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1 **An *in vitro* model to evaluate the impact of environmental fine particles (PM_{0.3-2.5}) on**
2 **skin damages**

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30 **Abstract**

31 Exposure to airborne particulate matter (PM) has significant effects on human health mainly
32 **leading to** cardio-respiratory diseases. However very few data are available regarding the
33 impact of PM on the skin, so to better understand the impact of fine particle (PM_{0.3-2.5}) on
34 both inflammatory response and epidermal structure, we exposed a reconstructed human
35 epidermis (RHE) to **several doses** of PM collected in Cotonou (Benin, West Africa). After
36 24h of exposure, inflammatory response, histological observations, and gene expression
37 **related to** oxidative stress, antioxidant defense and structural damages were determined. No
38 PM-linked changes in tissue morphology or membrane integrity were **observable**. PM was
39 however cytotoxic in a dose dependent manner. An inflammatory response appeared as
40 shown by the increase in IL-1 α and IL-8 cytokine productions. PM also **induced** oxidative
41 stress, leading to an increase in 4-HNE immunostaining and to the up-regulation of *HMOX1*,
42 *MTIG* and *MTIE*. Finally, PM had a negative impact on fundamental skin functions such as
43 tissue anchorage, cell differentiation, cornification / skin desquamation and apoptosis. Our
44 data show that airborne fine particles have an adverse effect on skin integrity, most probably
45 leading to accelerated ageing.

46 **Keywords**

47 Airborne fine particles; *In vitro* skin model; Inflammatory response; Oxidative stress;
48 Premature skin ageing

49 **Abbreviations**

50 4-HNE: 4-hydroxynonenal, BSA: bovine serum albumin, DAPI: 4',6-diamidino-2-
51 phenylindole dihydrochloride, PM: particulate matter, H&E: haematoxylin and eosin, MMP:
52 metalloproteinase, PBS: phosphate buffered saline, RHE: Reconstructed Human Epidermis,
53 RT-qPCR: real-time quantitative PCR

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59 useful comments and discussion.

60 **1. Introduction**

61 Air pollution is a degradation of air quality by introduction of harmful substances
62 including gases, chemicals, biological contaminants, and particles. Air pollutants include
63 particulate matter (PM), and are a major public health concern. PM is a heterogeneous and
64 complex mixture due to its various origins (natural: soil, forest fires or volcanoes, and
65 anthropogenic: human activities, industry, traffic...). Emitting sources and atmospheric
66 conditions are key parameters contributing to the chemical and physical properties of ambient
67 particulate matter (Kim et al., 2016). The role of the aerodynamic diameter of PM in the
68 increase in the risks of chronic illness and premature death is widely documented. Much less
69 recognized however, is that the chemical constituents adsorbed on PM also have significant
70 effects on various diseases (Dergham et al., 2015; Kim et al., 2016; Song et al., 2013). A large
71 number of studies have considered the effects of fine particles (PM_{2.5}) on human health, the
72 majority being focused on respiratory and cardiovascular diseases. In the case of airway
73 epithelium, for instance, the skin is in direct contact with airborne PM, and long-term
74 exposure to PM may induce or exacerbate pre-existing skin diseases such atopic dermatitis,

75 acne, psoriasis, or premature skin ageing (Mancebo and Wang, 2015; Drakaki et al., 2014;
76 Flohr and Mann, 2014; Kim et al., 2016). Different clinical studies have highlighted an
77 increased level of rash after exposure to polluted air, a relation between traffic-associated
78 pollution and eczema in children, a modification of the skin integrity, and a contribution of
79 traffic-based air pollution to facial lentigines (Huls et al., 2016; Krutmann et al., 2014;
80 Lefebvre et al., 2015; Vierkotter et al., 2010). At the cellular level, skin ageing modifies the
81 tissue structure with looseness of the epidermal-dermal junction, and of the dermis with the
82 reduction of blood vessels and nerves (Puizina-Ivic, 2008).

83 Oxidative stress seems to be one of the underlying mechanisms that induces adverse
84 health effects and it is known to play a role in skin damage after exposure to some
85 environmental factors like UV and pollutants (Carletto et al., 2000; Giacomoni and Rein,
86 2001; Krutmann et al., 2016; Lanuti and Kirsner, 2010; Makrantonaki et al., 2015; Valacchi et
87 al., 2012). A direct impact of the UV exposure has been shown on vitamin D production, loss
88 of elastic fiber properties, and oxidative stress (Gilchrest, 2013; Kammeyer and Luiten, 2015;
89 Sherratt, 2013). Airborne pollutants such as nitrogen oxides, hydrocarbons, fine particles and
90 ozone, also have a deleterious impact on skin, particularly sensitized skins which react more
91 strongly to stressors (Krutmann et al., 2014). Similarly, some studies have established a link
92 between tobacco smoke and skin alteration (Lecas et al., 2016; Morita et al., 2009; Urbanska
93 et al., 2012a; Urbanska et al., 2012b).

94 Generation of reactive oxygen species, upregulation of pro-inflammatory factors by the
95 arylhydrocarbon receptor (AhR), and keratinocytic VEGF up-regulation have been
96 demonstrated as responsible mechanisms in the genesis of certain cutaneous pathologies (Bae
97 et al., 2015; Krutmann et al., 2014; Li et al., 2003; Vierkotter and Krutmann, 2012). In order
98 to better explain the underlying mechanisms, studies have been mainly performed on animals.
99 A 6h exposure to controlled PM_{2.5} was shown to induce oxidative stress (*HMOX1* expression)

100 in neonatal and adult rat lungs and the stimulation of antioxidant enzymes (*SOD1* for
101 example) was observed for adults but not neonates, more susceptible to ultrafine particles
102 (Chan et al., 2013). Cytotoxicity and apoptosis of human keratinocytes were also associated
103 with combustion-generated organic nanoparticles (Pedata et al., 2012).

104 Mastrofrancesco et al. (2014) also reported that environmental pollution from diesel
105 exhaust plays a critical role in the development of some autoimmune diseases
106 (Mastrofrancesco et al., 2014). The authors showed that rat scleroderma skin keratinocytes
107 and fibroblasts expressed cytokine genes (*IL-1 α* , *IL-6*, *IL-8*, and *TNF- β*) at a higher level than
108 control cells when treated with carbon-based nanoparticles collected from diesel exhaust. In
109 addition, mRNA gene expressions of some *MMPs*, *Collagen types I and III*, and *VEGF* were
110 significantly higher in untreated scleroderma skin cells than controls, with a dose dependent
111 stimulation of all *MMPs* (Mastrofrancesco et al., 2014).

112 Taking into account that oxidative stress and inflammation play a major role in air
113 pollution mediated toxicity of the skin, the aim of the present work was to evaluate the impact
114 of environmental fine particles ($PM_{0.3-2.5}$), collected in a geographical area selected for its
115 natural wealth in metallic elements, on skin tissue. For this purpose, we used a sensitive *in*
116 *vitro* skin model (RHE) focusing on possible oxidative damage leading to morphological
117 changes and modulation of protein expression.

118 **2. Material and methods**

119 **2.1. Chemicals**

120 Culture media, maintenance medium for experiment days, and growth medium for non-
121 experiment days, were purchased from SkinEthic™, France. Penicillin-Streptomycin mixture
122 (penicillin: 100 μ g/mL; streptomycin: 100UI/mL) and Fungizon (Antibiotic-Antimycotic)
123 were from Gibco (Invitrogen, WI, USA), Phosphate-buffered saline (PBS) from Biosolve
124 Chimie (France), calcium chloride, goat serum, glycine, bovine serum albumin, glycerol,

125 sodium azide, Eukitt[®], sodium cacodylate, glutaraldehyde, hexamethyldisilazane, osmium
126 tetroxide, hydrochloric acid, and propylene oxide from Sigma-Aldrich (France). Acetic acid
127 and Triton X100 were purchased from Merck (Germany); paraffin from Labonord (France);
128 ethanol from VWR International (PA, USA). Xylene and paraformaldehyde (32%) were
129 provided by Euromedex (France); sodium chloride by Minisol (France); Mayer Haematoxylin
130 (RAL Diagnostics, France) and Eosin by Merck (Germany).

131 **2.2. *In vitro skin model***

132 A reconstructed human epidermis from normal human keratinocytes provided the RHE
133 model (SkinEthic[™], France) (Rosdy and Clauss, 1990; Rosdy et al., 1993) that was used as
134 described previously (Lecas et al., 2016). For all experiments, the same batch (MF22-5) was
135 used. Signed informed consent and ethical approval were obtained by the supplier.

136 Upon reception RHEs were placed in an incubator at 37 °C, 5% CO₂ and 90% relative
137 humidity. Experiments were carried out 24h after RHE-acclimation (Poumay et al., 2004).

138 **2.3. *Fine particles exposure (PM_{0.3-2.5})***

139 Detailed information about collection and physicochemical characteristics of the PM
140 collected in Cotonou city (Benin, West Africa) have been previously published (Table 1,
141 Table S1) (Cachon et al., 2014). After collection, PM_{0.3-2.5} were weighed and stored at -20°C
142 until experimentation. PM were suspended in the culture medium containing 1% fungizon and
143 sonicated at 50W during 10 min (Bath sonicator Branson CPXH, Dutscher, France) to avoid
144 the presence of aggregates (Fig. S1). RHEs were exposed to three PM concentrations: 3, 15
145 and 30 µg/cm² as published by Cachon (Cachon et al. 2014). 30 µL of suspension of the
146 desired PM concentration were deposited directly on the apical surface of RHE tissues. RHEs
147 were then placed on an orbital agitator for 10 mins to homogenize the PM suspension to the
148 apical surface and then incubated at 37°C, 5% CO₂ in a humidified incubator.

149 After 24h exposure to PM, culture media were collected to assess biological activity:
150 LDH release, cytokine and MMP quantifications. RHEs were harvested to perform MTT
151 assay, histological / immunohistochemical analysis and mRNA quantification by RT-qPCR.

152 Two controls were used: medium-control (named Control: 30 μ L of medium without PM
153 deposited on apical surface) and incubator-control (without medium on apical side). As no
154 difference was shown between these two controls, only the medium-control (Control) was
155 introduced in the continuation of the present work.

156 **2.4. Functional integrity**

157 Colorimetric MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay
158 relies on the quantification of mitochondrial succinate dehydrogenase activity. Viable cells
159 convert a soluble tetrazolium salt to a formazan product having a maximum photometric
160 absorbance at a wavelength of 490 nm (Persoz et al., 2010). Absorbance was read with a
161 microtiter plate reader (Multiskan-EX, Thermo Scientific). [Experiments were repeated at least](#)
162 [4 times in duplicate.](#)

163 **2.5. Cellular viability: LDH release**

164 Membrane integrity was determined by measuring the release of intracellular lactate
165 dehydrogenase (LDH) into the basal culture medium (Lactate dehydrogenase activity assay
166 Kit, Sigma). LDH [reduced](#) NAD to NADH, which is detected by colorimetric at 450nm using
167 a microtiter plate reader (Multiskan-EX, Thermo Scientific). LDH release is the reflection of
168 the cellular viability and was expressed as an arbitrary unit. [Experiments were repeated at](#)
169 [least 4 times in duplicate.](#)

170 **2.6. Proinflammatory mediator quantification**

171 Two cytokines, interleukin 1 α (IL-1 α #KAC1191) and interleukin 8 (IL-8 #KHC0081),
172 were assessed using an ELISA assay following manufacturer's instructions (Novex™,
173 France). Optical densities were determined using a microplate-reader (Multiskan-EX, Thermo

174 Scientific, USA) set to 450 nm. Calibration curves were established using Ascent Software[®]
175 (Thermo Scientific, MA, USA). Cytokine concentrations were expressed in pg/mL. The limits
176 of quantification were 3.9 and 15.6 pg/mL for IL-1 α and IL-8 respectively. [Experiments were](#)
177 [repeated 4 times in duplicate.](#)

178 **2.7. Matrix metalloproteinases assessment**

179 Assessment of matrix metalloproteinases was performed using a multiplex panel (Human
180 Magnetic Luminex Performance Assay Base Kit, MMP Panel [#LMPM000] R&D Systems,
181 France). Samples were incubated overnight with antibody-coated beads at 4°C under constant
182 shaking. The following day, plates were washed twice and secondary antibody was added to
183 the samples for one-hour incubation at room temperature under constant shaking. Then, plates
184 were incubated for 30 minutes with streptavidin labelled with R-Phycoerythrin at room
185 temperature under agitation. Washing process was repeated, before adding sheath fluid and
186 reading the results using Luminex[®] technology (Magpix[®], Bio-Rad, France) after 5 minutes
187 shaking. Concentrations were calculated out of standard curves using recombinant proteins
188 and expressed in pg/mL. [Experiments were repeated 2 times in triplicate.](#)

189 **2.8. Histological and immunochemical assessments**

190 Histological and immunohistochemical assessments were performed 24h after exposure.
191 RHEs were fixed in 4% paraformaldehyde for 4h and paraffin-embedded sections were sliced
192 for Mayer Haematoxylin and Eosin (H&E) and for immunohistochemistry.
193 Prior to Mayer H&E staining, RHE cross-sections (4 μ m) went through xylene
194 deparaffinization and rehydration with graded ethanol baths. Observations were made with an
195 inverted light microscope (Leica DM4000 B LED).

196 For immunohistochemistry, sections were blocked in 10% BSA/PBS before incubation
197 with anti-loricrin or anti-4-HNE primary antibodies (#ab24722 and #ab46545 respectively,
198 Abcam, UK). For both, an AlexaFluor[®]488-conjugated goat anti-mouse (#A-21121,

199 Invitrogen, USA) was used as a secondary antibody. Nuclei were stained with 4',6-diamidino-
200 2-phenylindole dihydrochloride (DAPI, #32670 Sigma, France). The resulting
201 immunofluorescence was observed under a Nikon Eclipse 50i fluorescence microscope and
202 pictures were taken using Nikon NIS Elements software. Quantification of both loricrin and
203 4-HNE fluorescence signals of tissue sections was performed using Visilog software version
204 7.0. Experiments were repeated in 3 independent experiments.

205 **2.9. RNA isolation and RT-qPCR**

206 Following the manufacturer's instructions, total RNA was extracted from tissues using
207 the RNeasy[®] miniKit (Qiagen, Germany). RNA quality and quantity were checked with the
208 2100-Bioanalyzer (Agilent Technologies, France), then RNA was reverse transcribed using
209 the Transcriptor Universal cDNA Master (Roche Diagnostics, Switzerland). PCR primers
210 were designed according to the Roche Universal Probe Library Assay Design Center
211 (www.universalprobelibrary.com). A LightCycler 480 system using LightCycler 480 Probes
212 Master (Roche, Germany) was used to perform quantitative RT-qPCR on 29-gene epidermal
213 function plates (Agilent RNA Nano chips #5067-1511, Roche Diagnostics, Switzerland). The
214 $2^{-\Delta\Delta Ct}$ method provided N-fold differential expression of mRNA gene expression and results
215 were obtained by calculating the fold change (FC) between two conditions, exposed *versus*
216 **control**, meaning the expression difference. mRNA expression was considered as increased
217 when $FC \geq 1.5$, while decreased when $FC \leq 0.7$. GAPDH and RPL13 housekeeping genes
218 were used as internal controls. Results are the mean of one experiment assessed in duplicate.

219 **2.10. Statistical analysis**

220 Data are displayed as mean \pm standard deviation. All experiments were repeated at least
221 in duplicate in two different experiments; the number of experiments conducted is indicated
222 in the legend of each Figure. Results were analyzed by one-way ANOVA followed by a

223 Tukey-Kramer post-test. Statistical analysis was performed using JMP v.10 (SAS division,
224 USA). $p < 0.05$ was considered significant.

225 **3. Results**

226 **3.1. Tissue morphology and cell functionality (Fig. 1-2)**

227 The SkinEthic reconstituted human epidermis (RHE) model consists of the four
228 characteristic layers *stratum germinativum*, *stratum spinosum*, *stratum granulosum*, and
229 *stratum corneum* as shown in the Figure 1A.

230 A 24h exposure of RHEs to increasing PM concentrations (3, 15 and 30 $\mu\text{g}/\text{cm}^2$) did not
231 induce structural changes whatever the PM-level tested (Figs. 1B1, 1B2, 1B3): RHEs exposed
232 to PM were similar to the Control. This observation is confirmed by the absence of LDH
233 release certifying good membrane integrity (Fig. 2B). Nevertheless, MTT assay revealed a
234 dose-dependent relation between the level of PM and cellular functionality, with a statistically
235 significant decrease after exposure of RHEs at 15 and 30 $\mu\text{g}/\text{cm}^2$ compared to Control (Fig.
236 2A). This last result would seem to indicate that the mitochondrial succinate dehydrogenase
237 function is disturbed without leading to cell death as indicated by LDH and morphology data.

238 **3.2. Inflammatory response (Fig. 3)**

239 To evaluate the inflammatory response of RHEs, the production of two cytokines, IL-1 α
240 and IL-8, was evaluated after RHE-exposure to PM at 3, 15 and 30 $\mu\text{g}/\text{cm}^2$. Compared to
241 Control, a 24h-exposure to PM at 3 $\mu\text{g}/\text{cm}^2$ did not induce significant difference in the
242 production levels of IL-1 α and IL-8 (Fig. 3). For higher doses of PM (15 and 30 $\mu\text{g}/\text{cm}^2$), a
243 significant dose-dependent increase in the production of the two cytokines tested was
244 observed. For IL-8, the increase was as high as 52-fold the level detected in the Control after
245 exposure of RHEs to 30 $\mu\text{g}/\text{cm}^2$ of PM.

246 *Remark:* These initial results concerning membrane integrity, functionality and inflammatory
247 responses, revealed significant cytokine production after RHE exposure at the highest dose of

248 PM (30 $\mu\text{g}/\text{cm}^2$) without membrane integrity disorder (LDH release) but with a loss of 50% of
249 cellular functionality, probably due to the oxidative stress caused by metallic compounds
250 adsorbed on the PM tested. In this context, the rest of the study has been focused on the
251 exposure of RHE tissue to PM at 30 $\mu\text{g}/\text{cm}^2$.

252 **3.3. Oxidative stress (Fig. 4)**

253 Two steps have been taken to assess oxidative damage on RHE tissue: 4-HNE
254 immunostaining with quantification (4-hydroxynonenal, one of the most important products
255 of lipid peroxidation) and three oxidant gene expressions evaluated by RT-qPCR.

256 Immunostaining of 4-HNE, a widely used marker of oxidative stress, showed a substantial
257 increase in labelling, 24h after exposure of RHE tissue to PM at 30 $\mu\text{g}/\text{cm}^2$, compared to
258 Control (Fig. 4A). The quantification of 4-HNE staining confirmed this significant increase
259 by 4-fold, demonstrating that PM exposure does stimulate oxidative stress in our model (Fig.
260 4B). To investigate the underlying effects on the molecular and biological activities of RHE,
261 the activity of three genes involved in oxidative stress (*HMOX1*, *MT1G* and *MT1E*) was
262 studied by quantification of their mRNA. mRNA expression levels of the genes were
263 stimulated by 1.6 to 3.0-fold respectively after exposure of RHEs to PM at 30 $\mu\text{g}/\text{cm}^2$ (Fig.
264 4C).

265 **3.4. Differentiation and morphology (Fig. 5-6)**

266 To evaluate the impact of PM on both extracellular matrix and tissue differentiation, two
267 approaches were considered: MMP level quantification and loricrin immunostaining with
268 quantification.

269 Immuno-quantification of matrix metalloproteinases panel showed that only two MMPs
270 from the nine MMPs tested (MMP-1, -2, -3, -7, -8, -9, -10, -12, -13) were significantly
271 modulated. Compared to Control, MMP-1 was induced by 1.5-fold and MMP-3 by 1.9-fold
272 after exposure of RHEs to 30 $\mu\text{g}/\text{cm}^2$ of PM (Fig. 5). In addition, immunostaining of loricrin,

273 a major protein component of terminally differentiated epidermal cells, showed a significant
274 decrease in labelling after PM-exposure (Fig. 6A). This result is confirmed by the
275 quantification of the intensity of loricrin labelling. Compared to the Control, a 1.9-fold loss of
276 loricrin staining was observed 24h after PM-exposure (Fig. 6B).

277 **3.5. Other biological impacts of PM on RHEs (Fig. 7)**

278 To investigate the underlying effects of PM at 30 $\mu\text{g}/\text{cm}^2$ on the biological activity of
279 RHEs, 12 genes were selected as the most representative to evaluate cellular and molecular
280 modulation. Differentiation genes were either weakly stimulated (*IVL*) or weakly decreased
281 (*KRT1*, *KRT10*) (Fig. 7). Expressions of cell-cell junction genes (*EVPL* (*ENVOPLAKIN*),
282 *GJA1*, *CDH1*, *CD44*), and anchorage genes (*COL7A1*, *COL4A1*) were suppressed after PM-
283 exposure. Apoptotic gene expression was also studied (*CASP3*, *CDKN2A*, *BIRC5*), and results
284 shown that upon PM exposure, *BIRC5* expression was reduced while *CDKN2A* expression
285 was doubled and *CASP3* was highly stimulated (6.3-fold) (Fig. 7).

286 **4. Discussion**

287 The aim of our study was to better understand the impact of airborne fine particles (PM)
288 on skin damage as suggested by other studies (Valacchi et al., 2012; Vierkotter and
289 Krutmann, 2012).

290 In this study we used particles collected in Cotonou, an urban city of West Africa
291 (Benin). Our objective was to generally test environmental particles rich in such metallic
292 inorganic elements as metals, positively correlated with intracellular ROS production
293 (Dergham et al., 2015), and not to specifically test Cotonou's PM. Having said this, the
294 interest of particle samples tested in our study does lie in their origin: in African countries like
295 Cotonou the soils are rich in iron oxide (Fe_2O_3) and aluminium oxide (Al_2O_3) (Kikouama et
296 al., 2009), with co-existing heavy metals at very high levels (Alford et al., 2010; Cheng,
297 2003). In comparison we can note that the level of metallic elements in PM collected in an

298 urban city of France (Dunkerque) was 620 ng/m³ versus 8,644 ng/m³ in Cotonou (Dergham et
299 al., 2015).

300 The presence of such elements contributes to atmospheric pollution leading to various
301 biological dysregulations in Human, such as oxidative stress, this latter being at the origin of
302 various pathologies (Cachon et al., 2014). The choice of the *in vitro* skin model was important
303 to our study. RHE tissue consists of an epidermis without dermis, obtained from keratinocytes
304 cultured at high cell density on a polycarbonate filter membrane with 0.4 µm pore diameter.
305 After 15 days of culture, keratinocytes formed a differentiated epidermis with histological
306 features similar to those observed *in vivo*: a basal layer, spinous layer, granular layer, and a
307 stratum corneum (Poumay et al, 2004). This model was used to assess the irritating and/or
308 corrosive properties of some chemical compounds (Kandárová et al, 2006). However, like all
309 *in vitro* models, RHE tissue has some limitations, principally the small experimentation
310 window (3 days) due to the model ageing quickly (Lecas et al, 2016). This led us to limit our
311 experiments to 24h of exposure. Nevertheless, the RHE model did allow the combined
312 implementation of 3D-reconstituted tissue and exposure at the air-liquid interface in a
313 controlled environment. Our work has thus highlighted the possibility of using this *in vitro*
314 3D-skin model to assess the importance of the chemical constituents of PM on the cellular
315 and molecular responses of exposed tissue.

316 Exposure to airborne particulate matter has toxic effects on human health as illustrated by
317 drastic respiratory and skin diseases (Beelen et al., 2014; Drakaki et al., 2014; Raaschou-
318 Nielsen et al., 2013). The skin can be impacted by several intrinsic and extrinsic phenomena
319 (Makrantonaki et al., 2015). External factors such as UV and air pollutants including tobacco
320 smoke can play an important role in skin ageing (Valacchi et al., 2012; Vierkotter and
321 Krutmann, 2012). In order to take into account all levels (tissue, cell and molecular level) and
322 have a better knowledge on the [underlying](#) mechanisms, an *in vitro* RHE skin model can be

323 used to evaluate the toxicity level of extrinsic exposure such as tobacco smoke (Lecas et al.,
324 2016) and airborne pollutants (Magnani et al., 2016). The mode of action (MoA) hypothesis
325 of the impact of airborne pollution on the premature skin ageing process, using the RHE 3D-
326 skin model exposed to fine particles, is illustrated in Figure 8.

327 While the PM did not induce obvious structural changes in RHE morphology and a loss
328 of membrane integrity, it did lead to a dose-dependent decrease in cellular functionality as
329 shown by MTT assay, evaluating mitochondrial succinate dehydrogenase activity, a
330 mitochondrial enzyme. These results - showing a loss of cellular functionality with absence of
331 both structural changes and tissue integrity injury - would appear contradictory, but is not.
332 Specifically, the complementary nature of the tests gives a more accurate view of the impact
333 of a xenobiotic on a targeted tissue. The oxidizing potential of PM, due to the presence of
334 metal elements adsorbed on their surface, could explain this result and constitute the key
335 event 1 (KE1, Fig. 8).

336 To consolidate this result, we observed that PM was able to stimulate IL-1 α and IL-8
337 production in a dose dependent manner, thus illustrating the inflammatory response of skin
338 tissue (AE1, Fig. 8). Oxidative stress was also induced, as illustrated by the increase of
339 4-HNE labelling in RHE (KE2, Fig. 8), and in accordance with the stimulation of *HMOX1*
340 expression. A similar induction of *HMOX1*, a sensitive marker for oxidative stress, was
341 observed upon exposure of a lung model to ultrafine particles (Chan et al., 2013; Li et al.,
342 2003). In reaction, the tissue induces antioxidants as illustrated by previous works on the lung
343 (Curjurić et al., 2012; Huang et al., 2009) and, in our case, by the induction of *MTIG* and
344 *MTIE* expressions which corresponding proteins are known to be involved in heavy metal
345 binding (KE3, Fig. 8).

346 Considering tissue integrity, no morphological changes appeared after PM-exposure, and
347 increased levels of MMP-1 and MMP-3 production suggest an accelerated degradation of the

348 extracellular matrix (KE5, Fig. 8). The level of loricrin, a keratinocyte specific protein, was
349 also decreased (AE2, Fig. 8). Moreover, cell-cell junction, anchorage, and terminal
350 differentiation genes, except *Involucrin (IVL)*, were drastically down-regulated on PM-
351 exposure (AE3, Fig. 8). *Involucrin (IVL)* is known to play an important role in cornified
352 envelope development (Hanel et al., 2013). Inhibition of *IVL* was observed in several studies,
353 including when the *in vitro* RHE skin model was exposed to tobacco smoke, illustrating the
354 disorganization of skin barrier function (Lecas et al., 2016). However, stimulation of *IVL* was
355 also found after exposure of HaCaT cells and atopic dermatitis to ambient PM_{2.5} leading to
356 the hypothesis that cell death is the primary cause of skin alteration rather than protein
357 modification (Hirao et al., 2003; Li et al., 2017). *KRT1* and *KRT10* expressions were
358 decreased, revealing the skin barrier dysfunction (Pan et al., 2015). This tissue slackening was
359 also illustrated by the decrease in the expression of cell-cell junction (*EVPL*, *GJAI*, *CDH1*
360 and *CD44*) and anchorage (*COL4A1* and *COL7A1*) genes. This is in line with the role of Gap
361 Junctions Intercellular Communications and Tight Junctions in the epithelial lining, which
362 modification might be implied in cancer development after PM₁₀ inhalation (Nemmar et al.,
363 2013).

364 In order to better understand the underlying mechanism of toxicity on the RHE model,
365 apoptosis-linked genes (*CASP3*, *CDKN2A* and *BIRC5 (SURVIVIN)*) were also investigated.
366 *BIRC5*, known to block apoptosis and to regulate the cell cycle, was downregulated.
367 Moreover, both *CASP3* and *CDKN2A*, pro-apoptotic genes, were upregulated in our study (6-
368 and 2-fold, respectively) (KE4, Fig. 8). Metal elements, being the main components of PM
369 from Cotonou, have been described as inducers of *CASP3* in hepatic cells and macrophages
370 (Andreau et al., 2012). *CDKN2A* (p16) is known to have an ageing-dependent expression and
371 therefore to be increased in the elderly (Ressler et al., 2006).

372 In conclusion, using an *in vitro* 3D-model of human reconstituted epidermis, our results
373 showed that exposure to fine particles lead to an increase in some indicators such as oxidative
374 stress and inflammatory response, contributing to a tissue slackening following the
375 degradation of the extracellular matrix and cell-cell junctions. The chemical composition of
376 airborne particulate matter, and more particularly the metallic elements adsorbed on the
377 surface of the PM, could contribute to various MoA hypotheses leading to the premature skin
378 ageing and the loss of the protective role of the cutaneous barrier. The present work is original
379 and innovative, and provides new development opportunities to better understand the role of
380 atmospheric particulate matter on skin damage.

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382 This research did not receive any specific grant from funding agencies in the public,
383 commercial, or not-for-profit sectors.

384 **Competing interests**

385 R. Fitoussi, N. Blanchet and K. Vié are full-time employees of Clarins, a major company
386 specialized in the design, manufacturing and marketing of cosmetic products.

387

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543 **Figure legends**

544 **Fig. 1. Morphological observations of RHEs after PM-exposure.**

545 RHE tissues were exposed to PM at 0 (Control), 3, 15, or 30 $\mu\text{g}/\text{cm}^2$ for 24h. After exposure
546 RHEs were fixed in 4% paraformaldehyde and cross-sections (4 μm) were stained with Mayer
547 Haematoxylin and Eosin (H&E) and observed using an inverted light microscope (Leica
548 DM4000B LED).

549 Scale bar represents 25 μm ; 40x magnification. Representative pictures of n=3 independent
550 experiments.

551 (A) RHE Control. 1/*Stratum germinativum*; 2/*Stratum spinosum*; 3/*Stratum granulosum*;
552 4/*Stratum corneum*.

553 (B) RHE exposed at (B1) 3 $\mu\text{g}/\text{cm}^2$; (B2) 15 $\mu\text{g}/\text{cm}^2$; (B3) 30 $\mu\text{g}/\text{cm}^2$.

554

555 **Fig. 2. Effects of PM on RHE functionality and membrane integrity.**

556 RHE tissues were exposed to PM at 0 (Control), 3, 15, or 30 $\mu\text{g}/\text{cm}^2$ for 24h.

557 Experiments were repeated at least four times in duplicate.

558 Results are presented as mean \pm SD ; ** $p < 0.01$; *** $p < 0.0001$ compared to the Control.

559 A. RHE functionality was assessed 24h after PM-exposure using MTT assay. Results are
560 expressed in absorbance (arbitrary units).

561 B. Membrane integrity of RHE was evaluated 24h after PM-exposure by intracellular lactate
562 dehydrogenase (LDH) release expressed in arbitrary unit per liter (U/L).

563 Maximal LDH activity was obtained after 5% SDS treatment (Positive control).

564

565 **Fig. 3. Inflammatory response of RHE after PM-exposure.**

566 RHE tissues were exposed to PM at 0 (Control), 3, 15, or 30 $\mu\text{g}/\text{cm}^2$ for 24h.

567 After exposure cytokine secretions [IL-1 α (plain bar) and IL-8 (dashed bar)] were measured
568 by immuno-quantification (ELISA test) in the culture medium in the basolateral
569 compartment.

570 Data are representative of 4 experiments repeated in duplicate.

571 Results are presented as mean \pm SD ; *** $p < 0.0001$ compared to the Control.

572

573 **Fig. 4. Changes in 4-HNE expression level after PM-exposure.**

574 RHE tissues were exposed to PM at 0 (Control) or 30 $\mu\text{g}/\text{cm}^2$ for 24h.

575 (A) After PM-exposure RHEs were fixed in 4% paraformaldehyde, embedded on paraffin and
576 sliced.

577 Cross-sections (4 μm) were blocked in BSA/PBS buffer and stained using 4-HNE (protein
578 produced when lipid peroxidation is) and DAPI (nucleus).

579 Scale bar represents 25 μm ; 200x magnification. Representative pictures of $n=3$ independent
580 experiments.

581 (B) Quantification of 4-HNE in comparison to Control using Visilog software version 7.0.

582 Data are representative of 3 independent experiments. Results are presented as mean \pm SD ;
583 *** $p < 0.0001$ compared to the Control.

584 (C) After exposure, total RNA was extracted from tissues using the RNeasy® miniKit and
585 reverse transcribed using the Transcriptor Universal cDNA Master. The gene expressions
586 were normalized with GAPDH housekeeping gene.

587 Results were obtained by calculating the fold change (FC) between two conditions: exposed
588 *versus* Control. Increased oxidant gene expression was observed: $\text{FC} \geq 1.5$.

589 Results are the mean from one RT-qPCR experiments assessed in duplicate.

590

591 **Fig. 5. Impact of PM exposure on MMP matrix metalloproteinase.**

592 After PM-exposure of RHEs ($30 \mu\text{g}/\text{cm}^2$), metalloproteinase secretion [MMP-1 (plain bar)
593 and MMP-3 (dashed bar)] were measured by immuno-quantification (Luminex® technology)
594 in the culture medium in the basolateral compartment.
595 Data are representative of 2 experiments repeated in triplicate.
596 Results are presented as mean \pm SD ; *** $p < 0.0001$ compared to the Control.

597

598 **Fig. 6. Changes in loricrin expression level after PM exposure.**

599 RHE tissues were exposed to PM at 0 (Control) or $30 \mu\text{g}/\text{cm}^2$ for 24h.

600 (A) After PM-exposure RHEs were fixed in 4% paraformaldehyde and embedded on paraffin.

601 Cross-section of RHE ($4 \mu\text{m}$) stained using loricrin (major protein of differentiated epidermal
602 cells) and DAPI (nucleus).

603 Scale bar represents $25 \mu\text{m}$; 200x magnification. Representative pictures of $n=3$ independent
604 experiments.

605 (B) Quantification of loricrin in comparison to control using Visilog software version 7.0.

606 Data are representative of 3 independent experiments. Results are presented as mean \pm SD ;
607 *** $p < 0.0001$ compared to the Control

608

609 **Fig. 7. Changes in mRNA expression levels of genes involved in differentiation, cell to
610 cell junction, anchorage and apoptosis after exposure to $30 \mu\text{g}/\text{cm}^2$ of PM.**

611 RHE tissues were exposed at $30 \mu\text{g}/\text{cm}^2$ of PM for 24hours. After exposure, total RNA was
612 extracted from tissues using the RNeasy® miniKit and reverse transcribed using the

613 Transcriptor Universal cDNA Master. The gene expressions were normalized with GAPDH
614 or RPL13 housekeeping gene.

615 Results were obtained by calculating the fold change (FC) between two conditions: exposed
616 *versus* Control).

617 mRNA expression was considered as increased when $FC \geq 1.5$ (dark grey) and decreased
618 when $FC \leq 0.7$ (light grey).

619 Results are the mean from one RT-qPCR experiments assessed in duplicate.

620

621 **Fig. 8. MoA hypothesis for PM-induced premature skin ageing.**

622 The mode of action (MoA) hypothesis of the airborne pollution on the premature skin ageing
623 process, using the RHE 3D-skin model exposed to fine particles, is subdivided into key
624 events, associative events and modulating factors. The initial key event is the increase of the
625 oxidative stress, associated with the modulation of the inflammatory response, which results
626 in an overexpression of various genes. Associative events include inhibition of the gap
627 junction communication and anchorage.

628

629 **Table 1. Sum of main metallic elements and organic components of fine particles ($PM_{0.3-2.5}$) from (Cotonou, Benin)** (adapted from Cachon et al., 2014).

630 Chemicals are expressed as mass composition per mass ($\mu\text{g}\cdot\text{g}^{-1}$) and volume unit (ng/m^3).

631 [Sampling of fine particles was](#) based on continuous collection using high volume samplers
632 (80 m^3/hr).

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637 **Supplementary data**

638

639 **Figure S1. Microscopic observation of the homogeneous dispersion of PM prior RHE-**
640 **exposure.**

641 Scale bar represents 100 μm ; 20x magnification.

642 Representative pictures of n=3 preparations

643

644 **Table S1. Chemical composition of the fine particles ($\text{PM}_{0.3-2.5}$) collected in (Cotonou,**
645 **Benin) (Cachon et al. 2014).**

646 PM were collected using a high volume cascade impaction air sampler as described by Billet
647 (2007) and the physicochemical characterization was carried out by studying their size
648 distributions, specific surface areas, organic and inorganic chemicals composition and ionic
649 species as described by Cazier (2016). Chemicals are expressed as mass composition per
650 volume unit ($\text{ng}\cdot\text{m}^{-3}$).

651 Billet, S. et al., 2007. Ambient particulate matter ($\text{PM}_{2.5}$): physicochemical characterization
652 and metabolic activation of the organic fraction in human lung epithelial cells (A549).
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655 of rural, urban and industrial areas: Organic compounds. J. Env Sci. S44, 45-56.

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659

660 **Highlights**

661 • Airborne particles induce skin [damages](#)

662 • Oxidative and inflammatory pathways modulation after skin exposure to PM

663 • Premature skin [ageing](#) after PM exposure

664

Fig. 1. Morphological observations of RHEs after PM-exposure.

RHE tissues were exposed to PM at 0 (Control), 3, 15, or 30 $\mu\text{g}/\text{cm}^2$ for 24h. After exposure RHEs were fixed in 4% paraformaldehyde and cross-sections (4 μm) were stained with Mayer Haematoxylin and Eosin (H&E) and observed using an inverted light microscope (Leica DM4000B LED).

Scale bar represents 25 μm ; 40x magnification.

Representative pictures of 3 independent experiments.

(A) RHE Control. 1/*Stratum germinativum*; 2/*Stratum spinosum*; 3/*Stratum granulosum*; 4/*Stratum corneum*.

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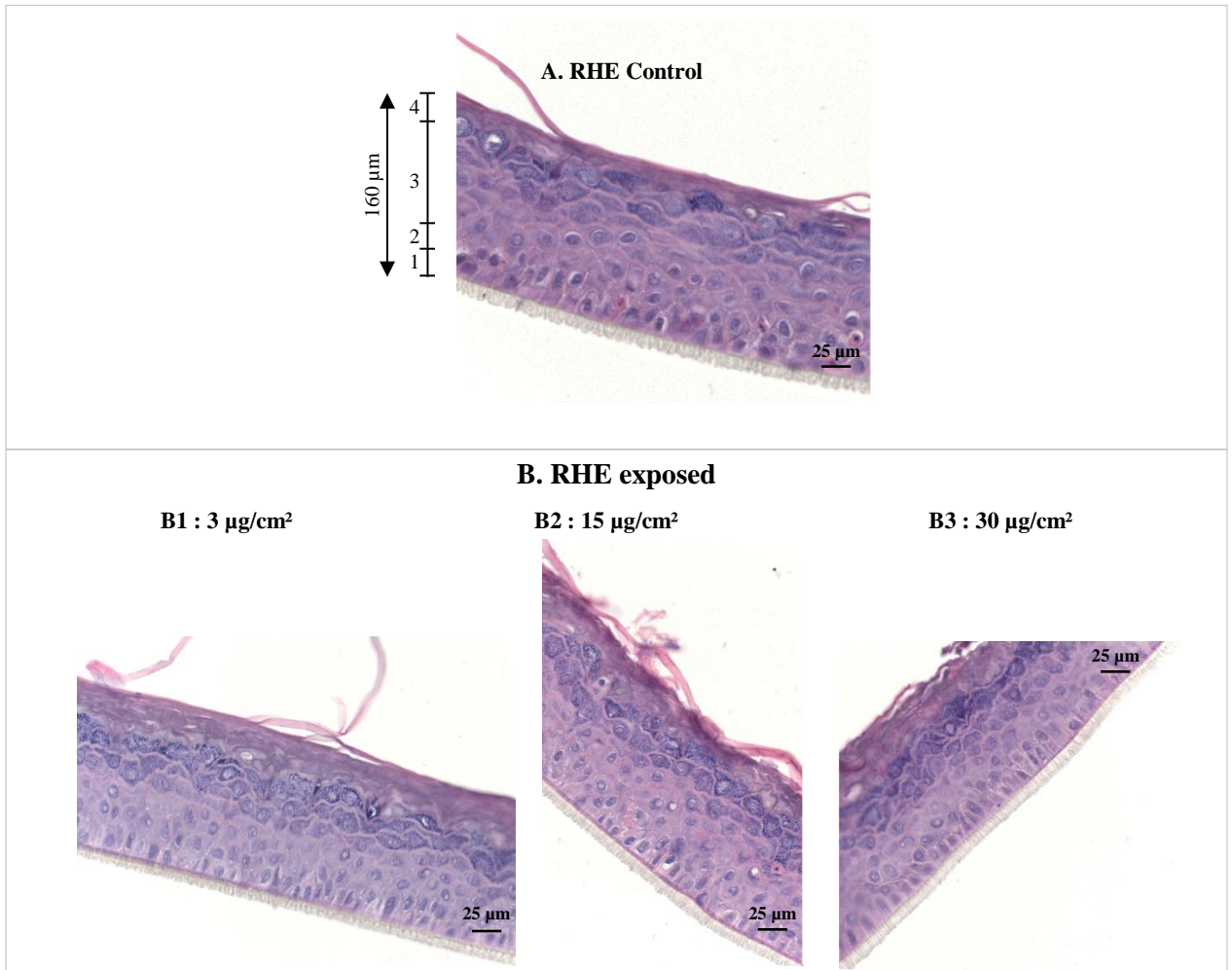


Fig. 2. Effects of PM on RHE functionality and membrane integrity.

RHE tissues were exposed to PM at 0 (Control), 3, 15, or 30 $\mu\text{g}/\text{cm}^2$ for 24h.

Experiments were repeated at least four times in duplicate.

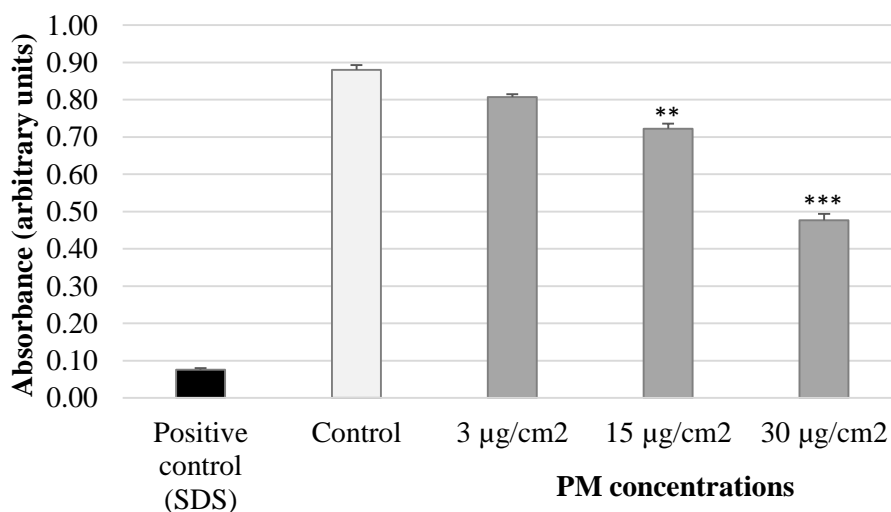
Results are presented as mean \pm SD ; ** $p < 0.01$; *** $p < 0.001$ compared to the Control

(A) RHE functionality was assessed 24h after PM-exposure using MTT assay. Results are expressed in absorbance (arbitrary units).

(B) Membrane integrity of RHE was evaluated 24h after PM-exposure by intracellular lactate dehydrogenase (LDH) release expressed in arbitrary unit per liter (U/L).

Maximal LDH activity was obtained after 5% SDS treatment (Positive control).

A.



B.

	Positive control (SDS)	Control	3 $\mu\text{g}/\text{cm}^2$	15 $\mu\text{g}/\text{cm}^2$	30 $\mu\text{g}/\text{cm}^2$
LDH release (U/L)	822 \pm 31***	15 \pm 5	20 \pm 10	30 \pm 18	41 \pm 21

Fig. 3. Inflammatory response of RHE after PM-exposure.

RHE tissues were exposed to PM at 0 (Control), 3, 15, or 30 $\mu\text{g}/\text{cm}^2$ for 24h.

After exposure cytokine secretions [IL-1 α (plain bar) and IL-8 (dashed bar)] were measured by immuno-quantification (ELISA test) in the culture medium in the basolateral compartment.

Data are representative of 4 experiments repeated in duplicate.

Results are presented as mean \pm SD ; *** $p < 0.0001$ compared to the Control

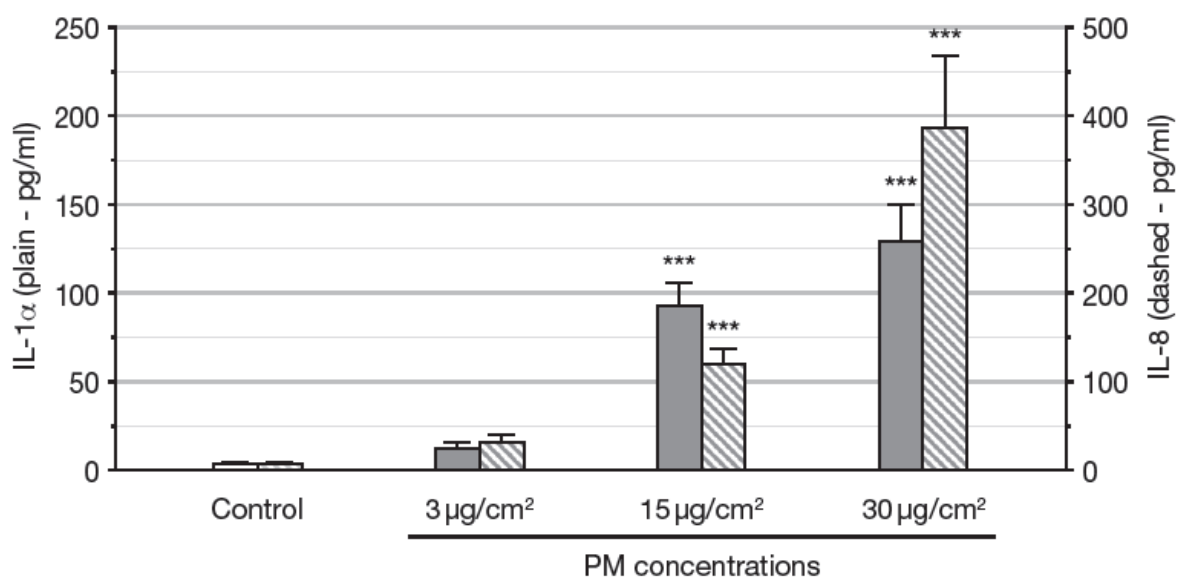


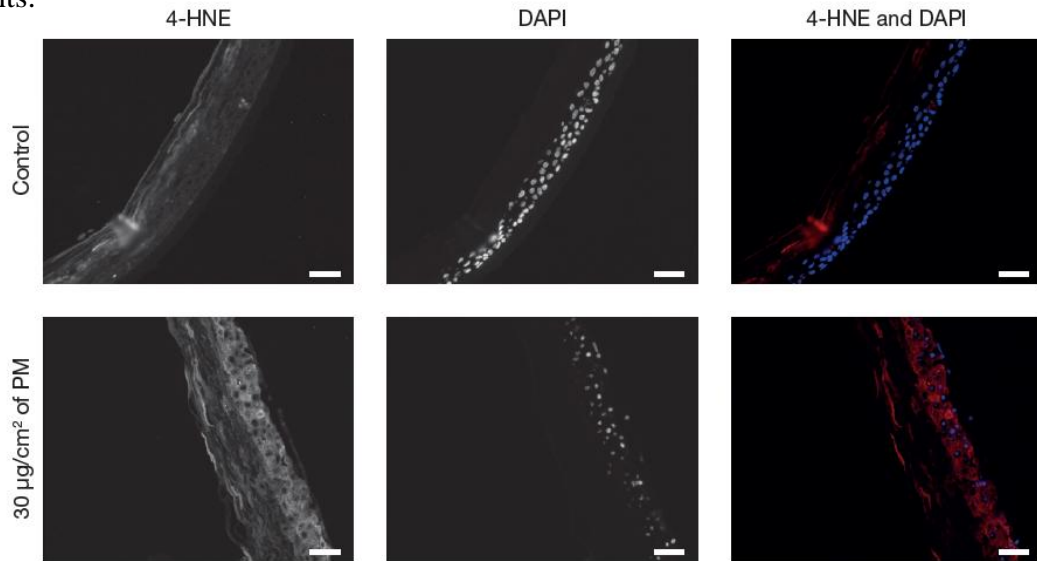
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RHE tissues were exposed to PM at 0 (Control) or 30 $\mu\text{g}/\text{cm}^2$ for 24h.

(A) After PM-exposure RHEs were fixed in 4% paraformaldehyde, embedded on paraffin and sliced.

Cross-sections (4 μm) were blocked in BSA/PBS buffer and stained using 4-HNE (protein produced when lipid peroxidation is) and DAPI (nucleus).

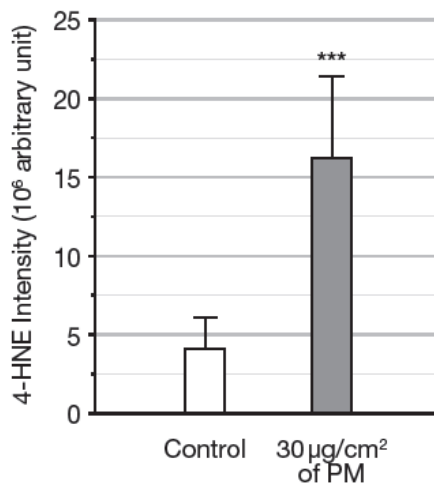
Scale bar represents 25 μm ; 200x magnification. Representative pictures of 3 independent experiments.



(B) Quantification of 4-HNE in comparison to Control using Visilog software version 7.0.

Data are representative of 3 independent experiments. Results are presented as mean \pm SD ;

*** $p < 0.0001$ compared to the Control.



(Fig. 4. continued)

(C) After exposure, total RNA was extracted from tissues using the RNeasy® miniKit and reverse transcribed using the Transcriptor Universal cDNA Master. The gene expressions were normalized with GAPDH housekeeping gene.

Results were obtained by calculating the fold change (FC) between two conditions: exposed *versus* Control.

Increased oxidant gene expression was observed: $FC \geq 1.5$.

Results are the mean from one RTqPCR experiments assessed in duplicate.

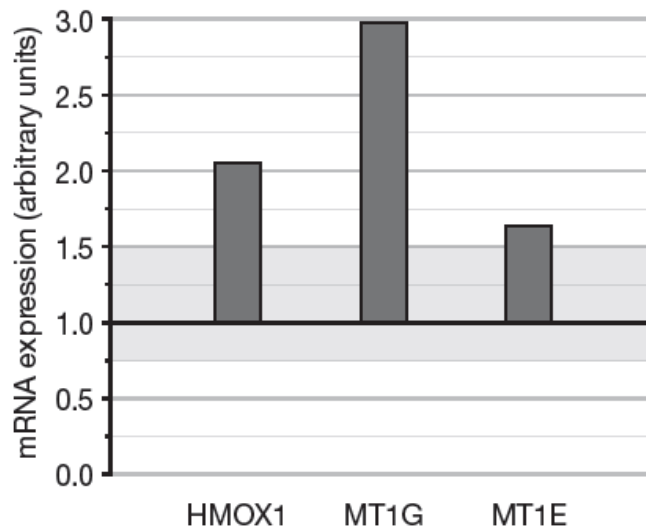


Fig. 5. Impact of PM-exposure on MMP matrix metalloproteinase.

After PM-exposure of RHEs ($30 \mu\text{g}/\text{cm}^2$), metalloproteinase secretion [MMP-1 (plain bar) and MMP-3 (dashed bar)] were measured by immuno-quantification (Luminex® technology) in the culture medium in the basolateral compartment.

Data are representative of 2 experiments repeated in triplicate.

Results are presented as mean \pm SD ; *** $p < 0.001$ compared to the Control.

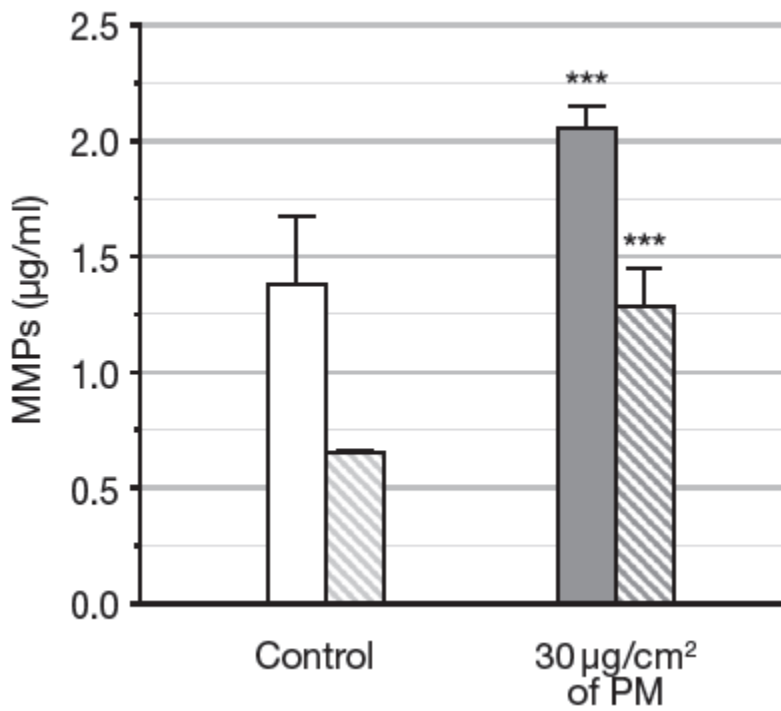
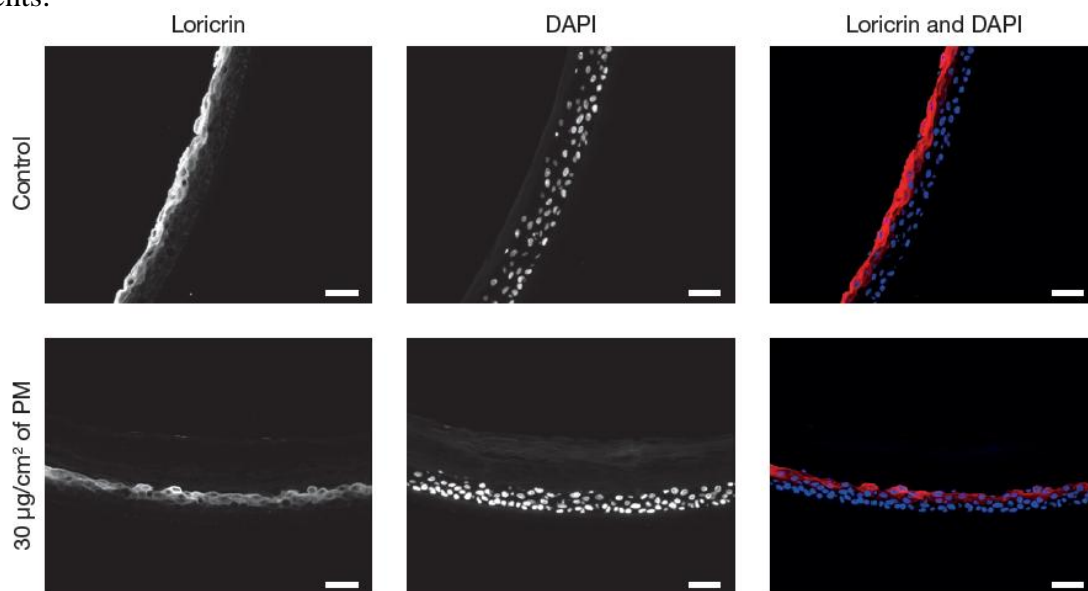


Fig. 6. Changes in loricrin expression level after PM-exposure.

RHE tissues were exposed to PM at 0 (Control) or 30 $\mu\text{g}/\text{cm}^2$ for 24h.

(A) After PM-exposure RHEs were fixed in 4% paraformaldehyde and embedded on paraffin. Cross-section of RHE (4 μm) stained using loricrin (major protein of differentiated epidermal cells) and DAPI (nucleus).

Scale bar represents 25 μm ; 200x magnification. Representative pictures of 3 independent experiments.



(B) Quantification of loricrin in comparison to control using Visilog software version 7.0.

Data are representative of 3 independent experiments. Results are presented as mean \pm SD ;

*** $p < 0.0001$ compared to the Control

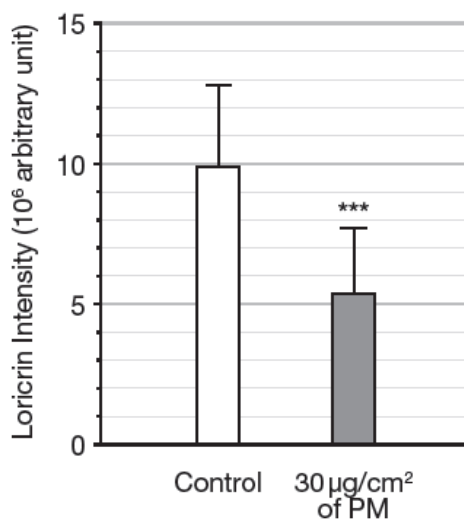


Fig.7. Changes in mRNA expression levels of genes involved in differentiation, cell to cell junction, anchorage and apoptosis after exposure to 30 µg/cm² of PM.

RHE tissues were exposed at 30 µg/cm² of PM for 24h. After exposure, total RNA was extracted from tissues using the RNeasy® miniKit and reverse transcribed using the Transcriptor Universal cDNA Master. Gene expressions were normalized with GAPDH or RPL13 housekeeping gene.

Results were obtained by calculating the fold change (FC) between two conditions: exposed *versus* Control). mRNA expression was considered as increased when FC ≥ 1.5 (dark grey) and decreased when FC ≤ 0.7 (light grey).

Results are the mean from one RTqPCR experiments assessed in duplicate.

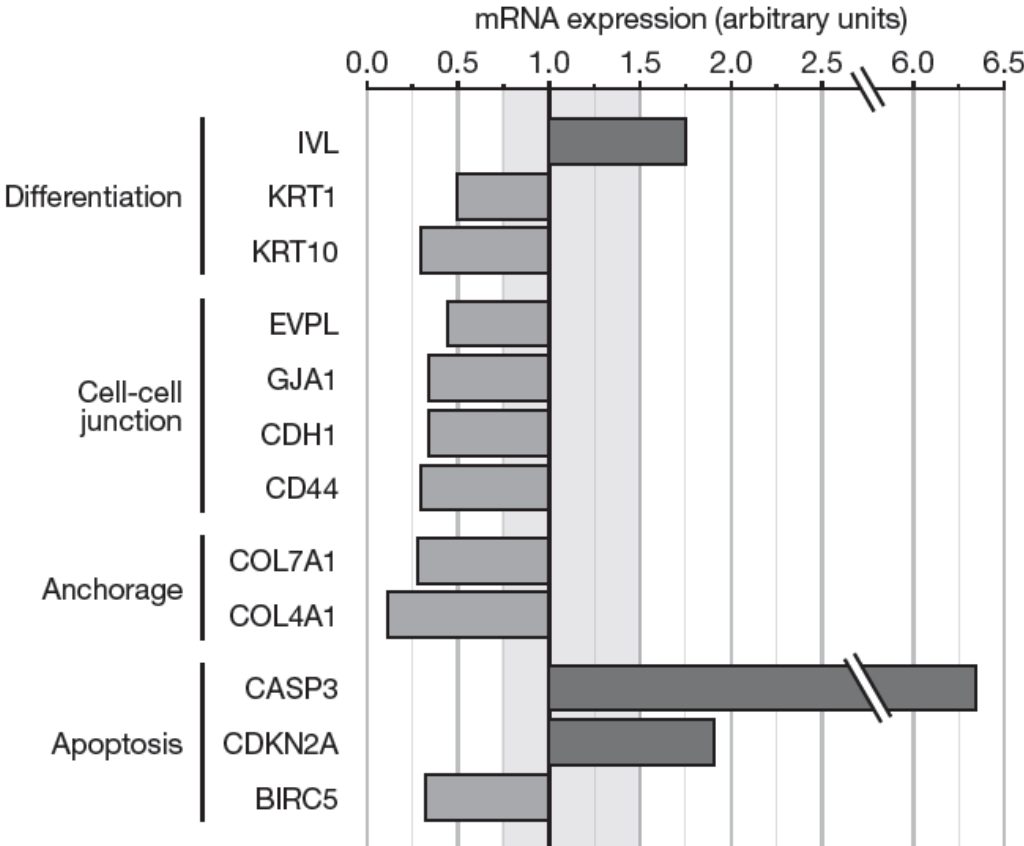


Fig. 8. MoA hypothesis for PM-induced premature skin aging

The mode of action (MoA) hypothesis of airborne pollution on premature skin ageing process, using the RHE 3D-skin model exposed to fine particles, is subdivided into key events, associative events, and modulating factors. The initial key event is the increase in oxidative stress, associated with the modulation of inflammatory response, which results in an overexpression of various genes. Associative events include inhibition of the gap junction communication and anchorage.

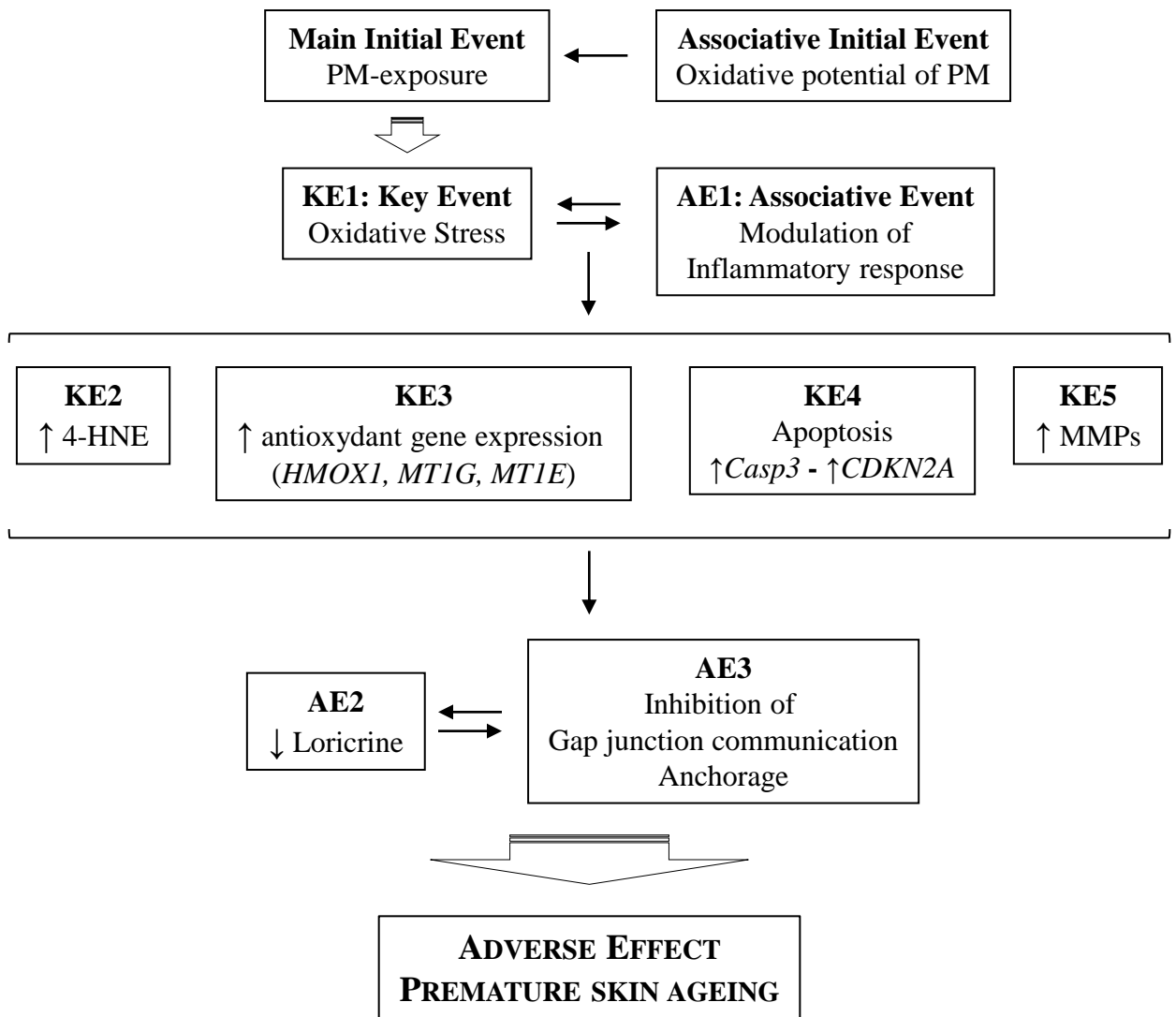


Table 1. Sum of main metallic elements and organic components of fine particles (PM_{0.3-2.5}) from (Cotonou, Benin) (adapted from Cachon et al., 2014).

Chemicals are expressed as mass composition per mass ($\mu\text{g}\cdot\text{g}^{-1}$) and volume unit ($\text{ng}\cdot\text{m}^{-3}$).

Samplings of fine particles were based on continuous collection using high volume samplers (80 m^3/hr).

	Chemical composition of fine particles PM_{0.3-2.5} Cotonou, Benin, West Africa	
	$\mu\text{g}\cdot\text{g}^{-1}$	$\text{ng}\cdot\text{m}^{-3}$
Major and trace elements		
Al, Ba, Cr, Cu, Fe, Mn, Ni, Pb, Ti, V, Zn		
Sum of elements	63,723	8,644
Organic compounds		
Benzene, Toluene, o,p-Xylene, Naphtalene, Phenanthrene, Alkyl-benzene, Benzo[a]pyrene, Benzo[g,h,i] perylene, <i>etc</i> ...		
Sum of organic compounds	78	11