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## Comparison of the chemical composition of aerosols from heated tobacco products, electronic cigarettes and tobacco cigarettes and their toxic impacts on the human bronchial epithelial BEAS-2B cells

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1 **Title Page**

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4 **Comparison of the chemical composition of aerosols from heated tobacco products,**  
5 **electronic cigarettes and tobacco cigarettes and their toxic impacts on the human**  
6 **bronchial epithelial BEAS-2B cells**  
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64

65

## Abstract

66

67 The electronic cigarettes (e-cigs) and more recently the heated tobacco products (HTP)  
68 provide alternatives for smokers as they are generally perceived to be less harmful than  
69 conventional cigarettes. However, it is crucial to compare the health risks of these different  
70 emergent devices, in order to determine which product should be preferred to **substitute**  
71 **cigarette**. The present study aimed to compare the composition of emissions from HTP, e-  
72 cigs and conventional cigarettes, regarding selected harmful or potentially harmful  
73 compounds, and their toxic impacts on the human bronchial epithelial BEAS-2B cells. **The**  
74 **HTP emitted less polycyclic aromatic hydrocarbons and carbonyls than the conventional**  
75 **cigarette**. However, amounts of these compounds in HTP aerosols were still higher than in e-  
76 cig vapours. Concordantly, HTP aerosol showed reduced cytotoxicity compared to cigarette  
77 smoke but higher than e-cig vapours. HTP and e-cig had the potential to increase oxidative  
78 stress and inflammatory response, in a manner similar to that of cigarette smoke, but after  
79 more intensive exposures. In addition, increasing e-cig power impacted levels of certain toxic  
80 compounds and related oxidative stress. This study provides important data necessary for  
81 risk assessment by **demonstrating that HTP might be less harmful than tobacco cigarette but**  
82 **considerably more harmful than e-cig**.

83

84 **Keywords:** heat-not-burn tobacco; e-cigarette; lung; PAHs; carbonyls; **toxicity**

85

## Abbreviations

86

87 ALI: air-liquid interface

88 e-cig: electronic cigarette

89 GSH: reduced glutathione

90 GSSG: oxidized glutathione

91 HTP: heated tobacco products

92 Mb18W: Modbox e-cig model set at 18 W

93 Mb30W: Modbox e-cig model set at 30 W

94 PAHs: polycyclic aromatic hydrocarbons

95 **1. Introduction**

96

97 The tobacco epidemic causes 8 million deaths each year worldwide. Responsible for almost  
98 30 % of cancer-related deaths (especially 90 % of lung cancers) and being the major risk  
99 factor for chronic obstructive pulmonary diseases, smoking is the main single cause of  
100 preventable deaths in the world (WHO, 2019). Out of the more than 7,000 chemicals present  
101 in tobacco smoke, at least 250 are known to be harmful and about 70 can cause cancer:  
102 benzene, formaldehyde, acetaldehyde, acrylamide, nitrosamines, arsenic, cadmium... There  
103 is no safe level of tobacco use and quitting smoking significantly reduces the risk of  
104 developing smoking-related diseases. Although the health benefits are greater for people  
105 who stop at earlier ages, benefits exist at any age (Babb et al., 2017). However, smoking  
106 cessation is a difficult and challenging task because of the addictive power of nicotine, which  
107 is naturally found in tobacco (Benowitz, 2010). Nicotine replacement therapy (mostly  
108 available as transdermal patch, nasal spray, inhaler, gum and sublingual tablets) can help to  
109 increase chances of sustainable smoking cessation (Stead et al., 2012). However, none of  
110 these devices mimics the feeling of satisfaction that occurs with the rapid and abundant  
111 delivery of nicotine during smoking. New alternatives to regular cigarettes, such electronic  
112 cigarettes (e-cigs) and heated tobacco products (HTP), also called heat-not-burn tobaccos,  
113 have emerged on the market over the past decade. The e-cigs and emergent tobacco  
114 products are generally perceived as low-risk substitutes for cigarette and have quickly gained  
115 popularity, well before sufficient scientific evidence would allow to determine their potential  
116 detrimental effects on users.

117

118 E-cigs comprise a battery-powered heating element that is designed to vaporize a solution  
119 (“e-liquid”) made of propylene glycol and/or glycerine and frequently flavouring and  
120 nicotine. The vapour is then inhaled by the user. Concerns raised about the levels of some  
121 harmful constituents found in e-cig aerosols, such as carbonyl compounds (including  
122 formaldehyde, acetaldehyde and acrolein) (Beauval et al., 2017, 2019; Belushkin et al., 2020)  
123 and polycyclic aromatic hydrocarbons (PAHs) (including benzo[a]pyrene) (Beauval et al.,  
124 2017; Belushkin et al., 2020), which may be produced by thermal decomposition of e-liquid  
125 components. The presence of several trace metals was also reported in e-cig emissions,  
126 likely released by cartomizer components (Beauval et al., 2017; Zhao et al., 2019). In parallel,

127 some studies investigated the effects of e-cig vapour exposure on human bronchial epithelial  
128 cells cultured at air-liquid interface, and found that e-vapours did not lead to significant  
129 cytotoxicity, but induced oxidative stress and/or increased the secretion of pro-  
130 inflammatory mediators (Anthérieu et al., 2017; Iskandar et al., 2016; Scheffler et al., 2015).  
131 In addition, transcriptomic modifications induced by e-cig vapour exposure were  
132 demonstrated to be lesser than those induced by tobacco smoke (Anthérieu et al., 2017;  
133 Iskandar et al., 2016). Evolution in e-cig devices (especially the development of new e-cig  
134 generation with **high power, sub-Ohm devices**) may increase the amounts of harmful and  
135 potentially-harmful compounds in emissions (Beauval et al., 2019; Belushkin et al., 2020;  
136 Farsalinos et al., 2018c; Zhao et al., 2019). Consequently, further research is needed to  
137 better understand the impacts of e-cig model and power output on cellular toxicity.

138  
139 Although e-cig could help to quit smoking (Kalkhoran et al., 2019; Nelson et al., 2015), some  
140 vapers could be unsatisfied because e-cig devices do not use real tobacco ingredients and  
141 lack the so-called “throat-hit” or authentic tobacco tastes that cigarettes offer. These  
142 elements may encourage some people to go back to conventional cigarettes (Staal et al.,  
143 2018). Therefore, tobacco companies developed new tobacco products to keep meeting the  
144 changing needs of their consumers. They launched HTP that taste more like conventional  
145 cigarettes while still delivering nicotine to consumers. In particular, Philip Morris  
146 International created the IQOS device: disposable tobacco sticks soaked in propylene glycol  
147 (called heatsticks) are inserted in a holder in the IQOS device and heated with an electric  
148 blade (Smith et al., 2016). These new devices are marketed by Philip Morris International as  
149 products that do not combust tobacco, as cigarettes do, but heat it to a lower temperature  
150 (less than 350 °C) with the aim to avoid the combustion-related production of harmful  
151 compounds. To support the health claims of IQOS, Philip Morris International published  
152 several peer-reviewed papers (Malinska et al., 2018; Schaller et al., 2016a, 2016b; Sewer et  
153 al., 2016; Smith et al., 2016; van der Toorn et al., 2015; Wong et al., 2016). They showed a  
154 reduction of most of the harmful constituents found in tobacco smoke (carbonyl  
155 compounds, PAHs, nitrosamines...), as well as a reduction of cytotoxicity and genotoxicity in  
156 comparison to cigarette smoke exposure (Schaller et al., 2016a). Today, most data about  
157 HTP are published by HTP or tobacco industries themselves and toxicity assessment is

158 limited to a comparison with cigarette smoke. However, it is crucial for smokers to know the  
159 toxicity of HTP compared to conventional cigarette and e-cigs.

160

161 The aim of this study is to compare the chemical composition and the toxicological effects of  
162 aerosols from HTP (iQOS model), conventional cigarette smoke (3R4F) and vapours from one  
163 nicotine-containing e-liquid vaporized by different e-cig models or conditions (a second  
164 generation model with 2.6 Ohms coil and 4.6 W-fixed power and a third generation  
165 “Modbox” model with 0.5 Ohms coil and set at 18 W (Mb18W) or 30 W (Mb30W) power).  
166 Chemical characterization analyses focused on nicotine, carbonyl compounds and PAHs. The  
167 toxicity of the aerosols was evaluated in the air-liquid interface-cultured BEAS-2B cell line,  
168 which is the most commonly used human bronchial epithelial cell line for respiratory  
169 toxicological studies. Cellular effects were assessed by measuring cytotoxicity, oxidative  
170 stress and inflammatory response, which are key mechanisms leading to chronic respiratory  
171 diseases.

172

173

## 174 2. Material and methods

175

### 176 2.1 Tobacco products and e-cig

177 The HTP used in this study was the iQOS model manufactured by Philip Morris (Neuchâtel,  
178 Switzerland), with iQOS heatsticks (amber box from Philip Morris) purchased in a local  
179 tobacco shop. IQOS was cleaned regularly after each 20<sup>th</sup> heatstick, as recommended by the  
180 manufacturer. Two models of e-cig from a French manufacturer (NHOSS<sup>®</sup> brand) were used  
181 in these experiments. The first one was the second generation “Lounge” model, equipped  
182 with a 2.8 Ω nichrome coil and 4.6 W power supply. The coil heating was triggered by air  
183 suction. The second one was the third generation “ModBox” model, used with the “Air Tank”  
184 clearomiser equipped with a 0.5 Ω kanthal coil and with a partially closed air flow. Heating  
185 was pre-activated 1 s prior the puff, as recommended by the manufacturer and accordingly  
186 with the CORESTA standard puffing method CRM81 (CORESTA, 2015). Modbox model was  
187 tested at two power settings, 18 W and 30 W. These settings correspond to the lower and  
188 upper range power supplies recommended by the manufacturer for the coils used. One e-  
189 liquid was used, “blond tobacco” flavoured (NHOSS<sup>®</sup> brand) and labelled as follows:



190 propylene glycol < 65 %; glycerol < 35 %; food flavourings; nicotine 16 mg/mL. 3R4F research  
191 cigarettes were purchased from the University of Kentucky (Lexington, KY, USA).

192

## 193 2.2 Aerosol generation

194 Aerosols from HTP, e-cig and 3R4F cigarette were generated with a Vitrocell® VC1 smoking  
195 machine (Vitrocell, Waldkirch, Germany), as described previously (Anthérieu et al., 2017;  
196 Beauval et al., 2017, 2019). All products were tested with Health Canada Intense (HCI) puff  
197 profile (55 mL puff volume, 2 s puff duration, 30 s puff period). For 3R4F cigarette, all  
198 ventilation holes were blocked using adhesive tape during the experiments to follow the  
199 recommendations of this smoking regime (WHO Tobacco Laboratory, 2012). Under these  
200 settings, one 3R4F cigarette was completely consumed after 10 puffs and one heatstick was  
201 limited to 12 puffs by the IQOS device.

202

## 203 2.3 Quantification of nicotine

204 Nicotine was collected from aerosols into two glass impingers with fritted nozzle placed in  
205 series containing 50 and 25 mL of methanol, respectively, maintained at -40 °C.  
206 Quantification of nicotine in aerosol extracts was performed as described previously  
207 (Beauval et al., 2017). Each collection was replicated four times.

208

## 209 2.4 Identification and quantification of carbonyl compounds

210 Carbonyls were collected from aerosols into two silica cartridges coated with 2,4-  
211 dinitrophenylhydrazine (DNPH) placed in series, as described previously (Beauval et al.,  
212 2019). Sep-Pak DNPH-Silica Plus Short Cartridges containing 350 or 750 mg of sorbent per  
213 cartridge (Waters, Guyancourt, France) were used for e-cig and IQOS aerosols or cigarette  
214 smoke, respectively. Each collection was replicated four times. Blank collections were  
215 performed using the smoking machine working without e-cig, HTP or conventional cigarette  
216 connected to and were taken into account for data analysis. DNPH cartridges were eluted with  
217 3 or 6 mL of acetonitrile for short and long cartridges, respectively. Elutions from both  
218 cartridges placed in series were pooled and then injected into a Thermo Scientific Dionex  
219 UltiMate 3000 UHPLC System with UV/VIS Detector (Thermo Scientific, Waltham, MA). The  
220 UHPLC instrument was operated at 28 C° at a constant flow rate of 0.4 mL/min, under a

221 gradient of acetonitrile and water during 15 min. Analysis was performed using Acclaim  
222 Carbonyl C18 RSCL 150 mm x 2.1 mm x 5  $\mu$ M (Thermo Fisher Scientific). Acquisition was  
223 performed at 360 nm wavelength. Instrument monitoring and data acquisition were done  
224 using Chromeleon 7.0 Data Acquisition System for LC (Thermo Scientific, Waltham, MA). The  
225 method allows the detection and quantification of nineteen compounds (Table 1) with limits  
226 of quantification (LOQs) ranging from 6 to 15 ng/mL. Acrolein was not measured in this study  
227 due to the unsuitability of using the DNPH-coated solid sorbent cartridge for its collection  
228 (Ho et al., 2011).

229

230 The number of puffs for each collection was determined in preliminary experiments in order  
231 to avoid saturation in the cartridges. Finally, for e-cig, HTP and 3R4F analyses, 20, 4 and 1  
232 puff(s) were respectively found to be the best compromise with satisfying efficiency without  
233 saturation of the cartridge (data not shown). Moreover, concerning e-cig, we measured  
234 carbonyl emissions in the twenty last puffs of one-hour-exposure session (100-120 puffs). To  
235 compare the different aerosol samples, the levels of the carbonyls were then expressed in  
236 mass of each compounds by puff.

237

## 238 2.5 Identification and quantification of PAHs

239 PAHs were collected from aerosols simultaneously with nicotine collection in the same  
240 impingers, as described previously (Beauval et al., 2017). Each collection was replicated four  
241 times. Blank collections were performed using the smoking machine working without e-cig,  
242 HTP or conventional cigarette connected to and were taken into account for data analysis.  
243 Methanolic samples were first concentrated under a gentle nitrogen flow in a water bath at  
244 45 °C to a final volume of 1 mL in order to subsequently extract PAHs using accelerated  
245 solvent extraction with acetonitrile. This extraction was followed by a second concentration  
246 step under a gentle nitrogen flow in a water bath at 60 °C to a final volume of 1 mL.  
247 Remaining products were then filtrated with a syringe membrane filter before injection into  
248 the chromatographic system. The analyses were performed on HPLC Alliance 2695 (Waters  
249 Corporation, MA) coupled with a multi-wavelength fluorescence detector. Chromatographic  
250 separation was achieved on a EC 250/2 Nucleosil 100-5 C18 PAH (Macherey Nagel, Hoerd, t,  
251 France). The method allows the detection and quantification of twenty-three compounds  
252 (Table 2) with LOQs ranging from 1 to 39 pg/mL.

253

## 254 2.6 Cell culture and cell exposure to aerosols

255 *In vitro* experiments were performed using the human bronchial epithelial BEAS-2B cell line  
256 obtained from the American Type Culture Collection (ATCC® CRL9609™). Cells were cultured  
257 in LHC-9 medium (Life Technologies, Courtabœuf, France) and seeded in CellBIND 75 cm<sup>2</sup>  
258 tissue culture flasks (Corning, Amsterdam, Netherlands) at 37 °C in a humidified incubator  
259 with 5 % CO<sub>2</sub> in air and 85 % relative humidity. Cell passaging was performed when cells  
260 reached 80-90 % confluence. Following subculture, cells were transferred to an air-liquid  
261 interface (ALI) system. Cells were seeded (18 000 cells/cm<sup>2</sup>) onto transwell clear culture  
262 inserts (4.67 cm<sup>2</sup>) with 0.4 µm pore size (Sigma Aldrich, Saint-Quentin Fallavier, France) pre-  
263 coated with 0.03 mg/mL type I collagen solution (Life Technologies). BEAS-2B cells were  
264 firstly maintained submerged, then ALI was established by removing medium from the apical  
265 surface, exposing only the basal surface to medium. Cells were then transferred to an  
266 exposure module (Vitrocell 6/4 CF module) and exposed to different doses (defined in puff  
267 number) of the undiluted HTP, e-cig or cigarette aerosol generated by the Vitrocell® system.  
268 Control cells were unexposed cells, which were left in the incubator. Each exposure was  
269 replicated in three independent cell cultures.

270

## 271 2.7 Cell viability

272 BEAS-2B cells were exposed to different puff number of undiluted aerosols (40, 80 and 120  
273 puffs for e-cig; 2, 12, 40, 80 and 120 puffs for HTP and 1, 2, 4 and 10 puffs for 3R4F cigarette)  
274 and cell viability was measured 24 h after exposure *via* the Cell Titer-Glo Luminescent Cell  
275 Viability assay kit (Promega, Charbonnières, France), as described previously (Anthérieu et  
276 al., 2017). Intracellular ATP was determined as percentages related to the ATP content in  
277 control cells arbitrarily set at a value of 100 %.

278

## 279 2.8 Glutathione content assay

280 The GSH/GSSG-Glo™ Assay (Promega) was used following the manufacturer's guidelines for  
281 the determination of total GSH and oxidized glutathione (GSSG). Finally, GSSG/GSH ratios  
282 were calculated and results are expressed as fold-change relative to the GSSG/GSH ratio in  
283 control cells arbitrarily set at a value of 1.

284

## 285 2.9 Gene expression analysis

286 The total RNA of BEAS-2B cells was extracted using the RNeasy plus mini kit (Qiagen,  
287 Courtaboeuf, France) following the manufacturer's instructions. Expression of target genes  
288 was measured by quantitative real-time PCR of corresponding reverse-transcribed mRNA.  
289 One  $\mu\text{g}$  of total RNA was reverse-transcribed into cDNAs using the High Capacity cDNA  
290 Reverse Transcription kit (Applied biosystems, CA, USA). qPCRs were carried out with the  
291 StepOnePlus thermocycler (Applied Biosystems), using the TaqMan Fast advanced Master  
292 Mix (Applied Biosystems) and the following TaqMan Assays: Hs99999901\_s1, *18S*;  
293 Hs01054797\_g1, *CYP1A1*; Hs00164383\_m1 *CYP1B1*; Hs01110250\_m1, *HMOX1*;  
294 Hs01045993\_g1, *NQO1*. Amplification curves were read with the StepOne software V2.1  
295 using the comparative cycle threshold method. The relative quantification of the steady-  
296 state mRNA levels was normalized against *18S* RNA. Results are expressed as fold-change  
297 relative to the levels in control cells arbitrarily set at a value of 1.

298

## 299 2.10 Measurement of secreted mediators of inflammation

300 Concentrations of ten secreted inflammation mediators were measured in the basolateral  
301 media of BEAS-2B cells: granulocyte-macrophage colony-stimulating factor (GM-CSF),  
302 growth regulated oncogene  $\alpha$  (GRO- $\alpha$ ), interleukin  $1\beta$  (IL- $1\beta$ ), interleukin 6 (IL-6), interleukin  
303 8 (IL-8), interleukin 13 (IL-13), monocyte chemoattractant protein 1 (MCP-1), macrophage  
304 inflammatory protein 1-alpha (MIP-1  $\alpha$ ), regulated on activation, normal T cell expressed and  
305 secreted (RANTES) and interferon gamma (INF- $\gamma$ ). The assay has been performed based on  
306 the recommendations of the Milliplex MAP Human Cytokine/Chemokine Magnetic Bead  
307 Panel kit (Merck Millipore, Molsheim, France) using the Luminex<sup>®</sup> xMAP<sup>®</sup> technology  
308 (Luminex Corp., Austin, TX). The capacity of BEAS-2B cells to secrete various mediators of  
309 inflammation had been previously tested by treating cells with lipopolysaccharide (Anthérieu  
310 et al., 2017).

311

312

## 313 2.11 Statistical analysis

314 Data were represented by the mean  $\pm$  SD of four independent measurements. Statistical  
315 analyses were performed using the non-parametric Mann-Whitney test. Data were  
316 considered significantly different when  $p < 0.05$ .

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### 3. Results and discussion

#### 3.1 Chemical characterization of aerosols

##### 3.1.1 Nicotine content

The potential of the HTP and e-cig to substitute smoking is expected to be at least dependent on the level of nicotine delivered in their aerosols and subsequently inhaled by the user (Farsalinos et al., 2018a). Therefore, the levels of nicotine present in the HTP aerosols were determined and compared with those in the tobacco cigarette smoke and vapours from different e-cig models **used with an e-liquid containing 16 mg/mL of nicotine.** Levels of nicotine delivered to the aerosols are presented in figure 1. HTP delivered about 30 % less nicotine to its aerosol (63 µg/puff) than the 3R4F cigarette (95 µg/puff) under the HCl puffing profile, as described in other studies (Belushkin et al., 2020; Farsalinos et al., 2018a; Li et al., 2019; Schaller et al., 2016a). For e-cig emissions, the second generation Lounge model provided strongly less nicotine amounts (8 µg/puff) than the two tobacco products tested, while the third generation Modbox model delivered 60 µg/puff at 18 W setting and 137 µg/puff at 30 W setting. Increasing power supply of e-cig has already been shown to increase nicotine level in vapour, mostly due to a more efficient vaporization of the e-liquid (Talih et al., 2015). In summary, the nicotine delivery is highly variable (from 8 to 137 µg of nicotine/puff) depending on the device in comparable conditions of use (under HCl puffing regime). This parameter is important, while it is expected a phenomenon of nicotine self-titration with smokers who want to switch from cigarette to nicotine-delivering alternatives, such as e-cig or HTP (Farsalinos et al., 2018c; Woodward & Tunstall-Pedoe, 1993). To compensate and obtain a similar nicotine amount from HTP or e-cig as from tobacco cigarette, they might adopt a more intense “puffing regime” and/or consume more puffs with HTP or e-cig. Consequently, the nicotine concentrations in emissions will be used to estimate a “normalized” exposure to other harmful and potentially harmful compounds.

##### 3.1.2 Carbonyl and PAH contents

348 Carbonyls and PAHs are part of principal compounds released from the tobacco combustion  
349 and many of them contribute significantly to the carcinogenic activity of tobacco smoke  
350 (IARC, 2004). Indeed, the carbonyl compounds, formaldehyde and acetaldehyde, are  
351 respectively classified as carcinogenic (Group 1) and possibly carcinogenic (Group 2B) to  
352 humans by the International Agency for Research on Cancer (IARC, 2016). Among the PAHs,  
353 the benzo[a]pyrene is classified in Group 1 and the dibenzo[a,h]anthracene in Group 2A as  
354 probably carcinogenic to humans. Within the framework of tobacco harm reduction, it  
355 appears interesting to measure and compare the levels of these harmful and potentially  
356 harmful compounds in the emissions of HTP and e-cig.

357  
358 The emission of nineteen carbonyl compounds in the aerosols of HTP, 3R4F cigarette and e-  
359 cigs was first analysed and results (expressed in mass of each compound per puff) are  
360 reported in Table 1. Fifteen compounds were quantified at levels between 2 and 15 times  
361 higher in cigarette smoke than in HTP aerosols. Hexanal was the only compound measured  
362 at a higher level in HTP emission (22.2 ng/puff) than in 3R4F tobacco smoke (10.5 ng/puff),  
363 and benzaldehyde was generated in almost equivalent amounts by HTP and cigarette. By  
364 contrast, all the carbonyl compounds were measured at very lower amounts in vapours from  
365 the different tested e-cig conditions in comparison to tobacco products, except for m-  
366 tolualdehyde and 2,5 dimethylbenzaldehyde which were only detected in aerosols from the  
367 Modbox device, at both low and high-power settings. Figure 2-A represents the sum of total  
368 carbonyl compounds measured in e-cig, HTP and 3R4F emissions. There are much less  
369 carbonyl compounds produced in one puff of HTP (35 µg/puff) than in one puff of cigarette  
370 smoke (230 µg/puff). Indeed, HTP emitted 84.7 % less carbonyl compounds than 3R4F  
371 cigarette (Figure 2-B). Levels of carbonyl compounds measured in vapours from different e-  
372 cig models were even at least 98.5 % weaker than in HTP aerosols. Comparison of data  
373 between different e-cig devices showed that Lounge and Mb18W emitted respectively 82.3  
374 % and 51.4 % less carbonyl compounds than Mb30W (0.4 µg/puff) (Figures 2-A and 2-B).

375  
376 These different tobacco products and e-cigs produced carbonyls at very different levels in  
377 emissions, probably because they generate aerosols *via* different processes and from diverse  
378 materials (tobacco fillers or e-liquid). During smoking, carbonyls are mainly produced by the  
379 pyrolysis of carbohydrates contained in tobacco (Seeman et al., 2002) at high temperature

380 (up to 900 °C). The aerosolization process of HTP operates at temperatures less than 350 °C  
381 with the use of heatsticks containing tobacco leaves soaked in propylene glycol. These  
382 devices are often referred to as “heat-not-burn” tobacco products with a reduction of the  
383 user’s exposure to carcinogenic chemicals usually produced by the combustion of tobacco  
384 (Schaller et al., 2016a). We effectively observed a reduction of about 90 % in carbonyl  
385 emissions in accordance with data from the IQOS manufacturer (Schaller et al., 2016) and  
386 two independent studies (Farsalinos et al., 2018b; Li et al., 2019). However, several harmful  
387 carbonyls were still measured in HTP aerosol, such as acetaldehyde and formaldehyde,  
388 which are carcinogenic compounds. The presence of high levels of acetaldehyde is the mark  
389 of pyrolysis and thermogenic degradation of tobacco (Auer et al., 2017). By contrast, the  
390 main source of carbonyls in e-cig emissions is the thermal degradation of glycerol and  
391 propylene glycol contained in the e-liquid (Uchiyama et al., 2020). Some carbonyls were  
392 quantifiable in the different vapours of tested e-cig, but several times far lower than in the  
393 emissions from both tested tobacco products (Table 1). **In addition, there is a relation**  
394 **between the operating power or the e-cig model and the concentrations of carbonyl**  
395 **compounds detected in the e-cig aerosols. The power of e-cig, and therefore ultimately the**  
396 **heat generated on the evaporation coil, has been reported to affect the quantity of**  
397 **carbonyls formed (Geiss et al., 2016; Kosmider et al., 2014; Talih et al., 2016).** Some studies  
398 reported higher carbonyl amounts in e-cig emissions (Goniewicz et al., 2014; Hutzler et al.,  
399 2014; Sleiman et al., 2016; Talih et al., 2015). However, these findings have been questioned  
400 as they could be the consequence of using unrealistic or extreme conditions (low interpuff  
401 interval or high power generating high temperatures, drypuff phenomenon) (Farsalinos et  
402 al., 2015, 2017). Indeed, e-cig can release high levels of aldehydes if the e-liquid is  
403 overheated, but the overheating generates an aversive taste that would secure such  
404 emissions to be avoided. To ensure realistic experimental conditions, two regular e-cig users  
405 tested the e-cigs used in our study for the generation of dry puffs, using the puff duration  
406 and power settings as tested with the smoking machine. The users confirmed no dry puff  
407 sensation and sufficient vapour production. In addition, the temperature of the generated  
408 aerosol was also considered as an indicator of experimental relevance and realism. The  
409 temperature of the aerosols generated from each device did not exceed 60 °C during all the  
410 collection periods (Supplemental Figure 1), following the recommendations of the French  
411 national organisation and standardization (AFNOR, 2016). Overall, our data thus

412 demonstrate that, at normal vaping temperatures, carbonyl content in e-cig emissions  
413 represents only a small fraction of levels inhaled by users of tobacco products.

414

415 In parallel, the emission of twenty-three PAHs in the aerosols of HTP, 3R4F cigarette and e-  
416 cigs was analysed and results expressed in ng/puff are reported in Table 2. A similar pattern  
417 than that seen for carbonyl compounds was observed for almost all emitted PAHs: the  
418 concentrations of twenty-one compounds were markedly lower in HTP emissions than in  
419 3R4F cigarette smoke (from 2 to 676 times depending on the compound), and were even  
420 lesser in e-cig vapours. Only benzo(c)phenanthrene was reported to be higher in HTP  
421 emissions, compared to all other aerosols. The sum of total PAHs measured was calculated  
422 for each aerosol (Figure 3-A) and the reduction rate is indicated in figure 3-B: HTP (0.7  
423 ng/puff) emitted 96.2 % less PAHs than 3R4F cigarette (19.6 ng/puff), but e-cig emitted 64.9  
424 to 78.2 % less PAHs than HTP. Comparison of e-cig models showed no significant difference  
425 in PAH content between Mb18W and Mb30W, and about 40 % less of total PAHs in Lounge  
426 than in the Modbox model. These results support that the pyrolysis process is limited with e-  
427 cigs. The e-liquid used for vaping is generally free of tobacco ingredients which contain the  
428 PAH precursors. **Moreover, the temperature required to produce an e-cig aerosol from a e-  
429 liquid is depending of the proportion in propylene glycol and glycerol. This temperature  
430 ranges from 188.6°C to 292 °C, but water and alcohol used as additives, decrease this boiling  
431 point (Duell et al., 2018). By comparison, IQOS operates at temperatures between 330°C and  
432 349°C (Davis et al., 2019).** PAH emissions released by HTP were lower than combustible  
433 cigarette but still contained harmful elements from thermal degradation that are also found  
434 in cigarette smoke (Li et al., 2019; Rodgman et al., 2000), including the carcinogenic  
435 benzo[a]pyrene.

436

437 The quantifications of carbonyl and PAH compounds were first presented in mass of  
438 analysed compounds per puff to compare devices with each other. However, users do not  
439 necessarily consume the same number of puffs when using HTP, e-cig or conventional  
440 cigarettes. They appear to self-regulate their consumption (number, frequency and volume  
441 of puffs, notably) according to their needed quantity of nicotine (Farsalinos et al., 2018c). To  
442 take into consideration this nicotine self-titration, it appears relevant to also report all the  
443 amounts of harmful and potentially harmful compounds per nicotine yield. Detailed data for



444 carbonyl and PAH levels, normalized by the level of emitted nicotine in aerosols, are  
445 reported in Tables S1 and S2 (supplementary materials), respectively. The comparison of  
446 total carbonyl compounds after nicotine normalization (Figures 2-C and 2-D) showed,  
447 according to previous conclusions, that the HTP emitted 76.9% less carbonyl compounds  
448 (497 ng/μg of nicotine) than the combustible cigarette (2308 ng/μg of nicotine), but at least  
449 97.9% higher levels than the e-cig vapours (< 10 ng/μg of nicotine). However, comparing the  
450 e-cig models, Lounge emitted more carbonyl compounds than the Modbox model and no  
451 significant difference was reported between Mb18W and Mb30W. The comparison of total  
452 PAHs after nicotine normalization (Figures 3-C and 3-D) also showed substantial reduction  
453 (94.3%) in the PAH content of HTP emissions (11 pg/μg of nicotine) in comparison to  
454 cigarette smoke (207 pg/μg of nicotine). The pattern of PAH content between the different  
455 e-cig models was different after nicotine normalization: the Lounge model emitted more  
456 PAHs (15 pg/μg of nicotine) than the Modbox model (79.2-90 %) and even 27.5 % more than  
457 HTP. These results showed that the way of expressing data (emissions per puff vs emissions  
458 per nicotine yield) can influence their interpretation. Today, there is no standardized manner  
459 to express the amount of emitted compounds in aerosols. Indeed, data can be expressed in  
460 amount per puff (Beauval et al., 2019), per mL of puff (Beauval et al., 2017), per cigarette or  
461 per IQOS heatstick (Li et al., 2019), per mass of nicotine (Farsalinos et al., 2018b), per liquid  
462 consumption for e-cig (Beauval et al., 2017), thus hampering comparisons between studies  
463 and making interpretations difficult. In addition, it is still unclear to which extent vapor  
464 generation, collection and analysis procedures could affect results of chemical  
465 characterization. Harmonized protocols to determine the chemical composition of emissions  
466 and to express results are crucially needed to establish and compare risk profiles of each  
467 emergent tobacco products in terms of chemical composition and user exposure.

468

### 469 3.2 *In vitro* toxicity

470 The apparent reduction of some harmful constituents in HTP and e-cig emissions in  
471 comparison to tobacco cigarette cannot be directly extrapolated to a proportionate harm  
472 reduction for smokers. Today, research is needed about toxicological impacts of these  
473 products on human airway epithelial cells in comparison with tobacco cigarette. The use of  
474 undiluted aerosols is described as a more sensitive method to compare responses from  
475 aerosols produced from emergent products, such as HTP and e-cig (Bishop et al., 2019).

476 Thus, human bronchial epithelial BEAS-2B cells cultured at ALI were exposed to undiluted  
477 emissions from HTP, e-cig and 3R4F cigarette and effects of those emissions were evaluated  
478 on cytotoxicity, oxidative stress and inflammatory response, which are key mechanisms  
479 leading to chronic airway diseases.

480

### 481 3.2.1 Cell viability

482 Cell viability was assessed 24 h after aerosol exposure by measuring intracellular ATP  
483 content, which is directly proportional to the number of living cells. Data displayed in **figure**  
484 **4** (A-E) show that e-cig vapours had no significant (Modbox model) or low (Lounge model: >  
485 75 % cell viability) cytotoxic effects up to a 120-puff-exposure. In comparison, HTP emissions  
486 caused intracellular ATP changes from 12 puffs (89 % viability) to a strong reduction of cell  
487 viability that reached 2 % after 120 puffs. Cigarette smoke demonstrated also a full dose-  
488 response curve, but its cytotoxicity appeared within fewer puffs (< 10 puffs). In order to  
489 better compare the different devices, the effective dose of aerosol which results in a 50 %  
490 reduction of cell viability (ED50, expressed here in puff number) was calculated. Although  
491 the e-cig vapours did not induce sufficient cell mortality to calculate an ED50 (whatever the  
492 tested e-cig power or model), ED50 was 45 puffs for HTP aerosol and 2 puffs for 3R4F  
493 cigarette smoke. Bishop *et al.* have exposed a 3D-reconstituted human airway epithelium to  
494 undiluted cigarette smoke and e-cig aerosol (Bishop et al., 2019). They found ED50 equal to 4  
495 puffs and 60 puffs under HCl regime for cigarette and e-cig exposures, respectively.  
496 However, they voluntarily used extreme conditions for e-cig exposure with an airflow vent  
497 closed to achieve a worst case for carbonyl production and, consequently, higher  
498 cytotoxicity. In a previous study using diluted aerosols (Anthérieu et al., 2017), we have  
499 tested the Lounge model with different e-liquids (with or without nicotine, flavoured or  
500 unflavoured) and demonstrated that none of the aerosols induced cytotoxicity in BEAS-B cell  
501 line up to an exposure of 576 puffs. Today, few *in vitro* studies have compared the relative  
502 cytotoxicity of HTP aerosols with both cigarette smoke and e-cig vapours, and most of these  
503 assays have been performed using submersed cultures exposed to aerosol extracts (Ito et al.,  
504 2019; Munakata et al., 2018; Sohal et al., 2019). ALI exposures provide a more pertinent  
505 approach to perform toxicological studies related to inhalation of emerging **e-cigs** or novel  
506 tobacco products (Johnson et al., 2009). Our present results are in agreement with a study  
507 performed in ALI-cultured human bronchial epithelial H292 cells, demonstrating that HTP

508 show reduced cytotoxicity relative to combustible cigarette, but higher cytotoxicity than e-  
509 cig (Leigh et al., 2018). There is no standardized approach to compare the *in vitro* toxicity  
510 between emergent tobacco products, e-cigs and tobacco cigarette. Some authors used the  
511 same exposure time or the same puff number between the different aerosols while some  
512 used comparable nicotine exposure (Wang et al., 2019). Therefore, we also reported cell  
513 viability as a function of emitted nicotine (Figure 4-F). The results were equivalent with or  
514 without nicotine normalization: a higher cytotoxicity for tobacco cigarette (ED50 = 0.2 mg of  
515 nicotine) than for HTP (ED50 = 2.8 mg of nicotine) and low cytotoxicity for e-cig. These  
516 differences in cytotoxic effects are probably attributable to lower concentrations of  
517 potentially harmful chemicals in HTP and especially e-cig aerosols. Based on these  
518 cytotoxicity data, sub-toxic (> 75 % cell viability) or toxic doses were chosen for the further  
519 analyses that assessed oxidative stress and inflammation: 40 and 120 puffs for e-cig; 2, 12  
520 and 40 puffs for HTP; 1 and 2 puffs for 3R4F cigarette.

521

### 522 3.2.2 Oxidative stress

523 The generation of oxidative stress was first assessed by measuring the intracellular content  
524 of oxidized (GSSG) and reduced (GSH) glutathione. GSH is considered to be one of the most  
525 important scavengers of reactive oxygen species (ROS), and the ratio GSSG/GSH may be used  
526 as a marker of oxidative stress. We have previously described that the generation of  
527 oxidative stress was transient and no change in glutathione levels was found in BEAS-2B cells  
528 3 h after the end of cigarette smoke exposure (Anthérieu et al., 2017). Consequently, we  
529 have measured GSH and GSSG contents immediately (0 h) after cell exposures (Figure 5). In  
530 these experimental conditions, HTP induced a significant increase of GSSG/GSH ratio in  
531 comparison to control cells (2.7 and 4.5 fold-changes after 12 and 40 puffs, respectively).  
532 3R4F cigarette induced also an increase of the GSSG/GSH ratio but already after only 1 puff  
533 (2.7 fold-change) and a 7.8 fold-change was observed after an exposure to 2 puffs. For e-cig  
534 exposures, anti-oxidative response was evidenced only after a longer exposure of 120 puffs  
535 with Mb30W (2.9 fold-change), although no significant change in the GSSG/GSH ratio was  
536 observed with Mb18W and Lounge.

537 The cellular defense mechanisms against toxic substances also include transcriptomic  
538 regulations of genes involved in detoxification processes and the anti-oxidative response.  
539 Thus, the expression of selected genes involved in xenobiotic metabolism (cytochrome P450

540 1A1, *CYP1A1*; cytochrome P450 1B1, *CYP1B1*) and the anti-oxidative response (heme  
541 oxygenase 1, *HMOX1*; NADPH Quinone Dehydrogenase 1, *NQO1*) was quantified 4 or 24 h  
542 after exposure to 120 puffs of e-cig vapours, 12 puffs of HTP aerosol or 1 puff of 3R4F  
543 cigarette smoke (Figure 6). For all the products and both time points, an increase of *CYP1A1*  
544 and *CYP1B1* expression was observed, with a higher induction for *CYP1A1* than for *CYP1B1*.  
545 PAHs contained in cigarette smoke are known to induce expression of *CYP1A1/1B1* via the  
546 Aryl Hydrocarbon Receptor (AhR) pathway and, subsequently, to affect the metabolism of  
547 tobacco carcinogens (Sacks et al., 2011). The *CYP1A1/1B1* expression is also induced in the  
548 lungs of smokers (Kim et al., 2004). E-cig aerosols can also induce *CYP1A1* and *CYP1B1* and  
549 enhance the metabolism of some PAHs (e.g. benzo[a]pyrene) to genotoxic products by  
550 activating AhR (Sun et al., 2019). Furthermore, the expression of the downstream target  
551 genes of the nuclear erythroid 2-related factor 2 (Nrf2), *NQO1* and *HMOX1*, was up-  
552 regulated 4 h after exposure to the Lounge model vapours and at both time points for all  
553 other emissions. The up-regulation of *NQO1* and *HMOX1* levels 4 h after exposure increased  
554 with the e-cig power, reaching a 3.3 or 95-fold-change, respectively, for Mb30W. Some  
555 smoke compounds, including carbonyls, PAHs, quinones, naphthoquinones and  
556 benzenediols, were identified as activators of the Nrf2/antioxidant response element (ARE)  
557 pathway and *HMOX1* induction in response to oxidative stress (Chan et al., 2013; Sekine et  
558 al., 2016; Zhang et al., 2019). Some of these different components of cigarette smoke were  
559 also found in aerosols of HTP or e-cig, which can explain the up-regulation of *NQO1* and  
560 *HMOX1* in BEAS-2B cells whatever the products. Almost all of these transcriptomic  
561 modulations were higher after 4 h exposure than after 24 h and correspond to early  
562 adaptive mechanisms set up by BEAS-2B cells in response to a cellular stress after aerosol  
563 exposure. It is important to note that these transcriptomic mechanisms were globally similar  
564 for all the devices tested, demonstrating that all products have the potential to induce  
565 detoxification and an anti-oxidative response. However, these molecular and cellular  
566 responses were observed for highly different exposure levels (from 120 puffs for e-cig, 12  
567 puffs for HTP and only 1 puff for tobacco cigarette). In addition, an exposure of 120 puffs in  
568 one hour is representative of an intense exposure session, compared to data from  
569 topography studies with e-cig users (Jones et al., 2020; Lee et al., 2018).

570

571 These results are in accordance with other studies demonstrating that the generation of ROS  
572 is observed in HTP emissions after a more intensive use than with cigarette smoke  
573 (Munakata et al., 2018). ROS would be mainly generated by exposure to chemicals derived  
574 from combustion processes (Kopa & Pawliczak, 2020) and, therefore, can be directly linked  
575 to the relative amounts of carbonyls and PAHs measured in HTP and cigarette smoke  
576 emissions (Figures 2 and 3). The increase in the amount of carbonyl compounds produced  
577 when Modbox was used under high power setting could explain, at least in part, the greater  
578 induction of ROS production that would contribute to alter the oxidative/antioxidative  
579 balance. Indeed, higher power leads to higher filament temperature, which enhances the e-  
580 liquid vaporization process, pyrolysis and chain reactions with the production of hydroxide  
581 and superoxide free radicals (Haddad et al., 2019; Son et al., 2019; Zhao et al., 2018).  
582 However, Son *et al.* reported that the dose of free radicals per puff associated with e-cig  
583 vaping was 10-1000 times lower than the reported dose generated by cigarette smoking  
584 (Son et al., 2019).

585

586

### 587 3.2.3 Inflammatory response

588 The inflammatory response of BEAS-2B cells was assessed by measuring the secretion of ten  
589 cytokines and chemokines 24 h after exposure to defined subtoxic doses of HTP, 3R4F  
590 cigarette smoke or e-cig emissions. Only four mediators were found in quantifiable levels (IL-  
591 6, IL-8, GRO $\alpha$ , and MCP-1) after exposure. GM-CSF, IL-13, IL-1 $\beta$ , MIP-1 $\alpha$ , RANTES and TNF- $\alpha$   
592 were not detected in cell culture medium of BEAS-2B cells exposed to aerosols, despite the  
593 capacity of these cells to secrete these mediators after treatment with lipopolysaccharide  
594 (Anthérieu et al., 2017). The results for the four detected mediators were expressed in fold-  
595 change relative to control cells (Table 3). A significant increase of IL-6 secretion was  
596 observed in the culture medium of cells exposed to Mb18W (for 120 puffs) and Mb30W (for  
597 both 40 and 120 puffs) aerosols, while no significant change was evidenced for IL-8  
598 secretion. Increase of IL-6 and/or IL-8 had already been described in different cell models  
599 after e-cig exposures (Merecz-Sadowska et al., 2020), and more remarkably in the plasma of  
600 e-cig users (Singh et al., 2019), demonstrating that e-cig vapors could induce a pro-  
601 inflammatory response. For HTP, a biphasic response was observed for IL-6 and IL-8 with a  
602 greater increase in secretion after 12 puffs (3 and 3.3 fold-change, respectively) than after 40

603 puffs (1.3 fold-change). A comparable biphasic response in IL-8 secretion was previously  
604 described in BEAS-2B cells exposed to HTP aerosol or tobacco smoke and a similar trend was  
605 also observed for GM-CSF (Munakata et al., 2018), which was undetectable in our study.  
606 These differences in results could be explained by the different protocols of cell exposure.  
607 Indeed, Munataka *et al.* exposed submerged BEAS-2B cells to aerosol extracts, while ALI-  
608 cultured cells were exposed directly to aerosols in our study. Finally, the secretion of MCP-1  
609 and GRO- $\alpha$  was significantly decreased for the longer exposures to HTP aerosol, 3R4F  
610 cigarette smoke and Mb30W vapour (Table 3). A down-regulation of GRO- $\alpha$  and MCP-1 was  
611 also demonstrated after exposure to cigarette smoke in human endothelial cells (Allam et  
612 al., 2013). GRO- $\alpha$  plays a significant role in the chemotaxis of neutrophils to the site of  
613 inflammation and MCP-1 is a potent chemoattractant for monocytes and macrophages. This  
614 recruitment and subsequent activation of monocytes into the inflamed tissues play a central  
615 role in determining the outcomes of the immune responses of the tissues. Thus, alteration of  
616 GRO- $\alpha$  and MCP-1 secretion after aerosol exposure could affect the tissue immune and  
617 protective responses.

618

619 These different modulations in inflammatory mediators can be explained partially by the  
620 carbonyl and PAH levels measured in the different emissions. Indeed, some carbonyls found  
621 in aerosols or smoke (such as acrolein, acetaldehyde and formaldehyde) are known strong  
622 irritants that may cause inflammation (Lino-dos-Santos-Franco et al., 2011; Shields et al.,  
623 2017). The benzo[a]pyrene is also known as an inducer of the secretion of pro-inflammatory  
624 cytokines (Chen et al., 2012). Other constituents of aerosols or smoke could play a major role  
625 in the inflammatory response. Metals are capable of causing inflammatory cytokine induction  
626 (Lerner et al., 2015). In addition, propylene glycol and glycerol produce a  
627 hygroscopic/hyperosmolar aerosol which could deposit on the surface of lung cells and  
628 trigger local inflammation (Chaumont et al., 2019).

629

630

631 Carbonyls and PAHs represent only a part of the complex mixture constituting the cigarette  
632 smoke or the HTP and e-cig aerosols. Therefore, these pollutants take part in the cellular  
633 response but all the changes observed in BEAS-2B cells (cytotoxicity, oxidative stress,  
634 inflammation) cannot be explained solely by PAH and carbonyl emissions. A multitude of

635 other harmful compounds comprising other volatile organic compounds, metals,  
636 nitrosamines etc. (Li et al., 2019; Schaller et al., 2016; Zhao et al., 2019) should take into  
637 account to better characterize the toxic profile of these novel tobacco products and e-cigs in  
638 comparison to tobacco cigarette.

639

640

#### 641 4. Conclusion

642

643 Within the framework of tobacco harm reductions, in which smokers ideally should be able  
644 to freely choose from a variety of alternatives for smoking, emerging tobacco products (such  
645 as HTP) and e-cig seem to have potential of a promising new offering. However, it is  
646 fundamental for smokers to know and compare the health risks of these different emergent  
647 devices in order to determine which product should be preferred for smoking cessation. Our  
648 study provides comparative data on both chemical composition of HTP, e-cig and tobacco  
649 cigarette emissions and their toxicological impacts on human bronchial epithelial cells. We  
650 first report that HTP deliver slightly less nicotine and emit much lower amounts of carbonyl  
651 and PAH compounds than tobacco cigarette. However, HTP emissions still contain  
652 carcinogenic compounds (e.g. formaldehyde, acetaldehyde and benzo[a]pyrene) and the  
653 amounts of carbonyls and PAHs in HTP aerosols are higher than in e-cig vapours. In  
654 accordance with the levels of toxic compounds in each aerosol, HTP aerosol exhibit reduced  
655 cytotoxicity compared to cigarette smoke but higher than e-cig vapours. HTP and e-cig have  
656 the potential to increase oxidative stress and inflammatory response, in a manner very  
657 similar to that of cigarette smoke, but only after a more intensive exposure. In addition, our  
658 data support that e-cig use at higher power settings emit higher carbonyl and PAH  
659 compounds and, consequently, generate more oxidative stress. Finally, this study  
660 contributes to a better understanding of HTP and e-cig emission properties and their related  
661 toxicological impacts and provides important data needed for risk assessment purposes, by  
662 demonstrating that HTP might be less harmful than tobacco cigarette but considerably more  
663 harmful than e-cig. Further long-term studies in animal models should be conducted to  
664 confirm these *in vitro* findings and to allow the assessment of chronic exposures to  
665 emergent tobacco products. In addition to the toxic impacts of these products, comparison of

666 their addictiveness is another key element to take into account in the tobacco harm-reduction  
667 strategy.

668

669

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673

674

## 675 **6. Declaration of interest**

676 The authors declare that they have no conflict of interest with tobacco, HTP or e-cig  
677 industries.



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## Figure legends

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987

988 **Figure 1.** Nicotine levels (in  $\mu\text{g}/\text{puff}$ ) in e-cig (Lounge, Mb18W or Mb30W), HTP and 3R4F  
989 cigarette aerosols. Data represent the mean  $\pm$  SD of four independent measurements. \* $p <$   
990 0.05.

991

992 **Figure 2.** Total content of carbonyl compounds in e-cig (Lounge, Mb18W or Mb30W), HTP  
993 and 3R4F cigarette aerosols. Data represent the mean  $\pm$  SD of four independent  
994 measurements (\* $p < 0.05$ ) and are expressed in  $\mu\text{g}/\text{puff}$  (A) or in  $\text{ng}/\mu\text{g}$  of nicotine (C). The  
995 corresponding reduction (%) in emissions of total carbonyl compounds (B and D,  
996 respectively) was compared to that of the 3R4F cigarette, HTP or e-cig.

997

998 **Figure 3.** Total content of PAHs in e-cig (Lounge, Mb18W or Mb30W), HTP and 3R4F  
999 cigarette aerosols. Data represent the mean  $\pm$  SD of four independent measurements (\* $p <$   
1000 0.05) and are expressed in  $\text{ng}/\text{puff}$  (A) or in  $\text{pg}/\mu\text{g}$  of nicotine (C). The corresponding  
1001 reduction (%) in emissions of total PAHs (B and D, respectively) was compared to that of the  
1002 3R4F cigarette, HTP or e-cig.

1003

1004 **Figure 4.** Cell viability after exposure of BEAS-2B cells to different puff numbers of e-cigs  
1005 [Lounge (A), Mb18W (B) or Mb30W (C)], HTP (D) and 3R4F cigarette (E). The viability was  
1006 assessed by measuring intracellular ATP content in cells 24 h after exposure. Results are  
1007 expressed as percentages relative to the ATP content in control cells arbitrarily set at a value  
1008 of 100 %. Data represent the mean  $\pm$  SD of three independent culture replicates. \* $p < 0.05$   
1009 compared to control cells. (F) Cell viability expressed as a function of nicotine content (mg)  
1010 in aerosols from each device. Nicotine content is determined by the nicotine concentration  
1011 per puff (depending of the device) multiplied by the number of puffs.

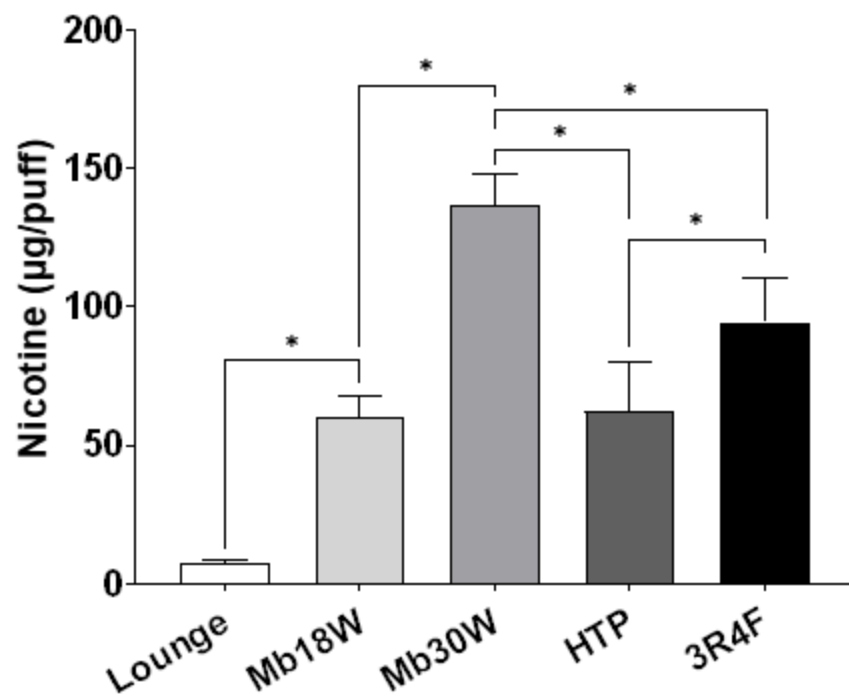
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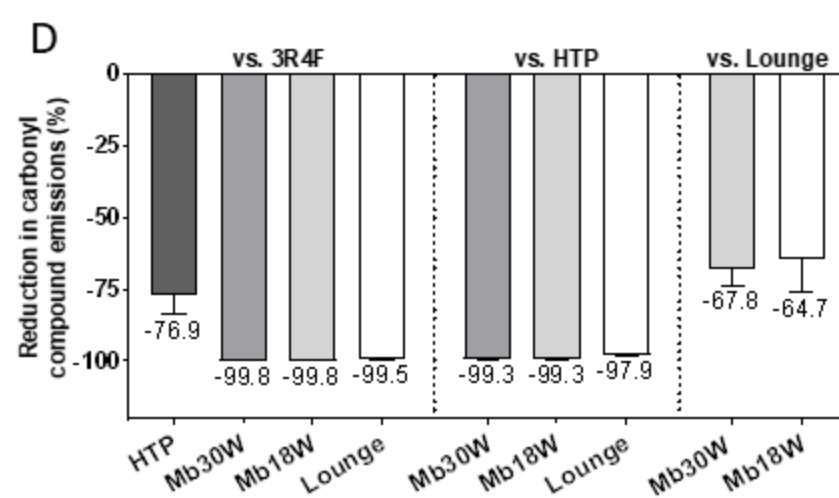
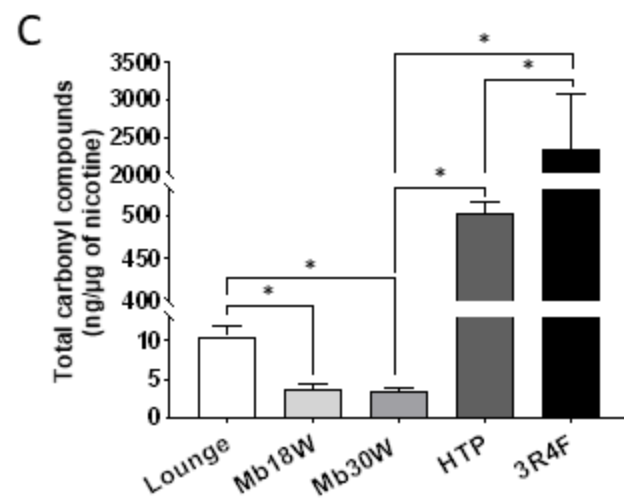
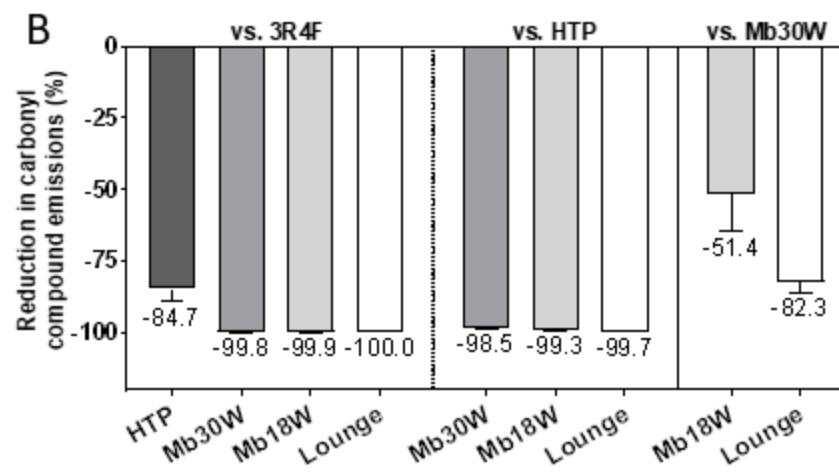
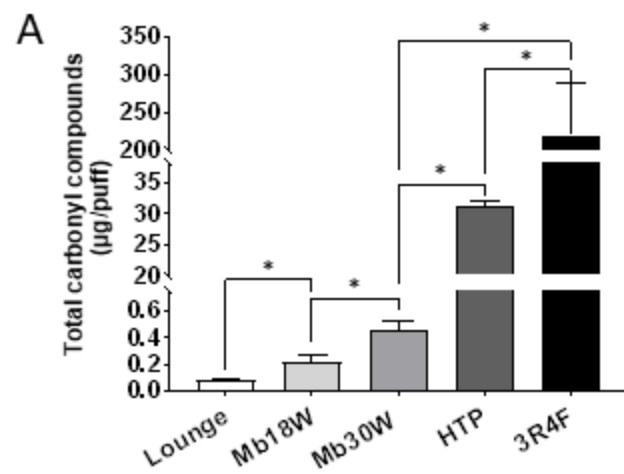
1013 **Figure 5.** Glutathione ratio (GSSG/GSH) in BEAS-2B cells after exposure to e-cig [Lounge (A),  
1014 Mb18W (B) or Mb30W (C)], HTP (D) and 3R4F cigarette (E) aerosols. The GSSG and GSH  
1015 contents were measured immediately after cell exposure. Results are expressed as fold-  
1016 change relative to the GSSG/GSH ratio in control cells arbitrarily set at a value of 1. Data

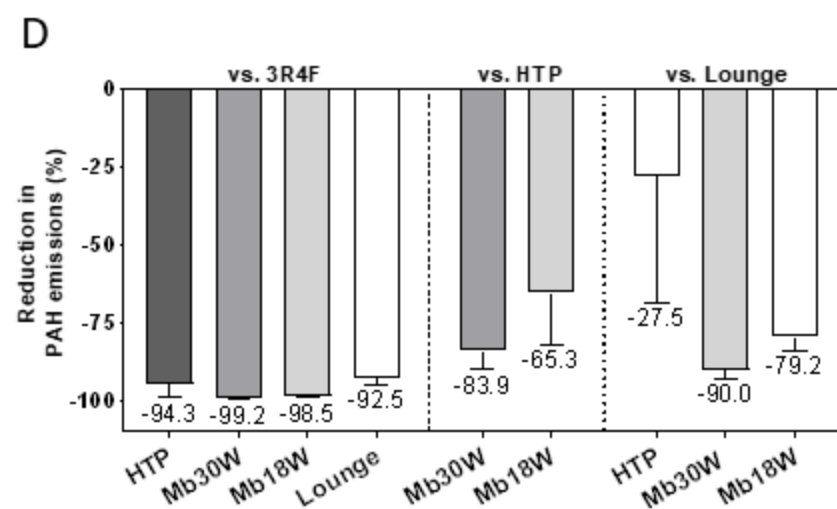
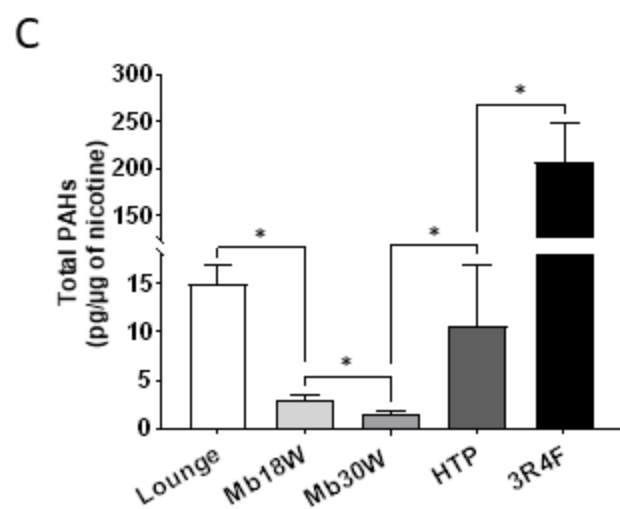
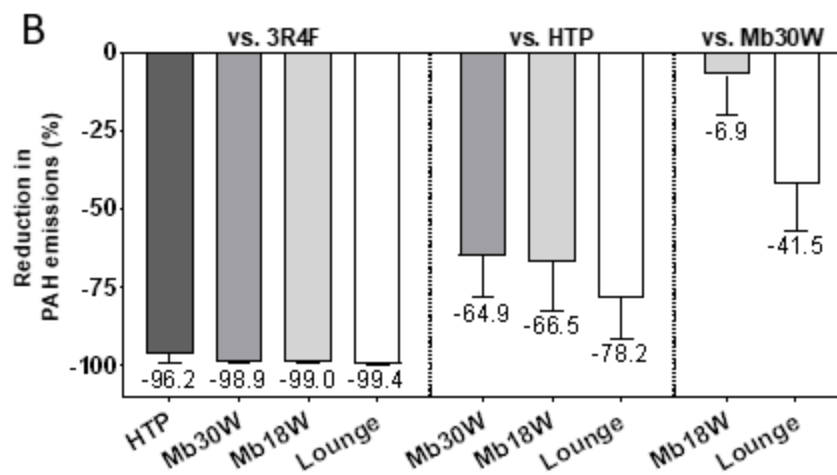
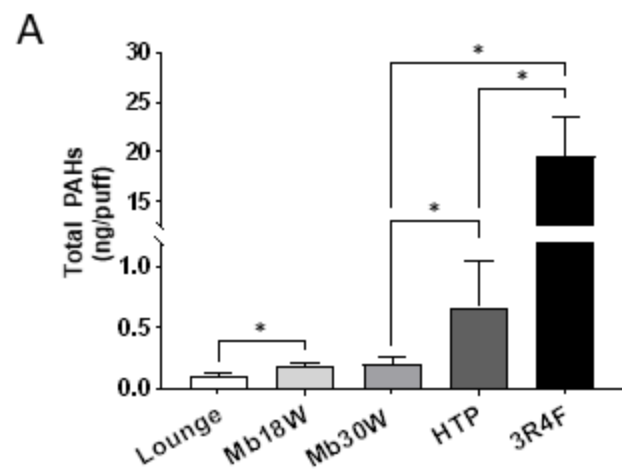
1017 represent the mean  $\pm$  SD of three independent culture replicates. \*  $p < 0.05$  compared to  
1018 control cells.

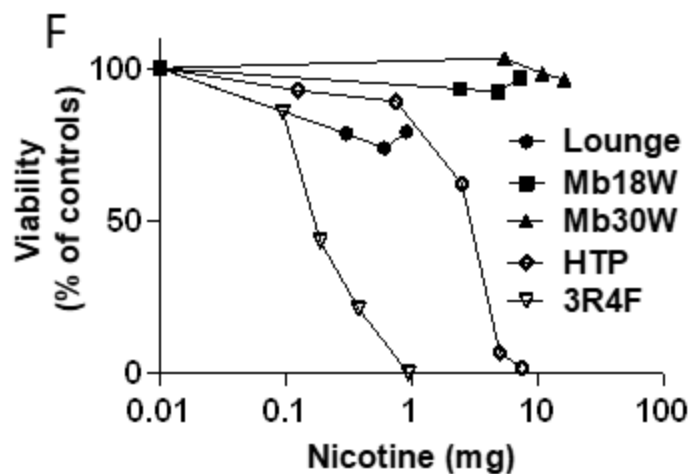
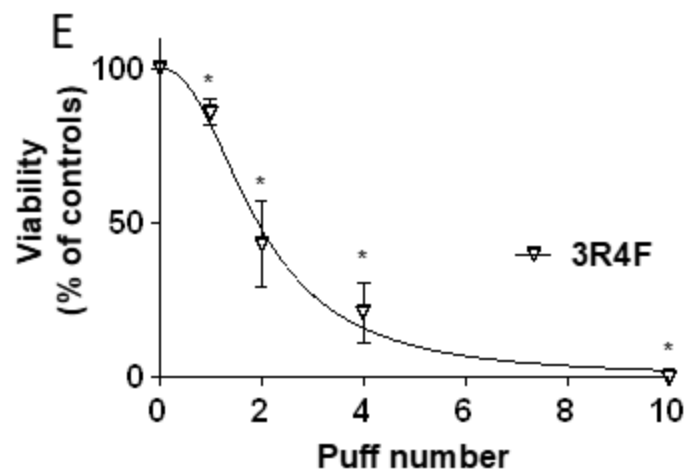
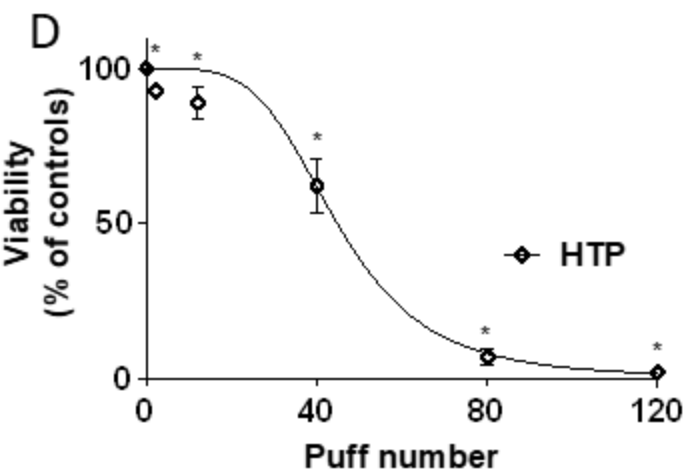
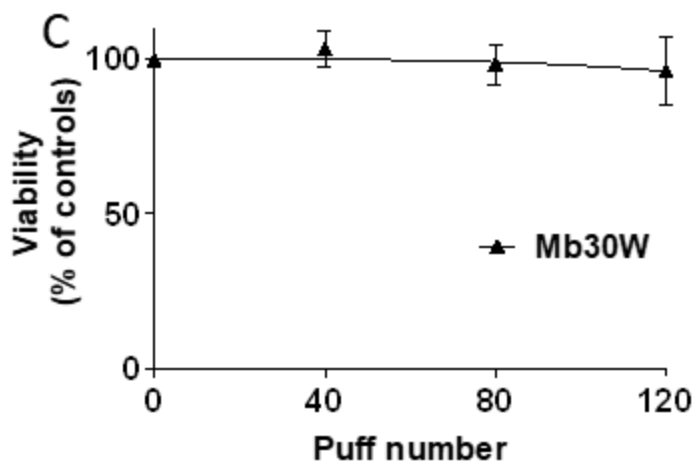
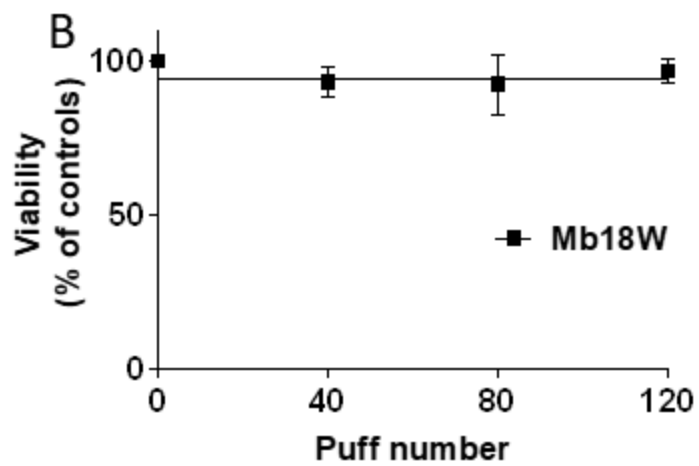
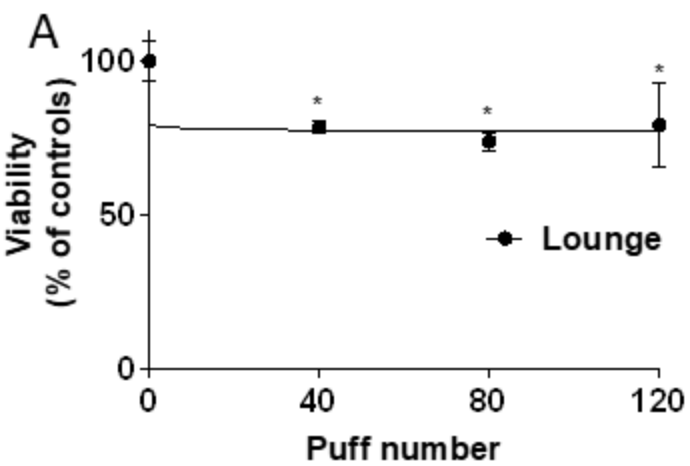
1019

1020 **Figure 6.** Expression of mRNAs encoding genes related to metabolism [*CYP1A1* (A), *CYP1B1*  
1021 (B)] and oxidative stress [*HMOX1* (C), *NQO1* (D)] in BEAS-2B cells. The gene expression was  
1022 analysed 4 h or 24 h after exposure to 120 puffs of e-cig (Lounge, Mb18W or Mb30W), 12  
1023 puffs of HTP or 1 puff of 3R4F cigarette. Data represent the mean  $\pm$  SD of three independent  
1024 culture replicates. Results are expressed as fold-change relative to control cells, arbitrarily  
1025 set at a value of 1. Data represent the mean  $\pm$  SD of three independent culture replicates. \*  
1026  $p < 0.05$  compared to control cells.

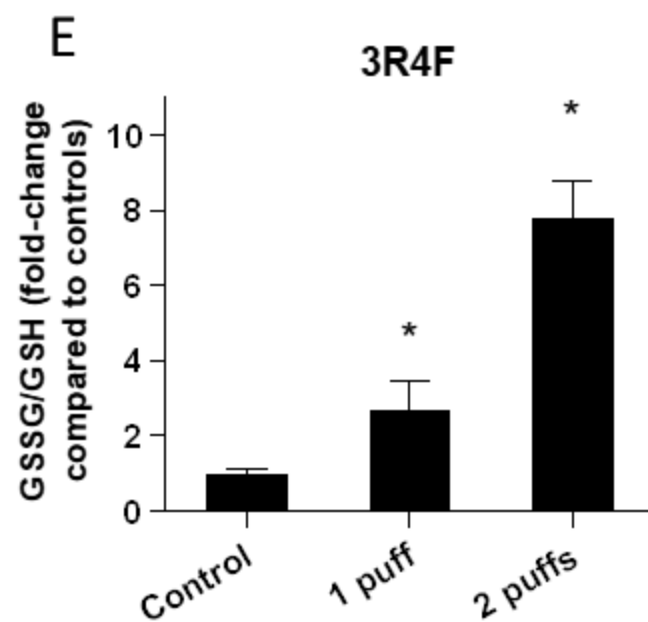
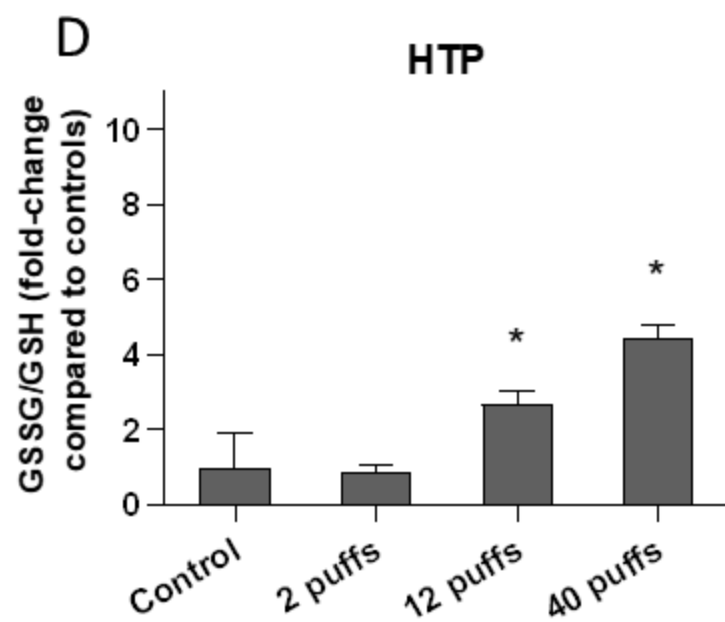
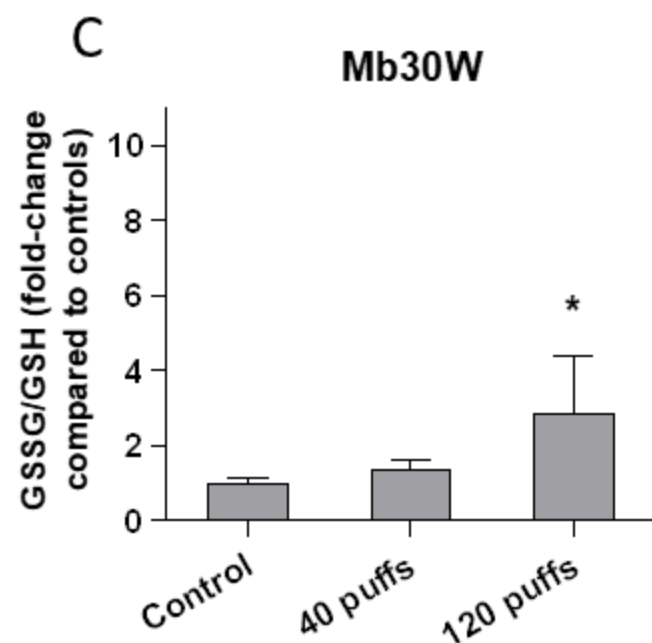
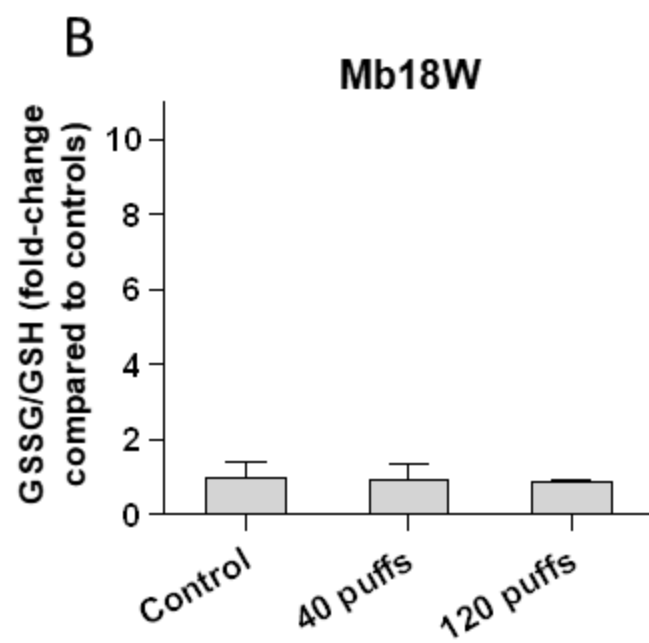
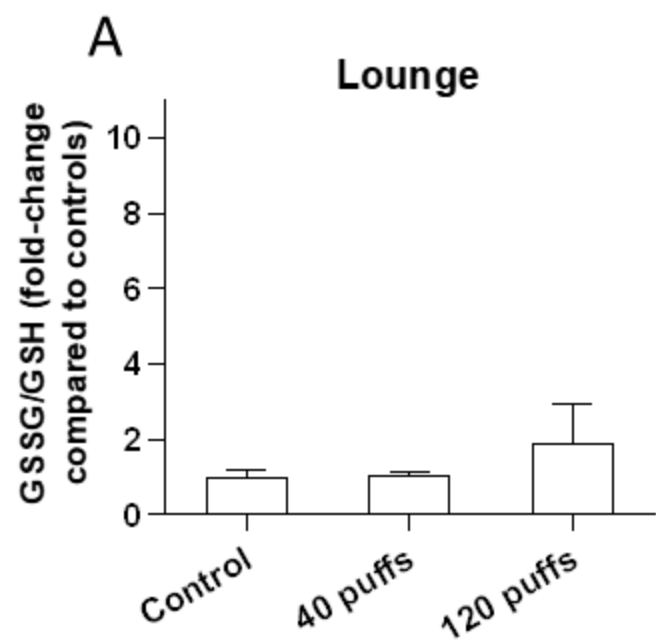


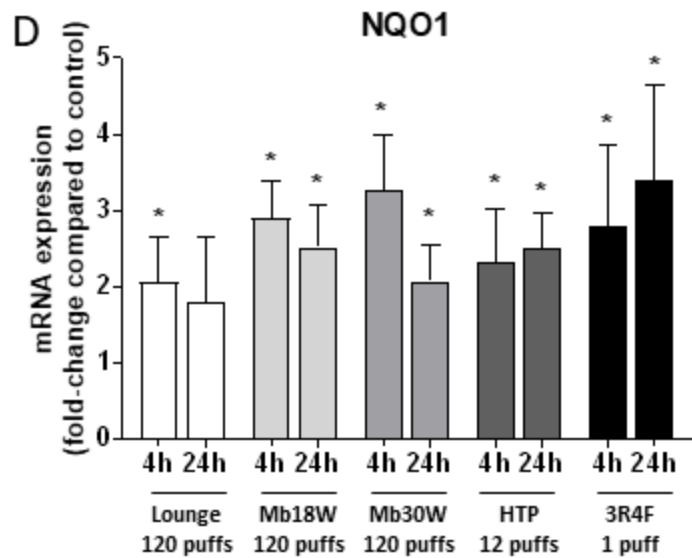
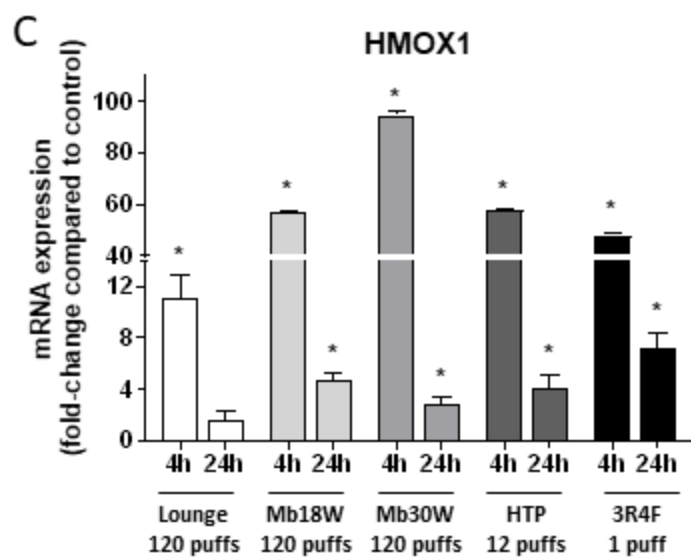
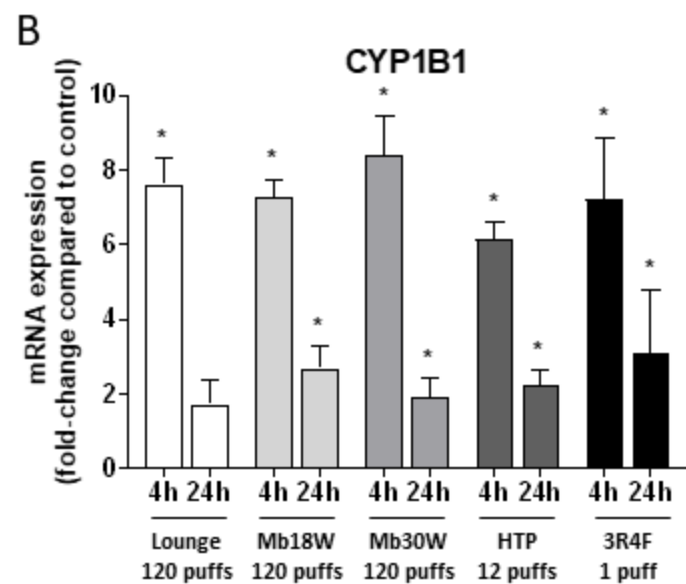
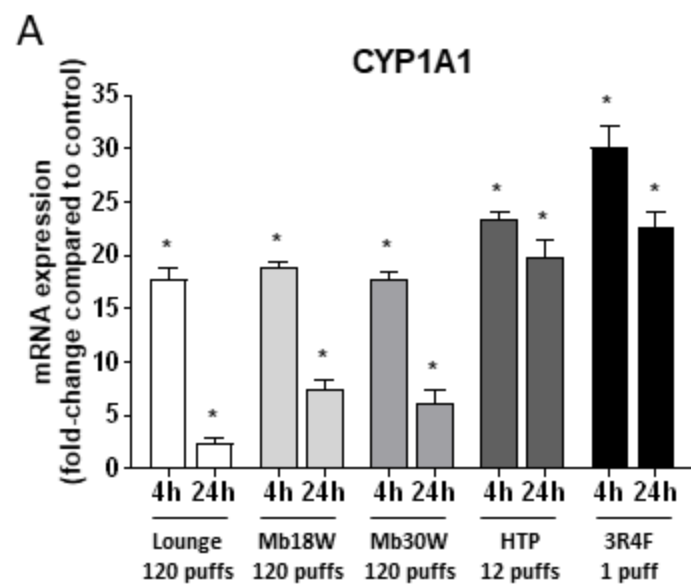












**Table 1.** Carbonyl concentrations (in ng/puff) in e-cig (Lounge, Mb18W or Mb30W), HTP and 3R4F cigarette aerosols. Data represent the mean  $\pm$  SD of four independent measurements. “~”: undetectable as < to LOD.

	Lounge		Mb18W		Mb30W		HTP		3R4F	
Formaldehyde	6.0	$\pm$ 0.7	25.8	$\pm$ 2.8	64.5	$\pm$ 23.7	156.9	$\pm$ 9.4	255.5	$\pm$ 60.8
Acetaldehyde	32.9	$\pm$ 5.4	63.0	$\pm$ 10.3	160.9	$\pm$ 46.4	26687.7	$\pm$ 657.8	166345.0	$\pm$ 59540.1
Propanone	3.9	$\pm$ 2.7	13.8	$\pm$ 3.0	28.5	$\pm$ 8.1	3132.3	$\pm$ 149.1	36075.8	$\pm$ 7896.5
Propanal	2.1	$\pm$ 0.7	8.4	$\pm$ 2.4	23.2	$\pm$ 5.6	1400.1	$\pm$ 205.8	6924.8	$\pm$ 1688.2
Methyl vinyl ketone	0.2	$\pm$ 0.1	6.4	$\pm$ 4.2	6.4	$\pm$ 2.1	443.1	$\pm$ 42.1	1341.1	$\pm$ 219.3
Crotonaldehyde	2.4	$\pm$ 0.1	16.1	$\pm$ 3.3	38.8	$\pm$ 8.1	139.9	$\pm$ 10.2	1697.4	$\pm$ 794.5
Methyl ethyl ketone	0.8	$\pm$ 1.6	34.7	$\pm$ 23.6	23.5	$\pm$ 9.5	625.6	$\pm$ 26.9	9005.1	$\pm$ 1097.8
Methylpropenal	~	$\pm$ ~	~	$\pm$ ~	~	$\pm$ ~	334.8	$\pm$ 20.6	842.4	$\pm$ 350.7
Butanal	0.1	$\pm$ 0.1	2.0	$\pm$ 0.1	2.4	$\pm$ 0.1	985.9	$\pm$ 94.7	3653.9	$\pm$ 1055.0
Benzaldehyde	0.5	$\pm$ 0.1	2.5	$\pm$ 0.3	3.2	$\pm$ 0.1	58.9	$\pm$ 2.8	63.6	$\pm$ 59.3
Isopentanal	0.7	$\pm$ 0.1	7.9	$\pm$ 1.1	11.5	$\pm$ 0.6	391.3	$\pm$ 37.6	2084.9	$\pm$ 599.0
Pentanal	0.5	$\pm$ 1.1	1.0	$\pm$ 0.2	0.4	$\pm$ 0.1	25.2	$\pm$ 1.4	172.0	$\pm$ 50.5
Glyoxal	0.6	$\pm$ 0.4	0.6	$\pm$ 0.0	0.7	$\pm$ 0.0	40.7	$\pm$ 9.2	308.2	$\pm$ 92.0
o-Tolualdehyde	0.7	$\pm$ 0.1	2.9	$\pm$ 0.5	2.8	$\pm$ 0.5	6.3	$\pm$ 0.4	29.0	$\pm$ 2.8
m-Tolualdehyde	~	$\pm$ ~	1.0	$\pm$ 0.6	1.1	$\pm$ 0.8	~	$\pm$ ~	~	$\pm$ ~
p-Tolualdehyde	1.7	$\pm$ 0.4	0.9	$\pm$ 0.6	0.6	$\pm$ 0.7	115.0	$\pm$ 26.4	291.8	$\pm$ 195.8
Methylglyoxal	25.2	$\pm$ 3.1	12.2	$\pm$ 1.1	44.1	$\pm$ 10.9	490.1	$\pm$ 69.8	982.0	$\pm$ 249.0
Hexanal	0.5	$\pm$ 0.1	1.5	$\pm$ 0.1	1.8	$\pm$ 0.1	22.1	$\pm$ 11.8	10.4	$\pm$ 12.1
2,5-Dimethylbenzaldehyde	~	$\pm$ ~	0.6	$\pm$ 0.1	0.7	$\pm$ 0.1	~	$\pm$ ~	~	$\pm$ ~
<b>Total carbonyl compounds</b>	<b>79</b>	<b><math>\pm</math> 10</b>	<b>201</b>	<b><math>\pm</math> 48</b>	<b>415</b>	<b><math>\pm</math> 63</b>	<b>35056</b>	<b><math>\pm</math> 825</b>	<b>230083</b>	<b><math>\pm</math> 70153</b>

**Table 2.** PAH concentrations (in pg/puff) in e-cig (Lounge, Mb18W or Mb30W), HTP and 3R4F cigarette aerosols. Data represent the mean  $\pm$  SD of four independent measurements. “~”: undetectable as < to LOD.

	Lounge			Mb18W			Mb30W			HTP			3R4F		
Naphthalene	61.5	$\pm$	9.5	75.9	$\pm$	5.6	92.2	$\pm$	6.2	71.2	$\pm$	38.8	3598.6	$\pm$	735.4
Acenaphthene	0.2	$\pm$	0.1	2.6	$\pm$	1.1	5.0	$\pm$	1.4	12.5	$\pm$	13.6	1318.2	$\pm$	397.5
Fluorene	6.7	$\pm$	3.3	6.7	$\pm$	1.5	5.0	$\pm$	1.3	26.0	$\pm$	22.4	1976.7	$\pm$	387.6
Phenanthrene	7.2	$\pm$	0.7	25.2	$\pm$	8.2	22.8	$\pm$	3.5	55.9	$\pm$	34.7	2829.4	$\pm$	533.3
Anthracene	0.6	$\pm$	0.1	1.7	$\pm$	0.4	2.8	$\pm$	3.7	4.7	$\pm$	2.3	1356.2	$\pm$	266.7
Fluoranthene	9.2	$\pm$	1.4	20.1	$\pm$	11.8	11.5	$\pm$	11.8	131.0	$\pm$	79.0	1463.5	$\pm$	288.7
Pyrene	17.9	$\pm$	4.3	30.9	$\pm$	9.2	30.9	$\pm$	10.9	153.0	$\pm$	98.6	1752.4	$\pm$	304.4
Benzo(c)phenanthrene	1.9	$\pm$	0.6	4.5	$\pm$	2.1	3.1	$\pm$	4.4	10.2	$\pm$	6.9	1.5	$\pm$	0.6
Benzo(a)anthracene	0.2	$\pm$	0.0	2.6	$\pm$	0.8	3.2	$\pm$	4.0	43.8	$\pm$	23.3	542.5	$\pm$	150.1
Chrysene	0.4	$\pm$	0.3	1.6	$\pm$	0.2	2.5	$\pm$	4.0	26.3	$\pm$	13.8	471.7	$\pm$	72.5
5-Methylchrysene	1.5	$\pm$	0.4	1.0	$\pm$	0.7	0.6	$\pm$	0.4	1.7	$\pm$	1.0	1130.5	$\pm$	293.9
Benzo(e)pyrene	1.9	$\pm$	0.2	6.1	$\pm$	2.5	5.3	$\pm$	3.6	22.9	$\pm$	17.1	1343.9	$\pm$	303.1
Benzo(b)fluoranthene	0.3	$\pm$	0.1	1.2	$\pm$	0.2	4.2	$\pm$	7.1	18.9	$\pm$	8.9	358.9	$\pm$	125.6
Benzo(k)fluoranthene	0.2	$\pm$	0.1	0.5	$\pm$	0.1	1.4	$\pm$	2.2	18.4	$\pm$	11.1	99.6	$\pm$	28.1
Benzo(a)pyrene	0.6	$\pm$	0.2	0.6	$\pm$	0.2	1.1	$\pm$	0.3	25.6	$\pm$	13.8	457.6	$\pm$	114.5
Dibenzo(a,l)pyrene	0.1	$\pm$	0.0	0.0	$\pm$	0.0	0.1	$\pm$	0.0	0.3	$\pm$	0.1	0.6	$\pm$	0.2
Dibenzo(a,h)anthracene	0.0	$\pm$	0.0	0.0	$\pm$	0.0	0.2	$\pm$	0.3	0.8	$\pm$	0.4	38.4	$\pm$	11.8
Benzo(g,h,i)perylene	1.5	$\pm$	0.7	0.9	$\pm$	0.2	4.8	$\pm$	3.1	16.6	$\pm$	8.9	276.2	$\pm$	55.9
Indeno(1,2,3-c,d)pyrene	0.3	$\pm$	0.1	0.2	$\pm$	0.0	1.5	$\pm$	2.0	6.6	$\pm$	5.6	214.0	$\pm$	81.4
Dibenzo(a,e)pyrene	0.1	$\pm$	0.0	0.0	$\pm$	0.0	0.2	$\pm$	0.3	0.5	$\pm$	0.3	92.4	$\pm$	43.5
Anthanthrene	0.3	$\pm$	0.1	0.2	$\pm$	0.1	0.4	$\pm$	0.1	11.7	$\pm$	6.7	233.9	$\pm$	52.6
Coronene	0.3	$\pm$	0.1	0.5	$\pm$	0.1	2.8	$\pm$	1.0	5.5	$\pm$	1.8	25.6	$\pm$	5.4
Cyclopenta(c,d)pyrene	~	$\pm$	~	~	$\pm$	~	~	$\pm$	~	~	$\pm$	~	~	$\pm$	~
<b>Total PAHs</b>	<b>113</b>	$\pm$	<b>16</b>	<b>183</b>	$\pm$	<b>29</b>	<b>202</b>	$\pm$	<b>57</b>	<b>664</b>	$\pm$	<b>389</b>	<b>19582</b>	$\pm$	<b>400</b>

**Table 3.** Profile of inflammatory mediators (IL-6, IL-8, MCP-1 and GRO $\alpha$ ) secreted by BEAS-2B cells 24 h after exposure to the emissions of e-cigs (Lounge, Mb18W or Mb30W), HTP or 3R4F cigarette. Data represent the mean  $\pm$  SD of three independent culture replicates. Results are expressed as fold-change relative to control cells, arbitrarily set at a value of 1. Data in bold are significantly different from controls ( $p < 0.05$ ).

	IL-6	IL-8	MCP-1	GRO- $\alpha$
<b>Lounge</b>				
40 puffs	2.1 $\pm$ 0.6	0.8 $\pm$ 0.3	0.7 $\pm$ 0.2	0.7 $\pm$ 0.1
120 puffs	2.2 $\pm$ 1.0	0.6 $\pm$ 0.1	0.6 $\pm$ 0.2	0.6 $\pm$ 0.1
<b>Mb18W</b>				
40 puffs	2.5 $\pm$ 1.1	1.1 $\pm$ 0.6	1.0 $\pm$ 0.1	1.0 $\pm$ 0.2
120 puffs	<b>2.3 <math>\pm</math> 0.6</b>	1.0 $\pm$ 0.3	0.8 $\pm$ 0.1	0.8 $\pm$ 0.1
<b>Mb30W</b>				
40 puffs	<b>3.3 <math>\pm</math> 0.4</b>	0.9 $\pm$ 0.1	0.8 $\pm$ 0.1	0.7 $\pm$ 0.2
120 puffs	<b>2.4 <math>\pm</math> 0.1</b>	0.6 $\pm$ 0.1	<b>0.6 <math>\pm</math> 0.1</b>	<b>0.4 <math>\pm</math> 0.1</b>
<b>HTP</b>				
2 puffs	0.4 $\pm$ 0.0	1.0 $\pm$ 0.0	<b>0.6 <math>\pm</math> 0.0</b>	0.9 $\pm$ 0.1
12 puffs	3.0 $\pm$ 3.0	<b>3.3 <math>\pm</math> 0.4</b>	<b>0.6 <math>\pm</math> 0.1</b>	<b>0.7 <math>\pm</math> 0.1</b>
40 puffs	1.3 $\pm$ 1.0	1.3 $\pm$ 1.5	<b>0.2 <math>\pm</math> 0.1</b>	<b>0.2 <math>\pm</math> 0.1</b>
<b>3R4F</b>				
1 puff	1.4 $\pm$ 0.5	1.4 $\pm$ 0.3	1.0 $\pm$ 0.1	<b>0.7 <math>\pm</math> 0.1</b>
2 puffs	1.5 $\pm$ 0.5	1.8 $\pm$ 0.8	<b>0.2 <math>\pm</math> 0.1</b>	<b>0.2 <math>\pm</math> 0.1</b>

