoE to tobacco smoke in a large cohort of smokers.

Smokers are a good study population to evaluate these associations because they voluntarily expose themselves to high concentrations of VOCs and PAHs on a daily basis. The exposure can be easily characterized by questionnaires (e.g., number of cigarettes per day) and by specific BoE to tobacco smoke (e.g., exhaled CO, nicotine metabolites, anabasine, and NNAL). Other factors that may have an influence on oxidative stress levels were tested, such as age, gender, body mass index, health status, sleep quality, physical activity, alcohol consumption, fruit and vegetables consumption, place of residence and occupations.

This study allowed us to determine which families of harmful compounds in tobacco smoke, as well as which lifestyle related factors, were associated with oxidative stress and what was their relative importance.

5.2 Overview of results and discussion

Associations between oxidative stress biomarkers and BoE to tobacco smoke were assessed by multiple linear regressions. Four models per oxidative stress biomarker were created: each with a different BoE. This was done because BoE were highly correlated with each other, as they originated from a common source (i.e., cigarette smoking). An overview of the associations found by the multiple linear regression analyses can be seen in Figure 3.

Urinary 8-oxodG concentrations increased by 10% with a 50% increase in TNE (molar sum of nicotine metabolites), by 1% with a 50% increase in ΣVOC score (logarithm sum of VOC metabolite concentrations), and by 4% with a 50% increase in ΣPAH score (logarithm sum of PAH metabolite concentrations). None of the other factors was significantly associated with urinary 8-oxodG concentrations (except a negative association between BMI and 8-oxodG in the ΣPAHs regression model only).

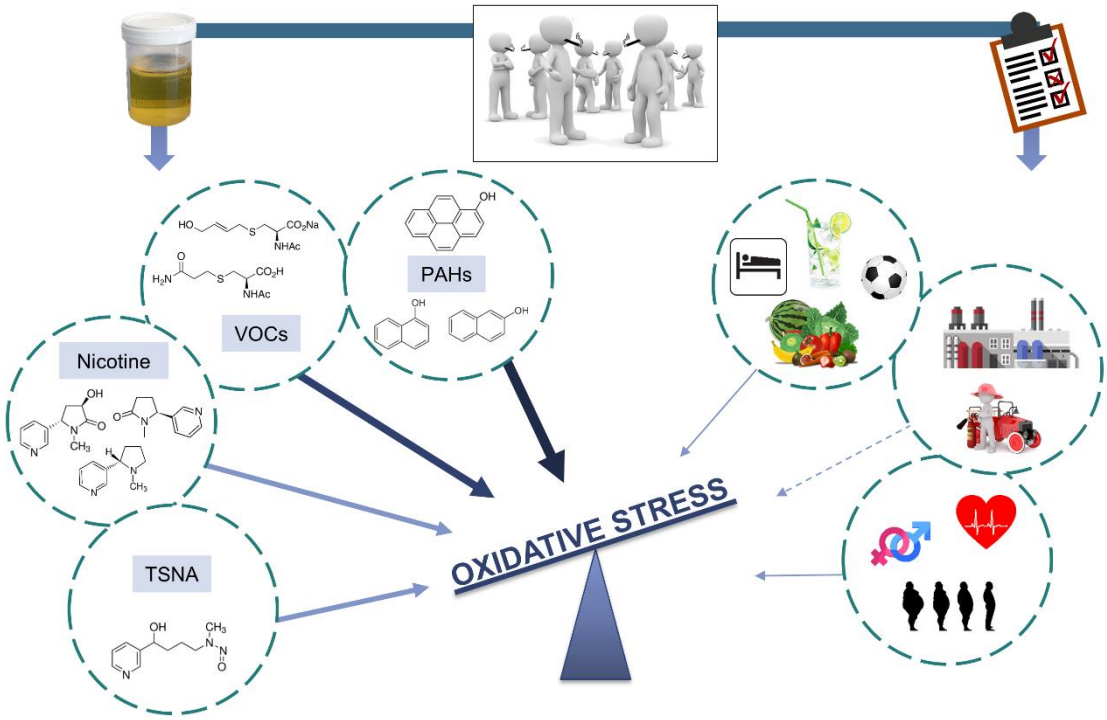

Figure 3 – Overview of associations between oxidative stress biomarkers with BoE and behavioral, environmental, and individual factors (arrow sizes reflect the degree of association).

Urinary concentrations of 8-isoprostane increased by 11%, 8%, 2%, and 5% with a 50% increase in TNE, NNAL, ΣVOC, and ΣPAH, respectively. A positive association between 8-isoprostane concentration and BMI was observed. Moreover, the daily consumption of fruits and vegetables was negatively associated with 8-isoprostane. None of the other factors was significantly associated with urinary 8-isoprostane concentrations.

The relative importance of the different associations was estimated using the effect size indicator partial R-squared (see Figure 4). ΣPAH and ΣVOC scores showed the strongest associations with both 8-oxodG and 8-isoprostane. The effect size of the association between 8-isoprostane and daily consumption of fruits and vegetables was small, indicating that the relative importance of smoking was much larger than the potential protective effect of fruit and vegetable consumption.

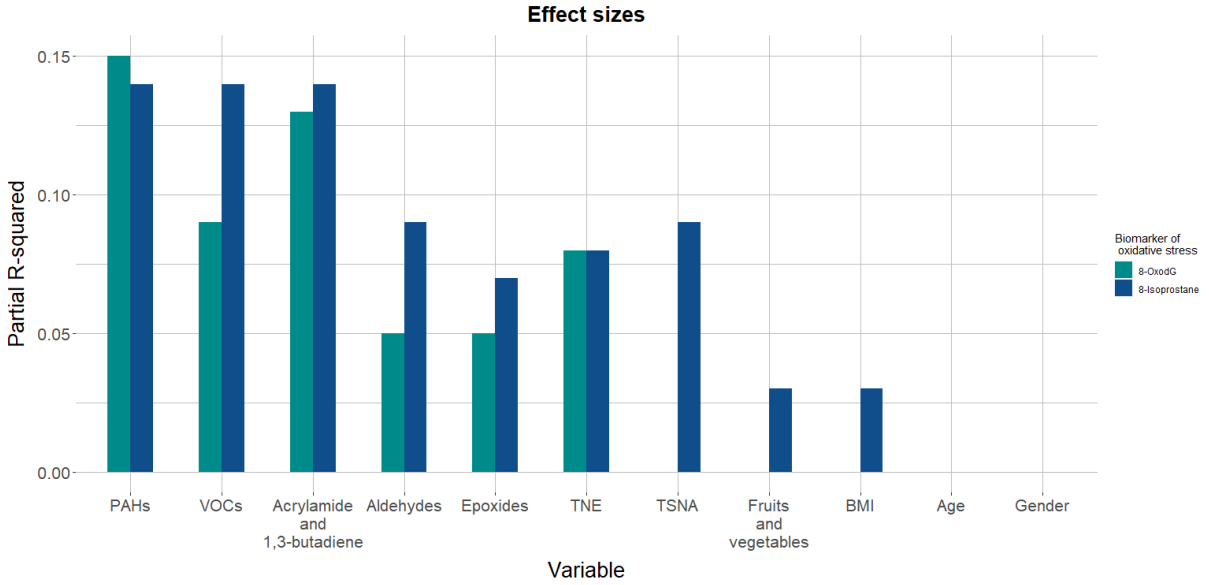

Figure 4 – Relative importance of the different associations between oxidative stress biomarkers (8-oxodG; green and 8-isoprostane; blue) and BoE and other factors, estimated by the effect size indicator partial R-squared.

The results suggested that exposure to PAHs and VOCs lead to an increase in oxidative stress, although the design of the study does not allow the establishment of a causal link.

5.3 Manuscript 3

See next page.

Associations between urinary biomarkers of oxidative stress and biomarkers of tobacco smoke exposure in smokers

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Abstract

The development of inflammation-related diseases have been associated with excessive oxidative stress levels defined as the imbalance between the production and elimination of reactive oxygen species (ROS). ROS concentrations are influenced by behavioral as well as environmental factors, such as exposure to airborne chemical substances, and in particular, polycyclic aromatic hydrocarbons (PAHs) and volatile organic compounds (VOCs). These substances are also common in tobacco smoke. Relationships between exposure biomarkers of these chemicals and effect biomarkers of oxidative stress have not been elucidated. We aimed to characterize the relationships between exposure biomarkers and oxidative stress biomarkers and to assess the relative importance of each family of compounds in modulating oxidative stress levels.

We selected a cohort of smokers as they have a known exposure source, which is quantifiable as biomarkers of nicotine and tobacco-specific nitrosamines (TSNAs). Two biomarkers of oxidative stress, 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) and 8-iso-prostaglandin $F_{2\alpha}$ (8-isoprostane), and 20 exposure biomarkers (VOC (11), nicotine (4), PAH (3), and TSNA (1) metabolites) were measured in urine (n=270) from smokers in an ongoing clinical study (ESTxENDS).

Participating smokers (153 men and 117 women, median age 44 years) had on average smoked 25 years and smoked, at the time of the study, about 17 cigarettes per day. Multiple linear regression results showed an association between 8-oxodG concentrations and the following metabolites in decreasing order: PAH (beta coefficient $\beta = 0.105$, p-value < 0.001, partial $R^2 = 0.15$) > VOC ($\beta = 0.028$, $p < 0.001$, partial $R^2 = 0.09$) > nicotine ($\beta = 0.226$, $p < 0.001$, partial $R^2 = 0.08$) metabolites, and between 8-isoprostane concentrations and PAH ($\beta = 0.117$, $p < 0.001$, partial $R^2 = 0.14$) > VOC ($\beta = 0.040$, $p < 0.001$, partial $R^2 = 0.14$) > NNAL ($\beta = 0.202$, $p = 0.003$, partial $R^2 = 0.09$) > nicotine ($\beta = 0.266$, $p < 0.001$, partial $R^2 = 0.08$) metabolites. Body mass index (BMI) and daily fruit and vegetable consumption had a weak although, statistically significant influence on 8-isoprostane (partial $R^2 = 0.03$ for both).

Tobacco smoke exposure biomarkers were strongly associated with both oxidative stress biomarkers.

Keywords: Biomonitoring; oxidative stress; tobacco smoke exposure; polycyclic aromatic hydrocarbons; volatile organic compounds

1. Introduction

The development of inflammation-related human diseases have been associated with excessive oxidative stress levels defined as the imbalance between the production or presence of high concentrations of reactive oxygen species (ROS) and the body's antioxidant defenses. ROS are highly reactive and attack cellular components (DNA, proteins, and lipids) when present in excess[1]. Oxidative stress can be both the cause and the consequence of inflammation as ROS are produced during normal metabolic processes as well as during the activation of the immune system. Indeed, high level of oxidative stress is involved in the onset of several diseases including cancers, cardiovascular diseases, and diabetes[2]. Environmental factors (e.g., air pollution and UV exposures) and behavioral factors (e.g., smoking, alcohol consumption, nutrition, and physical activity) influence oxidative stress levels[3].

Human biomonitoring of oxidative stress is based on the measurement of the products that result from the ROS attack on cellular components, as these compounds are more stable[4]. Two well-studied biomarkers of oxidative stress are 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG), a marker of DNA damage, and 8-iso-prostaglandin F2α (8-isoprostane), a marker of lipoperoxidation[5,6]. Both compounds are excreted in urine and can be measured by liquid (LC) or gas (GC) chromatography coupled with mass spectrometry (MS) or enzyme-linked immunosorbent assay (ELISA). LC-MS analysis is recommended as it is considered more reliable[7].

Oxidative stress is one of the mechanisms that can explain the adverse health effects of air pollution[8]. There are many environmental pollutants, including polycyclic aromatic hydrocarbons (PAHs) and volatile organic compounds (VOCs), and each may have a different effect on oxidative stress levels. Associations between exposure to these chemicals and effect biomarkers of oxidative stress have not been elucidated. These associations can give insight into mechanisms of the development of diseases, and an understanding of exposures that produce oxidative stress. Oxidative stress biomarkers are indicators of pre-clinical alterations and can be used to identify or monitor vulnerable individuals before the onset of symptoms[9–12]. However, it is not simple to find a large number of individuals exposed simultaneously to high concentrations of PAHs and VOCs from environmental or occupational sources, not to mention resources needed to establish such cohorts. An alternative is to study the relationship between PAH and VOC exposures and oxidative stress in smokers exposed to tobacco smoke that contain PAHs and VOCs.

In a cohort of smokers, study participants are exposed to a known source (cigarettes) and on a daily basis, making it easier to characterize the frequency, intensity and duration of exposure to these substances. Another advantage is that the exposure range is large (large variability in internal doses of PAHs and VOCs) depending on the number of cigarettes smoked per day. Moreover, a cohort of smokers can also give insight in the association between smoking, quantified as the internal dose of total nicotine equivalent (TNE), and ROS as this has been reported inconsistently[13]. The oxidative stress biomarkers, 8-oxodG and 8-isoprostane, are not specific to environmental exposures and tobacco smoking, but vary depending on an individual's health status, and lifestyle.

Tobacco smoke contains more than 7,000 identified chemicals, at least 70 of which are carcinogenic according to the International Agency for Research on Cancer (IARC)[14,15]. In addition, 93 are on the list of harmful and potentially harmful constituents (HPHC) in tobacco products and tobacco smoke issued by the U.S. Food and Drug Administration (FDA)[16]. These compounds are linked to one or more of the five major health effects caused by smoking: cancer, cardiovascular diseases, respiratory disorders, reproductive problems, and addiction. The list includes carcinogens such as PAHs, tobacco-specific nitrosamines (TSNAs), and some VOCs. Table 1 summarizes the selected biomarkers of tobacco smoke exposure for this study.

Table 1 – Urinary biomarkers of tobacco smoke exposure and their properties such as chemical family, chemical name, specificity to tobacco (yes/no), elimination half-life (h: hours, d: days), corresponding parent compound, and potential health effects (addictive (AD), carcinogen (CA), cardiovascular toxicant (CT), reproductive or developmental toxicant (RDT), and respiratory toxicant (RT))[16]. Only biomarkers selected for this study are presented. Nicotine and its metabolites can also be found in individuals using nicotine replacement therapy or electronic nicotine delivery systems. Therefore, there are not solely specific to tobacco exposure, that is why they have been marked with an asterisk (*).

Family	Biomarker	Tobacco specific	Half-life	Parent compound [1]	Potential health effects
Nicotine	Nicotine	Yes*	1–2 h [17]	-	RDT, AD
	Cotinine	Yes*	16–18 h [17]	Nicotine	
	Norcotinine	Yes*	-		
	3-OH-cotinine	Yes*	6.4 h [18]		
Anabasine	Anabasine	Yes	16 h [19]	-	AD
TSNAs	NNAL	Yes	42 d [17]	NNK	CA

Family	Biomarker	Tobacco specific	Half-life	Parent compound [1]	Potential health effects
VOCs	DHBMA	No	-	1,3-butadiene	CA, RT, RDT
	1-/2-MHBMA	No	> 9 h [20]		
	3-MHBMA	No	-		
	3-HPMA	No	9–12 h [21]	Acrolein	RT, CT
	AAMA	No	12–14h [21]	Acrylamide	CA
	GAMA	No	22 h [21]		
	CYMA	No	8 h [22]	Acrylonitrile	CA, RT
	HEMA	No	> 5h [23]	Acrylonitrile	CA, RT
				ethylene oxide	CA, RT, RDT
				vinyl chloride	CA
	SPMA	No	9 h [17]	Benzene	CA, CT, RDT
	HPMMA	No	-	Crotonaldehyde	CA
	2-HPMA	No	-	Propylene oxide	CA, RT
PAHs	1-Naphthol	No	4.3 h [24]	Naphthalene	CA, RT
	2-Naphthol	No	2.5 h [24]		
	1-OHP	No	20 h [17]	Pyrene	-

[1]All the parent compounds are on the HPHC list, except pyrene.

Biomarkers: Trans-3'-hydroxycotinine (3-OH-cotinine), 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), N-acetyl-S-(3,4-dihydroxybutyl)-L-cysteine (DHBMA), N-acetyl-S-(1-hydroxymethyl-2-propenyl)-L-cysteine (1-MHBMA), N-acetyl-S-(2-hydroxy-3-butenyl)-L-cysteine (2-MHBMA), N-acetyl-S-(4-hydroxy-2-buten-1-yl)-L-cysteine (3-MHBMA), N-acetyl-S-(3-hydroxypropyl)-L-cysteine (3-HPMA), N-acetyl-S-(2-carbamoylethyl)-L-cysteine (AAMA), N-acetyl-S-(2-carbamoyl-2-hydroxyethyl)-L-cysteine (GAMA), N-acetyl-S-(2-cyanoethyl)-L-cysteine (CYMA), N-acetyl-S-(phenyl)-L-cysteine (SPMA), N-acetyl-S-(3-hydroxypropyl-1-methyl)-L-cysteine (HPMMA), N-acetyl-S-(2-hydroxyethyl)-L-cysteine (HEMA), N-acetyl-S-(2-hydroxypropyl)-L-cysteine (2-HPMA), 1-hydroxypyrene (1-OHP).

Parent compound: 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK).

PAHs are generated during incomplete combustion of organic matter or pyrolysis processes[25]. They include a large family of organic compounds that contain only carbon and hydrogen atoms and have between two and more fused aromatic rings. Sources of PAHs include natural emissions (forest fires, volcanic eruptions) and human activities (traffic, industrial activities, power generation, incineration, residential heating, cooking, and smoking)[26]. In a cigarette, the dried tobacco leaves are burnt, and this will give rise to variable degrees of PAHs depending

on the cigarette brand and the puffing behavior[27]. Occupational exposures can occur among others in the metal industry, in construction (e.g., roads), among professional drivers, chimney sweeps and firemen[28–30]. The general population is also exposed to PAHs through diet (grilled or smoked meat and fish)[31]. Occupational PAH exposures are often assessed by measuring urinary concentrations of 1-hydroxypyrene (1-OHP); a pyrene metabolite, and 1-naphthol and 2-naphthol; two metabolites of naphthalene. Pyrene is not a carcinogen, but its metabolite, 1-OHP, is a sensitive biomarker that significantly correlates with total absorbed dose of PAHs in urine[32]. Both 1-naphthol and 2-naphthol concentrations are higher in smoker, but a better correlation between 2-naphthol and cotinine have previously been observed[33].

TSNAs are formed during the curing and processing of tobacco by the nitrosation of nicotine into N-nitrosonornicotine (NNN), nornicotine into nicotine-derived nitrosamine ketone (NNK), anatabine into N-nitrosoanatabine (NAT), and anabasine into N-nitrosoanabasine (NAB). Biomonitoring can be performed by measuring urinary concentration of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), a metabolite of NNK. There are no other sources than tobacco for TSNAs, making NNAL ideal for discriminating smokers from non-smokers.

VOCs are defined as compounds that have high vapor pressures at room temperature. VOCs commonly found in tobacco smoke are shown in Table 1. They consist of different chemical groups, including aldehydes, aliphatic hydrocarbons, amides, and epoxides. Acetaldehyde and formaldehyde are known carcinogens present in tobacco smoke. These aldehydes could not be included in our study, as no specific urinary biomarkers exist for these two compounds. Sources to VOCs are numerous. Occupational exposure to VOCs are found among others in nail stylists, printing workers, cleaning agents, and truck drivers[34–37]. Non-occupational sources of human exposure to VOCs include combustion processes, tobacco smoke, organic solvents, personal care products, cleaning agents, paints, adhesives, glues, wood preservatives, and air fresheners. Exposure to VOCs can be assessed using biomonitoring of urinary concentrations of mercapturic acids[38]. The mercapturic acid pathway is a major route for the biotransformation of electrophilic compounds and involves glutathione that plays a role in the regulation of oxidative stress[39].

Several studies reported a significant dose-response relationship between PAH or VOC metabolites and urinary 8-oxodG or 8-isoprostane[40–44]. Cao et al. (2020) showed that oxidative stress might be involved in the reduction of the lung function observed in a large cohort study of residents exposed to air pollution[45]. In this study, they quantified twelve urinary PAH metabolites (OH-PAHs) and two oxidative stress biomarkers (8-oxodG and 8-isoprostane) and reported associations between the sum of PAH metabolites (ΣOH-PAHs) and both 8-oxodG

and 8-isoprostane. Moreover, Kuang et al. (2021) found significant associations between 8-oxodG and 14 of the measured 27 VOC metabolites in urine from asthmatic and healthy children exposed to air pollution, and concluded that VOC exposures were positively associated to oxidative stress[46]. Similar relationships between VOC exposures and oxidative stress were previously reported in adults by Arif and Shah (2007) and Yoon et al. (2010), but exposure to VOCs was quantified in air samples in the first one (no urinary metabolites) and with different metabolites for the second one (no mercapturic acids)[47,48]. Only the study of Weinstein et al. (2017) analyzed both PAH and VOC metabolites and oxidative stress biomarkers at the same time, but dose-response relationships could not be demonstrated[49]. This study quantified four PAH metabolites and eight VOC metabolites, as well as 8-oxodG and 8-isoprotane, in urine samples from a small group of recently pregnant rural Guatemalan women who used woodstoves for cooking and heating. Compared to smokers, these women had higher PAH metabolite concentrations and lower VOC metabolite concentrations. However, the authors did not take into account the many known factors that influence oxidative stress that could mask a possible association between these exposures and oxidative stress biomarkers.

Typical factors that influence oxidative stress biomarkers include age, gender, body mass index (BMI), health status, sleep quality, physical activity, alcohol consumption, fruit and vegetable consumption, place of residence, and occupations[2,3].

Sleep disorders and night shifts were linked to an increase of oxidative stress and inflammation[50,51]. It has been proposed that sleep promotes anti-oxidative mechanisms, and therefore poor sleep quality can be expected to cause an increase in oxidative stress[52]. For example, 8-isoprostane was linked to obstructive sleep apnea, sleep duration and nightshift work[53,54]. On the contrary, the relationship between 8-oxodG and obstructive sleep apnea was not observed consistently across studies[55,56].

Prolonged or high-intensity physical activity (i.e., 65%-75% maximal oxygen consumption (VO_{2max})) increases production of ROS and may cause oxidative damage to skeletal muscles, while regular physical activity may be beneficial to protect the body from oxidative damages[57,58]. Elevated concentrations of 8-isoprostane after acute exercise (long duration and/or high intensity) were observed in blood, but not in urine[59,60].

Urinary 8-oxodG was shown not to be sensitive enough to detect exercise-induced DNA oxidation (for moderate and high-intensity exercise)[61].

Alcohol consumption can increase oxidative stress as ethanol metabolism induce the formation of ROS[62,63]. Both 8-oxodG and 8-isoprostane were linked to alcohol consumption[64,65].

Sleep disorders, strenuous physical activity, and alcohol consumption are factors that can modulate the associations between exposure biomarkers and oxidative stress biomarkers and therefore their influence needs to be assessed.

Elevated concentrations of oxidative stress biomarkers were not reported in all studies on smokers. Since cigarette smoke contains many toxic compounds, determining which ones are associated with the formation of oxidative stress biomarkers would provide a better understanding of the underlying mechanisms. In addition, it would allow discussion of the usefulness of these biomarkers in the context of studies on smoking and the diseases for which smoking is a main risk factor. Our objectives were to assess the associations between exposure biomarkers and oxidative stress biomarkers, and to evaluate simultaneously the effects of lifestyle related factors and their relative contribution to the urinary concentration of oxidative stress biomarkers.

2. Methods

2.1 Study population

Participants were selected from an on-going randomized controlled trial on smoking cessation: “Efficacy, Safety and Toxicology of Electronic Nicotine Delivery Systems as an aid for smoking cessation: the ESTxENDS multicenter randomized controlled trial” (ClinicalTrials.gov Identifiers: NCT03589989) approved by the ethics committees of Bern, Geneva, and Lausanne (Project-ID: 2017-02332), Switzerland. The study was conducted in accordance with the Swiss law and the ethical principles of the World Medical Association Declaration of Helsinki and the International Committee on Harmonization for Good Clinical Practice. All participants provided written informed consent.

Inclusion criteria for the ESTxENDS study were: aged 18 or older, current smoker who had consumed five or more cigarettes per day for at least 12 months, and were willing to try to quit smoking within the next three months.
Exclusion criteria can be found in supplementary information. Participants were invited to a first clinical visit at baseline before their chosen quit date. The day of each clinical visit, participants self-collected their full first-void urine sample (first morning urine sample) before their first cigarette and brought the sample to the study center. Due to logistical constraints, urine samples were stored at 4°C for one to seven days (on average five days) and were

transferred weekly to the laboratory in an icebox. Urinary metabolites (VOC, PAH, and nicotine metabolites, as well as NNAL) were shown to be stable during one week at 4°C[66–69]. They were then aliquoted and stored at -20°C until analysis.

We included 270 smokers in our study. All participants brought their first-morning urine void for their first clinical visit (baseline) in an on-going clinical trial, between July 2018 and November 2019. We measured urinary anabasine concentrations as well as concentrations of nicotine and its metabolites to verify smoking status. Cut-points of anabasine and cotinine to define smokers were set at 3 ng/mg creatinine and 30 ng/mg creatinine, respectively[70]. We calculated total nicotine equivalent (TNE 4) corresponding to the molar sum of nicotine, cotinine, norcotinine, and 3-OH-cotinine expressed in nmol/mg creatinine (see Equation 1) to estimate the daily intake of nicotine[71].

Equation 1:

$$TNE = \frac{\frac{[nicotine]}{MW_{nicotine}} + \frac{[cotinine]}{MW_{cotinine}} + \frac{[3\text{-}OH\text{-}cotinine]}{MW_{3\text{-}OH\text{-}cotinine}} + \frac{[norcotinine]}{MW_{norcotinine}}}{[creatinine]}$$

Where [*compound*] is the concentration in ng/mL (except for creatinine in mg/mL) and $MW_{compound}$ the molecular weight in g/mol.

Exhaled carbon monoxide (CO) was also measured during their first clinical visit (Micro⁺ or piCO⁺ Smokerlyzer, Bedfont, Anif, Austria), which were several hours after collecting the first-morning urine void. The exhaled CO cut-point was set at 10 ppm[70].

Urinary concentrations of NNAL may also be used to validate self-reported smoking status in addition to CO and urinary concentrations of anabasine and nicotine metabolites. We quantified urinary NNAL in 103 of the 270 participants due to cost limitations. We could therefore not use this biomarker to validate the smoking stats for all but a portion of our participants.

2.2 Analytical methods

All the biomarkers (Table 1) were quantified in first-void urine samples by liquid chromatography – tandem mass spectrometry (LC-MS/MS) methods.

All analyses were performed at the Unit of Forensic Toxicology and Chemistry, University Center of Legal Medicine (Lausanne – Geneva, Switzerland), except the NNAL analysis, which was performed at the Central Environmental Laboratory (CEL) at École Polytechnique Fédérale de Lausanne (EPFL, Lausanne, Switzerland).

Urinary creatinine was quantified with a routine clinical method based on Jaffe (1886) on a chemical analyzer (AU480 Chemistry Analyzer, Beckman Coulter, Nyon, Switzerland)[72].

TNE 4 included nicotine and three of its metabolites: cotinine, 3-OH-cotinine, and norcotinine. These metabolites, together with nicotine and anabasine, were analyzed with LC-MS/MS (Dionex Ultimate 3000 system + TSQ Quantiva, Thermo Scientific, Reinach, Switzerland; Thermo Fisher Scientific application note n°20709, 2013[73]). Sample preparation included a solid-phase extraction (SPE; Sola SCX 10 mg/1 mL, Thermo Scientific, Reinach, Switzerland). Limits of quantification (LOQs) were 1 ng/mL for all compounds. Two quality controls (QCs) were injected every 15 samples. Performance of the method was assessed twice a year by inter-laboratory tests (ISO17025 accreditation).

PAH metabolites were analyzed with a routine method. Briefly, urine (3 mL) was mixed with a solution (3 mL) containing internal standards (1-OHP-d9, 1-naphtol-d7, and 2-naphtol-d7), enzyme (β-glucoronidase from Helix pomatia type HP-2 ≥100,000 U/mL, Merck KGaA, Buchs, St. Gallen, Switzerland), and hydrochloric acid (4N) in sodium acetate buffer (32.8 g/L). The mixed solution was incubated at 37°C overnight. The target analytes were extracted with SPE (ABN 60 mg/3 mL, Biotage, Uppsala, Sweden). The extract was dried and reconstituted in water/acetonitrile (500 µL, 80:20, v/v) for LC-MS/MS analysis. Limits of detection (LODs) were 0.1 ng/mL and LOQs were 0.2 ng/mL for each metabolite. A QC was injected every 15 samples. Performance of the method was assessed twice a year by inter-laboratory tests (ISO17025 accreditation).

VOC metabolites were quantified with a LC-MS/MS according to the method of Alwis et al. (2012)[38]. Briefly, filtered urine samples were diluted 1:10 with buffer (100 µL urine + 50 µL internal standards + 850 µL 15 mM ammonium acetate pH 6.8). The diluted solution was directly injected in the LC-MS/MS. LOQs were 1.5 ng/mL for 3-HPMA, 2-HPMA and HPMMA, 2 ng/mL for AAMA, DHBMA, CYMA, 1-/2-MHBMA, 3-MHBMA and PMA, and 3 ng/mL for GAMA and HEMA. 1-MHBMA and 2-MHBMA could not be separated chromatographically. We thus report the sum of these here. A QC sample was injected every 10 samples. Performance of the method was regularly assessed by inter-laboratory tests (ISO17025 accreditation).

NNAL was quantified by LC-MS/MS (Acquity UPLC system + Xevo RQ MS, Waters, Baden-Dättwil, Switzerland). Samples were prepared at the department of occupational and environmental health (DSTE) of the Center for Primary Care and Public Health (Unisanté, Lausanne). The method was based on Biotage application note (n°AN884, 2017[74]) and the publications of Byrd and Ogden (2003), Kavvadias et al. (2009), and Hu et al. (2014)[75–77]. Briefly, urine samples (1.3 mL) were centrifuged (4000 rpm, 5 min) and mixed with internal standards (100 µL), buffer (200 µL, phosphate buffer 1 M) and enzyme (400 µL of β-glucoronidase ~5 mg/mL or ~5200 U/mL; β-glucoronidase from Escherichia coli type IX-A 125KU, Merck KGaA, Buchs, St. Gallen, Switzerland). After one night in the dark at 37°C, the mixture was extracted with a solid-supported liquid/liquid extraction (SLE cartridge; ISOLUTE® SLE+ 2 mL Sample, Biotage, Uppsala, Sweden) and the samples were analyzed by LC-MS/MS at EPFL. LOQ was 0.2 ng/mL.

8-oxodG and 8-isoprostane were analyzed with a previously described LC-MS/MS (Dionex Ultimate 3000 system + TSQ Quantiva, Thermo Scientific, Reinach, Switzerland) method[78]. Sample preparation included a SPE (Chromabond C18ec SPE 500 mg 3 mL, Macherey-Nagel, Oensingen, Switzerland). LOQs were 0.5 ng/mL for 8-oxodG and 0.1 ng/mL for 8-isoprostane. Two QCs were injected every 15 samples.

2.3 Health status, lifestyle and environmental exposure assessment

Self-reported questionnaires queried participants on health status and lifestyle, including smoking history[79,80], sleep quality (PSQI), physical activity (IPAQ), alcohol consumption (AUDIT-C questionnaire), and dietary habits (frequencies of vegetables and fruits consumption). We also asked participants to give their postal code and profession.
The purpose of including these factors in the statistical analysis was to assess if they modulate the association between exposure biomarkers and oxidative stress biomarkers in our samples of smokers.

1. Self-reported previous health conditions

Participants were asked whether they had an infection, fever or acute illness the day of urine collection (n=15). They also reported if they had allergies (n=101), hypertension (n=34), hypercholesterolemia (n=20), diabetes (n=9), cardiovascular diseases (n=33; CVD including previous myocardial infarction, percutaneous coronary intervention (PCI), heart failure, stroke, angina pectoris, peripheral arterial disease (PAD) or other self-reported CVD), and pulmonary diseases (n=49; including pulmonary embolism, deep vein thrombosis (DVT), chronic

obstructive pulmonary disease (COPD), chronic bronchitis, asthma or other self-reported pulmonary diseases). CVD and pulmonary diseases were grouped into two variables as few participants suffer from these diseases.

2. Smoking history

Three parameters were selected: daily cigarette consumption, years of smoking, and pack years. The latter was estimated by multiplying the number of cigarette packs per day (i.e., number of cigarettes per day divided by 20) by the number of years of smoking.

3. Sleep quality

Sleep quality can be assessed by the Pittsburgh sleep quality index (PSQI), which is a standardized self-report questionnaire[81]. Participants completed the PSQI questionnaire, and we calculated a score according to the authors' instructions[81]. Briefly, we grouped nineteen items in seven components of sleep: subjective sleep quality, sleep latency, sleep duration, habitual sleep efficiency, sleep disturbances, use of sleeping medication, and daytime dysfunction. Each component score ranged from 0 to 3, and all component scores were summed to yield a global score (from 0 to 21). A global score >5 indicates poor sleep quality.

4. Physical activity

International physical activity questionnaire (IPAQ) is a validated self-report questionnaire, and the short IPAQ form has been recommended for use in national monitoring surveys[82]. We included five IPAQ recommendations in our statistical analysis to assess physical activity in our cohort;

(1) Excluded data reported as "do not know" and missing data,

(2) Estimated Metabolic Equivalent Task minutes per week (MET-min/week) (a measure of total physical activity) by summing adjusted durations (minutes x days) for each type of activity (adjustment was performed with the following values: walking = 3.3 METs, moderate physical activity = 4.0 METs, and vigorous physical activity = 8.0 METs),

(3) Excluded participants who reported a total activity time greater than 960 minutes per day (i.e., intensive physical activity),

(4) Excluded physical activity reported for less than 10 minutes per day in any domains, and

(5) Truncated any domain exceeding 180 minutes to 180 minutes, as recommended by IPAQ[82].

We created a new variable including those with significant physical activity (vigorous MET-min/week > 3000, corresponding to approximately one hour of intense physical activity per day in one week; n=32) to analyze this separately.

5. Alcohol consumption

Alcohol consumption can be assessed by the alcohol use disorders identification test (AUDIT), a questionnaire developed in a multinational World Health Organization collaborative study and validated in different populations[83]. A short form focusing on alcohol consumption (AUDIT-C) was proposed as a screening test for heavy drinking or alcohol dependence[84]. Participants completed the AUDIT-C questionnaire, and a score from 0 to 12 was calculated by summing the score of each response (ranged from 0 to 4)[83]. A score ≥3 for women or ≥4 for men can reflect a problematic alcohol consumption and higher scores may indicate dependence.

6. Daily consumption of fruits and vegetables

Participants were asked: "How often do you eat fresh vegetables (carrots, green beans, salad, etc.)?" and "How often do you eat fresh fruit or fresh fruit juice?". We then separated the participants into two fruit and vegetables categories: daily consumption (n=59) and less than daily consumption (n=116). Information was missing for 95 participants and there was no information on the quantity.

7. Residence category (urban vs rural)

Participants were classified into two environmental categories according to their place of residence: urban or rural environment. We used the Swiss postal codes and classified them according to the FSO (Swiss Federal Statistical Office) system based on seven classes where 1 represents the city center up to 7 for the countryside (Table S1 in supplementary information). We grouped the classes 1 and 2 in the urban category (n=167) and classes 3 to 7 in the rural category (n=103). This allowed discriminating the ones living in city from the others.

8. Professions

We selected several jobs in which possible occupational exposures to PAHs or VOCs may occur including city cleaner, cleaning agent, cook, cab, bus or streetcar driver, truck driver, Uber driver, garage worker, gas station employee, firefighter, hairdresser, mechanic, policeman, postman, nail stylist, chimney sweep, street vendor, chemical industry worker, construction worker (building/roads), and metal industry worker. We grouped the participants working in these occupations in one category (cat. 1 – potentially exposed to PAHs or VOCs; n=20) and the others in a second category (cat. 2 – non-exposed to these pollutants; n=250).

2.4 Data presentation and statistical analysis

Biomarker concentrations below limit of quantification (LOQ) were substituted with: LOQ/sqrt(2)[85]. We flagged biomarkers with more than 40% of observations under LOQ and did not use it for statistical analysis. Oxidative stress biomarkers and biomarkers of exposure were creatinine-corrected, and presented as median with the interquartile range (IQR): 1st and 3rd quartiles (Q1–Q3).

We log-transformed creatinine-adjusted data for each biomarker because they were all right-skewed. We conducted factor analyses on the (log-transformed) PAH and VOC biomarkers to assess the validity of summary scores labeled as ΣPAHs and ΣVOCs. The consistency of the different exposure biomarkers (cigarettes per day, exhaled CO, urinary TNE, urinary NNAL, as well as ΣPAHs and ΣVOCs) was assessed using linear and partial correlation coefficients. Linear correlation coefficients give information on the degree of association between two variables, while partial correlation coefficients give the same information but remove the effect of other variables (i.e., controlling for potential confounding variables). In the case where two variables come from the same source (estimated by one or more other variables), the partial correlation coefficient would be close to zero. If it were not the case, it would indicate the presence of another source of the two variables studied (example in Figure S1 in supplementary information). We defined the correlation as weak (r=0.10–0.39), moderate (r=0.40–0.69) or strong (r=0.70–1.00). The associations between ΣPAHs and ΣVOCs with the place of living and the professions was assessed using multiple linear regression models adjusting for smoking parameters.

We investigated the association between oxidative stress biomarkers and biomarkers of exposure: TNE, ΣPAHs, ΣVOCs, or TSNAs with multiple linear regression. We constructed one model per biomarker of exposure to avoid multicollinearity, adjusted for creatinine and several other factors (mentioned above). *p*-values < 0.05 were considered statistically significant. The non-significant factors were not included in the models, except age, BMI, and gender. To compare the importance of the exposure biomarkers from the different models, we computed effect sizes. Effect sizes of the variables could then be compared by calculating the partial r-squared (partial R^2), which is the proportion of variance explained by each variable excluding the proportion of the variance due to the other variables in the model. In brief, this parameter allowed us to define the relative importance of each covariate, whether it is continuous or categorical, on the dependent variable. However, it gave no information on causality.

All calculations were performed with R version 4.0.2 (2020-06-22) - "Taking Off Again".

3. Results

3.1 Characteristics of participants

We verified participants' smoking status with exhaled CO, anabasine, and cotinine. Fifteen participants had concentrations of exhaled CO lower than 10 ppm (sensitivity 94%), and 42 had urinary anabasine concentration lower than 3 ng/mg creatinine (sensitivity 84%). However, no participant had cotinine below 30 ng/mg creatinine (sensitivity 100%). Therefore, all participants were considered as smokers.

Our sample had similar age and gender distributions to the 2017 Swiss Health Survey of tobacco consumption (Swiss Federal Statistical Office – FSO) and thus we deemed our sample to be representative of the smoking population of Switzerland (Table S2 available in supplementary information). Participant demographics are presented in Table 2.

Table 2 – Summary of participant demographics (number, age, body mass index (BMI)), cigarette consumption and history (years of smoking and pack years), total nicotine equivalent (TNE), exhaled CO, sleep quality (Pittsburgh Sleep Quality Index (PSQI) score), physical activity (International Physical Activity Questionnaire (IPAQ) score), and alcohol consumption (Alcohol Use Disorders Identification Test – Consumption (AUDIT-C) score). Characteristics are presented for all participants, and separately for men and women. Except for participant number, all characteristics are presented as median with interquartile range (1st quartile – 3rd quartile).

Characteristic	Total	Men	Women
Participants (-) [number (%)]	270 (100)	153 (57)	117 (43)
Age (years) [median (Q1–Q3)]	44 (32–54)	43 (32–54)	45 (33–54)
BMI (kg/m^2) [median (Q1–Q3)]	25.1 (22.4–27.6)	25.5 (23.4–27.6)	24.5 (21.3–27.2)
Cigarette consumption (cig/day) [median (Q1–Q3)]	17 (10–20)	20 (13–20)	15 (10–20)
Smoking history (years) [median (Q1–Q3)]	25 (16–36)	25 (16–35)	26 (16–36)
Pack years (-) [median (Q1–Q3)]	19.0 (11.2–31.5)	20.0 (13.5–32.3)	16.1 (9.3–29.7)
TNE (nmol/mg creatinine) [median (Q1–Q3)]	26.5 (17.3–37.3)	24.5 (17.3–36.7)	27.6 (17.9–38.4)
Exhaled CO (ppm)	25 (16–33)	26 (17–33)	23 (14–34)

Characteristic	Total	Men	Women
[median (Q1–Q3)]			
PSQI score (-) [median (Q1–Q3)]	5 (3–7)	5 (3–7)	5 (3–8)
IPAQ (MET-min/week) [median (Q1–Q3)]	3126 (1436–5118)	3552 (1388–5172)	2886 (1388–4981)
AUDIT-C score (-) [median (Q1–Q3)]	3 (2–5)	4 (2–6)	3 (2–4)

3.2 Urinary biomarkers of exposure

Concentrations of biomarkers of tobacco smoke exposure are presented in Table 3 separately for men and women.

Table 3 – Concentrations of urinary nicotine, tobacco-specific nitrosamine (TSNA), volatile organic compounds (VOC), and polycyclic aromatic hydrocarbons (PAH) metabolites in male and female smokers (normalized for creatinine, median with interquartile range (1st and 3rd quartiles); n=270). Percentages of samples above the limit of quantification (>LOQ) are also reported.

Family	Biomarker	>LOQ (%)	Men	Women
Nicotine (ng/mg creatinine) [median (Q1–Q3)]	Nicotine	100	685 (376 –1433)	746 (326 –1240)
	Cotinine	100	1230 (858–1784)	1194 (664–1617)
	Norcotinine	100	124 (89–187)	140 (82–170)
	3-OH-cotinine	100	2272 (1452–3429)	2853 (1462–3728)
Anabasine (ng/mg creatinine) [median (Q1–Q3)]	Anabasine [1]	98	7.50 (4.40–10.70)	7.34 (3.83–11.73)
TSNAs (pg/mg creatinine) [median (Q1–Q3)]	NNAL [2]	99	234 (147–383)	246 (107–337)
VOCs	DHBMA	100	420 (314–553)	404 (339–505)

Family	Biomarker	>LOQ (%)	Men	Women
(ng/mg creatinine) [median (Q1–Q3)]	1-/2-MHBMA [3]	70	3.22 (<LOQ–6.39)	2.94 (<LOQ–5.89)
	3-MHBMA	100	27.1 (17.0–38.91)	23.5 (15.90–36.88)
	3-HPMA	100	1355 (899–1979)	1026 (633–1787)
	AAMA	100	156 (112–208)	166 (108–226)
	GAMA	99	17.1 (13.0–25.6)	16.1 (13.3–24.0)
	CYMA	100	177 (102–306)	203 (103–448)
	SPMA [4]	31	<LOQ (<LOQ–3.01)	<LOQ (<LOQ–6.43)
	HPMMA	100	908 (642–1260)	762 (483–1127)
	HEMA [5]	68	3.69 (<LOQ–5.82)	4.76 (<LOQ–8.65)
	2-HPMA	100	59.8 (40.2–82.0)	51.5 (31.6–78.0)
PAHs (ng/mg creatinine) [median (Q1–Q3)]	1-Naphtol	100	8.83 (5.83–13.23)	8.27 (4.59–12.52)
	2-Naphtol [6]	100	16.1 (11.9–20.7)	16.4 (12.5–23.2)
	1-OHP	81	0.27 (0.20–0.42)	0.33 (0.23–0.45)

[1]Anabasine was analyzed by the same analytical method as for nicotine metabolites; [2]NNAL was analyzed for 103 participants only; [3]LOQ for 1-/2-MHBMA was 2 ng/mL; [4]the majority of the observations of SPMA (69%) were under LOQ (2 ng/mL); [5]LOQ for HEMA was 3 ng/mL; [6]Four values of 1-naphthol were not included in the statistical analysis because they were higher than 1000 ng/mg creatinine.

Biomarkers: Trans-3'-hydroxycotinine (3-OH-cotinine), 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), N-acetyl-S-(3,4-dihydroxybutyl)-L-cysteine (DHBMA), N-acetyl-S-(1-hydroxymethyl-2-propenyl)-L-cysteine (1-MHBMA), N-acetyl-S-(2-hydroxy-3-butenyl)-L-cysteine (2-MHBMA), N-acetyl-S-(4-hydroxy-2-buten-1-yl)-L-cysteine (3-MHBMA), N-acetyl-S-(3-hydroxypropyl)-L-cysteine (3-HPMA), N-acetyl-S-(2-carbamoylethyl)-L-cysteine (AAMA), N-acetyl-S-(2-carbamoyl-2-hydroxyethyl)-L-cysteine (GAMA), N-acetyl-S-(2-cyanoethyl)-L-cysteine (CYMA), N-acetyl-S-(phenyl)-L-cysteine (SPMA), N-acetyl-S-(3-hydroxypropyl-1-methyl)-L-cysteine (HPMMA), N-acetyl-S-(2-hydroxyethyl)-L-cysteine (HEMA), N-acetyl-S-(2-hydroxypropyl)-L-cysteine (2-HPMA), 1-hydroxypyrene (1-OHP).

3.3.1 Correlation between exposure biomarkers and indicators of tobacco consumption

We computed ΣPAHs and ΣVOCs scores (correlations between the scores and their individual components can be found in Table S3 and S4 in supplementary information – in "Annexes" section). SPMA was not included in the variable ΣVOCs as more than 69% of the observations were below the LOD. Table 4 presents correlation coefficients and partial correlation coefficients (gray cells) between the variables cigarettes per day, exhaled CO, anabasine, TNE, ΣVOCs, ΣPAHs, and NNAL.

Pearson's correlation coefficients between all the urinary biomarkers were high (r >0.5) signifying a common source: cigarette smoking. We found weak/moderate correlations between the exposure biomarkers and the number of cigarettes per day (r ~ 0.3–0.5) and exhaled CO. Partial coefficients were greatly reduced compared to correlation coefficients for most relationships, although some were still moderate (r ~0.3–0.5).

Table 4 – Pearson's correlation and partial correlation analysis between exposure biomarkers (log-transformed values), including reported number of cigarettes per day (cig/day) and exhaled CO (ppm).

	Cig/day	CO	Anabasine	TNE	ΣPAHs	ΣVOCs	NNAL
Cig/day		0.45 (<0.001)	0.33 (<0.001)	0.45 (<0.001)	0.34 (<0.001)	0.34 (<0.001)	0.45 (<0.001)
CO	0.35 (<0.001)		0.34 (<0.001)	0.35 (<0.001)	0.32 (<0.001)	0.33 (<0.001)	0.27 (0.007)
Anabasine	-0.01 (0.522)	0.11 (0.050)		0.69 (<0.001)	0.6 (<0.001)	0.71 (<0.001)	0.63 (<0.001)
TNE	0.26 (<0.001)	0.01 (0.943)	0.30 (<0.001)		0.68 (<0.001)	0.75 (<0.001)	0.6 (<0.001)
PAHs	0.03 (0.736)	0.06 (0.410)	0.04 (0.496)	0.18 (0.005)		0.78 (<0.001)	0.51 (<0.001)
VOCs	-0.04 (0.718)	0.01 (0.692)	0.31 (<0.001)	0.33 (<0.001)	0.51 (<0.001)		0.59 (<0.001)
NNAL [1]	0.28 (0.003)	-0.08 (0.513)	0.32 (<0.001)	0.11 (0.637)	0.00 (0.728)	0.13 (0.252)	

Upper triangle: Pearson's correlation coefficients (with *p*-value), lower triangle: partial correlation coefficients (with *p*-value); [1]Partial correlation coefficients were calculated separately for NNAL as many observations (n=167) were missing. Biomarkers of exposure: total nicotine equivalent (TNE), logarithm sum of polycyclic aromatic hydrocarbons (ΣPAHs), logarithm sum of volatile organic compounds (ΣVOCs), 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL).

3.3.2 Potential other sources of PAHs and VOCs

The selected VOCs and PAHs are not specific to cigarette smoke exposures. We investigated other covariates that could be associated with these biomarkers such as the residence category and the participant's profession. We used multiple linear regression analysis for both ΣPAHs and ΣVOCs. Covariates with significant positive associations were TNE and exhaled CO. Residence category and profession did not show any significant influence in our sample of smokers.

3.3 Urinary biomarkers of oxidative stress

Table 5 shows descriptive data for the two biomarkers of oxidative stress. Correlation coefficient between 8-oxodG and 8-isoprostane was high (r = 0.69).

Table 5 – Concentrations of 8-oxodG and 8-isoprostane in smokers, and separately in men and women (normalized for creatinine, median with interquartile range (1st and 3rd quartiles); n=270). Percentages of samples above the limit of quantification (>LOQ) are also reported.

Biomarkers	>LOQ (%)	Total	Men	Women
8-oxodG (ng/mg creatinine) [median (Q1–Q3)]	100	4.33 (3.46–5.76)	4.15 (3.30–5.76)	4.42 (3.57–5.76)
8-isoprostane (ng/mg creatinine) [median (Q1–Q3)]	100	0.22 (0.17-0.31)	0.23 (0.17-0.32)	0.22 (0.16-0.30)

Biomarkers: 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) and 8-iso-prostaglandin F2α (8-isoprostane)

We modeled the concentrations of the exposure biomarkers (i.e., nicotine, ΣPAH, ΣVOC and TSNA metabolites) separately, as they were highly correlated. The results from the four regression models for 8-oxodG are shown in Table 6. Associations between urinary concentrations of 8-oxodG and exposure biomarkers were calculated with equation 2 as a percentage increase (for log-transformed predictor only; more details on the calculation in the supplementary information).

Equation 2:

$$\%_{outcome} = \left(\left(\frac{\%_{covariate}}{100} + 1\right)^{\beta} - 1\right) * 100$$

Where $\%_{outcome}$ is the percent increase or decrease in the concentration of an oxidative stress biomarker, $\%_{covariate}$ is the percent increase or decrease in the concentration of an exposure biomarker (or other log-transformed covariates), and β is the regression coefficient (estimate). For instance, a 50% increase of TNE ($\%_{covariate}$) using an estimate (β) of 0.226 (Table 6) resulted in a 10% increase of 8-oxodG ($\%_{outcome}$). Similarly, a 50% increase in the ΣVOC and ΣPAH scores yielded 1% (β = 0.028) and 4% (β = 0.105) increases in 8-oxodG concentration, respectively. TNE, ΣPAHs and ΣVOCs were all associated with urinary 8-oxodG.

We did not find any change of the measures of association with age and gender in our study sample, but found a negative association between BMI and 8-oxodG for the ΣPAHs regression model only. Other covariates, such as residence category, professions, diseases, sleep quality, physical activity, alcohol consumption, vegetable and fruit consumption, did not change of the measures of association with 8-oxodG concentrations (not included in the final models).

We assessed the respective effect sizes of the covariates with partial R^2. This allowed us to determine the relative importance of the covariates for 8-oxodG formation. We found that, besides creatinine, ΣPAHs was the most associated with 8-oxodG concentrations (partial $R^2 = 0.15$), followed by ΣVOCs (partial $R^2 = 0.09$) and TNE (partial $R^2 = 0.08$).

Table 6 – Multiple linear regression models for 8-oxodG and 8-isoprostane (estimate (coefficient beta (β)) [95% confidence interval] and *p*-value (* means significant: <0.05)). All models were adjusted for biomarker of exposure (adjusted for creatinine), age, gender, body mass index (BMI), and other significant covariates if any. Covariates significantly associated with the outcome (*) are in bold.

8-oxodG	TNE (n=270)	ΣPAHs (n=266)	ΣVOCs (n=270)	NNAL (n=103)
Biomarker of exposure	**0.226 [0.134, 0.318] <0.001 ***	**0.105 [0.075, 0.135] <0.001 ***	**0.028 [0.017, 0.038] <0.001 ***	0.037 [-0.084, 0.159] 0.541
Age	0.001 [-0.003, 0.005] 0.536	0.002 [-0.002, 0.005] 0.413	0.000 [-0.003, 0.004] 0.831	0.001 [-0.006, 0.008] 0.724
Gender	-0.017 [-0.120, 0.086] 0.742	0.005 [-0.093, 0.103] 0.923	-0.009 [-0.110, 0.093] 0.8671	-0.051 [-0.227, 0.126] 0.570
BMI	-0.007 [-0.018, 0.004] 0.220	**-0.013 [-0.023, -0.003] 0.011 ***	-0.008 [-0.019, 0.002] 0.1173	-0.011 [-0.029, 0.006] 0.193

8-isoprostane	TNE (n=270)	PAHs (n=266)	VOCs (n=270)	NNAL (n=103)
Biomarker of exposure	**0.266 [0.158, 0.374] <0.001 ***	**0.117 [0.081, 0.153] <0.001 ***	**0.040 [0.028, 0.052] <0.001 ***	**0.202 [0.068, 0.335] 0.003 ***
Age	0.003 [-0.002, 0.007] 0.248	0.003 [-0.002, 0.007] 0.200	0.001 [-0.003, 0.006] 0.555	0.000 [-0.008, 0.008] 0.991
Gender	0.055 [-0.067, 0.176] 0.377	0.090 [-0.022, 0.209] 0.135	0.058 [-0.059, 0.175] 0.332	0.110 [-0.084, 0.303] 0.264
BMI	**0.020 [0.007, 0.033] 0.003 ***	**0.014 [0.001, 0.026] 0.030 ***	**0.019 [0.007, 0.031] 0.003 ***	**0.022 [0.003, 0.041] 0.027 ***
Fruits and vegetables [1]	**-0.170 [-0.326, -0.015] 0.032 ***	**-0.170 [-0.324, -0.015] 0.032 ***	**-0.181 [-0.334, -0.028] 0.021 ***	-0.020 [-0.266, 0.225] 0.869

Biomarker concentrations were log-transformed. Creatinine concentration (log-transformed) was added as covariate in the models (not showed). [1]The effect of the daily consumption of fruits and vegetables was added separately as 95 observations were missing.

Biomarkers: 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) and 8-iso-prostaglandin F2α (8-isoprostane), total nicotine equivalent (TNE), polycyclic aromatic hydrocarbons (PAHs), volatile organic compounds (VOCs), 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL).

Table 6 shows the results from the four regression models for 8-isoprostane. All biomarkers of exposure had a positive relationship with urinary 8-isoprostane concentrations. A 50% increase in the following exposure biomarkers: TNE, NNAL, ΣVOC, and ΣPAH was associated with a 11%, 8%, 2%, and 5% increase in 8-isoprostane concentrations, respectively. In our smoker cohort, we observed a positive association between 8-isoprostane concentration and BMI, but no change of the measure of association with age and gender. Daily consumption of fruits and vegetables was negatively associated to 8-isoprostane (concentration were reduced by 16%).

We found that ΣPAHs and ΣVOCs were the most associated with 8-isoprostane concentrations (partial $R^2 = 0.14$ for both) followed by NNAL and TNE (partial $R^2 = 0.09$ and 0.08, respectively). The effect size of fruit and vegetable consumption was low (partial $R^2 = 0.03$; insignificant for the NNAL model) and similar to BMI (partial $R^2 \sim 0.03$; for all models). The relative importance of smoking was thus much larger than the potential protective effect from fruit and vegetable consumption.

4. Discussion

In this analysis of urinary biomarkers of oxidative stress and biomarkers of tobacco smoke exposure among 270 longtime smokers, exposures to PAHs and VOCs were strongly associated with both urinary 8-oxodG and 8-isoprostane concentrations.

We verified the participants' smoking status with exhaled CO, anabasine, and cotinine, and we showed that only cotinine had a 100% sensitivity to detect smokers. However, it has been previously reported that TNE should by preferred to cotinine, as it accounts for cotinine metabolites and differences in metabolic rate and genetic variation among smokers[86]. The number of metabolites included in TNE vary across studies. We used nicotine in addition to three of its metabolites (cotinine, 3-OH-cotinine, and norcotinine) to calculate TNE. The gold standard to estimate daily nicotine intake is TNE 7, which is the molar sum of nicotine and six of its metabolites (cotinine, 3-OH-cotinine, nornicotine, norcotinine, nicotine N-oxide, cotinine N-oxide, including their glucuronide conjugates). The analysis of TNE 7 is technically difficult and costly. Benowitz et al. (2020) proposed to use TNE 3 (molar sum of nicotine, cotinine, and 3-OH-cotinine) as it is strongly correlated to TNE 7 ($r = 0.99$) and insensitive to individual metabolic variation[71]. In our study, we used TNE 4 (nicotine, cotinine, 3-OH-cotinine, and norcotinine) as norcotinine is also part of the routine laboratory method.

The exposure biomarker concentrations (nicotine, TSNA, VOC, and PAH metabolites) were in the same range as reported by Goniewicz et al. (2018) who analyzed 2,411 urine samples from smokers[85]. Only three biomarkers could not be quantified in 100% of the samples: SPMA (benzene metabolite; 31%), HEMA (metabolite of acrylonitrile, ethylene oxide, vinyl chloride; 68%), and 1-/2-MHBMA (1,3-butadiene metabolite; 70%). This is because LOQs (2 ng/mL for SMPA and 1-/2-MHBMA, and 3 ng/mL for HEMA) were not low enough to quantify concentrations in all samples. Therefore, we do not recommend analyzing SPMA (benzene metabolite) if LOQ is higher than 0.6 ng/mL (corresponding to ~70% >LOQ in smokers)[85]. 1-/2-MHBMA and HEMA LOQs do not necessarily need to be reduced, but these two biomarkers should be used in conjunction with others in statistical analysis.
VOC metabolites (except SPMA) showed moderate/strong correlations with each other (average $r = 0.45–0.50$; Table S4). This was expected because they are all tobacco smoke exposure biomarkers. VOC metabolites follow a similar phase two elimination (mercapturic acid pathway), but have different half-lives, which could explain why we did not observe higher

correlations. This reasoning is also valid for the three PAH metabolites, which also showed moderate correlations between them (Table S3).

The reason for the weak to moderate correlations between cigarettes per day and urinary nicotine metabolites, which we and others have previously found, could potentially be explained by puffing behaviors in smokers[87]. Indeed, parameters such as puff volume, puff duration and interpuff interval can modulate the exposure to toxic compounds present in cigarette smoke. Other explanations are the metabolisms, which present inter-individual variations. For example, a given level of nicotine intake can lead to a twofold different level of cotinine in different individuals[88]. The time between the last cigarette and the urine collection, as well as the number of voids in between, also influence the biomarkers concentrations, as they are urinary metabolites. Finally, inaccurate number of cigarettes reported by the participants could also weaken the correlations. No single parameter can account for all these factors. TNE allowed us taking into account the smokers' puffing behaviors and to dispense with the need to rely on a value reported by the participants. Nevertheless, it does not take into account the differences in metabolisms of the other biomarkers, and the timing of collection should be as similar as possible for all participants (e.g., first-void urine collection).
CO measurement and urine collection were not performed simultaneously, which could explain the weak correlation between exhaled CO and other biomarkers of exposure ($r = 0.3–0.35$). Urine samples were collected the morning before the first cigarette, while CO concentrations were measured at the clinical visits several hours afterwards. Exhaled CO measurement is not essential if anabasine and cotinine are used for biochemical verification of tobacco exposure.

We observed moderate partial correlation between ΣPAHs and ΣVOCs scores ($r = 0.51$), which could potentially suggest the presence of other sources of exposure such as environmental sources (traffic, industrial activities). We suggest that there was no other important source of VOCs and PAHs for our smokers, because the correlations between anabasine (a tobacco-specific biomarker) and score of ΣVOC and ΣPAH were strong. Therefore, we explain the moderate partial correlation by the fact that VOCs and PAHs are formed during the incomplete combustion of cigarettes, while the other compounds (nicotine, anabasine) are inherent in the tobacco leaves. Moreover, other factors, such as the puffing behavior or the varying amount of nicotine in different brand of cigarettes, may also play a role. These hypotheses are supported by the correlation between ΣVOCs and ΣPAHs that was the strongest observed ($r = 0.78$).

We tested the effect of some professions where exposures to VOCs and PAHs are possible (list of nineteen professions, grouped together) but did not find any association. However, very

few participants were included in the exposed category (n=20) and the activities were diverse (12 different professions); consequently, we could not draw any conclusion.
We found no association with residence category of the participants (urban vs rural) in which different traffic and anthropogenic activities generating PAHs and VOCs can occur. These results were not surprising, as tobacco smoke remains the major well-known source of these compounds. Still, air pollution remains a major problem in most cities and industrial areas, but exposure is globally lower than smoking[89].

Urinary concentrations of 8-oxodG and 8-isoprostane in our participants were similar to concentrations reported in smokers (normalized for creatinine) in two recent systematic literature reviews[90,91]. For 8-oxodG, the systematic review reported urinary concentration of 22.2 (3.0–41.4) ng/mg creatinine (median with interquartile range (1st quartile – 3rd quartile)) for smokers with a BMI ≤ 25 and 4.0 (3.5–4.5) ng/mg creatinine (median with interquartile range (1st quartile – 3rd quartile)) for smokers with a BMI > 25. Our results showed similar 8-oxodG concentrations (Table 5) for smokers with a BMI > 25, but were 4 times lower for smokers with BMI ≤ 25 compared to the systematic review. However, Graille et al. (2020a) specified that the high concentrations for smokers with BMI ≤ 25 in the meta-analysis originated from only one study and thus these results need to be confirmed.

Graille et al. (2020b) mentioned that no consistent effect of smoking was observed for 8-isoprostane unlike for 8-oxodG. BMI, however, influenced 8-isoprostane concentrations. In their study, subgroups with a mean BMI > 25 had urinary 8-isoprostane of 0.55 (0.51–0.65) ng/mg creatinine (median with interquartile range (1st quartile – 3rd quartile)) while subgroups with a mean BMI ≤ 25 had half of this concentration (0.25 (0.24–0.41) ng/mg creatinine (median with interquartile range (1st quartile – 3rd quartile))). This BMI effect was not observed in our study although BMI had a significant positive association with 8-isoprostane. According to our multiple linear regression models, 8-isoprostane concentrations increased by 1% per unit of BMI; however, the effect size was small (partial R^2=0.03), indicating that BMI was not strongly associated with 8-isoprostane urinary concentrations in smokers.

For 8-oxodG, none of the tested factors (the place of residence, diseases, sleep quality, physical activity, alcohol consumption, and fruits and/or vegetables consumption) had any significant influence in addition to cigarette smoking. Results from the multiple linear regression showed that NNAL was not associated with 8-oxodG. Even if we had fewer observations than for the other exposure biomarkers (n=103), the effect size was very small (partial $R^2 < 0.01$) and therefore we would not expect to observe a consistent association with 8-oxodG with more participants. We did not observe any change of the measure of association with gender or age,

but found a small negative association with BMI (significant for ΣPAHs model only), i.e., an 8-oxodG decrease of 1% when BMI increased by one unit.
The link between BMI and 8-oxodG is not clearly established, as conflicting results are reported in several studies[64,92]. As we found an association with BMI only in the PAH model, one hypothesis would be that some of the inhaled PAHs are stored in the adipose tissue due to their high lipophilicity and are gradually released[93]. The body's defense mechanisms would then be less likely overwhelmed.

For 8-isoprostane, BMI was significant in all models of the regression analysis. Several studies have reported that oxidative stress increases with BMI[94]. We observed an association with hypercholesterolemia, but we did not include this factor in the multiple linear regression model as it was highly correlated with BMI.

We observed a significant negative association of 8-isoprostane concentrations with the daily consumption of fruits and vegetables, which could be due to their natural antioxidant content[95]. According to the estimate found in the multiple linear regression (Table 6), it reduced the 8-isoprostane concentrations of about 15–16% in smokers. In other studies, 8-isoprostane excretions in women (n_{tot}=246) were reduced with consumption of fruits and vegetables, and an inverse association between urinary 8-isoprostane concentration and fruit consumption in a study on polyphenols contained in the Mediterranean diet was reported[96,97]. The link between fruit and vegetable consumption and oxidative stress is still being studied, as several studies have not found conclusive results[98–100].

Tobacco smoke exposure biomarkers we included in this study, were associated positively with 8-isoprostane and 8-oxodG (except NNAL). We assessed their relative importance on oxidative stress levels and observed the following order for 8-oxodG: PAHs, VOCs, and TNE, and for 8-isoprostane: VOCs, PAHs, NNAL, and TNE.
1-OHP is a biomarker that correlates with the total absorbed dose of PAHs. If we consider a 50% increase of this biomarker, we would expect an 11–13% increase of 8-oxodG and 8-isoprostane. Concerning the VOCs, we regrouped the metabolites of aldehydes (acrolein, crotonaldehyde), epoxides (ethylene oxide, propylene oxide), and others (acrylamide, 1,3-butadiene) and calculated the effect sizes (Table 7) for these to understand if any of these groups of biomarkers were in particular related to oxidative stress.

Table 7 – Effect sizes (partial R^2) for three groups of volatile organic compounds (VOCs) on oxidative stress biomarkers.

Metabolite groups	Partial R^2 (8-oxodG) [1]	Partial R^2 (8-isoprostane) [1]
Aldehydes [2]	0.05	0.09
Epoxides [3]	0.05	0.07
Acrylamide and 1,3-butadiene [4]	0.13	0.14

[1]Partial R^2 were calculated by replacing the variable ΣVOCs by the variables Aldehydes, Epoxides or Others in the multiple linear regression models; [2]Aldehydes was the logarithm sum of 3-HPMA and HPMMA; [3]Epoxides was the logarithm sum of HEMA and 2-HPMA; [4]Acrylamide and 1,3-butadiene was the logarithm sum of AAMA, GAMA, DHBMA, 1-/2-MHBMA, and 3-MHBMA.

Biomarkers: 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) and 8-iso-prostaglandin F2α (8-isoprostane), N-acetyl-S-(3,4-dihydroxybutyl)-L-cysteine (DHBMA), N-acetyl-S-(1-hydroxymethyl-2-propenyl)-L-cysteine (1-MHBMA), N-acetyl-S-(2-hydroxy-3-butenyl)-L-cysteine (2-MHBMA), N-acetyl-S-(4-hydroxy-2-buten-1-yl)-L-cysteine (3-MHBMA), N-acetyl-S-(3-hydroxypropyl)-L-cysteine (3-HPMA), N-acetyl-S-(2-carbamoylethyl)-L-cysteine (AAMA), N-acetyl-S-(2-carbamoyl-2-hydroxyethyl)-L-cysteine (GAMA), N-acetyl-S-(3-hydroxypropyl-1-methyl)-L-cysteine (HPMMA), N-acetyl-S-(2-hydroxyethyl)-L-cysteine (HEMA), N-acetyl-S-(2-hydroxypropyl)-L-cysteine (2-HPMA).

Acrylamide and 1,3-butadiene had an effect size twice that of the other groups, which meant that they were the most associated with increased concentrations of oxidative stress biomarkers. 1,3-Butadiene and acrylamide are oxidized by cytochrome P450 enzymes, known to generate free radicals, before conjugation with glutathione[101,102]. The others are not. This might explain the larger effect sizes for 1,3-butadiene and acrylamide compared to aldehydes or oxides. Both aldehydes and oxides react directly with glutathione and cause an indirect increase of free radicals by decreasing the antioxidant defenses of the body[103]. This would explain why their effect sizes were similar.

Although acrylonitrile can also be oxidized by cytochrome P450 enzymes, the effect size was lower than most of the other compounds (not included in Table 7). One hypothesis is that CYMA (one of acrylonitrile metabolites) results from the direct conjugation to glutathione, and it might not be linked to the cytochrome P450 enzyme activity[104]. Moreover, the second metabolite HEMA, resulting from oxidation by the cytochrome P450 enzymes, is not specific to acrylonitrile, as it is also a metabolite of ethylene oxide and vinyl chloride. Thus, the importance of this compound on the formation of oxidative stress biomarkers is probably masked.

Our study has several strengths. We measured a large number of exposure biomarkers (20 biomarkers of tobacco smoke exposure) and two oxidative stress biomarkers. We also had data on possible factors that influence possible relationships between the exposure biomarkers and effect biomarkers, such as health status and lifestyle habits. This was particularly important as the oxidative stress biomarkers are not specific to exposures and can vary greatly between individuals. Another strength was that all participants were smokers, so we were able to characterize exposure biomarkers and their variability from a single exposure source. This allowed us to assess the relative importance of all factors influencing oxidative stress levels in smokers. The statistical analysis were robust, as we had sufficient participants (n=270) to detect possible associations between exposure and oxidative stress. One limitation is that urine collection and exhaled CO measurement were not collected at the same time. Another limitation is the lack of information on cigarette brand and puff parameters for each participant. This made the interpretation of the partial correlation analysis between biomarkers of tobacco smoke exposure difficult, which was not the case when assessing associations with oxidative stress biomarkers.

In conclusion, we found significant associations between two oxidative stress biomarkers and four different families of tobacco exposure biomarkers (TNE, ΣVOCs, ΣPAHs, and NNAL) in smokers. These biomarkers reflected cigarette smoking and were associated with of 8-oxodG and 8-isoprostane concentrations. We also showed that BMI had an inverse association with 8-oxodG concentration and a positive association with 8-isoprostane concentration. We observed that daily consumption of fruits and vegetables was negatively associated with the concentration of 8-isoprostane in smokers, but the effect size was small compared to the ones of the exposure biomarkers. Consequently, exposures to carcinogens and irritants in cigarette smoke have the greatest influence on the increase in oxidative stress levels, and in particular exposures to PAHs and VOCs.

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Chapter 6 – Urinary biomarkers analysis

This chapter is a descriptive analysis of concentrations of BoE to tobacco smoke and oxidative stress biomarkers at baseline and at 6-month follow-up and changes between the two clinical visits of the ESTxENDS study. The results must remain confidential until the main paper of the ESTxENDS clinical trial is published in 2022.

6.1 Introduction

Smokers expose themselves to high concentrations of harmful compounds during tobacco use due to their addiction to nicotine. These harmful compounds include cardiovascular, reproductive or developmental, and respiratory toxicants. Many are also carcinogens, such as NNAL, 1-naphthol, 2-naphthol, 1,3-butadiene, acrylamide, acrylonitrile, benzene, crotonaldehyde, and propylene oxide. Smokers are strongly encouraged to quit smoking to reduce exposure to these harmful compounds and their related adverse health effects.

ENDS have been proposed as a substitute for cigarettes to reduce exposure to harmful compounds. ENDS aerosols generated in laboratory contained harmful compounds at lower concentrations than in cigarette smoke (Chapter 3). However, exposure to these compounds can vary greatly from one individual to another depending on e-liquid consumption and puffing topography. Human biomonitoring takes into account these factors, as well as other sources of exposure (e.g., environmental, diet, or occupational), which allows evaluating the total internal dose. Changes in urinary concentrations of BoE following smoking cessation would therefore reflect the extent of reduction in exposure to harmful compounds.

Exposures to PAHs and VOCs present in tobacco smoke were associated with oxidative stress level (Chapter 5). Biomarkers of oxidative stress have the potential to reflect the effects of all compounds, identified of not, present in ENDS aerosols. As oxidative stress was associated to the development of several diseases and is involved in inflammatory processes, biomarkers of oxidative stress might give valuable information on short-term effects of ENDS use, or long-term effects if chronic oxidative stress is induced. Urinary concentrations of oxidative stress biomarkers following smoking cessation would therefore help to observe the changes in oxidative stress level according to the participants' final smoking status.

6.2 Method

Both BoE and oxidative stress biomarkers previously described (Chapter 5) were quantified in urine samples obtained six months after baseline. In addition, analysis of urinary concentrations of heavy metals and trace elements were performed both in urine samples of twenty ex-smokers and twenty ENDS users from both baseline and 6-month follow-up. Metal analysis was added to assess exposure to metals that were identified in ENDS aerosols.

6.2.1 Study population

The same sample of participants as in Chapter 5 was analyzed. Briefly, the selection consisted of 273 participants who completed the clinical follow-up visit between January 2019 and March 2020: all ex-smokers, ENDS users, and dual users from this period were included. A sample of smokers of similar size was randomly selected to complete the study population. Only participants who collected and brought their urine sample to the study center were included in this sub-study.

Smoking status at 6-month follow-up was determined based on the participant's smoking preference during the 7 days before the follow-up clinical visit (Table 15). Participant self-reported status was then verified with urinary anabasine (cutpoint; 3 ng/mg creatinine), cotinine (cutpoint; 30 ng/mg creatinine), and NNAL (if applicable, cutpoint; 31 pg/mL) concentrations, as well as exhaled CO concentrations (cutpoint; 10 ppm) (Benowitz et al., 2020). Urinary samples with creatinine concentrations outside the normal range (0.3 – 3 mg/mL) were excluded (n=25). Of the 273 participants (546 samples), only 238 participants (505 samples) were ultimately included in the statistical analyses (see Table 16).

Table 15 – Determination of smoking status based on tobacco and ENDS use in the last seven days before the follow-up clinical visit.

	Had not smoked in the last 7 days	Had smoked in the last 7 days
Had not vaped in the last 7 days	Ex-smokers	Smokers
Had vaped in the last 7 days	ENDS users	Dual users

Table 16 – Biological verification of self-reported smoking status at baseline and follow-up. Cut-points of anabasine, cotinine, exhaled CO, and NNAL were set at 3 ng/mg creatinine, 30 ng/mg creatinine, 10 ppm, and 31 pg/mg creatinine, respectively. We expect concentrations to be higher than the cut-points for smokers (including dual users) and lower for ex-smokers. For ENDS users, only cotinine concentrations could be higher than the cut-point (except when vaping nicotine-free e-liquid).

Self-reported smoking/vaping status	Number of participants below / above the cutpoint of anabasine	Number of participants below / above the cutpoint of cotinine	Number of participants below / above the cutpoint of exhaled CO	Number of samples excluded and justifications
Smokers – baseline	40 / 217	**2** / 255	18 / 249	**2** → two participants stopped smoking before urine collection
Smokers – follow-up	12 / 49	**1** / 60	14 / 49	**1** → one participant was not smoking before urine collection
Dual users – follow-up	9 / 25	0 / 34	14 / 21	-
ENDS users – follow-up	77 / 15	11 / 81	93 / **3**	**3** → one participant reported cannabis use and two had urinary NNAL concentrations above cut-point
Ex-smokers – follow-up	68 / 8	57 / 19	72 / **5**	**8** → three participants smoked before urine collection (two of whom reported cannabis use), three reported using smokeless tobacco, one reported using a heated tobacco product, and two had urinary NNAL concentrations above cut-point

6.2.2 Analytical methods

For the analysis of urinary creatinine, nicotine metabolites, PAH metabolites, VOC metabolites, NNAL, 8-oxodG and 8-isoprostane by LC-MS/MS, please refer to the method section of Chapter 5.

The concentrations of 20 metals were quantified in urine samples: Be, Al, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Se, Mo, Pd, Ag, Cd, Sn, Sb, Pt, and Pb. The same metals were analyzed in the ENDS emissions (Chapter 3). The samples came from 20 ex-smokers (baseline + 6-month follow-up) and 20 ENDS users (baseline + 6-month follow-up) randomly selected. The metal quantification was performed using inductively coupled plasma mass spectrometry (ICP-MS; iCAP TQ, Thermo Scientific, Reinach, Switzerland). Urine samples (500 µL) were diluted 10 times in a solution of 0.5% nitric acid (HNO_3, 5 mL; 69% solution from SCP Science, Marktoberdorf, Germany) and internal standard (Yttrium; 50 µL at 100 µg/L) was added. Purified water was prepared in the laboratory with a water purification system (MilliQ

Advantage, Merck, Schaffhausen, Switzerland). Standard solutions of metals for calibration curves were bought from Labkings (Hilversum, Netherlands), except Fe that was obtained from SCP Science (Marktoberdorf, Germany). LODs and LOQs are presented in supplementary data, as they are different for each metal. Details of the ICP-MS method can be found in supplementary data of Chapter 3 (see Annexes).

6.2.3 Data presentation

Biomarker concentrations under LOQs were substituted with LOQ/sqrt(2) to allow log-transformation for statistical tests (Goniewicz et al., 2018). Oxidative stress biomarkers and BoE were creatinine-corrected to take into account the hydration status. Concentrations are presented as median with the interquartile range (IQR): 1st and 3rd quartiles (Q1–Q3) because median is less sensitive to outliers than mean. Biomarker concentrations at 6-month follow-up are presented in boxplots and changes from baseline to 6-month follow-up, expressed as percentage of baseline (%), are presented in bar charts (median with median absolute deviation).

6.3 Results and discussion

Participant characteristics at 6-month follow-up are presented for ex-smokers, ENDS users, dual users, and smokers in Table 17. Characteristics at baseline can be found in Chapter 5.

Table 17 – Summary of participants' characteristics (number, percentages of men and women, age, body mass index (BMI)), consumption of cigarettes (cigarettes per day), ENDS use (milliliters e-liquid per week), and exhaled CO (ppm). Characteristics are presented for the four groups formed at 6-month follow-up according to their smoking status: ex-smokers, ENDS users, dual users, smokers. All characteristics are presented as median with interquartile range (1st quartile – 3rd quartile), except for participant number and percentages of men and women.

Characteristic	Ex-smokers	ENDS users	Dual users	Smokers
Participant (-) [number (%)]	77 (28)	97 (36)	35 (13)	64 (23)
Percentage of men / women (%)	55 / 45	55 / 45	77 / 23	52 / 48
Age (years) [median (Q1–Q3)]	43 (34-55)	44 (33-54)	43 (31-59)	40 (30-43)

Characteristic	Ex-smokers	ENDS users	Dual users	Smokers
BMI (kg/m^2) [median (Q1–Q3)]	25.8 (23.2-28.3)	26 (23.2-28.9)	25.3 (24.2-27.8)	24.3 (22.0-28.5)
Cigarette consumption (cig/day) [median (Q1–Q3)]	-	-	3 (1-11)	10 (7-15)
E-liquid consumption (mL/week) [median (Q1–Q3)]	-	15 (10-28)	8 (5-14)	-
Exhaled CO (ppm) [median (Q1–Q3)]	3 (2-4)	3 (1-5)	13 (6-23)	18 (11-30)

A higher proportion of men among dual users was observed compared to the other three groups. Age and BMI were not different between groups. Smokers reduced their daily consumption of cigarettes compared to baseline (10 cig/day vs 17 cig/day; medians). Dual users reported a lower consumption of cigarettes than smokers did (3 cig/day; median), and among them 23 participants reported smoking ≤5 cigarettes per day. Concerning ENDS use, the median of e-liquid consumption per week was 15 mL, which is about 2 mL per day. However, the range was very wide, from 1 mL to 84 mL per week. Dual users also reported a lower ENDS use compared to ENDS only users. The concentrations of exhaled CO were consistent with the smoking status and the number of cigarette smoked per day. Exhaled CO concentrations were similar for ex-smokers and ENDS users, and higher in both smokers and dual users.

Figure 5 presents the boxplots of urinary TNE in ex-smokers, ENDS users, dual users, and smokers at 6-month follow-up (left plot), expressed as nmol/mg creatinine, and bar charts of changes of urinary TNE from baseline to 6-month follow-up (right plot), expressed as percentage of baseline (%). Dashed lines correspond respectively to medians of urinary TNE concentrations at baseline (left plot) and baseline urinary TNE concentrations (100%; right plot). Most ex-smokers were no longer exposed to nicotine. High concentrations were observed in some ex-smokers, mainly due to the use of NRT (data not shown). Smokers reported a 40% decrease in cigarettes per day from baseline to 6-month follow-up. TNE decreased by only 20%, which implied that participants might adjust their puffing regimes to absorb a similar dose of nicotine with a reduced number of cigarettes. This also indicated that a reduction in number of cigarettes per day is not necessarily associated with a proportional reduction in exposure to harmful compounds. Urinary TNE concentrations in ENDS users were significantly lower than in smokers at 6-month follow-up. One hypothesis would be that

smokers with lower nicotine addiction (i.e., who need less nicotine to satisfy craving) are more likely to use ENDS. To test this hypothesis, urinary TNE concentrations at baseline were compared between smokers (median 29.6 nmol/mg creatinine) and ENDS users (median 24.7 nmol/mg creatinine). The difference in TNE at baseline was not significant. This can be interpreted as nicotine addiction is not the only factor that may explain why ENDS users had lower TNE concentrations compared to smokers at 6-month follow-up. Nicotine addiction is also not uniquely related to nicotine dose. Dual users were the only group in which no difference in urinary TNE concentration from baseline was observed.

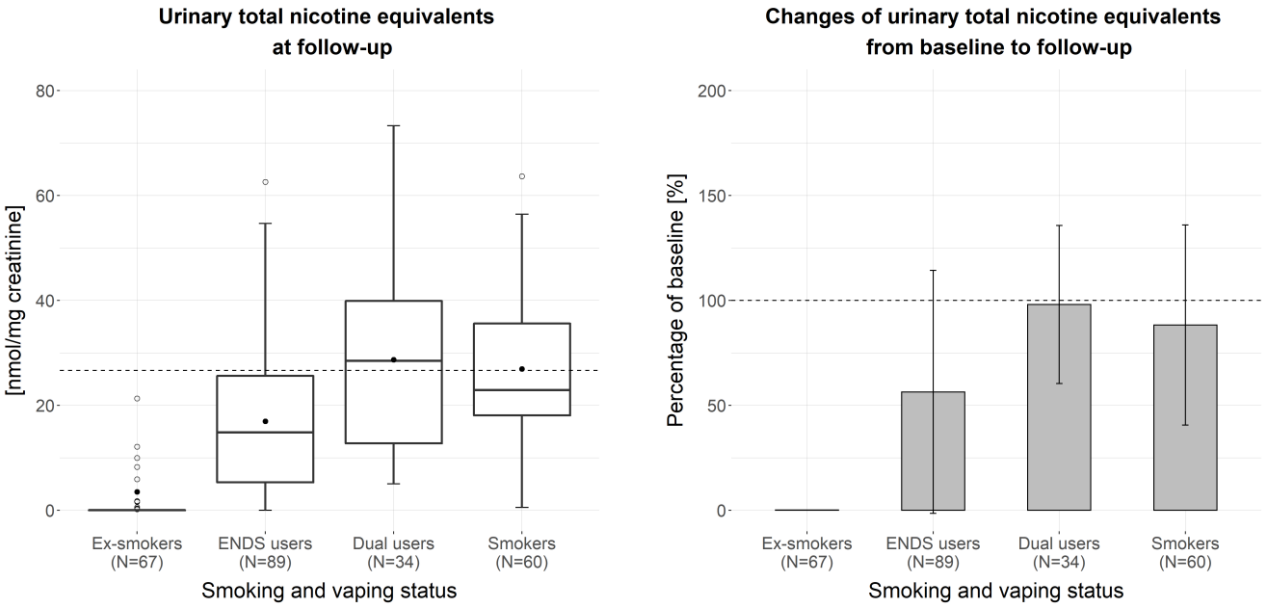


Figure 5 – Boxplots of urinary concentrations of total nicotine equivalent (TNE) in ex-smokers, ENDS users, Dual users, and smokers at 6-month follow-up (left) and bar charts of changes of urinary TNE concentrations from baseline to 6-month follow-up, expressed as percentage of baseline (right).

(left) Medians of urinary TNE concentrations at baseline are displayed as a dashed line. Outliers are displayed as empty circles. Note that y-axis has been truncated to ensure good result readability; several outliers may not be displayed in the graphs. Mean values of TNE concentrations are displayed as black circle.

(right) Medians with median absolute deviation are presented. Baseline concentrations (100%) are displayed as a dashed line.

Metabolites and parent compounds: TNE (nicotine).

The absence of tobacco use needed to be verified in both ex-smokers and ENDS users. Two highly specific BoEs for tobacco smoke exposures were used: anabasine and NNAL (Figure 6). Urinary anabasine concentrations <3 ng/mg creatinine is considered a non-tobacco user (Benowitz et al., 2020). A few participants were above this limit, as shown in Figure 1. The half-life of anabasine is relatively short (16 h), thus this identifies only recent exposures

(Jacob et al., 2002). NNAL, the NNK metabolite, has a longer half-life (10–18 days), which allows for the detection of exposure that took place 6 to 12 weeks prior to the sample time (Goniewicz et al., 2009). Cut-point for NNAL for distinguishing smokers from non-smokers was reported to be 14.4 pg/mL (Benowitz et al., 2018), which is half of the LOQ value in our method. A LOQ higher than the cut-point decrease the sensitivity of the biomarker and lead to misclassifications. Urinary NNAL and anabasine concentrations were greatly reduced in ex-smokers and ENDS users compared to dual users and smokers. Therefore, anabasine and NNAL are both effective biomarkers to verify the absence of tobacco use.

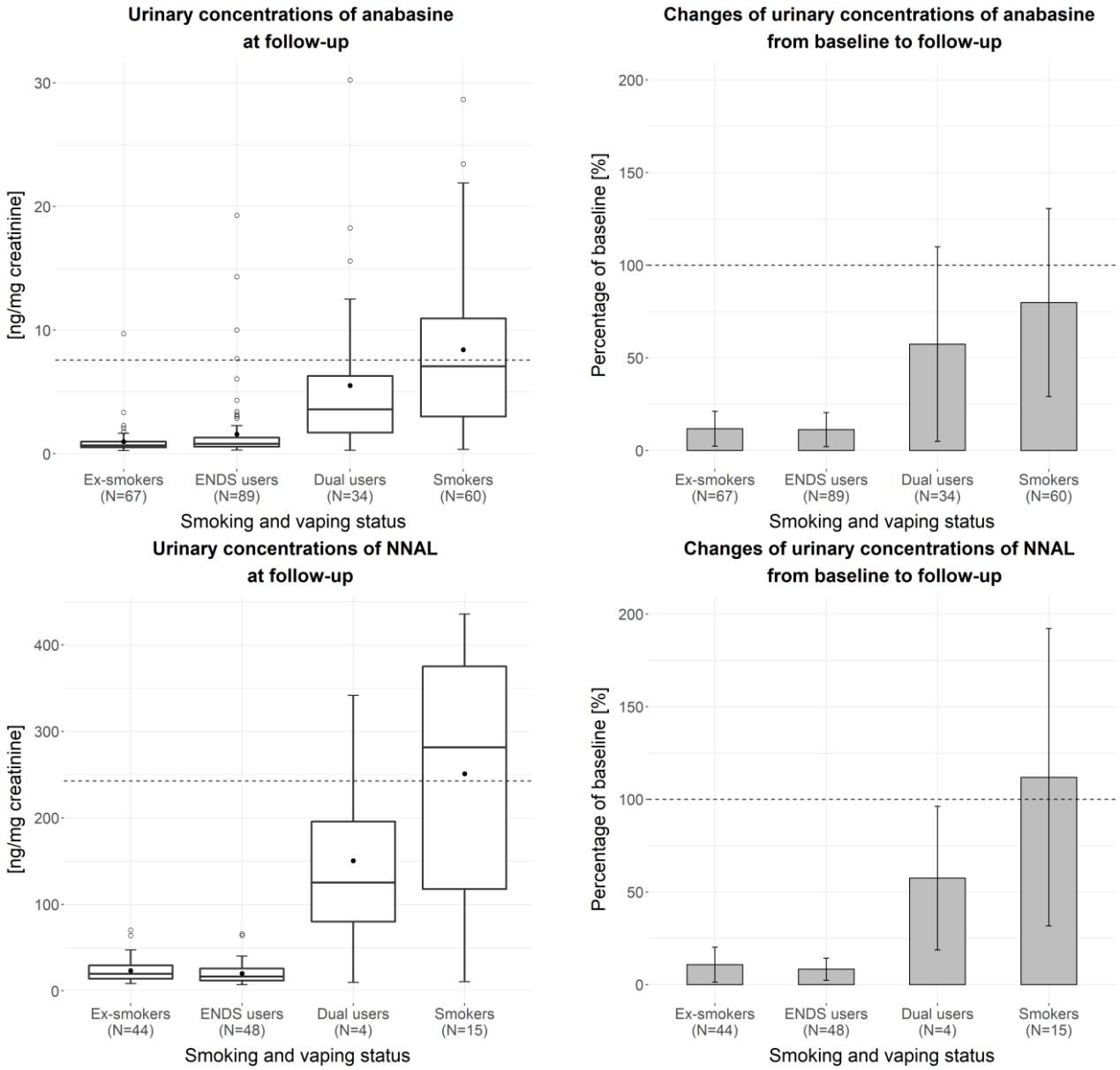


Figure 6 – Boxplots of urinary concentrations of anabasine and NNAL in ex-smokers, ENDS users, Dual users, and smokers at 6-month follow-up (left) and bar charts of changes of urinary anabasine and NNAL concentrations from baseline to 6-month follow-up, expressed as percentage of baseline (right).

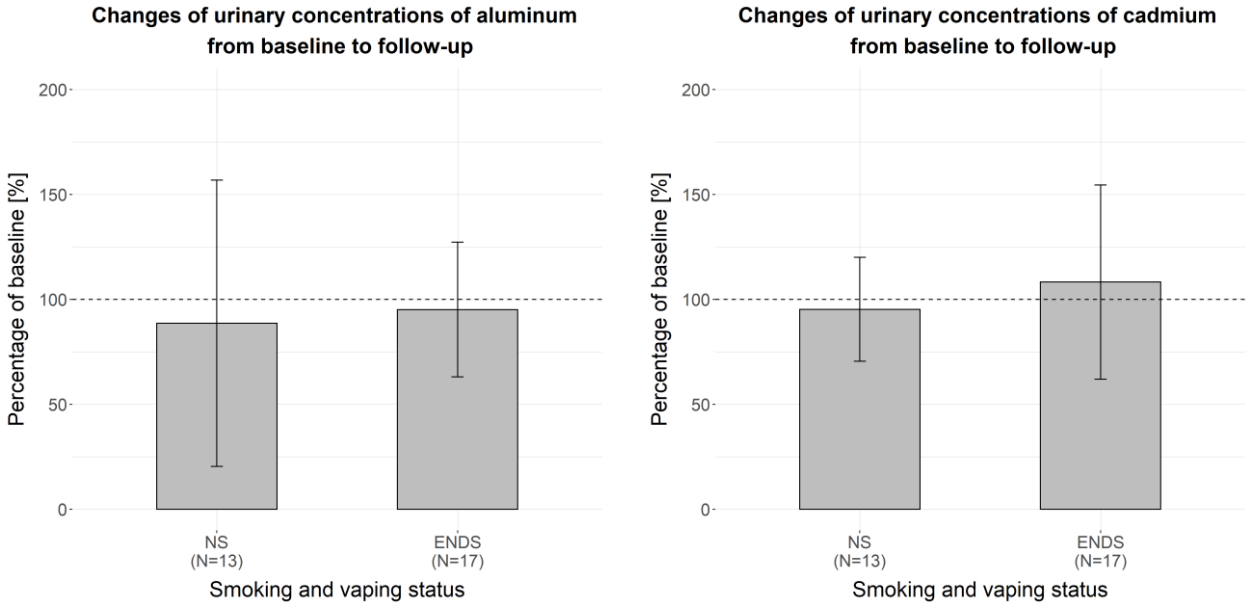
(left) Medians of urinary anabasine and NNAL concentrations at baseline are displayed as a dashed line. Outliers are displayed as empty circles. Note that y-axis has been truncated to ensure good result readability; several outliers may not be displayed in the graphs. Mean values of anabasine and NNAL concentrations are displayed as black circle.

(right) Medians with median absolute deviation are presented. Baseline concentrations (100%) are displayed as a dashed line.

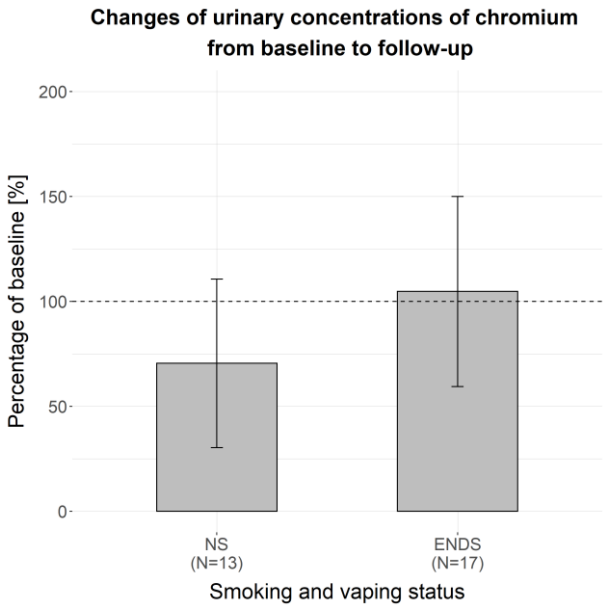
Metabolites and parent compounds: anabasine (-), NNAL (NNK).

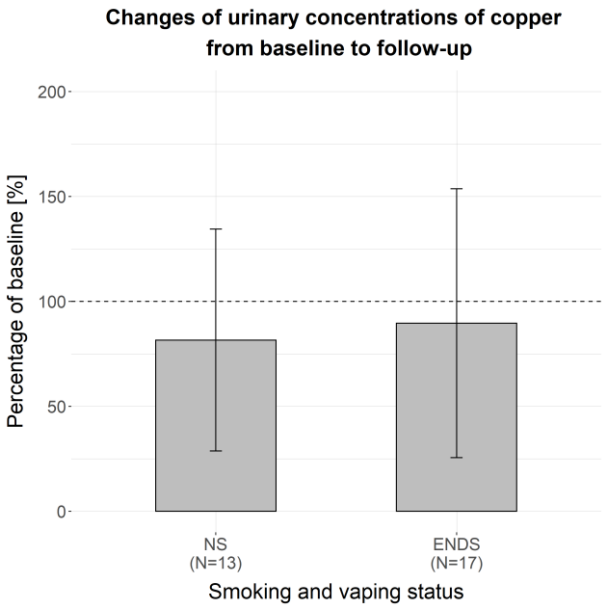
Furthermore, urinary anabasine concentrations were reduced in dual users and smokers compared to baseline, which is in agreement with the decreased number of cigarettes per day reported. Urinary NNAL concentrations were not reduced in dual users and smokers compared to baseline despite the cigarette consumption reduction; however, this could be related to the smaller sample sizes as urinary NNAL concentrations were not analyzed in all participants' urine samples. Only 127 samples (47%) were analyzed for urinary NNAL concentrations due to financial constraints.

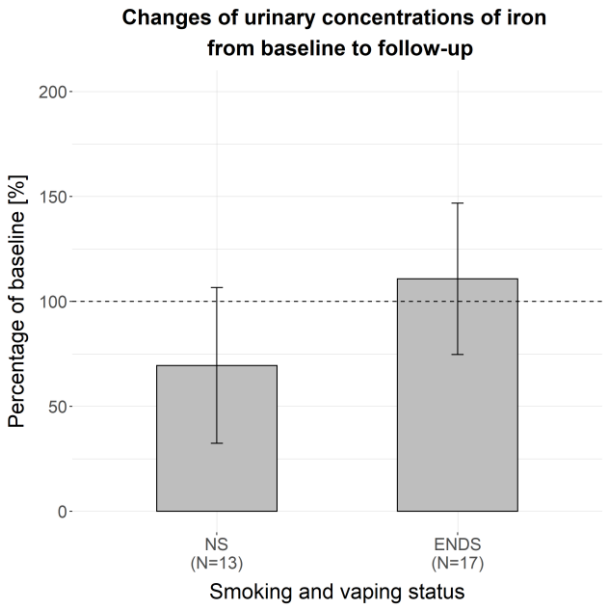
Heavy metals and trace elements were found in ENDS emissions (Chapter 3). In particular, concentrations of Al, Fe, Ni, Cu, Zn, and Pb were above 100 ng/g e-liq in the aerosols. None of these metals was present in greater amounts in urine of ENDS users compared to ex-smokers (Figure 7). Overall, the median concentrations of the majority of metals were below LOQs, with the exception of As (100% above LOQ), Cd (50% above LOQ), Cu (100% above LOQ), Fe (100% above LOQ), Mo (100% above LOQ), Ni (100% above LOQ), Se (100% above LOQ), Sn (50% above LOQ), and Zn (100% above LOQ). However, the concentrations were not different between groups. These metals are essential trace elements or additional trace elements, except arsenic (Bhattacharya et al., 2016). Median concentrations were equivalent or lower than the reference values (RV_{95} in µg/l) reported in the Canadian and other populations (Saravanabhavan et al., 2017). No differences in urinary metal concentrations were found between ex-smokers and ENDS users as reported in several studies (Subchapter 1.8). However, the small sample size (i.e., thirteen ex-smokers and seventeen ENDS users) could lead to decreased statistical power. Other sources of exposure to metals (e.g., diet, environment, or occupation) may be more important than exposure to cigarette smoke or ENDS aerosol.

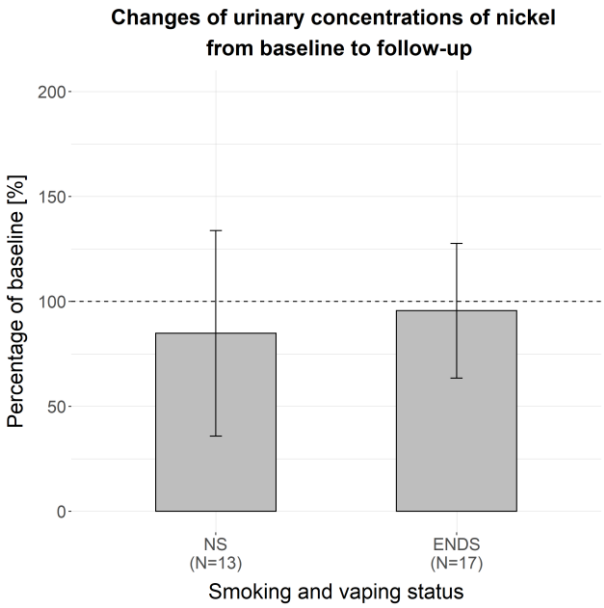

Changes of urinary concentrations of aluminum from baseline to follow-up
Percentage of baseline [%]
200
150
100
50
0
NS (N=13)
ENDS (N=17)
Smoking and vaping status

Changes of urinary concentrations of cadmium from baseline to follow-up
Percentage of baseline [%]
200
150
100
50
0
NS (N=13)
ENDS (N=17)
Smoking and vaping status


Changes of urinary concentrations of chromium from baseline to follow-up
Percentage of baseline [%]
200
150
100
50
0
NS (N=13)
ENDS (N=17)
Smoking and vaping status


Changes of urinary concentrations of copper from baseline to follow-up
Percentage of baseline [%]
200
150
100
50
0
NS (N=13)
ENDS (N=17)
Smoking and vaping status


Changes of urinary concentrations of iron from baseline to follow-up
Percentage of baseline [%]
200
150
100
50
0
NS (N=13)
ENDS (N=17)
Smoking and vaping status


Changes of urinary concentrations of nickel from baseline to follow-up
Percentage of baseline [%]
200
150
100
50
0
NS (N=13)
ENDS (N=17)
Smoking and vaping status

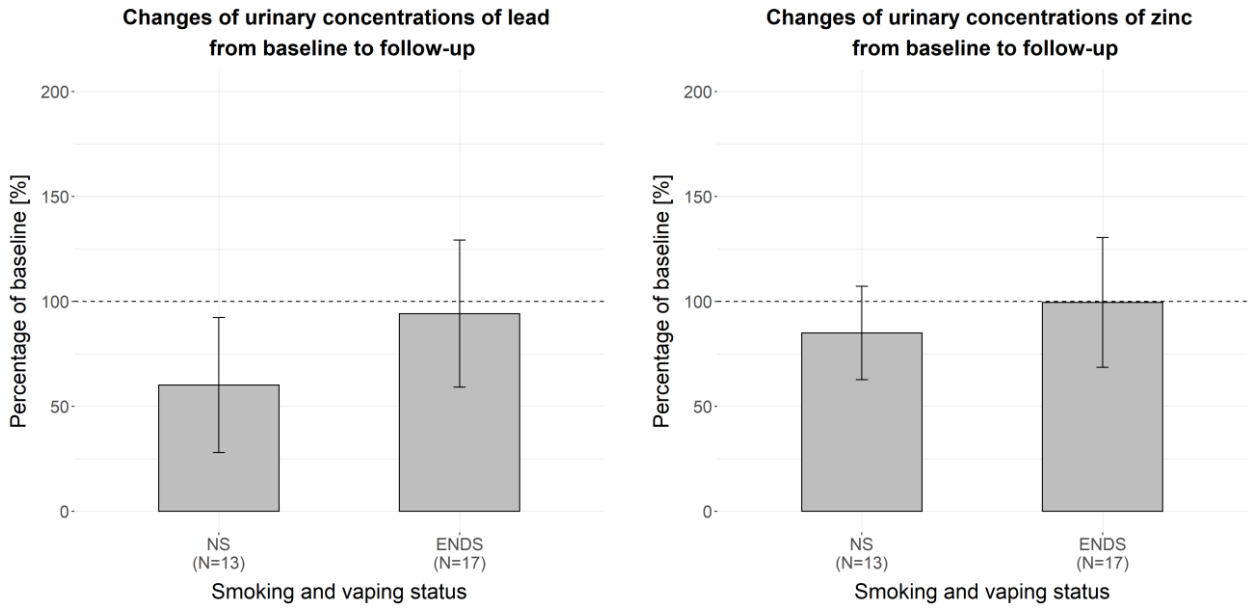
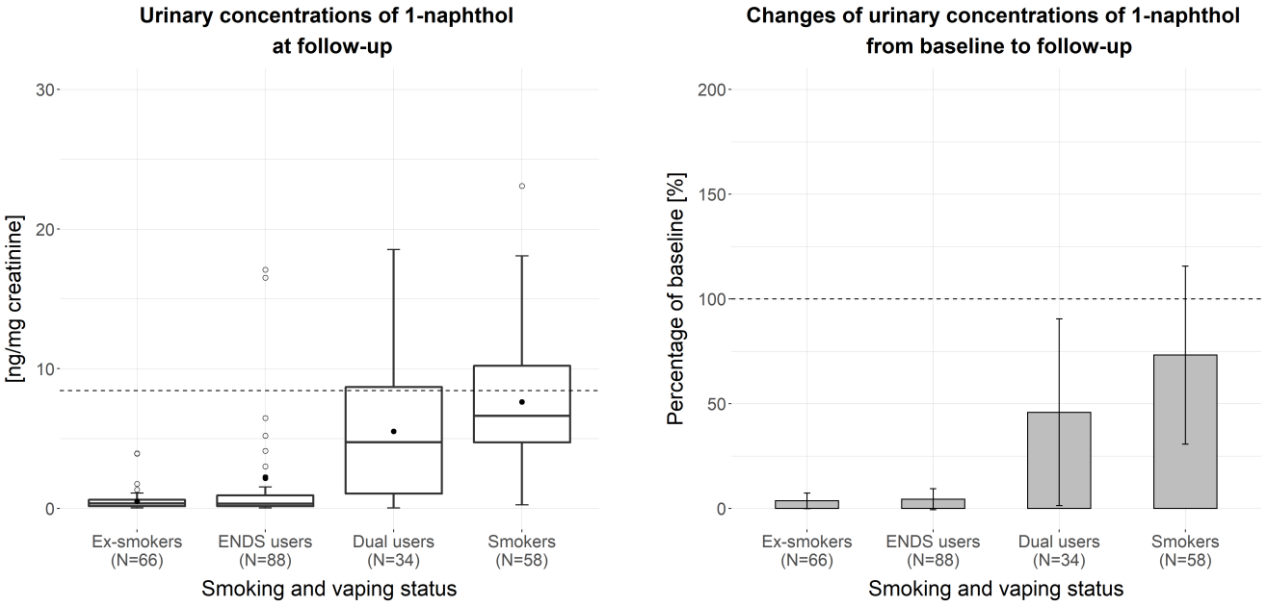


Figure 7 – Bar charts of changes of urinary metal concentrations from baseline to 6-month follow-up, expressed as percentage of baseline (%), in ex-smokers, ENDS users, Dual users, and smokers. Medians with median absolute deviation are presented. Baseline concentrations (100%) are displayed as a dashed line.

Urinary concentration of BoE to PAHs were similar between ex-smokers and ENDS users (Figure 8), which is consistent with the characterization of ENDS aerosols (Chapter 3) as naphthalene (parent compound of both 1-naphtol and 2-naphtol) was not detected. PAH metabolite concentrations were higher in dual and in smokers (see Table S2). Median urinary concentrations of 1-OHP were under LOQ for ex-smokers, ENDS users and dual users, which limits the use of this biomarker in exposure assessment in this sub-study.



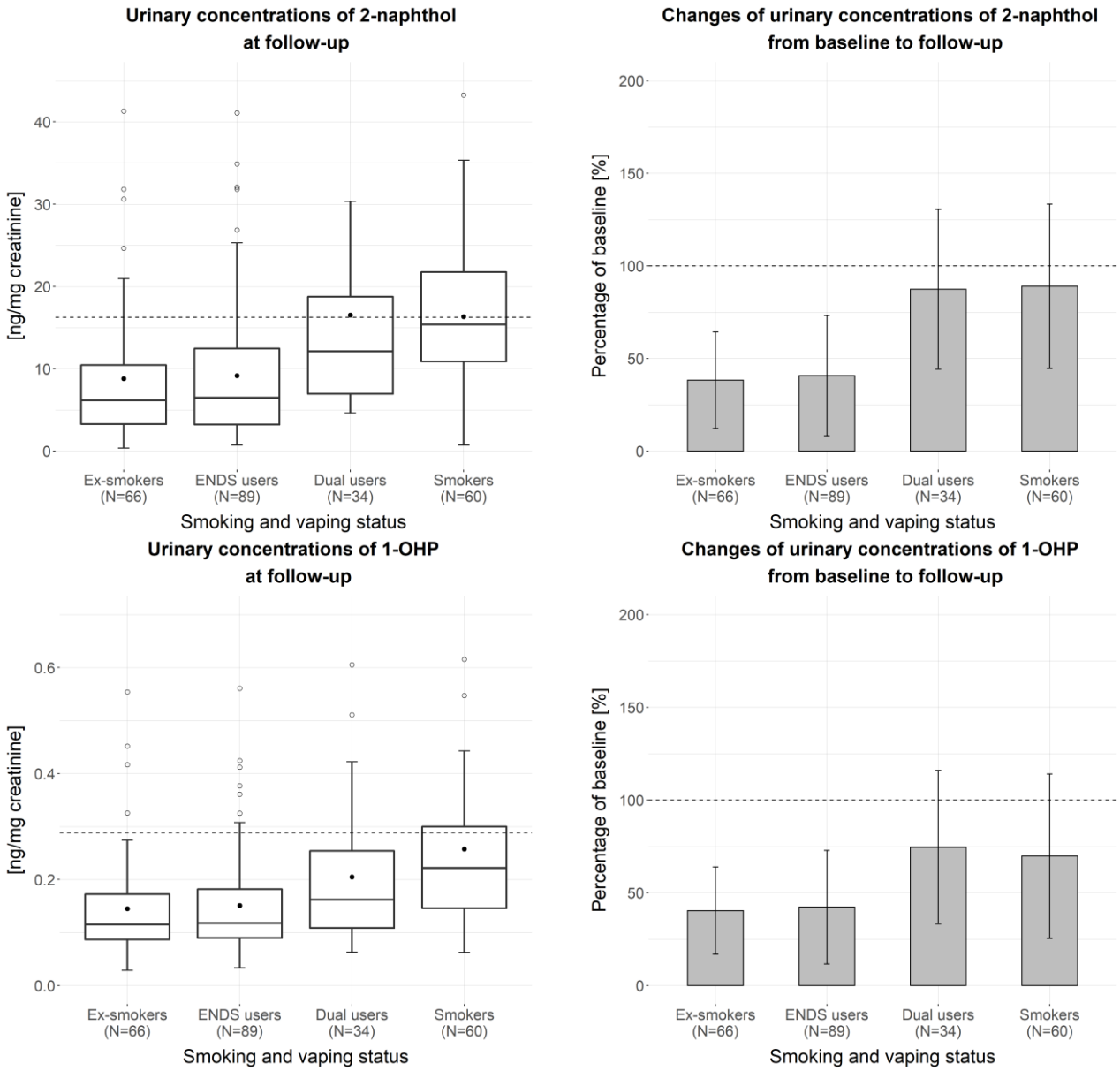


Figure 8 – Boxplots of urinary PAH concentrations in ex-smokers, ENDS users, Dual users, and smokers at 6-month follow-up (left) and bar charts of changes of urinary PAH concentrations from baseline to 6-month follow-up, expressed as percentage of baseline (right).

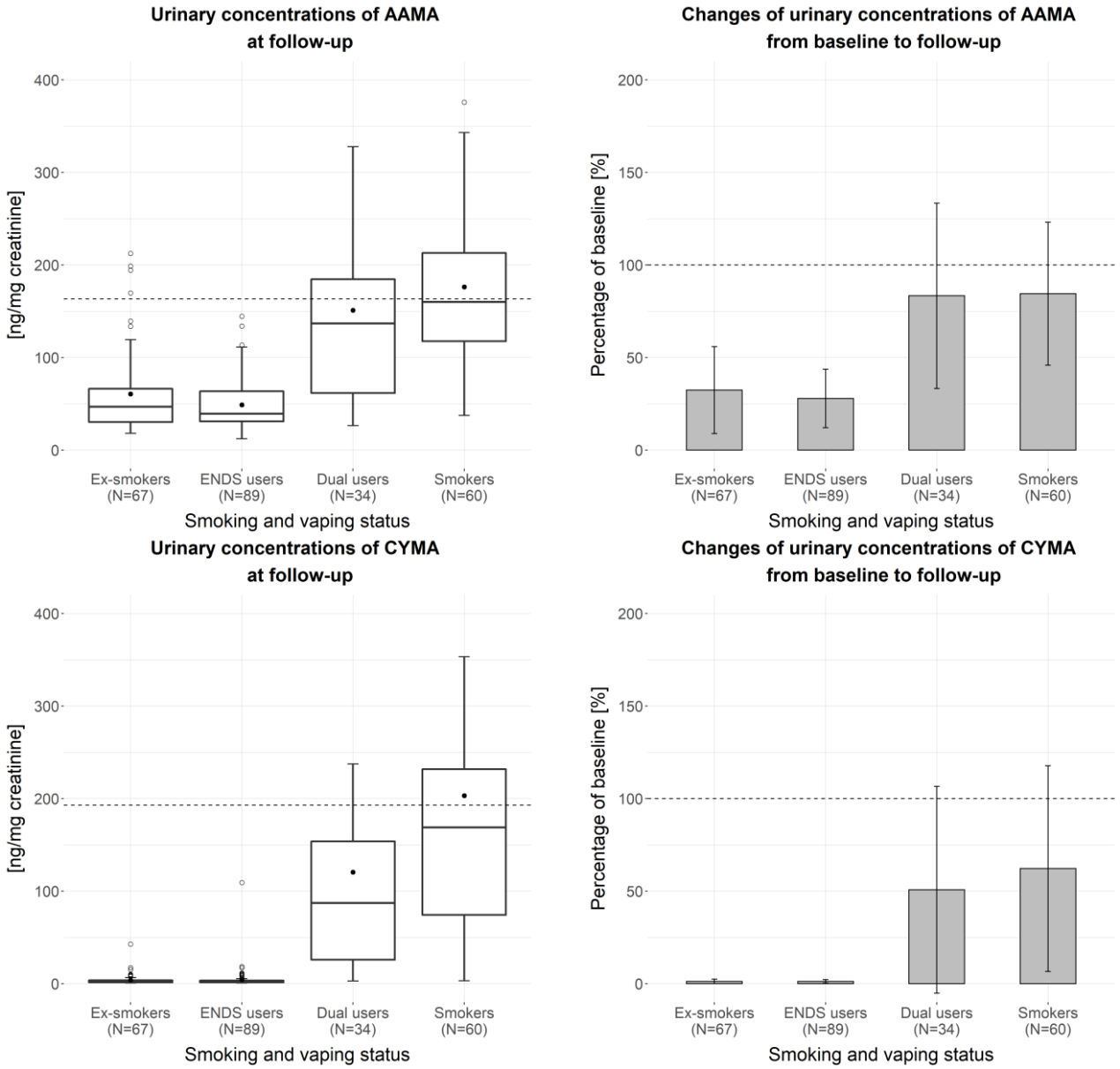
(left) Medians of urinary PAH concentrations at baseline are displayed as a dashed line. Outliers are displayed as empty circles. Note that y-axis has been truncated to ensure good result readability; several outliers may not be displayed in the graphs. Mean values of PAH concentrations are displayed as black circle.

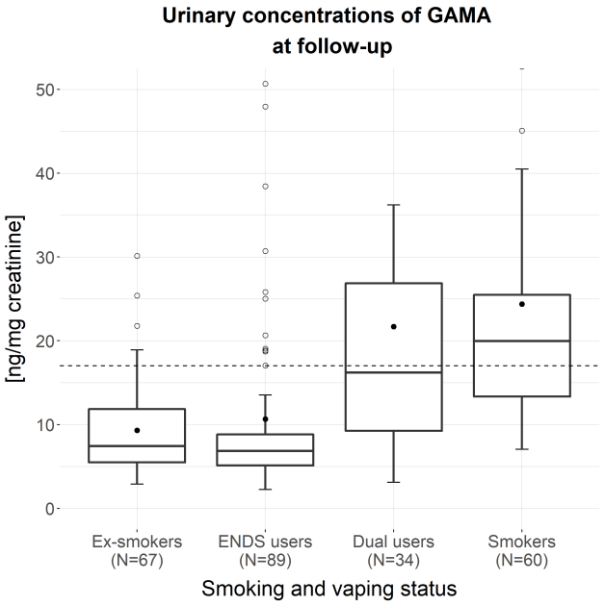
(right) Medians with median absolute deviation are presented. Baseline concentrations (100%) are displayed as a dashed line.

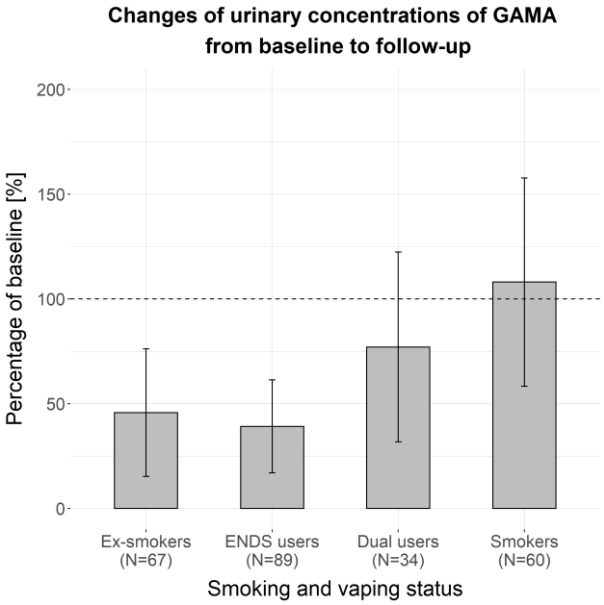
Metabolites and parent compounds: 1-naphtol and 2-naphtol (naphthalene), and 1-OHP (pyrene).

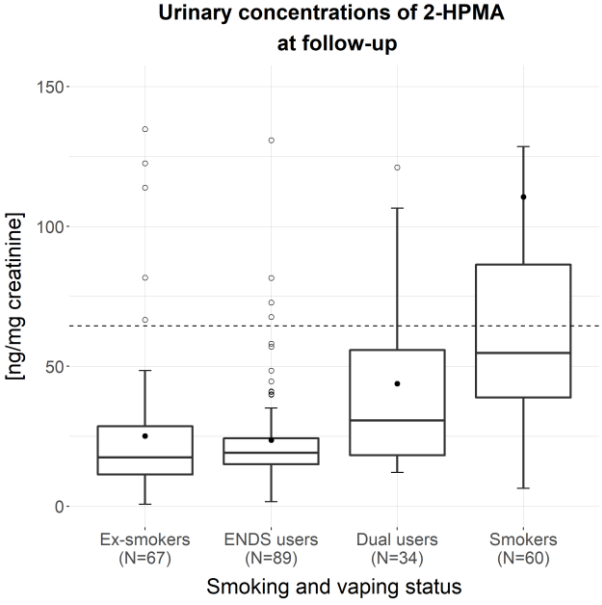
Acrolein, 1,3-butadiene, acrylamide, acrylonitrile, and crotonaldehyde were not detected in ENDS aerosols (Chapter 3). Urinary concentrations of their metabolites (3-HPMA, 3-MHBMA,

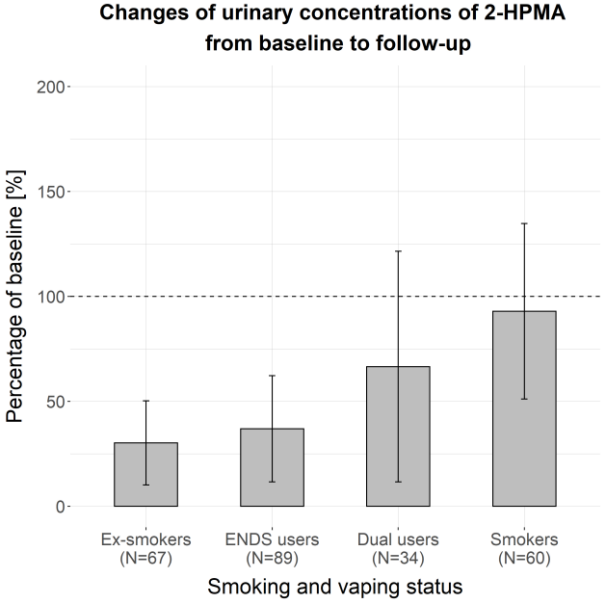
AAMA, GAMA, CYMA, and HPMMA) were similar between ex-smokers and ENDS users (Figure 9), which is in agreement with the laboratory results. These two groups not exposed to cigarette smoking still had quantifiable concentrations of VOC metabolites, highlighting the importance of environmental exposure in human biomonitoring studies. Similarly, VOC metabolite concentrations were higher in smokers and dual users (see Table S2). Median urinary concentrations of SPMA (benzene metabolite) of the four groups were under LOQ.

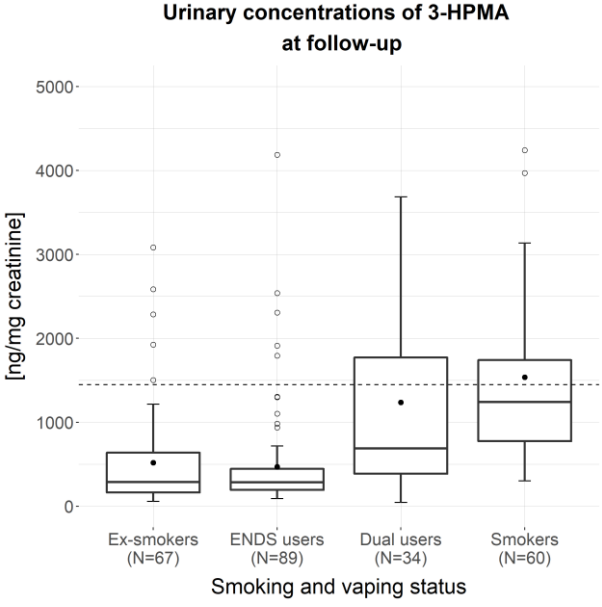


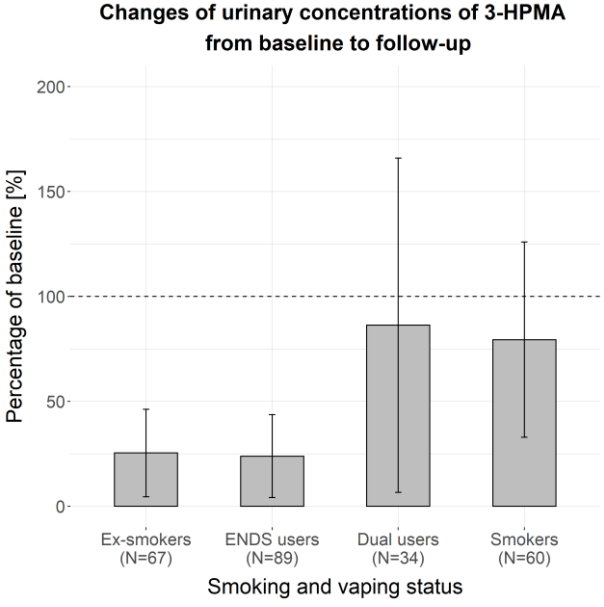

Urinary concentrations of GAMA
at follow-up
[ng/mg creatinine]
0
10
20
30
40
50
Ex-smokers (N=67)
ENDS users (N=89)
Dual users (N=34)
Smokers (N=60)
Smoking and vaping status


Changes of urinary concentrations of GAMA
from baseline to follow-up
Percentage of baseline [%]
0
50
100
150
200
Ex-smokers (N=67)
ENDS users (N=89)
Dual users (N=34)
Smokers (N=60)
Smoking and vaping status


Urinary concentrations of 2-HPMA
at follow-up
[ng/mg creatinine]
0
50
100
150
Ex-smokers (N=67)
ENDS users (N=89)
Dual users (N=34)
Smokers (N=60)
Smoking and vaping status


Changes of urinary concentrations of 2-HPMA
from baseline to follow-up
Percentage of baseline [%]
0
50
100
150
200
Ex-smokers (N=67)
ENDS users (N=89)
Dual users (N=34)
Smokers (N=60)
Smoking and vaping status


Urinary concentrations of 3-HPMA
at follow-up
[ng/mg creatinine]
0
1000
2000
3000
4000
5000
Ex-smokers (N=67)
ENDS users (N=89)
Dual users (N=34)
Smokers (N=60)
Smoking and vaping status


Changes of urinary concentrations of 3-HPMA
from baseline to follow-up
Percentage of baseline [%]
0
50
100
150
200
Ex-smokers (N=67)
ENDS users (N=89)
Dual users (N=34)
Smokers (N=60)
Smoking and vaping status

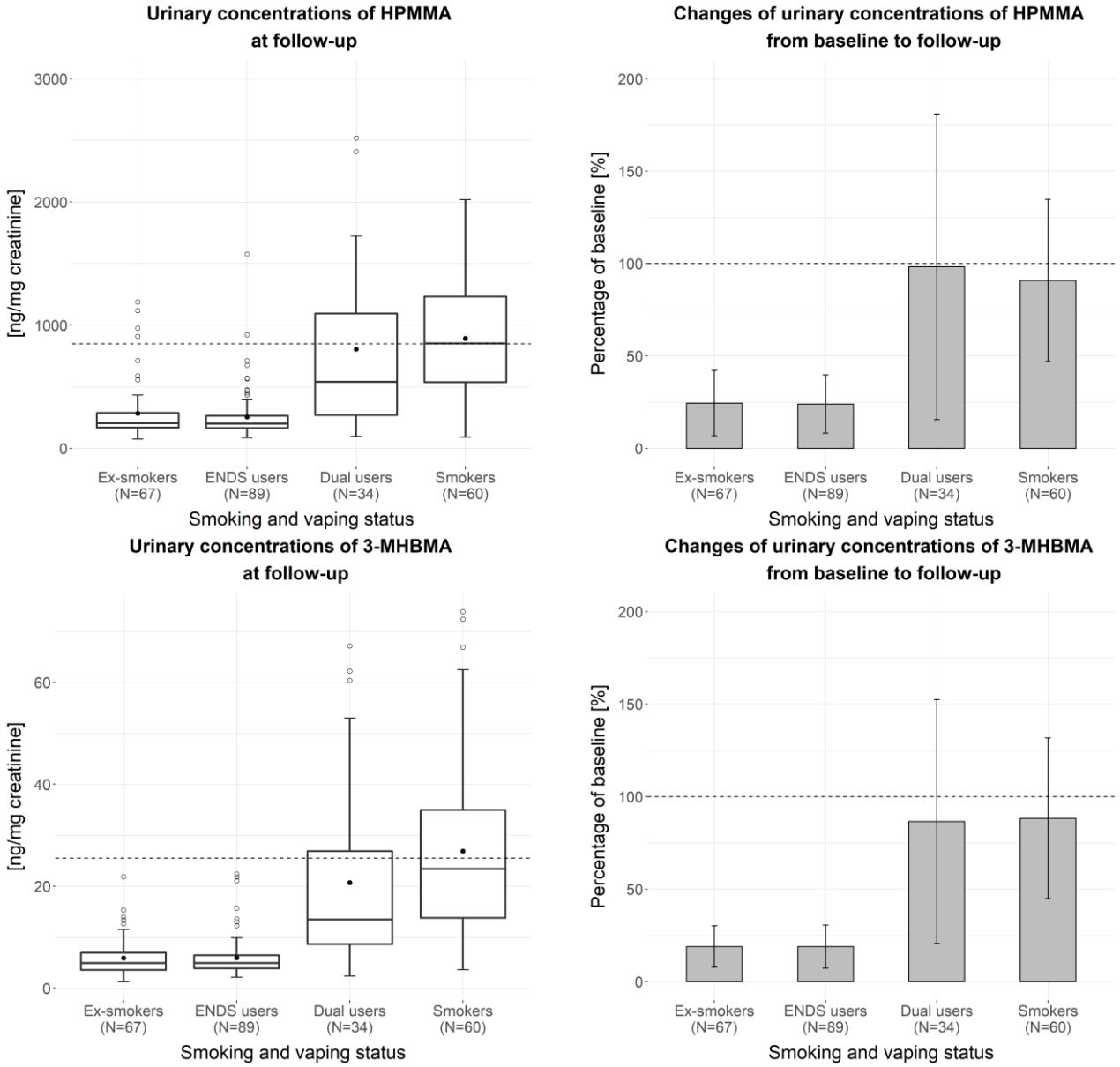


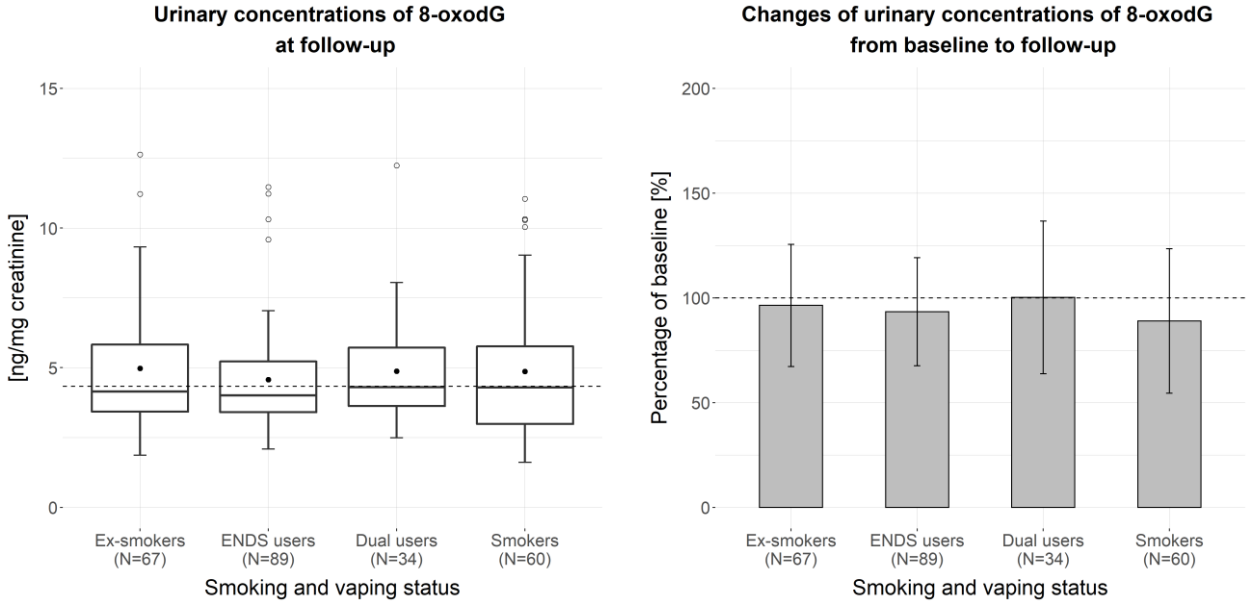
Figure 9 – Boxplots of urinary VOC concentrations in ex-smokers, ENDS users, Dual users, and smokers at 6-month follow-up (left) and bar charts of changes of urinary VOC concentrations from baseline to 6-month follow-up, expressed as percentage of baseline (right).

(left) Medians of urinary VOC concentrations at baseline are displayed as a dashed line. Outliers are displayed as empty circles. Note that y-axis has been truncated to ensure good result readability; several outliers may not be displayed in the graphs. Mean values of BoE concentrations are displayed as black circle.

(right) Medians with median absolute deviation are presented. Baseline concentrations (100%) are displayed as a dashed line.

Metabolites and parent compounds: 3-MHBMA (1,3-butadiene), 3-HPMA (acrolein), AAMA and GAMA (acrylamide), CYMA (acrylonitrile), HPMMA (crotonaldehyde), 2-HPMA (propylene oxide).

Positive associations between biomarkers of oxidative stress and BoE to tobacco smoke were previously observed (Chapter 5), suggesting that exposure to PAHs and VOCs might increase oxidative stress level. In the context of smoking cessation, exposure to these harmful compounds drops drastically and therefore concentrations of oxidative stress biomarkers can be expected to drop to some extent (e.g., a 50% decrease in ΣPAH score would result in a 4% decrease in 8-oxodG concentration). However, urinary concentrations of oxidative stress biomarkers were similar between baseline and 6-month follow-up and did not differ between groups (Figure 10). In a recent meta-analysis, urinary 8-isoprostane concentration median for the general adult population was reported to be 0.249 ng/mg creatinine (0.236–0.407 ng/mg creatinine; IQR: 25%-75%) for smokers (BMI ≤25 kg/m^2) and 0.232 ng/mg creatinine (0.159–0.276 ng/mg creatinine; IQR: 25%-75%) for non-smokers (BMI ≤25 kg/m^2), which is similar to what was found in this sub-study (Graille et al., 2020a). However, no consistent effect of smoking was observed, which might explain why a reduction of oxidative stress levels following smoking cessation was not observed. In a second meta-analysis, urinary 8-oxodG concentration median for the general adult population was reported to be 22.2 ng/mg creatinine (3–41.4 ng/mg creatinine; IQR: 25%-75%) for smokers (BMI ≤25 kg/m^2) and 4.3 ng/mg creatinine (2.9–5.5 ng/mg creatinine; IQR: 25%-75%) for non-smokers (BMI ≤25 kg/m^2) (Graille et al., 2020b). Median concentrations measured in this substudy were similar to those of non-smokers. While the authors reported that concentrations in smokers were indeed greater than in non-smokers, they cautioned that, this result (for BMI ≤25 kg/m^2) was based on a single study and still needed to be confirmed.



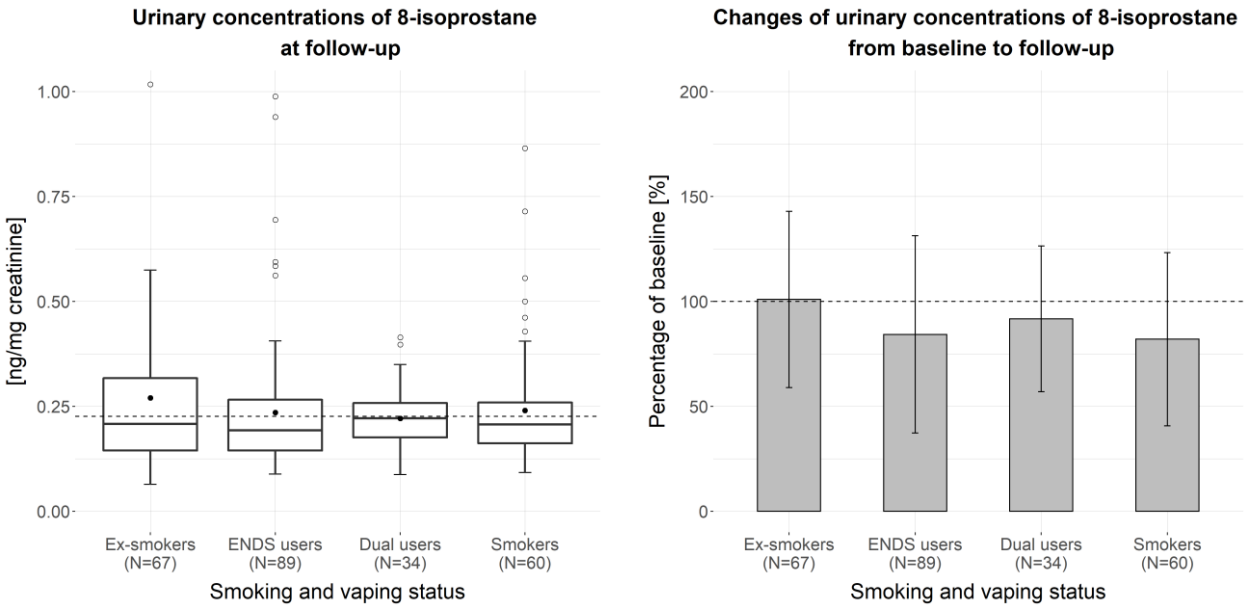


Figure 10 – Boxplots of urinary concentrations of oxidative stress biomarkers in ex-smokers, ENDS users, Dual users, and smokers at 6-month follow-up (left) and bar charts of changes of urinary concentrations of oxidative stress biomarkers from baseline to 6-month follow-up, expressed as percentage of baseline (right).

(left) Medians of urinary concentrations of oxidative stress biomarkers at baseline are displayed as a dashed line. Outliers are displayed as empty circles. Note that y-axis has been truncated to ensure good result readability; several outliers may not be displayed in the graphs. Mean values of oxidative stress biomarkers concentrations are displayed as black circle.

(right) Medians with median absolute deviation are presented. Baseline concentrations (100%) are displayed as a dashed line.

These results highlight the fact that oxidative stress biomarkers are not exposure specific, and they are influenced by other individual, behavioral or environmental factors. Cigarette smoke contains free radicals and induces free radical formation in the body. However, the increased presence of free radicals will not necessarily lead the organism into a state of oxidative stress. Indeed, the body has several means of defense (e.g., antioxidant molecules or enzymes) to maintain the cellular redox homeostasis. In such case, damages to DNA and lipids leading respectively to the formation of 8-oxodG and 8-isoprostane may not be increased up to a certain point (e.g., the development of a disease).

Although, the majority of urinary biomarkers analyzed showed significant differences between smokers and ex-smokers; however, none of these 40 biomarkers (nicotine metabolites not included) could distinguish ex-smokers from ENDS users. Heavy metals, trace elements, and oxidative stress biomarkers were not different between smoking statuses. Switching from cigarettes to ENDS resulted in greatly reduced urinary biomarker concentrations, and

consequently, by reducing exposures to many harmful compounds, including carcinogens, the overall risk of disease decreases. The selected oxidative stress biomarkers, 8-oxodG and 8-isoprostane, did not show any effect of smoking or vaping on oxidative stress levels in our population. The underlying mechanisms of induction of oxidative stress are complex and influenced by multiple factors and further studies with large sample sizes should explore the relationship between oxidative stress biomarkers and environmental and lifestyle factors. These steps are necessary before these markers can be evaluated as clinical diagnostics.

Chapter 7 – General discussion

7.1 Exposure assessment to harmful compounds of ENDS aerosols

The composition of e-liquid, the fuel of ENDS, is not complex: it is mainly composed of a mixture of propylene glycol (PG) and vegetable glycerin (VG), in which flavorings and nicotine are added. Except nicotine, all ingredients have low oral toxicity: they are generally recognized as safe (GRAS) for ingestion and used as food additives (FDA & FEMA). However, the heating element in contact with e-liquid behaves like a small chemical reactor. Although the temperatures reached are between 250 and 350°C, some thermolabile molecules (e.g., flavorings) can be degraded and form other compounds not present in the original mixture. In the last 10 years, ENDS have been subject of numerous researches to identify harmful compounds in their emissions. Most that have been previously looked for – not necessarily found – in ENDS emissions were the same that have been identified and quantified in cigarette smoke from the FDA's list of harmful and potentially harmful compounds in tobacco products. They included the following chemical families: aldehydes, metals, PAHs, TSNAs, and VOCs. Most studies reported lower concentrations of these compounds in ENDS aerosols compared to cigarette smoke, mainly because combustion is absent in these new products (Chapter 1).

Nevertheless, some aldehydes were found in ENDS emissions, including formaldehyde (carcinogenic to human – Group 1 IARC), acetaldehyde (possibly carcinogenic to human – Group 2B IARC), and sometimes acrolein (probably carcinogenic to human – Group 2A IARC). These compounds result from the thermal degradation of PG and VG and are the mark of the (weak) pyrolysis present in ENDS. The concentrations measured in the emissions of the device we selected were low (<10 µg/g e-liq in average). As there are no "safe doses" for carcinogens, the precautionary principle would imply not selling and using these products. However, we calculated that the participants in our study were exposed, on average, to 7 times and 200 times more formaldehyde and acetaldehyde, respectively, by smoking cigarettes compared to vaping. Therefore, in a risk reduction approach, smokers should be encouraged to vape instead of smoking. We also compared our results with environmental values (i.e., indoor air) and occupational limits (i.e., short-term exposure limit (STEL) in Switzerland) to facilitate the interpretation of aldehyde concentrations. Although these were low, all exposures are cumulative, and the sources of exposure should be avoided whenever possible. In addition, the personal preferences in vaping the ENDS induce a high inter-individual variability. For

example, the calculations were based on an average e-liquid consumption per day of 3 mL/day, while the maximum reported consumption was four times higher (12 mL/day). Therefore, ENDS users could be exposed to formaldehyde concentrations similar to those of light smokers (5 cig/day). In such instances, ENDS users should use e-liquid with increased concentrations of nicotine with the aim of reducing their consumption. Nevertheless, the daily exposure resulting from vaping 12 mL of e-liquid per day (84 µg/day) would still be at least 10 times lower than the daily exposure resulting from breathing (1 mg/day) and from food (1.5-14 mg/day) estimated by the WHO for an average adult (World Health Organization. Regional Office for Europe, 2000). Regular change of coils is also important to avoid the device from overheating. With repeated uses, they can become clogged, which can reduce the supply of e-liquid and create areas of high temperatures (dry puffs). The aerosols would contain thermally degraded by-products, including aldehydes. Although ENDS users should experience a degradation of flavors, even a burnt taste, they should be informed of the need to prevent these dry puffs.

Metals were also identified and quantified in ENDS emissions. Indeed, metals on the surface of the coil or other parts of the device can leach in solution and end up in the aerosol droplets after vaporization. Intake of metals have been linked to cardiovascular diseases (CVD), kidney toxicity, neurotoxicity, and cancer. The six metals we found in concentrations greater than 100 ng/g e-liq in aerosols (Al, Fe, Ni, Co, Zn, and Pb) were below the minimal risk levels (MRLs), the permitted daily exposures (PDEs) and the recommended exposure limits (RELs). Therefore, metal concentrations were likely to be without appreciable risk of adverse health effects for the users according to the Agency for Toxic Substances and Disease Registry (considering only this source of exposure and without considering cancer risks). Moreover, the tested device would meet the European Medicines Agency requirements regarding the metal emissions, if it were considered a medical product. Metal intake via food can be greater than what we found in ENDS emissions. For example, the European Food Safety Authority has estimated the daily lead dietary exposure of an adult to be 0.5 µg/kg b.w. per day (European Food Safety Authority, 2012). For an adult of 70 kg, the contribution of ENDS would represent only 2% of what is ingested through food. However, as mentioned in the last paragraph, the exposures are cumulative and ENDS manufacturers have a responsibility to use good quality raw materials and ensure a design that minimizes metal leaching.

Several research groups have also investigated impurities present in the e-liquid ingredients and flavorings. While the concentrations of impurities are generally very low or even negligible when the ingredients are of good quality, flavorings are present in high concentrations (2.8 mg/mL; median concentration) (Krüsemann et al., 2021). Flavorings are generally

recognized as safe for ingestion, but their inhalation safety is not assured. Thus, toxicological risk evaluations of these compounds should be conducted to better regulate them and restrict their use if necessary. Users should be made aware of the lack of information regarding inhaled compounds, especially with the emergence of "do it yourself" (DIY) e-liquids in which users dose themselves concentrates of flavorings. In addition, aldehydes (including some flavorings) can react with propylene glycol to form hemiacetals whose toxicological properties remain unknown at this time.

Daily exposure to ENDS emissions is a chronic exposure. The effects from multiple exposures, even at low concentrations, are unknown. Thus, potential long-term effects of vaping cannot be excluded and cohorts of ENDS users should be followed to characterize them. Paradoxically, ENDS users who never smoked would be the most appropriate study population to evaluate these effects, because years of smoking are an important confounding factor. Indeed, the effects of smoking appear mostly after decades, which explains the many years of exposures needed to establish causal links between smoking and disease developments.

Human biomonitoring is a powerful tool to track exposure to harmful compounds from ENDS emissions. The concentrations of these compounds inhaled from the ENDS users varied greatly because ENDS emissions are dependent on multiple factors, such as battery settings (power, voltage), coil resistance and material, temperature, type of device, type of e-liquid and consumption, and puffing regime. Not all these parameters can be replicated in a laboratory for practical and financial reasons. Analysis of BoE to harmful compounds in the urine samples of the ESTxENDS participants indicated no differences between the biomarker concentrations in ex-smokers and ENDS users. This suggested that vaping does not expose participants to higher concentrations of harmful compounds than they are exposed to on a daily basis (e.g., from the environment or from food). This is consistent with the absence of tobacco related-PAHs, TSNAs, and VOCs in the ENDS emissions analyses. However, formaldehyde and acetaldehyde, which were quantified in ENDS emissions, cannot be measured by biomonitoring because there are no specific biomarkers for these substances. Concerning metals, the contribution from ENDS is probably not sufficiently large compared to the contribution from food, which may explain why no differences were observed between ENDS users and ex-smokers. The small number of participants for this analysis (<20 participants per group) may also explain why this small difference was not significant. Moreover, the medians of half of the metals were below the limits of quantifications, which means that only analytical instruments with high precision (pg/mL) can quantify them.

Self-reported smoking status of participants was verified with urinary concentrations of anabasine, cotinine, exhaled CO, and NNAL. After removing fourteen misclassified participants, sensitivity (i.e., the ability to identify smoking status in smokers or dual users) and specificity (i.e., the ability to identify non-smoking status in ex-smokers or ENDS users) were calculated for each biomarker based on the cut-points chosen in Chapter 6.

Cotinine and NNAL were the biomarkers with the highest sensitivities: 100% and 93-99%. Sensitivities of exhaled CO (78-93%) and anabasine (80-84%) were lower, which implied a greater risk of misclassification of smokers. Biomarker sensitivities decreased to 60 to 75% for dual users, except for cotinine (100%). Dual users in ESTxENDS reported a low cigarette per day consumption (3 cig/day, median), which may explain the decreased sensitivities observed. Exhaled CO and NNAL were the biomarkers with the highest specificity: 100% for both. Specificity of anabasine was lower (84-92%). Concerning cotinine, this parameter was not applicable to ENDS users as most e-liquids contained nicotine. Specificity of cotinine for ex-smokers was 75% and it can be explained by the use of nicotine replacement therapy (NRT). Therefore, considering both sensitivity and specificity, NNAL appeared to be the biomarker of choice for distinguishing between dual users/smokers from ex-smokers/ENDS users. However, nicotine metabolites (e.g., cotinine) are the only biomarkers that can separate ex-smokers and ENDS users, although ENDS users vaping e-liquid without nicotine or ex-smokers using NRT would by misclassified.

Several biomarkers of exposure to PAHs and VOCs required high sensitivity of the analytical instrument (in pg/mL), such as 1-hydroxypyrene, HEMA, 1-MHBMA + 2-MHBMA, and SPMA. Most of their medians were under the limits of quantification in our study, indicating that our analytical instrument was not sufficiently sensitive. 1-hydroxypyrene is an important PAH biomarker, because it represents the overall PAH mixture exposure. Both 1-naphthol and 2-naphthol were greatly influenced by cigarette smoking. All three PAH biomarkers are quantified in the same method; therefore, their number does not need to be reduced although naphthols reflect the same information (effect of smoking). The same situation applies to VOCs that can be analyzed in a single analytical method.

The ESTxENDS study allowed investigating the effects of smoking cessation on exposure to harmful compounds with three main advantages: the use of a single device and six selected e-liquids, the collection of two urine samples per participants (at baseline and at 6-month follow-up) and its randomized controlled trial design. Based on the results obtained, smokers are recommended to stop smoking completely as is the recommendation today. Smokers could lower their exposures to hazardous compounds with the help of ENDS if needed. The

use of ENDS by non-smokers should be strongly discouraged since these devices still emit concentrations of harmful compounds. Regarding the analysis of ENDS emissions, the focus should now be on flavorings and their potential degradation products. These compounds are present in high concentrations (>0.5 mg/mL) in e-liquids and it is therefore important to ensure that they do not induce long-term health effects following chronic exposure via inhalation. Their oral toxicity was shown to be low, but most were not tested for inhalation toxicity.

In conclusion, the selected ENDS did emit less harmful compounds (tobacco-related aldehydes, PAHs, TSNAs, and VOCs) than cigarettes and the urinary biomarkers of exposure to these harmful compounds were reduced 6-month after smoking cessation when using the selected ENDS. The design of this study was unique, as it was the first time that both harmful compounds in ENDS emissions and their metabolites in participants' urine have been analyzed together in a randomized controlled trial (RCT) on smoking cessation. Moreover, it was only the second RCT in which analysis of biomarkers of exposure to tobacco smoke were added. Future RCT on smoking cessation using ENDS should build on this design.

7.2 Oxidative stress biomarkers and ENDS

Characterization of ENDS emissions showed that concentrations of selected harmful compounds were reduced compared to tobacco smoke. However, ENDS emissions are complex mixtures as other harmful compounds are still likely to be formed during vaporization. Their short- and long-term effects on the body are not known. Biomarkers of potential harm (BoPH) are useful in investigating the mechanisms of action of substances before the onset of disease. Oxidative stress biomarkers, a type of BoPH, could provide information on potential health effects of vaping, as oxidative stress was associated with the development of several diseases. These biomarkers reflect the systemic oxidative stress level that may be influenced by the harmful compounds present in ENDS emissions. However, their clinical utility is currently limited to research, because they are influenced by many factors and are unspecific.

The LC-MS method we developed for the simultaneous quantification of 8-oxodG and 8-isoprostane was successfully applied in analyzing participant's urine samples. This was the first time that associations between internal doses of oxidative stress biomarkers and BoE to tobacco smoke were investigated in a large cohort of smokers (baseline data). Furthermore, this was the first time that relative importance of BoE on oxidative stress levels (calculate with effect size indicators) was defined. The strongest associations were for PAH and VOC

exposures with both oxidative stress biomarkers, highlighting the importance of environmental factors on oxidative stress. However, the randomized clinical trial design of the study did not allow establishing a cause and effect relationship.

Despite these findings, urinary 8-oxodG and 8-isoprostane concentrations were not different between groups at 6-month follow-up. In addition, smoking cessation appeared to have no effect on oxidative stress biomarker concentrations, as they did not vary between baseline and follow-up. Yet systematic reviews have found an association between oxidative stress biomarker concentrations and smoking status (Ellegaard and Poulsen, 2016; van der Plas et al., 2019). In the first systematic review, 18 studies reported a significant effect of smoking on oxidative stress levels (measured with 8-oxodG), while 19 showed no effects. They concluded that 8-oxodG was related to smoking status as no study showed negative results (i.e., higher concentrations in non-smokers than in smokers). In the second review, high heterogeneity between studies was present, but most showed a positive association between smoking and 8-isoprostane concentrations. ROS homeostasis involves complex mechanisms and oxidative stress caused by smoking and/or vaping (if any) may perhaps have other effects than DNA damage or lipoperoxidation. Indeed, the human body is equipped with antioxidant defense networks to limit the presence of free radicals. The formation or presence of ROS could be neutralized by these endogenous antioxidants, and damages to cellular components would become more significant only when the body is no longer able to maintain cellular redox homeostasis. Therefore, other biomarkers of oxidative stress, such as enzymatic or non-enzymatic antioxidant biomarkers, should be analyzed to observe if an antioxidant depletion is observed.

Based on the results obtained in this sub-study and the content of the meta-analyses on 8-oxodG and 8-isoprostane, the recommendation would be to include them in human biomonitoring studies on ENDS only in conjunction with other biomarkers of potential harm to obtain meaningful data. Both 8-oxodG and 8-isoprostane lack a predictive validity, meaning that elevated concentrations were not systematically linked to a particular disease or condition. On the contrary, other BoPH were linked to cardiovascular diseases (CVD) and cancers, such as white blood cells count, C-reactive protein, interleukins, fibrinogen, and oxidized low-density lipoprotein (LDL) (Chang et al., 2019). These could help investigate potential future health effects of ENDS use without having to wait for diseases to occur.

7.3 Limitations

A limitation of this work is that only one ENDS device with six e-liquids were analyzed. As there are many devices of different generations and types, as well as a multitude of e-liquids with various flavors, the results obtained in this study can only be generalized with caution. We could therefore expect that the more powerful devices will generate more thermal degradation products.

Only known tobacco-related harmful compounds were measured in ENDS aerosols. However, unidentified harmful compounds generated by thermal degradation may be present, as well as harmful compounds for which toxicological data are missing (e.g., flavorings, acetals). Moreover, some known harmful compounds, such as formaldehyde and acetaldehyde, have no specific BoE, which complicates their exposure assessments. In addition, the range of e-liquid consumption was wide, which suggests that participants might have different daily exposure to harmful compounds present in ENDS emissions.

The method of oxidative stress biomarker analysis that we developed and validated was not cross-validated. Cross-validation is a comparison of data from the method used by at least two laboratories (e.g., with spiked quality control samples). This step would be necessary in the event that the sample analysis of a clinical trial would be carried out at several study centers, but also to ensure that the results obtained with methods used are comparable between studies (provided that the analysis methods are similar).

Urinary NNAL and metals were quantified on a sub-set of the 273 selected participants due to budgetary constraints. Therefore, the small sample size in this study could potentially not have reached sufficient statistical power. Some metals might accumulate in the human body and depending on their half-life, they could be released month or years later. Therefore, they may come from prior tobacco product use, passive exposure, or environmental sources.

Only descriptive analysis of the 6-month analysis results was performed within the realm of the PhD study. Additional statistical analyses could be performed in the future, such as assessing associations between oxidative stress biomarkers and BoE and other factors, assessing associations between BoE and reported e-liquid use, or identifying factors that influence BoE and oxidative stress biomarker concentrations.

7.4 Perspectives and future works

There is growing evidence that ENDS emissions contain lower concentrations of harmful compounds compared to tobacco smoke. However, other harmful compounds not identified yet or without known toxicological data could cause future adverse health effects. Non-targeted analysis coupled with high-resolution mass spectrometry is a powerful tool to identify products contained in ENDS emissions. In addition, the degradation of flavorings present in e-liquids and with different functional groups should be evaluated under different ENDS conditions of use. These compounds are present in large quantities (>0.5 mg/mL) and are therefore likely to form degradation products at significant concentrations.

One of the current debates on ENDS is the presence or not of long-term effects. Measured concentrations of BoE are reassuring, but biomarkers of potential harm might be useful to investigate the potential health effects of exposure to ENDS aerosol. Clinical markers, such as respiratory symptoms, indicators of cardiovascular health and many others also provide important information to assess ENDS safety. However, smoking history (i.e., pack years) is an important confounding factor. Therefore, future studies evaluating long-term effects of ENDS use should focus on ENDS users without a history of tobacco smoking.

The two oxidative stress biomarkers, 8-oxodG and 8-isoprostane, were not changed by smoking/vaping in the population studied in this work. Other biomarkers of potential harm could be tested to assess potential health effects of ENDS use, such as C-reactive protein, inerleukin-6, interleukin-8, and oxidized low-density lipoprotein that have all a predictive validity to lung cancer. Associations between oxidative stress biomarkers and BoE to tobacco smoke should be assessed at 6-month follow-up to confirm findings previously found (Chapter 5). These two biomarkers could also be quantified in exhaled breath condensate (EBC), which may better represent oxidative stress level in the respiratory system.

7.5 Conclusion

Assessment of exposure to harmful compounds emitted by ENDS was achieved both through laboratory characterization of ENDS emissions and analysis of BoE in participants' urine samples. ENDS emissions contained aldehydes and metals, but their concentrations were low when compared to cigarette smoke, environmental exposure, occupational limits, MRLs, and PDEs. Analysis of participants' urine samples at 6-month follow-up indicated that

concentrations of BoE to tobacco smoke were not different between ENDS users and ex-smokers, while they were greatly reduced compared to those of smokers. Quitting smoking with or without ENDS does not expose participants to the selected harmful compounds. Smokers would therefore greatly reduce their exposure to harmful compounds from the switch from cigarettes to ENDS. However, formaldehyde and acetaldehyde, two carcinogens identified in ENDS aerosols, have no specific BoE. ENDS users are still exposed to harmful compounds when vaping, but to concentrations lower than in cigarette smoke. Non-smokers should refrain to use ENDS to avoid exposure to these harmful compounds.

The association between oxidative stress and smoking was investigated. A new LC-MS/MS analytical method was successfully developed and validated. This method was used to quantify both 8-oxodG and 8-isoprostane in a total of 546 urine samples. Potential associations between oxidative stress biomarkers and BoE to tobacco smoke were assessed in 273 smokers. Both 8-oxodG and 8-isoprostane were associated to PAH and VOC from tobacco smoke exposure. The same method was applied to measure the strength of association between urinary oxidative stress and BoE biomarker concentrations in the same 273 participants at 6-month follow-up. No differences in urinary oxidative stress biomarker concentrations were observed between baseline and six months later. Moreover, the four smoking status groups presented similar urinary oxidative stress biomarker concentrations. These oxidative stress biomarkers might not be suitable for monitoring oxidative stress changes following smoking cessation or they might not be influenced by smoking or vaping. Future work should determine whether association between oxidative stress biomarkers and BoEs are still observable at 1-year and 2-year post baseline.

We recommend the use of ENDS to help smokers reduce their exposures to harmful compounds. ENDS use by non-smokers should be strongly discouraged to avoid exposures to ENDS emitted harmful compounds, such as aldehydes and metals. Future studies should focus on compounds identified in ENDS emissions for which no toxicological data are available and on potential future health effects of ENDS.

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Annexes

a) Chapter 3 – Supplementary data

- Aldehydes – HPLC-UV parameters

Aldehydes derivatives were analyzed with a high performance liquid chromatography – ultraviolet (HPLC-UV) instrument equipped with a Hypersil Gold column (1.9 µm, 100 x 0.1 mm, Thermo Scientific, Reinach, Switzerland). Injection volume was 2 µL and column temperature was maintained at 40°C. The mobile phase consisted of: eluent A composed of water/tetrahydrofuran (H_2O/THF, 95:5, v/v) and eluent B acetonitrile (ACN). The solvent gradient is showed in Table S1. UV detector had working wavelength of 360 nm (bandwidth of 2 nm). The limit of quantification was 0.05 µg/mL for all compounds. The retention times of the aldehyde derivatives were: formaldehyde 2.2 min, acetaldehyde 3.1 min, acrolein 4.5 min, propanal 5.1 min, crotonaldehyde 6.3 min, butyraldehyde 7.4 min, benzaldehyde 8.6, isovaleraldehyde 9.6 min, valeraldehyde 10.0 min, o-tolualdehyde 10.7 min, m-/p-tolualdehyde 10.9 min, hexanal 12.7 min, and 2,5-dimethylebenzaldehyde 12.9 min.

Table S1 – Solvent gradient for the analysis of aldehyde derivatives on a Hypersil Gold column (1.9 µm, 100 x 0.1 mm) by high performance liquid chromatography – ultraviolet (HPLC-UV).

Time [min]	Flow [mL/min]	Eluent A [%]	Eluent B [%]
0	0.6	68	32
5	0.6	60	40
13	0.6	42	55
13.2	0.6	20	80
14.5	0.6	20	80
14.7	0.6	68	32
17	0.6	68	32

Eluent A was composed of water/tetrahydrofuran (H_2O/THF, 95:5, v/v) and eluent B of acetonitrile (ACN).

- Volatile organic compounds (VOCs) – GC-MS parameters

VOCs were analyzed by a gas chromatography – mass spectrometry (GC-MS) instrument equipped with a VF-624ms column (60 m, 0.25 mm, 1.4 µm; Agilent, Basel, Switzerland). Volume injection was 3 µm, the split flow was 20 mL/min, the spit ratio was 13, and the inlet temperature 250°C. The temperature gradient was the following: 40°C for 5 min, increase of 15°C/min to 250°C, and 1 min at 250°C. Helium flow rate was 1.5 mL/min. Electron impact (EI) was used. The ion source temperature was 240°C and the MS transfer line 240°C. LOQs were 40 ng/mL for each compound. MS parameters and retention time can be found in Table S2.

Table S2 – Mass (MS) parameters for the analysis of volatile organic compounds (VOCs) by gas chromatography – mass spectrometry (GC-MS): quantification peak, confirmation peaks, and retention time.

Compounds	Quantification (m/z)	Confirmation 1 (m/z)	Confirmation 2 (m/z)	Retention time (min)
1,3-butadiene	54	53	39	4.0
Isoprene	68	68	39	6.2
Acrylonitrile	53	52		7.9
Benzene	78	70	51	10.0
Toluene	91	92	65	13.0
Acrylamide	44	70	55	13.7
Naphthalene	128	127		16.6

- Metals – ICP-MS parameters

Inductively coupled plasma mass spectrometry (ICP-MS) instrument was operated at 1550 W, with an argon cool flow of 14 L/min. The nebulizer flow was 1.1 L/min, and the auxiliary flow was 0.8 L/min. The spray chamber temperature was 2.7°C. Collision cell was operated with kinetic energy discrimination (KED). Sampling depth was 5 mm.

The standard solutions of metals for calibration curves were bought from Labkings (Hilversum, Netherlands), except Fe from SCP Science (Marktoberdorf, Germany) and their certified concentrations are shown in Table S3. Limits of quantification (LOQs) for each metals are presented in Table S4. Calibration ranges went from 1'000'000-fold to 200-fold dilution of the standard solutions (for several metals, LOQs were higher than the lowest points of the calibration curves).

Table S3 – Certified concentrations of metal stock solutions used to prepared calibration standard solutions for inductively coupled plasma mass spectrometry (ICP-MS) analysis, expressed in micrograms per milliliter (µg/mL).

Metals	Certified Concentration (µg/mL)	Metals	Certified Concentration (µg/mL)
Be	5.010	**As**	20.01
Al	20.01	**Se**	50.02
V	20.01	**Mo**	19.99
Cr	5.002	**Pd**	4.986
Mn	10.00	**Ag**	2.002
Fe	1000	**Cd**	5.002
Co	10.01	**Sn**	2.009
Ni	10.01	**Sb**	9.984
Cu	500.5	**Pt**	1.993
Zn	998.2	**Pb**	100.1

Table S4 – Limits of quantification (LOQs) of metals for electronic nicotine delivery systems (ENDS) aerosols and e-liquids, expressed in nanograms per milliliter (ng/mL).

Metals	LOQs in e-liquids (ng/mL)	LOQs in aerosols (ng/mL)
Be	0.05	0.005
Al	5	5
V	0.02	0.02
Cr	0.05	0.005
Mn	0.01	0.01
Fe	0.1	5
Co	0.1	0.1
Ni	0.1	0.01
Cu	0.5	0.5
Zn	1	1
As	0.02	0.02
Se	0.5	0.5
Mo	0.02	0.05
Pd	0.01	0.01
Ag	0.002	0.002

Metals	LOQs in e-liquids (ng/mL)	LOQs in aerosols (ng/mL)
Cd	0.005	0.005
Sn	0.02	0.005
Sb	0.01	0.01
Pt	0.002	0.002
Pb	0.1	0.1

- Tobacco-specific nitrosamines (TSNAs) – LC-MS/MS parameters

TSNAs were analyzed with a liquid chromatography – tandem mass spectrometry (LC-MS/MS) instrument equipped with a Zorbax Eclipse Plus C18 column (3 x 50 mm, 1.8 µm, Agilent, Basel, Switzerland). Temperature of the column was maintained at 35°C. Injection volume was 5 µL. The mobile phase consisted of: eluent A composed of water with ammonium acetate (50 mM) and eluent B of acetonitrile with ammonium acetate (50 mM). The gradient was the following: 0 min 0% B, 2 min 100% B, 2.5 min 100% B, 3 min 0% B, and 7 min 0% B. Retention times were: N-nitrosonornicotine (NNN) 4.3 min, 4-(methylnitrosamino)-1-(3-bipyridyl)-1-butanone (NNK) 4.4 min, N-nitrosoanatabine (NAT) 4.6 min, and N-nitrosoanabasine (NAB) 4.7.

Electrospray ionization (ESI) mode was positive, with a voltage of 1500 V. MS parameters were the following: sheath gas 50 Arb (arbitrary units), aux gas 5 Arb, sweep gas 0 Arb, ion transfer tube temperature 400°C, and vaporizer temperature 400°C. Ion source parameters, as well as m/z transitions for the multiple reaction monitoring (Table S5), were determined by infusion of aqueous standard of NNN, NNK, NAB, and NAB (5 µg/mL). Pressure of the CID gas was 1.5 mTorr.

Table S5 – Multi-reaction monitoring parameters for the analysis of NNN, NNK, NAT, and NAB by liquid chromatography – tandem mass spectrometry (LC-MS/MS): precursor mass to charge ratio, product mass to charge ratio, collision energy voltage (italic), and RF lens voltage.

Compound	Precursor (m/z)	Products (m/z) and *collision energy (V)*				RF lens (V)
NNN	178.1	79.1 *44*	105.1 *33*	119.1 *30*	148.1 *10*	35
NNN-d4	182.1	152.1 *10*				35

Compound	Precursor (m/z)	Products (m/z) and *collision energy (V)*			RF lens (V)
NAT	190.1	79.1 *28*	106.1 *18*	160.1 *10*	36
NAT-d4	194.1	164.1 *10*			36
NAB	192.1	79.1 *40*	106.1 *33*	133.1 *25*	53
NAB-d4	196.1	166.1 *10*			53
NNK	208.1	79.1 *37*	122.1 *13*	178.1 *10*	42
NNK-d4	212.1	126.1 *13*			42

b) Chapter 4 – Supplementary data

Description of the standard stock solutions

Stock solutions of 8-oxodG (1 mg/mL) and 8-isoprostane (1 mg/mL) in water/methanol (8:2) were stored at -20°C. Intermediate stock solutions of 8-oxodG (5 µg/mL) and 8-isoprostane (5 µg/mL) in water were prepared from the stock solution and stored at 4°C during the validation process. Internal standard (IS) stock solutions were made with [$^{15}N_5$]-8-oxodG (2.5 µg/mL) and 8-isoprostane-d_4 (2.5 µg/mL) in water and stored at 4°C during the validation process. Working solutions of 8-oxodG were freshly prepared at 100 ng/mL and 10 ng/mL in water, and 8-isoprostane at 100 ng/mL, 10 ng/mL and 1 ng/mL in water from the intermediate stock solutions before each sequence. Working solutions of [$^{15}N_5$]-8-oxodG (50 ng/mL) and 8-isoprostane-d_4 (25 ng/mL) in water were also freshly prepared before each sequence.

Description of the MS parameters (Table S1)

Table S1 – Multi-reaction monitoring parameters

Compounds	Polarity	Mass transitions [m/z][1]	Collision energy [V]	RF lens [V]
8-oxodG	Positive	284.1 → 140.0	28.8	37
		284.1 → 168.1	10	37
		284.1 → 243.0	10.2	37
[$^{15}N_5$]-8-oxodG	Positive	289.1 → 173.1	10	40
8-isoprostane	Negative	353.2 → 193.1	25	80
		353.2 → 291.0	20	80
		353.2 → 309.2	20	80
8-isoprostane-d_4	Negative	357.2 → 313.2	25	78

[1] Mass transitions in bold are quantification transitions, others are confirmation transitions.

ESI parameters under positive detection mode (for 8-oxodG) were optimized at 3700 V, and under negative mode (for 8-isoprostane) at 3400 V. The vaporizer temperature was maintained at 350°C and the ion transfer tube at 390°C. The sheath gas and the auxiliary gas pressures were set at 45 and 17 Arb (arbitrary unit), respectively. The argon pressure was set at 1.5 mTorr.

Description of the method validation parameters

LODs were determined by injecting decreasing concentrations of analyte in water until obtaining a signal to noise ratio (S/N) of three, and LOQs until a signal to noise ratio (S/N) of ten. In urine, LOQs, corresponding to the lowest calibration points, were chosen according to the reported concentrations of the analyte. Criteria for linearity was a coefficient of determination R^2 greater than 0.999 for the urinary calibration curve. Intra-day precision and accuracy were determined from three replicates measurements of three concentrations with two different urine samples on the same day. Inter-day precision and accuracy were determined on three different days. The precision was expressed as the coefficient of variation. The accuracy was calculated as the ratio of the mean of the calculated concentrations of the spiked samples to the theoretical concentrations, and was expressed as a percentage. Extraction recovery was calculated by dividing the IS signal area of sample spiked before and after SPE, and was expressed as a percentage. Absolute matrix effects were calculated by dividing the IS signal area of sample spike after SPE and the IS signal area in spiked water without SPE, and was expressed as a percentage. Relative matrix effects were calculated by comparing slopes of calibration curves in three different urine samples and expressed as the coefficient of variation. Calibration curves in water and urine were also compared (ratio of the slope in water and in urine, expressed as a percentage). The stability of the compounds had been previously studied [1–3]. We investigated the stability of the analytes in urine after being frozen at -20°C for 6 months by analyzing QC aliquots (low and high) and monitoring the concentration changes along time. Twenty-one aliquots were analyzed over the 6-month period. We also investigated the stability of processed samples at room temperature (12 h) by injecting three times the same QC aliquot seven hours apart (on 21 different days).

Description of the optimization of the SPE (Table S2)

We investigated several SPE cartridges for the sample clean up and these are represented in Table S2. The two anion exchange SPE cartridges gave good recoveries for 8-oxodG but not for 8-isoprostane. During the tests with anion exchange cartridges, the samples were adjusted to basic pH ranges with ammonium hydroxide (0.05%). Chromabond C18 endcapped performed the best of the four other reversed-phase cartridges tested as the two analytes were well retained. We tested two different phase quantities (200 mg and 500 mg) and selected the bigger. We optimized the washing step and found that a small part of 8-oxodG (4 6%) was eluted during a washing step with 10% methanol. We chose a high volume of methanol (3 mL) for the elution to recover the total 8-isoprostane quantity.

Table S2. Summary of tested SPE cartridge during method development.

SPE cartridge	Features
EVOLUTE AX	Mixed-mode hydrophobic and strong anion exchange
CHROMABOND Easy	Polar modified polymer with weak anion exchange
Bond Elut C18 OH	Non-polar non-endcapped sorbent
Bond Elut NEXUS	Non-polar polymeric sorbent
ISOLUTE C18	Non-polar sorbent
CHROMABOND C18 ec	Non-polar endcapped sorbent

Comments on matrix effects observed in "dark urine"

"Matrix effects were also observed for "light urine" (67% for 8-oxo-dG and 83% for 8-isoprostane) and "dark urine" (4% for 8-oxodG and 25% for 8-isoprostane), estimated by the IS variation. A simple dilution by a factor two of "dark urine" reduced matrix effects to 19% for 8-oxodG and 58% for 8-isoprostane." It is important to mention that the dilution did not reduce the MS response of 8-oxodG and 8-isoprostane by two: 8-oxodG signal increased by 39% and 8-isoprostane decreased by only 21%.

We observed signal suppression due to matrix effects. This signal suppression was proportional to the concentration of the urine sample (i.e., the presence of co-eluting matrix components). This relationship was, however, not linear. This explains why we observed a signal increase after sample dilution. For example, the matrix effect for 8-oxodG ("dark urine") changed from 4% to 19% with a two-fold dilution. If we assume that the MS signal of the undiluted sample was 1, then the theoretical signal would be 25 (corresponding to 100%; no matrix effect). Diluting by two (and considering a linear response of the instrument with a slope of 1) then the theoretical signal of the two-fold diluted sample would be 12.5 (100%). Applying the matrix effect of 19% would give a signal of 2.4, which is effectively higher than the signal of the undiluted sample (1).

Signal suppression was different between the two analytes. There are more compounds co-eluting with 8-oxodG (4.7 min) than with 8-isoprostane (10.2 min). Generally, the closer the compounds are to the solvent elution (short retention times), the stronger the matrix effects. To counter balance this effect, appropriate internal standard are used for correcting for signal suppression. Stable isotopically labeled internal standards are preferred since their retention times are very close to those of the analytes and will undergo similar matrix effects as the biomarker.

Comments on matrix effects and method performance

The parameters that are directly impacted by matrix effects are LODs and LOQs. Indeed, several researchers report the LODs in aqueous solution (e.g., Wu et al. [2016]), some of them mentioning that this limit may vary in biological fluids (e.g., Martinez and Kannan [2018]) [1,4]. In reality, these parameters will change according to the urine samples, and they should be considered with precaution when assessing a method's performance. Calibration range, especially the lower calibration point, is more important as it delimits to which concentration the method is effective. However, analyte MS responses of samples must not be lower than the lower calibration point response, in which case they should be reinjected or reported as "under LOQ". This requires special attention because, due to matrix effects, a sample with the same concentration as the lowest calibration standard may have a lower MS response for the analyte and its internal standard (with the same analyte/IS ratio).

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c) Chapter 5 – Supplementary data

1. Exclusion criteria of ESTxENDS study

- Known hypersensibility or allergy to contents of e-liquids
- Participation in another study with investigational drug within 30 days preceding the baseline visit and during the present study where interactions are to be expected
- Woman who are pregnant or breast feeding
- Intention to become pregnant during the course of the scheduled study intervention, i.e., within the first 6-month of the study
- Persons having used ENDS or tobacco heating systems regularly in the 3 months preceding the baseline visit
- Persons having used nicotine replacement therapy (NRT) or other medications with demonstrated efficacy as an aid for smoking cessation such as varenicline or bupropion within the 3 months preceding the baseline visit
- Persons who cannot attend the 6-month follow-up visit for any reason
- Persons who cannot understand instructions delivered in person or by phone, or otherwise unable to participate in study procedures

2. Typology of Swiss urban area – classification by the Federal Statistical Office (FSO; 2014)

Table S1 – Typology of urban areas proposed by the FSO

Class 1	Agglomeration center community (city center)
Class 2	Agglomeration center community (main center)
Class 3	Agglomeration center community (secondary center)
Class 4	Agglomeration ring community
Class 5	Multi-oriented community
Class 6	Non-urban center community
Class 7	Rural community without urban character

3. Comparison of ESTxENDS smokers with the Swiss smoking population

Table S2 – Gender and age distributions in a random subset of the Swiss smoking population and the participants included in our study

		Swiss smoking population [%] [1]	ESTxENDS smokers [%]
Gender	Female	46 (n=3248)	43 (n=117)
	Male	54 (n=2718)	57 (n=153)
Age group	15-24 [2]	16 (n=930)	5 (n=14)
	25-34	17 (n=983)	26 (n=70)
	35-44	18 (n=1081)	21 (n=56)
	45-54	20 (n=1186)	24 (n=65)
	55-64	17 (n=967)	18 (n=48)
	65-74	9 (n=514)	6 (n=15)
	75+	3 (n=195)	1 (n=2)

[1]Based on a random subset of the Swiss population, n=22'131 (2017 Swiss health survey of tobacco consumption by the Swiss Federal Statistical Office); [2]In ESTxENDS study, the participants were at least 18 years old (inclusion criteria).

6. Correlation between the PAH and VOC scores with their respective components

Table S3 – Correlation coefficients between ΣPAHs and the three PAH metabolites

	ΣPAHs	**1-Naphtol**	**2-Naphtol**	**1-OHP**
ΣPAHs	**1**	**0.89**	**0.87**	**0.83**
1-Naphtol		1	0.66	0.6
2-Naphtol			1	0.59
1-OHP				1

Table S4 – Correlation coefficients between ΣVOCs and the 10 VOC metabolites (SPMA not included)

	ΣVOCs	**GAMA**	**HEMA**	**AAMA**	**DHBMA**	**3-HPMA**	**CYMA**	**2-HPMA**	**1-/2-MHBMA**	**3-MHBMA**	**HPMMA**
ΣVOCs	**1**	**0.78**	**0.68**	**0.86**	**0.81**	**0.83**	**0.69**	**0.81**	**0.72**	**0.88**	**0.9**
GAMA		1	0.42	0.8	0.73	0.51	0.4	0.63	0.53	0.64	0.59
HEMA			1	0.52	0.47	0.44	0.42	0.53	0.51	0.52	0.52
AAMA				1	0.78	0.65	0.55	0.68	0.55	0.67	0.7
DHBMA					1	0.67	0.43	0.63	0.47	0.68	0.7
3-HPMA						1	0.59	0.66	0.48	0.77	0.87
CYMA							1	0.41	0.33	0.57	0.63
2-HPMA								1	0.59	0.68	0.69
1-/2-MHBMA									1	0.63	0.58
3-MHBMA										1	0.87
HPMMA											1

7. Simplified graphical explanation of partial correlation analysis

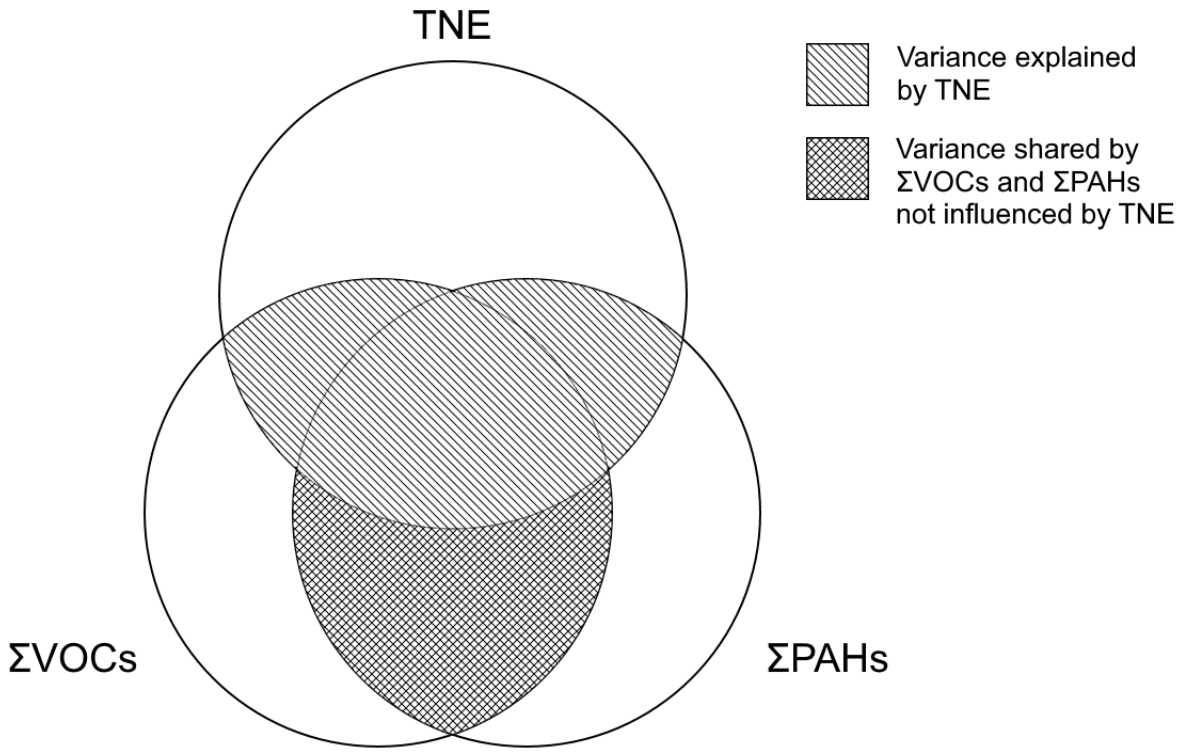

Figure S1 – Example of partial correlation between ΣPAHs and ΣVOCs controlled for TNE.

8. Calculation of the % increase from multiple linear regression analysis

Let us define a = *covariate concentration*, b = *covariate concentration* + **x % increase**, c = outcome *concentration*, and d = *outcome concentration* + **y % increase**.

The y % increase in the outcome (associated to the x % increase in the covariate) is what we want to determine using multiple linear regression analysis (beta coefficients, β). We can write:

$$\log(d) = \log(c) + \beta * (\log(b) - \log(a))$$

$$\log(d) - \log(c) = \beta * (\log(b) - \log(a))$$

$$\log\left(\frac{d}{c}\right) = \beta * \log(\frac{b}{a})$$

$$\frac{d}{c} = \exp(\beta * \log\left(\frac{b}{a}\right))$$

$$\frac{d}{c} = \exp(\log\left(\frac{b}{a}\right))^{\beta}$$

$$\frac{d}{c} = (\frac{b}{a})^{\beta}$$

To obtain the % increase, we should transform the ratio of concentration in % increase:

$$x\ \%\ increase = \left(\frac{b}{a} - 1\right) * 100$$

$$y\ \%\ increase = \left(\frac{d}{c} - 1\right) * 100$$

If we transform our previous equation, we obtain:

$$\frac{y\ \%\ increase}{100} + 1 = \left(\frac{x\ \%\ increase}{100} + 1\right)^{\beta}$$

$$\boldsymbol{y\ \%\ increase} = \left(\left(\frac{x\ \%\ increase}{100} + 1\right)^{\beta} - 1\right) * 100$$

d) Chapter 6 – Supplementary data

Table S1 – Limits of detection (LODs) and limit of quantification (LOQs) of metals for the analysis of urine samples, expressed in nanograms per milliliter (ng/mL).

Metals	LODs (ng/mL)	LOQs (ng/mL)
Be	0.002	0.005
Al	1.67	5
V	0.25	0.75
Cr	0.083	0.25
Mn	0.083	0.25
Fe	0.83	2.5
Co	0.17	0.5
Ni	0.17	0.5
Cu	0.5	1.5
Zn	2	6
As	0.42	1.25
Se	0.33	1
Mo	0.33	1
Pd	0.05	0.15
Ag	0.17	0.5
Cd	0.067	0.2
Sn	0.05	0.15
Sb	0.2	0.6
Pt	0.007	0.02
Pb	0.33	1

Table S2 – Concentrations of urinary nicotine, tobacco-specific nitrosamine (TSNA), polycyclic aromatic hydrocarbon (PAH), and volatile organic compound (VOC) metabolites, anabasine, metals, and biomarkers of oxidative stress in ex-smokers, ENDS users, dual users and smokers at follow-up (normalized for creatinine, median with interquartile range (1st and 3rd quartiles); n=273). Total number and percentages of samples above the limit of quantification (>LOQ) are also reported. Urinary metal concentrations of smokers were not measured at follow-up and were replaced by baseline results (in italics) to facilitate comparison.

Biomarkers	Ex-smokers		ENDS users		Dual users		Smokers	
	number (>LOQ)	median (Q1–Q3)	number (>LOQ)	median (Q1–Q3)	number (>LOQ)	median (Q1–Q3)	number (>LOQ)	median (Q1–Q3)
Nicotine metabolites (ng/mg creatinine)								
Total nicotine equivalent (TNE 4) (nmol/mg)	67 (-)	0.01 (0.02–0.49)	97 (-)	14.95 (5.39–26.23)	35 (-)	28.7 (13.2–41.8)	64 (-)	22.4 (17.7–35.4)
Nicotine	67 (52%)	0.61 (<LOQ –7.53)	97 (98%)	354 (115–783)	35 (100%)	800 (260–1687)	64 (100%)	646 (257–1044)
Cotinine	67 (42%)	1.00 (<LOQ –7.53)	97 (96%)	679 (230–1226)	35 (100%)	1085 (562–1455)	64 (100%)	1114 (763–1201)
Norcotinine	67 (29%)	<LOQ (<LOQ–3.75)	97 (91%)	58.6 (17.5–99.4)	35 (100%)	127.9 (54.3–172.2)	64 (100%)	128.3 (87.2–175.0)
Trans-3'-hydroxycotinine	67 (61%)	1.29 (<LOQ –59.88)	97 (99%)	1375 (503–2430)	35 (100%)	2286 (1183–4027)	64 (100%)	2156 (1482–3278)
Minor tobacco alkaloids (ng/mg creatinine)								
Anabasine	77 (17%)	<LOQ (<LOQ – 1.25)	97 (39%)	<LOQ (<LOQ – 1.44)	35 (97%)	3.65 (1.73–6.79)	64 (91%)	6.62 (2.93–10.65)
Tobacco-specific nitrosamine (TSNA) metabolite (pg/mg creatinine)								

Biomarkers	Ex-smokers		ENDS users		Dual users		Smokers	
	number (>LOQ)	median (Q1–Q3)	number (>LOQ)	median (Q1–Q3)	number (>LOQ)	median (Q1–Q3)	number (>LOQ)	median (Q1–Q3)
4-methylnitrosamino)-4-(3-pyridil)-1-butanol (NNAL)	52 (13%)	<LOQ (<LOQ–34.6)	55 (7%)	<LOQ (<LOQ–<LOQ)	4 (75%)	125.3 (79.9–195.8)	16 (88%)	270.0 (85.7–372.4)
Heavy metals and trace elements (ng/mg creatinine) [1]								
Aluminum (Al)	20 (55%)	5.33 (<LOQ–7.70)	20 (40%)	<LOQ (<LOQ –5.62)	-	-	*39 (49%)*	*<LOQ (<LOQ –9.81)*
Antimony (Sb)	20 (0%)	<LOQ (<LOQ –<LOQ)	20 (5%)	<LOQ (<LOQ –<LOQ)	-	-	*39 (0%)*	*<LOQ (<LOQ –<LOQ)*
Arsenic (As)	20 (100%)	6.81 (2.62–12.62)	20 (100%)	6.28 (4.08–18.12)	-	-	*39 (100%)*	*7.50 (3.96–30.43)*
Beryllium (Be)	20 (0%)	<LOQ (<LOQ –<LOQ)	20 (5%)	<LOQ (<LOQ –<LOQ)	-	-	*39 (8%)*	*<LOQ (<LOQ –<LOQ)*
Cadmium (Cd)	20 (45%)	0.21 (<LOQ–0.25)	20 (55%)	0.21 (<LOQ–0.32)	-	-	*39 (51%)*	*0.20 (<LOQ–0.31)*
Chromium (Cr)	20 (3%)	<LOQ (<LOQ –<LOQ)	20 (40%)	<LOQ (<LOQ –0.29)	-	-	*39 (33%)*	*<LOQ (<LOQ –0.29)*
Cobalt (Co)	20 (20%)	<LOQ (<LOQ –<LOQ)	20 (35%)	<LOQ (<LOQ –0.58)	-	-	*39 (23%)*	*<LOQ (<LOQ –<LOQ)*
Copper (Cu)	20 (100%)	16.1 (10.7–19.5)	20 (100%)	13.9 (9.2–20.6)	-	-	*39 (100%)*	*15.5 (11.1–31.0)*
Iron (Fe)	20 (100%)	14.1 (8.6–24.2)	20 (100%)	14.5 (12.6–19.5)	-	-	*39 (100%)*	*17.2 (11.1–22.6)*

Biomarkers	Ex-smokers		ENDS users		Dual users		Smokers	
	number (>LOQ)	median (Q1–Q3)	number (>LOQ)	median (Q1–Q3)	number (>LOQ)	median (Q1–Q3)	number (>LOQ)	median (Q1–Q3)
Lead (Pb)	20 (20%)	<LOQ (<LOQ –<LOQ)	20 (10%)	<LOQ (<LOQ –<LOQ)	-	-	*39 (38%)*	*<LOQ (<LOQ –1.23)*
Manganese (Mn)	20 (50%)	0.28 (<LOQ–0.40)	20 (45%)	<LOQ (<LOQ –0.40)	-	-	*39 (44%)*	*0.29 (<LOQ–0.61)*
Molybdenum (Mo)	20 (100%)	31.3 (19.0–54.0	20 (100%)	31.2 (14.7–47.1)	-	-	*39 (100%)*	*31.9 (18.5–54.6)*
Nickel (Ni)	20 (100%)	1.65 (1.06–2.75)	20 (100%)	1.55 (1.02–3.17)	-	-	*39 (97%)*	*1.63 (0.94–2.80)*
Palladium (Pd)	20 (0%)	<LOQ (<LOQ –<LOQ	20 (5%)	<LOQ (<LOQ –<LOQ)	-	-	*39 (0%)*	*<LOQ (<LOQ –<LOQ)*
Platinum (Pt)	20 (0%)	<LOQ (<LOQ –<LOQ	20 (10%)	<LOQ (<LOQ –<LOQ)	-	-	*39 (5%)*	*<LOQ (<LOQ –<LOQ)*
Selenium (Se)	20 (100%)	17.2 (14.8–21.7)	20 (100%)	17.1 (13.9–21.0)	-	-	*39 (100%)*	*17.4 (14.1–22.6)*
Silver (Ag)	20 (0%)	<LOQ (<LOQ –<LOQ)	20 (5%)	<LOQ (<LOQ –<LOQ)	-	-	*39 (100%)*	*<LOQ (<LOQ –<LOQ)*
Tin (Sn)	20 (55%)	0.16 (<LOQ–0.24)	20 (50%)	0.17 (<LOQ–0.39)	-	-	*39 (67%)*	*0.17 (<LOQ–0.44)*
Vanadium (V)	20 (0%)	<LOQ (<LOQ –<LOQ)	20 (5%)	<LOQ (<LOQ –<LOQ)	-	-	*39 (5%)*	*<LOQ (<LOQ –<LOQ)*
Zinc (Zn)	20 (100%)	316 (180–435)	20 (100%)	251 (180–366)	-	-	*39 (100%)*	*297 (177–398)*

Biomarkers	Ex-smokers		ENDS users		Dual users		Smokers	
	number (>LOQ)	median (Q1–Q3)	number (>LOQ)	median (Q1–Q3)	number (>LOQ)	median (Q1–Q3)	number (>LOQ)	median (Q1–Q3)
Polycyclic aromatic hydrocarbon (PAH) metabolites (ng/mg creatinine)								
1-Naphtol	76 (75%)	0.39 (<LOQ–0.71)	97 (74%)	0.35 (<LOQ–1.11)	35 (91%)	4.86 (1.16–8.89)	64 (100%)	6.63 (4.65–10.31)
2-Naphtol	76 (100%)	6.19 (3.32–10.88)	97 (99%)	6.49 (3.41–13.11)	35 (100%)	12.51 (6.99–18.97)	64 (100%)	15.12 (10.39–21.66)
1-Hydroxypyrene	76 (47%)	<LOQ (<LOQ –<LOQ)	97 (36%)	<LOQ (<LOQ–0.2)	35 (31%)	<LOQ <LOQ–0.27)	64 (64%)	0.21 (0.14–0.29)
Volatile organic compound (VOC) metabolites (ng/mg creatinine)								
N-acetyl-S-(2-carbamoylethyl)-L-cysteine (AAMA)	77 (100%)	47.3 (30.4–73.3)	97 (100%)	39.6 (31.1–64.8)	35 (100%)	137.3 (63.9–195.6)	64 (100%)	157 (112–207)
N-acetyl-S-(2-cyanoethyl)-L-cysteine (CYMA)	77 (51%)	<LOQ (<LOQ –4.66)	97 (64%)	2.17 (<LOQ –4.14)	35 (100%)	88.4 (29.1–169.9)	64 (100%)	152.2 (70.4–228.8)
N-acetyl-S-(3,4-dihydroxybutyl)-L-cysteine (DHBMA)	77 (100%)	325 (227–447)	97 (100%)	337 (280–424)	35 (100%)	371 (256–441)	64 (100%)	397 (317–560)
N-acetyl-S-(2-carbamoyl-2-hydroxyethyl)-L-cysteine (GAMA)	77 (94%)	8.24 (5.38–12.22)	97 (96%)	6.98 (5.26–10.68)	35 (97%)	16.24 (9.29–25.78)	64 (98%)	19.3 (13.3–25.2)
N-acetyl-S-(2-hydroxyethyl)-L-cysteine (HEMA)	77 (18%)	<LOQ (<LOQ –<LOQ)	97 (15%)	<LOQ (<LOQ –<LOQ)	35 (54%)	3.22 (<LOQ –4.69)	64 (72%)	3.78 (<LOQ –8.04)
N-acetyl-S-(2-hydroxypropyl)-L-cysteine (2-HPMA)	77 (99%)	18.9 (11.1–29.9)	97 (98%)	19.3 (15.0–24.5)	35 (97%)	30.5 (17.7–55.8)	64 (100%)	53.9 (37.5–82.0)

Biomarkers	Ex-smokers		ENDS users		Dual users		Smokers	
	number (>LOQ)	median (Q1–Q3)	number (>LOQ)	median (Q1–Q3)	number (>LOQ)	median (Q1–Q3)	number (>LOQ)	median (Q1–Q3)
N-acetyl-S-(3-hydroxypropyl)-L-cysteine (3-HPMA)	77 (100%)	326 (169–704)	97 (100%)	299 (194–465)	35 (100%)	691 (409–1828)	64 (100%)	1212 (715–1664)
N-acetyl-S-(3-hydroxypropyl-1-methyl)-L-cysteine (HPMMA)	77 (100%)	205 (164–313)	97 (100%)	202 (164–278)	35 (100%)	553 (271–1190)	64 (100%)	798 (500–1232)
N-acetyl-S-(1-hydroxymethyl-2-propenyl)-L-cysteine + N-acetyl-S-(2-hydroxy-3-butenyl)-L-cysteine (1-MHBMA + 2-MHBMA)	77 (18%)	<LOQ (<LOQ –2.27)	97 (18%)	<LOQ (<LOQ –<LOQ)	35 (51%)	<LOQ (<LOQ –2.88)	64 (66%)	2.74 (<LOQ –6.55)
N-acetyl-S-(4-hydroxy-2-buten-1-yl)-L-cysteine (3-MHBMA)	77 (96%)	5.17 (3.63–7.74)	97 (95%)	5.24 (3.98–7.19)	35 (100%)	13.86 (9.03–28.39)	64 (100%)	22.9 (13.7–35.0)
N-acetyl-S-(phenyl)-L-cysteine (SPMA)	77 (6%)	<LOQ (<LOQ –<LOQ)	97 (12%)	<LOQ (<LOQ –<LOQ)	35 (9%)	<LOQ (<LOQ –<LOQ)	64 (22%)	<LOQ (<LOQ –<LOQ)
Oxidative stress biomarkers (ng/mg creatinine)								
8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG)	77 (100%)	4.22 (3.44–5.67)	97 (100%)	4.02 (3.47–5.30)	35 (100%)	4.47 (3.63–5.64)	64 (100%)	4.24 (3.00–5.59)
8-iso-prostaglandin F2α (8-isoprostane)	77 (100%)	0.21 (0.14–0.31)	97 (100%)	0.20 (0.14–0.27)	35 (100%)	0.22 (0.18–0.26)	64 (100%)	0.20 (0.14–0.26)

[1]Only the urinary metal concentrations of ex-smokers and ENDS users were analyzed at follow-up. The urinary concentrations presented for smokers are baseline results. This was done to facilitate comparison.

Table S3 – Follow-up to baseline ratios of the concentrations of urinary nicotine, tobacco-specific nitrosamine (TSNA), polycyclic aromatic hydrocarbon (PAH), and volatile organic compound (VOC) metabolites, anabasine, metals, and biomarkers of oxidative stress in ex-smokers, ENDS users, dual users and smokers (expressed in percentage, median with interquartile range (1st and 3rd quartiles); n=273). Sample numbers are also reported.

Biomarkers	Ex-smokers (n=77)		ENDS users (n=97)		Dual users (n=35)		Smokers (n=64)	
	number	median (Q1-Q3)	number	median (Q1-Q3)	number	median (Q1-Q3)	number	median (Q1-Q3)
Nicotine metabolites								
Total nicotine equivalent (TNE 4)	76	0.1 (0.1–2.3)	97	61 (32–98)	35	98 (70–123)	64	82 (56–113)
Nicotine	76	0.2 (0.1–2.0)	97	55 (12–112)	35	102 (67–206)	64	71 (36–149)
Cotinine	76	0.1 (0.0–2.4)	97	60 (24–100)	35	89 (66–118)	64	79 (57–114)
Norcotinine	76	1.0 (0.4–4.1)	97	43 (19–74)	35	84 (67–119)	64	91 (58–111)
Trans-3'-hydroxycotinine	76	0.1 (0.0–4.8)	97	66 (30–98)	35	11 (73–138)	64	83 (60–13)
Minor tobacco alkaloids								
Anabasine	76	13 (8–33)	97	14 (7–28)	35	64 (31–108)	64	73 (45–119)
Tobacco-specific nitrosamine (TSNA) metabolite								
4-methylnitrosamino)-4-(3-pyridil)-1-butanol (NNAL)	46	11 (8–23)	46	10 (5–15)	4	57 (44–71)	7	67 (58–169)

Biomarkers	Ex-smokers (n=77)		ENDS users (n=97)		Dual users (n=35)		Smokers (n=64)	
	number	median (Q1-Q3)	number	median (Q1-Q3)	number	median (Q1-Q3)	number	median (Q1-Q3)
Heavy metals and trace elements								
Aluminum (Al)	20	76 (36–135)	19	95 (73–121)	-	-	-	-
Antimony (Sb)	20	85 (49–129)	19	106 (69–126)	-	-	-	-
Arsenic (As)	20	62 (29–135)	19	90 (26–125)	-	-	-	-
Beryllium (Be)	20	79 (24–114)	19	95 (66–117)	-	-	-	-
Cadmium (Cd)	20	90 (79–107)	19	99 (70–137)	-	-	-	-
Chromium (Cr)	20	86 (58–141)	19	99 (67–157)	-	-	-	-
Cobalt (Co)	20	88 (74–202)	19	103 (56–173)	-	-	-	-
Copper (Cu)	20	64 (44–139)	19	92 (47–170)	-	-	-	-
Iron (Fe)	20	82 (35–129)	19	119 (80–135)	-	-	-	-
Lead (Pb)	20	60 (45–89)	19	105 (54–123)	-	-	-	-

Biomarkers	Ex-smokers (n=77)		ENDS users (n=97)		Dual users (n=35)		Smokers (n=64)	
	number	median (Q1-Q3)	number	median (Q1-Q3)	number	median (Q1-Q3)	number	median (Q1-Q3)
Manganese (Mn)	20	43 (32–104)	19	90 (56–138)	-	-	-	-
Molybdenum (Mo)	20	66 (37–131)	19	91 (56–148)	-	-	-	-
Nickel (Ni)	20	106 (51–183)	19	113 (86–191)	-	-	-	-
Palladium (Pd)	20	48 (30–83)	19	94 (62–119)	-	-	-	-
Platinum (Pt)	20	79 (41–100)	19	94 (62–111)	-	-	-	-
Selenium (Se)	20	106 (82–136)	19	94 (77–143)	-	-	-	-
Silver (Ag)	20	85 (49–114)	19	95 (66–119)	-	-	-	-
Tin (Sn)	20	78 (39–141)	19	94 (72–133)	-	-	-	-
Vanadium (V)	20	79 (43–129)	19	095 (62–119)	-	-	-	-
Zinc (Zn)	20	96 (63–140)	19	99 (86–155)	-	-	-	-
Polycyclic aromatic hydrocarbon (PAH) metabolites								

Biomarkers	Ex-smokers (n=77)		ENDS users (n=97)		Dual users (n=35)		Smokers (n=64)	
	number	median (Q1-Q3)	number	median (Q1-Q3)	number	median (Q1-Q3)	number	median (Q1-Q3)
1-Naphtol	76	4 (2–12)	97	5 (2–13)	35	46 (33–84)	64	73 (49–107)
2-Naphtol	76	39 (23–61)	97	42 (21–69)	35	87 (60–124)	64	90 (60–131)
1-Hydroxypyrene	76	41 (28–70)	97	42 (26–70)	35	77 (40–97)	64	67 (42–107)
Volatile organic compound (VOC) metabolites								
N-acetyl-S-(2-carbamoylethyl)-L-cysteine (AAMA)	76	34 (19–70)	97	28 (20–44)	35	84 (43–104)	64	84 (65–122)
N-acetyl-S-(2-cyanoethyl)-L-cysteine (CYMA)	76	2 (1–4)	97	1 (1–2)	35	57 (21–106)	64	64 (40–102)
N-acetyl-S-(3,4-dihydroxybutyl)-L-cysteine (DHBMA)	76	75 (53–98)	97	80 (58–101)	35	95 (62–118)	64	102 (73–151)
N-acetyl-S-(2-carbamoyl-2-hydroxyethyl)-L-cysteine (GAMA)	76	51 (31–87)	97	43 (26–76)	35	77 (54–131)	64	104 (75–146)
N-acetyl-S-(2-hydroxyethyl)-L-cysteine (HEMA)	76	51 (29–81)	97	49 (31–90)	35	87 (62–122)	64	80 (49–122)
N-acetyl-S-(2-hydroxypropyl)-L-cysteine (2-HPMA)	76	35 (21–52)	97	37 (21–69)	35	63 (31–118)	64	93 (63–120)
N-acetyl-S-(3-hydroxypropyl)-L-cysteine (3-HPMA)	76	27 (15–57)	97	26 (14–50)	35	89 (35–152)	64	78 (55–130)

Biomarkers	Ex-smokers (n=77)		ENDS users (n=97)		Dual users (n=35)		Smokers (n=64)	
	number	median (Q1-Q3)	number	median (Q1-Q3)	number	median (Q1-Q3)	number	median (Q1-Q3)
N-acetyl-S-(3-hydroxypropyl-1-methyl)-L-cysteine (HPMMA)	76	27 (17–46)	97	24 (17–36)	35	102 (39–134)	64	91 (61–121)
N-acetyl-S-(1-hydroxymethyl-2-propenyl)-L-cysteine + N-acetyl-S-(2-hydroxy-3-butenyl)-L-cysteine (1-MHBMA + 2-MHBMA)	76	60 (28–92)	97	40 (23–85)	35	57 (41–93)	64	76 (43–137)
N-acetyl-S-(4-hydroxy-2-buten-1-yl)-L-cysteine (3-MHBMA)	76	21 (14–35)	97	19 (13–32)	35	86 (41–126)	64	79 (58–118)
N-acetyl-S-(phenyl)-L-cysteine (SPMA)	76	89 (55–29)	97	83 (34–124)	35	90 (46–144)	64	82 (44–120)
Oxidative stress biomarkers								
8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG)	76	96 (78–128)	97	93 (76–111)	35	99 (76–124)	64	97 (74–122)
8-iso-prostaglandin F2α (8-isoprostane)	76	100 (72–131)	97	84 (61–122)	35	92 (72–129)	64	83 (57–110)