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Impact of cigarette smoke on physical-chemical and molecular proprieties of human skin in an *ex vivo* model

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20

21 **ABSTRACT**

22 Background: This is a study about the skin aging exposome, focusing on the effect of cigarette smoke.
23 Human living skin explants (HSE) were exposed to cigarette smoke (CS) of two cigarettes for 2 hours
24 using a custom-made exposure chamber, the Pollubox®. Effects on the surface physico-chemistry and
25 molecular properties of the skin were analyzed and reported for the first time.

26 Basic Procedures: To this end, transcriptomic study followed by immunohistochemistry,
27 malondialdehyde dosage (MDA) and surface physio-chemistry data (surface free energy
28 determination, TEWL, skin pH and FTIR spectroscopy of the explant) were collected from non-treated
29 and treated HSE.

30 Main Findings: Results showed a decrease of the total surface free energy of the treated HSE. This
31 decrease reflected higher interactions with polar compounds from the environment and consequently a
32 decrease of the surface hydrophobicity. Additionally, an increase of TEWL and skin pH was observed
33 after treatment.

34 The transcriptomic analysis showed downregulation of mitochondrial genes (PON2-NDUFA4L2-
35 ATP1A1-ALDH2-PRODH) combined with an increase of MDA in CS-treated HSE.

36 Conclusions: CS-induced oxidation of lipids at HSE surface alters the skin barrier: interactions with
37 polar products are enhanced and the lipid chain packing at the surface is modified. Consequently, skin
38 permeability could increase which correlated with repression of CA9 and AQP1 genes. Beside
39 activation of AHR-NRF2 pathway in CS-exposed HSE, our results suggested that mitochondrial
40 functions were strongly impacted and oxidized lipids failed to be eliminated promoting skin barrier
41 alteration. A mitophagy activity was suggested through the confirmation of PINK1 accumulation in
42 the epidermis by immunostaining.

43

44 **KEYWORDS:**

45 cigarette smoke-skin exposome –mitochondrial alterations -surface physico chemistry-transcriptomic
46 analysis

47

48 INTRODUCTION

49 The exposome includes the totality of external factors to which human beings are exposed
50 during their life [1]. The concept of the skin aging exposome has been defined by Krutman
51 and collaborators [2] as the whole external and internal factors to which humans are exposed
52 daily and the response of the human body to these factors promoting an accelerated skin aging
53 process.

54 It has been proposed that the skin aging exposome is composed of seven different categories,
55 including sun radiation, tobacco, temperature, nutrition, stress, lack of sleep and air pollution.

56 **Environmental cigarette smoke** (CS) is one of the major harmful components of the human
57 skin exposome.

58 **Most commonly called secondhand smoke, it is a complex and reactive mixture which**
59 **contains over 5000 chemicals including hazardous amines, carbonyls, hydrocarbons and**
60 **metals among others emitted from the burning tobacco made up of the mainstream smoke**
61 **exhaled by smokers and sidestream smoke diluted with ambient air [3].**

62 Many studies were concerned with the detection and characterization of the hazardous
63 components in mainstream smoke [4, 5], and their effects on human health have been largely
64 documented **in response to active smoking [6].**

65 **Additionally, environmental CS exposure can cause several illnesses in non-smokers**
66 **including ischaemic heart diseases in adults, lower respiratory infections, and asthma in adults**
67 **and children, among other adverse health effects [7,8,9].**

68 Furthermore, the macroscopic effects of CS on skin are largely described and recognized.
69 From the beginning of the seventies it has been demonstrated that wrinkle formation is a main
70 clinical symptom related to tobacco consumption [10] and ever since the visible impact of
71 tobacco smoke on human skin has been reported by different studies **in vitro** and **in vivo** [11,
72 12]. However, the molecular mechanisms impacted by CS in cutaneous tissue have been
73 investigated only recently.

74 For the first time in 2001, Lahmann and collaborators [13] have indicated that the skin of
75 smokers expresses higher levels of matrix metalloproteinase 1 (MMP1) compared to non-
76 smokers. The negative effects of CS on the extracellular matrix (ECM) were confirmed by
77 studies in mice showing that the skin exposed to CS extracts presents a decreased level of
78 collagen staining [14] and exhibited an alteration of collagen biosynthesis. In parallel, it has
79 been shown that CS can increase reactive oxygen species (ROS) formation in different cell

80 types, including fibroblasts [15]. ROS are responsible for the induced expression of different
81 MMPs and activity resulting in degraded extracellular matrix and accelerated skin aging.

82 Most recently, the implication of the aryl hydrocarbon receptor (AhR) in skin responses to CS
83 have been also established *in vitro* [16]. In particular, it has been shown that upon exposure to
84 tobacco extracts, keratinocytes express higher levels of MMP-1 and CYP1B1 in an AhR-
85 dependent manner. Likewise, the role of AhR in CS-induced skin hyperpigmentation was also
86 investigated [17].

87 Moreover, the relationship between smoking and the development of several cutaneous skin
88 diseases, including atopic dermatitis [18], psoriasis [19] and impaired wound healing [20]
89 have been studied.

90 Given the importance and the necessity to characterize in more detail the impact of CS on the
91 different organs in humans, including skin, several experimental systems have been
92 developed for the exposure and the delivery of tobacco smoke *in vitro* [21]. Indeed, Lecas and
93 collaborators [22] have implemented a reconstructed epidermis-based model in order to
94 investigate the impact of tobacco smoke on the epidermis. This study has shown that CS
95 exposure triggers different events in human epidermis including an oxidative stress evaluated
96 by 4-hydroxynonenal immunostaining, an overexpression of ECM degrading enzymes such as
97 MMP-1 and MMP-3, and an increase of the pro-inflammatory interleukins, IL1 alpha, IL-8
98 and IL-18. Interestingly, they observed also a repression of a group of genes encoding
99 proteins involved in skin barrier functions, cell-cell junctions and cell adherence.

100 The surface physico-chemistry is an important parameter to determine skin interactions with
101 its surrounding environment [23]. Mavon et al. [24] have demonstrated that *in-vivo* human
102 skin surface is a hydrophobic monopolar basic surface. Thus, it may strongly interact with
103 bipolar substances and with monopolar ones with opposite sign. This behavior depends on the
104 specific topography and lipid composition (*stratum corneum*) of the area of the body. Despite
105 this interest, so far, no studies investigated the impact of CS on this parameter of human skin.
106 Interestingly, Eudier et al. [23] have recently highlighted that HSE behaved in a very similar
107 way as *in vivo* skin in terms of surface physico-chemistry and are a precious alternative to
108 study skin surface interactions with urban pollutants or CS.

109 Thus, our aim was to study the effects of environmental cigarette smoke on the physico-
110 chemical properties of the stratum corneum (SC). To that purpose, HSE were exposed to CS
111 using the exposure chamber Pollubox® which reproduces a condition of passive smoking.
112 The physico-chemical properties of the surface of the HSE were measured before and after
113 exposure. For the first time the surface free energy parameters of non-treated and treated HSE

114 are documented. The parameters relevant to the skin barrier function modifications included
115 measurements of trans-epidermal water loss, skin pH and the lipid chain packing in the
116 *stratum corneum*. Additionally, the impact of CS on the human skin transcriptome was
117 investigated.

118

119

120 **Materials and methods**

121 **1. Preparation of human skin explants**

122 Human living skin explants were obtained from plastic surgery of the abdominal area of six
123 different donors (Table S1), in accordance with the Declaration of Helsinki and after the
124 patients had given informed consent. HSE have been prepared as described by Percoco et al.
125 2013 [25]. For surface characterization square HSE (2cm x 2cm) have been used.

126

127 **2. Exposure of human skin explants to cigarette smoke**

128 In order, to define whether exposure of HSE with 1 or 2 cigarettes can induce an efficient
129 stress activation in HSE the expression of AHR, NRF2 target genes and expression of
130 MMP1/MMP3 as well as pro-inflammatory genes, IL1b and IL6 were evaluated first (Table
131 S2). The following results were obtained after 5 days of culture with HSE, exposed to smoke
132 from 2 cigarettes of a US brand for 2 hours using the exposure chamber PolluBox®.

133 For microarrays, immunostaining and malondialdehyde (MDA) dosage, HSE were sampled
134 24hrs after CS exposure (See supplement data, Materials and methods). Explants from three
135 different donors have been analyzed (Table S1) and for each condition (control and CS-
136 exposed sample) 3 explants were used, except for MDA dosage where 4 samples per
137 condition were analyzed.

138 For surface characterization after CS exposure, HSE were mounted on a specific support in
139 order to stretch the explant surface and subsequently sent to URCOM laboratory for further
140 analysis after 48 hours following CS exposure. For each condition 5 explants were used
141 (Table S1 and supplement data, Materials and methods).

142

143 Results

144 1. Contact angle measurements and surface free energy determination

145 Skin surface physico-chemistry is an important means to evaluate interactions of the surface
146 of the skin, our first barrier, with its environment and, as a consequence, skin permeability
147 [24]. As detailed above, the Pollubox®) allows nebulization of CS similar to a real skin
148 exposure. Contact angle measurements were performed 48h after previous exposure to CS
149 (2h) or on control explants, using three reference liquids (water, diiodomethane, and
150 formamide). The values of these angles are reported in figure 1. Contact angle measurements
151 characterize the wettability of a surface by a liquid which depends on the intermolecular
152 interaction between the two. Thus, the smaller the angle, the higher the liquid spreads,
153 indicating a better intermolecular interaction.

154 According to ANOVA performed on recorded data (Fig. 1a), CS treatment has a significant
155 impact on explant physico-chemistry. Indeed, the water contact angle decreased by more than
156 15° after CS treatment ($p < 0.0001$) indicating a decreased hydrophobicity; while the
157 diiodomethane contact angle increased by 8° ($p < 0.0001$). The formamide contact angle
158 slightly decreased not significantly. Those changes indicated that upon CS treatment,
159 interactions with polar liquids such as water were reinforced and interactions with apolar
160 liquid such as diiodomethane were weakened. This was a first indication that CS treatment
161 modified skin polarity.

162 The Van Oss model was used to determine the surface free energy (SFE) [26,27]. This model
163 allows splitting SFE into two components: the Lifshitz-van der Waals component (γ^{LW}),
164 which represents the van der Waals (apolar) interactions (Keesom, Debye, London), and the
165 acid-base component (γ^{AB}), representing the acidic and basic interactions, according to the
166 Lewis concept. In this model, acid-base component is composed of two parameters: γ^+
167 (electron acceptor, Lewis acid) and γ^- (electron donor, Lewis base). γ^{AB} component includes
168 the hydrogen bonding and the π -electron interactions.

169

170 Surface free energy parameters calculated from those measurements also showed a significant
171 impact of CS treatment (Fig. 1b). γ^{LW} component significantly decreased by 6 mJ/m². This
172 means that the dispersive interactions of CS-treated explants appear weaker than of control

173 explants. In parallel, the basic component (γ^-) was increased by more than 10 mJ/m².
174 Nevertheless, as the acidic component (γ^+) remained close to zero, the increase of γ^- did not
175 significantly impact the γ^{AB} value ($p > 0.05$). On the other hand, the increase in the electron
176 donor component means that the monopolar basic behavior of skin surface was reinforced
177 with an increase of the electron density at the extreme surface. This specific behavior was
178 already evidenced by Eudier et al. [28] dealing with squalene oxidation by urban pollutants.
179 The described surface free energy parameters modifications induced a significant lowering of
180 the total surface free energy of explants ($p = 0.002$).
181 Taken together, these data suggested that electron density was increased on skin surface after
182 CS treatment, and reduced apolar interactions by lowering dispersive interactions [29].

183

184

185 2. Biometrological probes and FT-IR spectroscopy

186 CS-treated explants were then analyzed using biometrological probes and infrared
187 spectroscopy in order to characterize skin barrier function modifications induced by cigarette
188 smoke.

189 The mean pH value obtained for control explants was 5.42 ± 0.74 , consistent with pH values
190 *in vivo* [30]. The average pH obtained for CS-treated skin increased significantly and reached
191 a value of 5.92 ± 0.68 .

192 In addition, the mean TEWL value measured for control explants was 15.5 ± 4.8 g/h/m²
193 compared to a significant ($p < 0.05$) increase to 21.4 ± 6.9 g/h/m² for CS treated explants.

194 Finally, infrared spectroscopic confirmed the CS impact on skin barrier function. We focused
195 analyses on CH₂ symmetric stretch wavelength ($\nu_{\text{sym}}\text{CH}_2$). According to Mendelsohn et al.
196 [31], the corresponding wavelength allows quantitative monitoring of chain conformation
197 order and packing of lipid bilayers in the *stratum corneum*. As highlighted by temperature
198 induced modifications, solid-solid transitions can be observed through the evolution of
199 $\nu_{\text{sym}}\text{CH}_2$. A first orthorhombic \rightarrow hexagonal transition is observed at a wavelength around
200 2849.5 cm^{-1} followed by a hexagonal \rightarrow disorder packing transition observed around 2852
201 cm^{-1} [31]. In the present study, as shown on fig. 2, the mean $\nu_{\text{sym}}\text{CH}_2$ observed for control
202 explants was $2851.01 \pm 0.35 \text{ cm}^{-1}$, corresponding to a hexagonal packing. Such packing is
203 usually observed at the skin surface [31]. However, the mean $\nu_{\text{sym}}\text{CH}_2$ observed for CS treated
204 explants was significantly increased ($2851.43 \pm 0.46 \text{ cm}^{-1}$, $p < 0.05$). This change in

205 wavelength is associated with more flexible chain of lipids and more disordered lipid chain
206 packing.

207 3. Impact of cigarette smoke on skin transcriptome and protein expression

208

209 To investigate how the physico-chemical parameters reflect alterations of gene expression,
210 transcription profiles were analyzed. An efficient stress activation in HSE after 1CS or 2CS
211 exposure was indeed observed through the induction of AHR and NRF2 -target genes, as well
212 as MMP1, MMP3 genes encoding for metalloproteinases (Table S2) as described in several
213 studies [13, 16, 17, 22]. In contrast to the AHR target genes (CYP1A1 and CYP1B1) for
214 which induction levels were quite close following 1CS or 2CS exposure, the induction of the
215 majority of NRF2 target genes was higher with smoke from 2 cigarettes. Similarly, the
216 induction of the MMP1 and MMP3 genes confirmed that the stress generated is higher with 2
217 CS. However, pro-inflammatory genes were not significantly modulated in CS-exposed
218 explants. In contrast to oxidative stress, it is possible that the absence of modulated pro-
219 inflammatory genes cannot be detected at the chosen time window (24hrs after CS exposure).

220 Thus, the following results are based on the 2CS exposed HSE from 4 volunteers.
221 Noteworthy, our molecular analyses showed modulations of specific genes, which should
222 cause mitochondrial dysfunction as well as an altered elimination of oxidized lipids as
223 illustrated by the downregulation of genes such as NDUFA4L2, ATP1A1, ALDH7A1,
224 ALDH2 and PRODH (Fig. 3 and Table S4). This observation was supported by the increase
225 of MDA (+37%, $p < 0.0001$), a marker of lipid peroxidation, detected in the culture medium of
226 CS-treated HSE (Fig. 4).

227 The NDUFA4L2 protein is located in the mitochondria and is considered to be a crucial
228 mediator that regulates cell survival [32]. For instance, it has been shown that silencing of
229 *NDUFA4L2* (NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 4-like 2) affects cell
230 viability, increases mitochondrial mass, and induces ROS generation in hypoxia in various
231 cancer cells (non-small cell lung cancer, renal cell carcinoma) [33]. ALDH2 (aldehyde
232 dehydrogenase 2) is a mitochondrial enzyme metabolizing acetaldehyde and other toxic
233 aldehydes, such as 4-hydroxynonenal (4-HNE). Inhibition of *ALDH2* by siRNA altered cell
234 functions together with the impairment of bioenergetic functions and a decrease in
235 mitochondrial respiration. Additionally, loss of ALDH2 reduced cell proliferation and
236 migration and increased paracellular permeability [34]. Associated with a regulation of
237 mitochondrial respiration, *PRODH* (proline dehydrogenase/oxidase), which encodes the first

238 enzyme of the pathway of proline catabolism can be channeled to produce ATP for cell
239 survival under low glucose conditions with or without concurrent hypoxia [35]. *PRODH*
240 expression is often repressed in various tumors and in situations that limiting mitochondrial
241 proline utilization. Proline availability for *PRODH*-dependent ATP or ROS generation
242 depends on the activity of prolylase and on the use of proline during collagen biosynthesis
243 [36]. Indeed, the dermal architecture should be likely altered in response to CS since we
244 observed a repression of *COL1A2*, *COL9A3* and *LOX* (lysyl oxidase) genes together with an
245 induction of metalloproteinases genes *MMP1* and *MMP3*. Recent works have shown that
246 knockdown of *ATP1A1*, an α -subunit of Na⁺/K⁺ ATPase, induces mitochondrial dysfunction
247 by disrupting ion homeostasis that results in apoptosis [37]. This α subunit has 10
248 transmembrane segments known as FXYD segments, with variable binding affinity for
249 different regulators or modulators, particularly lipid molecules. Na⁺/K⁺-ATPase activity is
250 compromised by high production of ROS and in particular by lipid peroxidation products
251 [38]. Lipid peroxidation and oxidative stress can be negatively regulated by induction of
252 *ANGPTL4* [39]. Its downregulation in our study might indicate that these processes are not
253 negatively controlled at least 24 h after CS exposure. Similarly, bioactive oxidized lipids may
254 be degraded by antioxidant enzymes; such as paraxonase 2 encoded by the *PON2* gene [40],
255 which is widely expressed in a variety of tissues, including intestinal epithelial cells, vascular
256 endothelial cells, kidney, cardiomyocytes, and keratinocytes. Overexpression of *PON2* reduces
257 oxidative status, prevents cell apoptosis and inhibits cell-mediated low-density lipoprotein
258 oxidation. In contrast, *PON2* knock-down caused vast reactive oxygen species formation and
259 stimulated mitochondrial cell death signaling [41]. *PON2* expression was decreased both at
260 the gene and protein level (Fig. 3 and 4) under CS treatment supporting ongoing prooxidative
261 activities. According to these observations at a transcriptional level, our results highlight the
262 ability of CS to promote the production of ROS as a result of the decrease of mitochondrial
263 activities in HSE. These findings were confirmed by immunohistochemistry, as revealed by
264 the increase of PINK-1, a marker of damaged mitochondria, in the viable epidermis (Fig. 4).
265 This mitochondrial alteration is responsible for changes in other processes such as lipid
266 peroxidation, decreased collagen biosynthesis, and increased collagen degradation via the
267 induction of metalloproteinases. In line with reported observations [42, 43], the induction of
268 several NRF2 target genes (*HMOX1*, *AKR1C1*, *AKR1C3*, *PRDX1*, *SLC7A11*, *SRXN1*,
269 *TXNRD1*) was detected in HSE exposed to CS. Among them, NRF2 and AKR1C3 protein
270 expressions were also confirmed by immunostaining (Fig. 4).

271 In addition, repression of *CA9* (carbonic anhydrase IX) and *AQP1* (aquaporin 1) genes was
272 observed in our transcriptomic analysis. The cell surface pH regulatory enzyme, CA9,
273 catalyzes the reversible hydration of carbon dioxide to acidify the cellular microenvironment
274 and also provides an extracellular pH control mechanism [44]. In normal skin, the highest
275 expression of *CA9*, whose inhibition can contribute to increase cellular pH, was detected in
276 hair follicles, sebaceous glands, and basal parts of epidermis [45]. *AQP1* expression which
277 was found to be increased with pH [46], is correlated with the regulation of epidermal
278 hydration and might influence skin water transport [47].

279

280

281

282 Discussion

283 The observed alteration of the skin surface physico-chemistry and the skin surface free energy
284 can result from a modification of the lipid composition as highlighted by Mavon *et al.* [24].
285 Especially, the basic component γ^- results from the amount of amphiphilic lipids, namely the
286 free fatty acids (FFAs), present in the skin surface because their carbonyl groups enriched in
287 electrons induce basic interactions.

288 This observation can also be explained by the oxidation of surface lipids caused by CS
289 exposure [48,49]. Indeed, it has been shown that CS triggers an oxidative stress favoring the
290 peroxidation of skin lipids thus reducing its anti-oxidant content. This oxidation leads to the
291 formation of new oxygenated chemical species and amphiphilic molecules such as epoxides,
292 peroxides or carbonyls [50].

293 The chemical composition of such oxidized compounds, their possible chemical structures
294 and the mechanisms of their formation were deeply described in the study of Eudier *et al.* [28]
295 in the case of squalene oxidation. As a consequence, the corresponding compounds, enriched
296 in electrons, are able to establish additional hydrogen bonds thus increasing interactions with
297 polar liquids such as water, as illustrated by contact angle measurements on CS treated
298 explants (Fig. 1). It is well established that those compounds are responsible for the increase
299 of basic interactions [24] and the decrease of dispersive interactions [29].

300 By modifying skin surface physico-chemistry, CS treatment alters the way the skin interacts
301 with its environment. A significant increase of skin wettability can impact skin barrier
302 function and skin interactions with topic products [24].

303 The rise of pH ($p < 0.0001$) was consistent with the physico-chemical data indicating an
304 enhanced basic behavior of treated skin surface. These observations confirmed the suggested
305 influence of CS on skin permeability. Indeed, skin pH can influence the diffusion of drugs
306 and their distribution in skin layers [30].

307 Parallel with the pH increase, the mean TEWL increases also significantly after CS treatment.
308 According to Elkeeb *et al.* [51], the TEWL measurement is an accurate *in vitro* approach to
309 evaluate skin water barrier function. An increase in TEWL following CS exposure indicates
310 also an alteration of the skin barrier.

311 Finally, FTIR results demonstrated a lipid chain packing disorder. This chain packing reflects
312 skin barrier function integrity [50]. Highly ordered lipids provide impermeability needed for
313 the water barrier function whereas disordered lipids bilayers may increase skin permeability
314 [31]. Consequently, skin barrier function was disrupted in CS treated explants, consistent with

315 the TEWL results [51,52] as proposed on fig. S1. Such a disorganization of lipids chain
316 bilayers can be explained by the formation of lipid oxidation compounds, perturbing the
317 original lipid bilayer organization [53].

318 Predictable, genes involved in the AHR-pathway were induced in CS-exposed HSE, our
319 molecular analysis suggested that mitochondrial functions were strongly impacted and
320 oxidized lipids failed to be eliminated promoting skin barrier alteration.

321 Indeed, the various environmental toxicants are known to induce the AhR translocation to the
322 nucleus and interact with AhR nuclear translocator (ARNT) and binds to the Xenobiotic
323 Response Element DNA domain (XRE). Once the complex is bound, it initiates the
324 transcription of genes such as CYP1A1, CYP1B1, which are involved in cell detoxification
325 [54]. However, the AhR-dependent protection could not shield cells from constant oxidative
326 stress [55] and prolonged ROS production by the repression of several key enzymes (i.e
327 NDUFA4L2 and PRODH), as illustrated in our study (Fig. 3), would indeed enhance
328 oxidative damage to mitochondria and *in fine* the elimination of oxidized lipids might be
329 impacted. Downregulation of ATP1A1 (repressed in our study), whose activity is
330 compromised in response to a high generation of ROS or by lipid peroxidation products,
331 induces also mitochondrial dysfunction by disrupting iron homeostasis. The presence of lipid
332 peroxidation products was also confirmed by an increase in MDA of 37% ($p < 0.0001$).
333 Furthermore, oxidized lipids can be degraded by paraxonase 2 encoded.
334 Overexpression of this gene reduces the oxidative status and inhibits cell-mediated low-
335 density lipoprotein oxidation. Since expression of this gene is repressed, it can be expected
336 that its activity should also decrease leading to LDL/HDL oxidation and mitochondrial
337 apoptosis/oxidation (see Fig. 3). Therefore, according to these findings, besides an oxidative
338 stress favoring the peroxidation of skin lipids and triggered by the modulation of these genes,
339 it can be assumed that the activities of the encoded protein will produce in turn new
340 oxygenated chemical species and amphiphilic molecules such as epoxides, peroxides, or
341 carbonyls.

342 Exposure of mitochondria to cellular stress alters their fusion/fission dynamics and promotes
343 excessive ROS production, an accelerator of cellular senescence. Whereas mild stress induces
344 a prosurvival response termed stress-induced mitochondrial hyperfusion, severe stress results
345 in mitochondrial fragmentation and mitophagy [56]. In the present study, the mitophagy was
346 illustrated through the induction of *HMOX1* and confirmed by immunostaining of LC3B and
347 PINK1 (Fig. 4). In response to the mitochondrial dysfunction, induction of NRF2-regulated

348 genes was not surprising since such an induction is an indicator of the activation of a cellular
349 protection against the pro-oxidative process and the need to neutralize ROS in the outer layers
350 of the epidermis.

351 Polyunsaturated fatty acids in cells and organelles membranes are primary targets for ROS
352 attack, which may lead to lipid peroxidation and generation of reactive lipids. Indeed, the
353 presence of lipids such as 4-hydroxynonenal was supported by an increase in MDA of 37%
354 ($p < 0.0001$).

355 Thus, the CS-induction of lipid oxidation is detected by the physico-chemistry approach,
356 results in an oxidative stress due to the modulation of genes expression.

357 Moreover, the repression of genes such as *CA9* and *AQP1* provided some clues to explain the
358 alterations of the skin barrier after CS exposure through an increase of the cellular pH [45]
359 and the altered water transport [47], respectively.

360 Although the major intake of tobacco smoke toxicants is through the inhalation of secondhand
361 smoke, our results show that the accumulation of toxicants in the skin is equally relevant as
362 shown by *ex vivo* CS exposure. Epidermal absorption is usually overlooked as a possible
363 pathway of exposure, but it is especially pertinent in the case of environment pollution where
364 nonsmokers are exposed to smoke toxicants. As observed by Weschler and Nazaroff, many
365 semi-volatile organic compounds like nicotine can be dermally absorbed and transported to
366 the dermal blood supply [57].

367 Despite the absence of an inflammatory response that may be also transient compared to the
368 conditions of active smoking, this is the first time that an *ex vivo* study demonstrates
369 alterations in response to smoking, probably closer to the physiological conditions of passive
370 smoking which may contribute to skin aging.

371 In the future, it would be interesting to evaluate with this *ex vivo* model the effects on the skin
372 of e-cigarettes via the Pollubox® device and to study the impact of cigarette smoke extracts
373 on human skin explants *ex vivo* following systemic application (in the culture medium) in
374 order to mimic more closely active smoking environment.

375

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389 **Author contributions**

390 GP, AP, FE, CP, GS and PB wrote the main manuscript.

391 GP, EL, AP, FE, GS and CP developed the study design.

392 AP, TB and PB performed the genomic experiments.

393 GP was in charge to carry out the microscope analysis.

394 FE, MG, GS and CP realized the physical-chemical characterization of human skin surface.

395 All the authors took part in the scientific discussions of the obtained results and have read and
396 approved the manuscript.

397 The study was conducted in accordance with the ethical policies of Experimental
398 Dermatology. The Bio-EC Laboratory possesses an authorization from the French Minister
399 for Health.

400

401 **Conflict of interest**

402 The authors have declared no conflicting interests.

403

404

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564

565 **FIGURE LEGENDS**

566

567 **Figure 1:** (a) Advancing contact angle measured on control and CS treated explants with
568 water, diiodomethane and formamide and (b) surface free energy parameters calculated
569 according to Van Oss model. γ^{LW} is the Lifshitz–van der Waals (apolar) component of the
570 surface free energy of the liquid, γ^- and γ^+ are the electron-donor and electron-acceptor (polar)
571 parameters of the acid–base components of the liquid surface free energy, respectively.

572 **Figure 2:** Mean FTIR Spectra of control and CS-treated living skin explant with a zoom of
573 the lipid chain packing area.

574 **Figure 3:** Schematic representation of functional networks deduced from PredictSearch®
575 analysis of the set of genes modulated in skin explants 24 h after exposure to CS. The legend
576 of this figure is indicated at the bottom left corner. Induced genes are noted in blue and
577 repressed genes in red.

578 **Figure 4:** Microscopical analysis and MDA dosage of skin explants upon CS exposure.

579 Abbreviations: SC, *stratum corneum*; E, epidermis; PD, papillary dermis.

580 Scale bar: 50 μm .

581 **Figure S1:** Proposed explanation for TEWL increase and skin barrier degradation in CS
582 treated explants.

583 **Table S1:** List of the different skin donors used in the present work.

584 **Table S2:** List of key genes evaluated in response to 1CS and 2 CS to determine dose effect
585 on the stress stimulation on human skin explants

586

587 **Table S3:** List of the primary antibodies used in the present work. Abbreviations: HIER (Heat
588 Induced Epitope Retrieval).

589

590 **Table S4:** List of modulated genes in response to CS2 and correlated in functional networks.
591 Fold change (FC) were calculated from the mean values of 3 explants per condition (CS2vsT)
592 generated from each donor (V1, V2, V3 and V4), $p < 0.05$

593

594