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Abstract

The skin is an essential barrier, protecting the body against the environment and its numerous pollutants. Several environmental pollutants are known to affect the skin, inducing premature aging through mechanisms including oxidative stress, inflammation, and impairment of skin functions. Even climate conditions can impact the skin. Therefore, using a Reconstructed Human Epidermis (RHE), we tested the effect of two samples of fine particulate matters ($PM_{0.3-2.5}$ – one metals-rich sample and the other organic compounds-rich), two Volatile Organic Compounds mixtures (VOCs – from a solvent-based paint and a water-based paint) and Tobacco Smoke (TS). All pollutants affected cellular functionality, but to a lesser extent for the water-based paint VOC. This effect was enhanced when RHE were preconditioned for two hours by a semi-dry airflow (45% relative humidity) before pollutants application, compared to preconditioning by a humid airflow (90% relative humidity). In the absence of preconditioning, IL-1α, IL-6, IL-8, and RANTES were almost systematically induced by pollutants. When RHE were preconditioned by a semi-dry or humid airflow before being subjected to pollutants, the increase of IL-1α, IL-8, and RANTES falls into two groups. Similarly to RHE not treated with pollutants, RHE treated with VOCs after preconditioning by a semi-dry airflow showed increased IL-1α, IL-8, and RANTES release. On the contrary, RHE treated with PM or TS after preconditioning by a semi-dry airflow show a lower increase in IL-1α, IL-8, and RANTES compared to preconditioning by a humid airflow. The effect of real environmental relative humidity conditions of the air, combined with acute exposure to various environmental pollutants, seemed to relate mainly to structural changes of the skin, determining the outcome of

47 the inflammatory response depending on the physicochemical characteristics of
48 pollutants.

Keywords

3D-Skin model

Air Liquid exposure

Environmental pollutants

Environmental humidity

Inflammatory response

Abbreviations

DMSO: dimethyl sulfoxide, IL: Interleukin, MMPs: matrix metalloproteinases, MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylte-trazolium bromide, PM: particulate matter, RH: relative humidity, RHE: Reconstructed Human Epidermis, ROS: reactive oxygen species, SDS: sodium dodecyl sulfate, TS: tobacco smoke, VOCs: volatile organic compounds.

1. Introduction

Air pollution – the degradation of air quality due to harmful gases, chemicals, biological contaminants, and/or particles – is associated with a variety of acute and chronic illnesses leading to increased morbidity and mortality (Hoek et al., 2013; Willers et al., 2013). Like the airway epithelium, the skin is constantly exposed to the environment and its pollutants. It is essential in protecting the body and many factors can affect it.

An obvious intrinsic factor that affects the skin is aging. It is visually characterized by the appearance of wrinkles, thinner epidermis, cutaneous dryness, ptosis, loss of elasticity, dark spots, and uneven pigmentation. Central to the process are the increase of oxidative stress, the increase in the activity of matrix metalloproteinases (MMPs) leading to degradation of the extracellular matrix, and the increase in the expression of cytokines and interleukins (Haydont et al., 2019; Parrado et al., 2019). If intrinsic factors can accelerate these processes, extrinsic factors are also important (Makrantonaki et al., 2015). The effect of UVs is well known (Jackson et al., 2001; Flament et al., 2013). Airborne pollutants also exert negative effects on the skin, especially on sensitive skin that can overreact to external aggression (Krutmann et al., 2014).

For several years, works agreed that Tobacco Smoke (TS) has a strong impact on the skin. TS is a complex mixture of over 4,000 compounds (CO, HCN, benzene, formaldehyde, nicotine, phenol, polycyclic aromatic hydrocarbons, *etc.*) and only the particulate phase, which represents approximately 5% of the cigarette's total output, is visible as smoke (Hoffmann et al., 1997; Löfrth, 1989; Auer et al., 2017). This complex mixture leads to premature skin aging, wrinkling, poor wound healing, squamous cell carcinoma, and chronic dermatoses (Ortiz and Grando, 2012). *In vivo* studies revealed

86 that TS induces reactive oxygen species (ROS), affects collagen production, and
87 increases the production of tropoelastin as well as MMPs, leading to the degradation of
88 the extracellular matrix (Morita, 2007). We previously corroborate these results, using
89 Reconstructed Human Epidermis (RHE), showing that, if two TS exposures are
90 sufficient to alter the *stratum corneum*, a single one is enough to induce inflammatory
91 cytokines, MMPs, and oxidative stress, as well as down-regulation of essential skin
92 functions (Lecas et al., 2016).
93 Outdoor air pollution is made up of gases (O_3, CO_2, CO, SO_2, and NO_x) and particulate
94 matters (PM). It is considered the world's largest environmental health risk factor due to
95 fine ($PM_{2.5}$) and ultrafine ($PM_{0.1}$) particles (WHO, 2018; Loomis et al., 2014). In
96 addition to the respiratory diseases and premature death they induce (Beelen et al.,
97 2014; Raaschou-Nielsen et al., 2013), PM are responsible for premature skin aging,
98 appearance of pigmentation spots, decrease in squalene (a major lipidic component of
99 sebum) and vitamin E, as well as of the increase of lactic acid and melanin, these later
100 indicating oxidative stress (Schikowski and Hüls, 2020). Using RHE we showed that
101 exposure to $PM_{0.3\text{-}2.5}$ affects cellular functionality, induces the release of inflammatory
102 cytokines, and leads to oxidative stress (Verdin et al., 2019). PM also have a negative
103 impact on fundamental skin functions: anchorage, cell differentiation, cornification/skin
104 desquamation, and apoptosis.
105 Another source of pollution under scrutiny are Volatile Organic Compounds (VOCs).
106 Defined as organic compounds with a high vapor pressure at normal room temperature
107 (EU, 2010), they represent a numerous, varied, and ubiquitous group of substances.
108 Because of the time people spend inside, VOCs emanating from indoor air pollution are
109 of particular concern. Their sources are numerous including building material,

equipment, cleaning products, or even combustion processes such as cooking (Kostiainen, 1995; WHO, 2010). Some of them (toluene, benzene, ethylbenzene, and xylene for instance) are known to have serious adverse effects on human health. So far, the effects of VOCs on the skin is poorly studied. Still, using keratinocytes, Dezest et al. (2017) suggested that VOCs would play a role in premature skin aging. Indeed, they show that VOCs inhibit the proteasome, inducing apoptosis, DNA damage, and protein oxidation.

Oxidative stress, inflammation, and metabolic impairments seem to be common mechanisms of skin aging and pollution-induced premature skin aging. Even climate conditions – relative humidity, temperatures – impact skin properties and barrier function and may modify skin reactions (Singh and Maibach, 2013; Cau et al., 2017). Therefore, we evaluated the effect of several pollutants using an RHE system, focusing on inflammation markers. The pollutants tested are two $PM_{0.3\text{-}2.5}$ samples with different characteristics, VOCs samples from two different paints and TS. We especially focused on how semi-dry/humid conditions can affect the inflammation response induced by pollutants.

2. Materials and Methods

2.1. In vitro skin model

The *in vitro* skin model used is a reconstructed human epidermis system (RHE) from SkinEthic™ (Lyon, France; Rosdy and Clauss, 1990; Rosdy et al., 1993). It provides a differentiated 3D epidermal tissue from human keratinocytes for which signed informed consent and ethical approval were obtained by the supplier. All experiments were conducted on a single batch (MF22-5).

Culture media, growth medium for non-experiment days, and maintenance medium for experiment days were supplemented with 1% of Penicillin-Streptomycin mixture (penicillin: 100 µg/mL; streptomycin: 100UI/mL). Upon reception, RHEs were placed in a 24-well plate with growth medium on the basal side and maintained in an incubator at 37 °C, 5% CO_2, and 95% relative humidity. Experiments were carried out 24h after RHE-acclimation.

2.2. Fine particulate matters ($PM_{0.3-2.5}$)

Two samples of fine PM, having the same aerodynamic diameter (0.3 to 2.5µm, $PM_{0.3-2.5}$) and similar granulometric profile, but differing in their sampling origin and their chemical composition were tested: PM from Cotonou (Benin, West Africa) that are rich in metals and inorganic elements (PMm – metal-rich PM), and PM from Dunkerque (France) that are rich in organic compounds (PMo – organic-rich PM). The collection and characterization of both PM were already published (Cachon et al., 2014, Dergham et al., 2015). A summary of their composition is presented in Table 1 and detailed analysis in Table S1.

Aliquoted and kept at -20°C after collection, PM were thawed and suspended in the maintenance culture medium containing 1% of Penicillin-Streptomycin and 1%

150 fungizone (Thermo Fisher Scientific, Waltham, MA, USA). Twenty-four hours before
151 experiments, PM suspension was sonicated (50W, 10 min, Bath sonicator Branson
152 CPXH, Dutscher, France) and 30 µL were deposited onto the apical side of RHEs.
153 Three concentrations – 3, 15, and 30 µg/cm^2 – as previously described (Verdin et al.,
154 2019) were tested. Medium without PM was used as a negative control.

155 ***2.3. Volatile organic compounds (VOCs)***

156 VOC atmospheres were generated from two different paints, which containers have
157 never been opened). VOCwb (water-based) are from an aqueous paint (Brillant Intérieur
158 Acrylique, Auchan, France) bought in 2014, after the French regulation governing
159 construction and decoration labeling (decrees 19/04/2011 and 20/04/2012 that set the
160 upper limit of VOCs to 20 g/L; European Union, 2010 and the French decree 2011-321
161 (March 23, 2011) related to the mandatory labeling of VOCs emission of building and
162 decoration products. VOCsb (solvent-based) are from an alkyd paint (Brillant, Orion
163 line, Guittet, France) manufactured before 2007, and thus before the above-mentioned
164 decrees.

165 The protocol applied to generate VOCs atmospheres was inspired by the emission
166 standard test (ISO 16000-9, ISO, 2006a) and the paints were prepared according to
167 ISO 16000-11 (ISO, 2006b). Briefly, paint was spread onto a 150 cm² glass plate that
168 was then placed in an 11 L sealed generation chamber under a steady airflow (20-25°C,
169 79 ± 8% humidity and a rate of 0.5 L/h (Ricquebourg et al., 2015). The VOCs
170 atmosphere was stabilized for 3 days before exposure. Samplings of VOCs from paint
171 were chemically controlled by vacuum vial SPME sampling (Desauziers and Auguin,
172 2012) followed by GC/MS/FID analysis. The concentrations of VOCwb and VOCsb

were determined as toluene equivalent from the FID signal (Badji et al., 2018) and are summarized in Table 2 and detailed in Table S2.

On the day of exposure, the generation chamber was connected to the Vitrocell® dynamic exposure system (Vitrocell System® GmbH, Waldkirch, Germany). This system contains six exposure chambers, each one with an insert with the RHE tissue and a trumpet. For one hour, a 2mL/min airflow was created to deliver the VOC atmosphere through the trumpet, onto the apical side of the RHE that was maintained at 37°C thanks to a water bath (Persoz et al., 2010). An atmosphere without VOCs, delivered with the same dynamic system, was used as a negative control.

2.4. Tobacco smoke (TS)

TS that is a complex mixture containing polycyclic aromatic hydrocarbons, volatile organic compounds, and PM present in air pollution was used as positive environmental pollution control. For this purpose, mainstream tobacco smoke (TS) was generated by the combustion of a French brand cigarette composed of 87% tobacco, 6.5% flavors agents, and 6.5% cigarette paper. It produces 7 mg of tar, 0.6 mg of nicotine, 9 mg of carbon monoxide and comprises two parts, gas and particulate matter.

Using a static system adapted from the Vitrocell Cloud™ chamber (Vitrocell System® GmbH, Germany), fresh TS was delivered to the apical face of RHE at a rate of 25 mL per puff with a total of 10 puffs (Lecas et al., 2016; Bardet et al. 2016). The dose delivered corresponds to one cigarette, with an average of 200 $\mu g/cm^2$ of particulate matters. Similarly to RHEs treated with VOCs, the air atmosphere used to generate TS was used as a negative control.

2.5. Exposure of RHEs to pollutants

196 To compare the effect of the sole pollutants, RHEs were exposed to pollutants as
197 described above, then incubated for 24h in classical culture conditions (incubator at
198 37°C, with 5% CO_2, and 90% relative humidity). RHEs not subjected to pollutant were
199 used as negative controls (Fig. 1A, 1C).
200 For all experimental series, at the end of the incubation period, culture media were
201 collected to assess the immune response by quantifying cytokines/chemokines release,
202 and RHEs were harvested to perform MTT assay.

203 ***2.6. Preconditioning of RHE to semi-dry/humid airflow followed by exposure to***
204 ***pollutants***

205 To assess the role of humidity on the impact of pollutants, RHE were preconditioned by
206 a semi-dry or humid airflow for 2h before being incubated with pollutants. RHE
207 maintained at 37°C thanks to a water bath, were subjected to different airflows. The
208 airflow was delivered using the Vitrocell® Humidification Station (Vitrocell Systems®
209 GmbH, Waldkirch, Germany) that was supplying semi-dry air (24°C, 5% CO_2 and 45%
210 relative humidity (RH) similarly to European conditions like in Dunkerque city) or
211 humid air (24°C, 5% CO_2 and 90% RH representative of conditions in South America,
212 Asia or Africa, like in Cotonou city). Then, RHEs were exposed to pollutants as
213 described above, and further incubated for 24 h in classical culture conditions (37°C,
214 with 5% CO_2, and 95% RH) before performing MTT assays on RHE and quantifying
215 cytokines/chemokines released in the culture media.
216 The protocol of exposure is described in Fig. 1B. Various controls were considered and
217 are summarized in Fig. 1C. Negative controls: RHEs preconditioned by the humid or
218 semi-dry airflow but not exposed to pollutants, and RH controls: RHEs exposed to the

pollutants but not to preconditioning by the humid/semi-dry airflow (kept in the incubator at 37°C with 90% RH).

2.7. Cellular functionality: MTT assay

Cellular functionality was assayed on the basal side of RHE relying on the ability of the mitochondrial succinate dehydrogenase to convert MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylte-trazolium bromide) into a formazan product that has a maximum photometric absorbance at 490 nm (Persoz et al., 2010). Briefly, 24h after exposure, the culture medium was removed, 500 µL of MTT was added to the bottom of the well containing the RHE. After two hours of incubation (37°C, with 5% CO_2, and 95% relative humidity), DMSO (500µL) was added to the bottom of the well, reagents were homogenized by shaking and transferred to 96-well plates. Absorbance was read using a microtiter plate reader (Multiskan® EX, Thermo Scientific, Waltham, MA, USA).

2.8. Mapping of cytokine production: Q-Plex™ analysis

Q-Plex™ quantitative ELISA-based chemiluminescent assay (Quansys, Logan, UT, USA) were performed according to the manufacturer's instructions, using the profiling service of Tebu-bio (Le Perray-en-Yvelines, France). Nine cytokines (Q-Plex Human Cytokine-Inflammation #110433HU – IL-1α, IL-1β, IL–2, IL-4, IL-6, IL-8, IL-10, IFNy and TNFα) and eight chemokines (Q-Plex Human Chemokine Inflammation #110451HU – Eotaxin, GROα, I-309, IP-10, MCP-1, MCP-2, RANTES and TARC) were measured. The analysis was performed by quantifying the intensity of the chemiluminescent signals on the Tebu-bio technological platform using both Q-View™ and Quansys Software. Sensitivity ranges between 1 pg/mL and 30 pg/mL depending on the considered analyte.

2.9. Specific cytokine/chemokine production: ELISA analysis

Two cytokines and one chemokine were assessed using Human DuoSet ELISA (R&D Systems, Minneapolis, MN, USA) interleukin 1α (IL-1α/IL-1F1, #DY200), interleukin 8 (IL-8/CxCL8, #DY208) and RANTES (CCL5/RANTES, #DY278). Absorbance was determined using an ELISA-reader (Multiskan® EX, Thermo Scientific, Waltham, MA, USA) at 450 nm and 540 nm to correct the optical imperfections in the plate. Calibration curves were calculated using Ascent Software® (Thermo Fisher Scientific, Waltham, MA, USA). Concentrations are expressed in pg/mL and the lower limits of quantification are 31.26, 7.81, 156 pg/mL for IL-8, IL-1α, and RANTES, respectively.

2.10. Statistical analysis

All results are reported as mean ± standard deviation. Except for Q-Plex™ analysis that was performed only once with duplicated quantification, all other experiments were performed at least in two independent experiments, each with duplicated quantification. The exact number of independent experiments (n) is indicated in the legend of the figures. After verifying the Normal distribution of results using the Shapiro-Wilk test ($p<0.05$), statistical differences were analyzed using one-way ANOVA followed by Tukey HSD (Honestly Significant Difference) test. $p<0.05$ was considered significant.

3. Results

3.1. RHE functionality upon exposure to pollutants

RHEs were exposed to various environmental pollutants: two $PM_{0.3-2.5}$ samples – PMm, rich in metals and inorganic elements, and PMo, rich in organic compounds – two VOC mixtures – VOCwb, emanating from a water-based paint, and VOCsb, from a solvent-based paint – and TS.

Results of the MTT assay (Fig. 2) reveals that the pollutant which most affects RHE is TS, inducing a significant -43.9% ($p<0.0001$) decrease in cell functionality compared to the negative control. Moreover, both PM were found to alter the functionality of RHEs. The effect is dose-dependent and statistically significant from the lowest exposure doses to 3 µg/cm^2 of PMm (-10.5%, $p=0.0155$) or 15 µg/cm^2 of PMo (-15.4%, $p<0.0001$). The negative effect is higher when RHEs are exposed to PMm than to PMo, reaching -39.4% ($p<0.0001$) for exposure to 30 µg/cm^2 of PMm, while the decrease induced by PMo is of -26.7% ($p<0.0001$). Among the two VOCs tested, only VOCsb affects RHEs, leading to -8.7% ($p=0.0213$) in cell functionality, the -4.4% decrease induced by VOCwb being not significant ($p=0.4543$).

3.2. RHE functionality upon preconditioning in a semi-dry or humid airflow followed by exposure to pollutants

To evaluate the impact of airflow with different relative humidity on the effect of pollutants, we preconditioned RHEs by submitting them to a semi-dry (45% relative humidity – RH) or a humid (90% RH) airflow for 2 hours prior exposure to pollutants. Controls included negative controls, preconditioned by the semi-dry or humid airflow but not subjected to pollutants, and RH controls, incubated with pollutants but not to

preconditioning by the humid/semi-dry airflow (kept in the incubator at 37°C with 90% RH).

Results (Fig. 3) showed that, for the negative control, preconditioning by the humid airflow does not affect RHE compared to the RH control. This lack of effect of preconditioning by the humid airflow is also observed when comparing RHE incubated with pollutants with their respective RH controls. Only preconditioning by the semi-dry airflow affects the results of the MTT test for both, the negative control and the RHE treated with the various pollutants. The decrease is always significant when results are compared to that of RHE preconditioned by the humid airflow and almost always when compared to the respective RH controls (the exception being PMm). Still, within series, the decrease in cellular functionality induced by the semi-dry preconditioning compared to preconditioning by the humid airflow remains generally mild (negative control: -12.9%, $p=0.0443$; PMm: -16.7%, $p=0.0242$; PMo: -19.8%, $p=0.0014$; VOCwb: -23.9%, $p<0.0001$; VOCsb: -26.6%, $p<0.0001$), except for TS for which it reaches -34.1% ($p=0.0007$).

3.3. Inflammatory response upon exposure to pollutants

We have then investigated the inflammatory response induced by pollutants by monitoring the production of nine cytokines (IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IFNα, TNFα) and eight chemokines (Eotaxin, GROα, I-309, IP-10, MCP-1, MCP-2, RANTES, TARC). The results are presented in Fig. 4.

IL-1α, IL-6, IL-8, and RANTES are the only cytokines/chemokines almost systematically and significantly induced by a factor of 1.3 to 2.7 by pollutants: PM at 15 $\mu g/cm^2$ and over, VOCs and TS. Even if VOCwb does not significantly induce IL-1α/IL-6 and TS does not significantly induce IL-8, the probabilities are at the limit of

306 significance for IL-1α and VOCwb (p=0.0535), as well as for IL-8 and TS (p=0.0903).
307 For both PM a dose-dependent effect is clearly observed for IL-1α, IL-6, IL-8, and
308 RANTES. Besides, the induction of the three cytokines/chemokines seems to be higher
309 for PMm (x2.2 folds for IL-1α, x2.7 folds for IL-6, x2.7 folds for IL-8 and 2.1 folds for
310 RANTES with 30 µg/cm^2) than for PMo (x2.1 folds for IL-1α, x1.9 folds for IL-6, x2.1
311 folds for IL-8 and 1.8 folds for RANTES with 30 µg/cm^2).
312 In these experimental series, none of the pollutants induced IL-1β, IL-4, Eotaxin, I-309,
313 MCP-2 or TARC, and the induction of IL-2, IL-10, IFNα, TNFα, GROα, IP-10, MCP-1
314 depends on the pollutant. Still, overall PMm is the pollutant inducing the most
315 cytokines and chemokines (10 among the 17 tested) while VOCwb is the one inducing
316 the least (5), VOCsb, and TS being intermediate.

317 ***3.4. Inflammatory response upon preconditioning in a semi-dry or humid airflow***
318 ***followed by exposure to pollutants***

319 To analyze the effect of airflows with different relative humidity on the inflammatory
320 response induced by pollutants, we preconditioned RHEs for two hours using a semi-
321 dry or humid airflow before subjecting them to pollutants. For this analysis, we focused
322 on IL-1α, IL-8, and RANTES that were induced by most pollutants tested. Similarly to
323 the study of the cellular functionality of both, semi-dry/humid airflow and pollutants,
324 the controls encompassed negative controls, subjected to preconditioning by the
325 different airflows but not to pollutants, and RH controls, subjected to pollutants but not
326 to preconditioning by the airflows. The results are presented in Fig. 5.
327 In the negative controls, comparison of the amount of cytokines/chemokine released by
328 the RH controls and by RHEs preconditioned by the 90% RH airflow shows that
329 preconditioning by the humid airflow significantly increases the release of IL-1α and

IL-8 by a factor of 1.9, while, for RANTES, the increase is not significant. The increase in IL-1α and IL-8 is also observed when comparing RH controls treated with pollutants and RHEs which underwent both, preconditioning by the humid airflow and incubation with pollutants, but, in that case, the increase is also significant for RANTES. In addition, the fold change compared to RHE treated with pollutants but not preconditioned by the humid airflow is much higher, reaching over 10 folds for IL-8 induction by any of the PM and 3 to 5 folds for RANTES inductions by whatever VOCs.

For all negative controls, preconditioning in the semi-dry airflow significantly increases the release of IL-1α, IL-8 and RANTES compared to the RH controls. Except for IL-1α, this increase is significantly higher than that obtained upon preconditioning by the humid airflow. When RHEs are treated with both, preconditioning by the semi-dry airflow and either PM, the release of IL-1α, IL-8 and RANTES is higher than in the RH controls, but overall significantly lower than when RHEs are preconditioned by the humid airflow. For TS, combination with preconditioning by the semi-dry airflow acts similarly to PM on IL-1α, IL-8, and RANTES. Contrary to PM and TS, preconditioning by the semi-dry airflow and either VOC, VOCwb, or VOCsb, leads to an increase in IL-1α, IL-8, and RANTES compared to preconditioning by the humid airflow.

4. Discussion

In recent years, several studies were devoted to the biological effect of air pollutants. Many focused on small particulate matters ($PM_{<2.5}$) due to their role in pulmonary morbidity and mortality. But PM are not the only source of air pollution and, with the increasing time spent inside, VOCs are also important to consider. Besides, the majority of the studies focuses on the pulmonary epithelium. Little is known about the fate of the skin despite the fact it is the largest human organ and it is also constantly exposed to the environment. Therefore, we wanted to better understand the impact of air pollutants on the skin and, especially, to investigate how an environmental factor such as humidity affects the response to pollutants knowing that changes in relative humidity are sufficient to impact the skin (Singh and Maibach, 2013; Cau et al., 2017).

Among the pollutants we tested, TS was primarily used as a positive control. This pollutant is a complex mixture containing over 4,000 gaseous and particulate compounds, including VOCs and PM. Its effect on premature skin aging is already well documented and we previously showed, using the very same RHE system, that it induces morphological alterations, oxidative stress, leading to the production of inflammatory cytokines (Lecas et al., 2016). Therefore, our primary interest was the response induced by two types of pollutants, fine particles ($PM_{0.3-2.5}$) and VOCs, with two different samples for each. Both PM samples present similar granulometric profiles, but show very different chemical compositions, therefore enabling us to evaluate the impact of chemicals adsorbed to the particles. PMm are originating from a highly polluted urban region of Sub-Saharan Africa (Cotonou, Benin). They are characterized by their extreme richness in metallic elements as the region where they were collected is surrounded by soils with high concentrations of iron oxide, aluminum oxide, and heavy

372 metals (Cachon et al., 2014). On the contrary, PMo are from a region of France
373 (Dunkerque) with intense industrial activities and heavy vehicle traffic, which their
374 richness in organic compounds reflects (Dergham et al., 2015). The last two pollutants
375 we tested are VOCs. Much less studied, we focused on VOCs from a solvent-based
376 paint that was manufactured before the French regulation limiting VOCs in construction
377 and decoration products and that liberates large amounts of various VOCs. The second
378 source of VOCs is a recent water-based paint manufactured after the French regulation
379 and showing reduced VOCs emissions.
380 All these pollutants are real environmental pollutants. In addition, the exposure system
381 we set up is realistic in the sense that the pollutants are delivered to the apical side of
382 RHEs, therefore mimicking a real-life situation. Still, it is inhomogeneous in its
383 exposition due to the physical nature of the different pollutants tested. As a matter of
384 fact, PM are applied topically and remain on RHE for 24 hours before analysis, making
385 it a static system. On the other hand, VOCs are delivered dynamically, via a one-hour
386 airflow followed by a 24-hours incubation before analysis. The system we used for TS
387 is intermediate. Fresh TS is generated in a chamber where its compounds are allowed to
388 interact with, and sediment onto RHEs. Therefore, it is more of a static system that
389 favors the action of the PM constituents that will stay on RHEs, rather than that of its
390 gaseous phase for which the contact is time-limited. Another source of inhomogeneity is
391 the dose of pollutants tested. If the doses of PM and TS we tested are much higher than
392 normal exposures, as frequently in *in vitro* studies, those of VOCs are, on the contrary,
393 very low: 6.1 µg/liter for VOCwb and 183.5 µg/liter for VOCsb, therefore 0.73 µg and
394 22.0 µg respectively during the one-hour exposure. These low doses are due to the fact
395 that we followed the ISO standards to generate the VOCs samples.

Not only does our experimental setup show heterogeneity in the testing of pollutants, but the pollutants we tested are very different and interact differently with the skin. PM are a complex mixture of insoluble particles – the physical part – and soluble components absorbed to their surface – the chemical part. If PM can induce their effects through the release of soluble components that can enter the stratum corneum, the tiniest PM can enter the skin. Indeed, Jin et al. (2018) showed that PM can enter the skin by accumulating in hair follicles and sweat glands. In RHE, they were found to enter the upper layers in less than 24 hours (Magnani et al., 2015), which is not surprising regarding the fact that PM break the skin barrier by down-regulating filaggrin, thereby disrupting the tight skin junctions (Pan et al., 2015; Lee et al., 2016). RHEs are deprived of hair follicles and sweat glands, but we incubated them with PM for 24 hours. Therefore, it seems likely that the toxicological endpoint we observe is due to both, the prolonged contact and/or diffusion of soluble components through the surface of the stratum corneum, as well as to the direct release of compounds absorbed to their surface within the upper layers of RHE. On their side, VOCs must have directly diffused into the upper layers of RHE. Indeed, the toxicity of hexane and toluene, two VOCs, relates to their lipophilicity and their accumulation in the lipid bilayer of cellular membranes (Dreiem et al., 2003; Pariselli et al., 2009). Besides, VOCs are also known to affect the skin due to the ability of UV and NOx to transform them into photochemical oxidants, which seems unlikely to have played a major role considering our experimental setup.

Whatever the entry route of the pollutants, inhibition of the succinate dehydrogenase revealed by the MTT assay shows that PM, TS, and VOCsb affect RHEs. For the two PM, the alteration is dose-dependent, and the effect is stronger when RHE are treated

with PMm than with PMo. This may result from the high level of transition metals and other metals adsorbed on PMm (*e.g.* Al, Cu, Fe, Mn, Zn, Pb, *etc*.) that are powerful ROS inducers. Using the same exposure system, we previously showed that PMm are powerful inducers of oxidative stress (Verdin et al., 2019). The fact that polycyclic aromatic hydrocarbons absorbed to PM bind the aryl hydrocarbon receptor (AhR) to induce a signaling pathway leading to ROS formation and proinflammatory gene expression is now well established (Esser et al., 2018; Parrado et al., 2019). Oxidative stress was also demonstrated for PMo using BEAS-2B bronchial epithelial cells (Dergham et al., 2015). Evidence of oxidative stress was also found for keratinocytes exposed to different VOCs (Dezest et al., 2017). Oxidative stress and inflammation are tightly linked and, if ROS are not controlled, they lead to inflammatory cell and/or tissue injury (Lonkar and Dedon, 2010). In our case, all pollutants tested induce RANTES and almost all of them IL-1α as well as IL-8. Similarly to the cellular functionality, the effect is more pronounced for PMm, which also induces more cytokines and chemokines, than PMo. The induction of cytokines and chemokines production is also observed with the two VOCs and TS even if the range of cytokines and chemokines induced is more limited, especially for VOCwb.

When testing the influence of preconditioning by a humid *versus* a semi-dry airflow, results show that the semi-dry airflow impacts cellular functionality, whatever pollutant tested. Negative controls also revealed increased levels of IL-8 and RANTES upon preconditioning by the semi-dry airflow. If airflows differ between them by their relative humidity, they also differ from the negative control by the temperature (24°C). Still, RHEs are maintained at 37°C and it is only their apical side that is subjected to this lower temperature. Hence, it sounds reasonable to assume that most of the effect we

444 observed is due to the differences in relative humidity, and that the increased levels of
445 IL-8 and RANTES could be the first consequence of the initial increase in
446 Transepidermal Water Loss (TEWL) followed by inflammatory cytokine stimulation as
447 described in studies performed in dry conditions (Singh and Maibach, 2013). Still,
448 results show that the short preconditioning period in semi-dry or humid air is sufficient
449 to affect the reaction of RHE towards pollutants. Contrary to cellular functionality, the
450 different preconditioning is also sufficient to reveal different effects depending on the
451 type of pollutants. For both PM and TS, preconditioning by the semi-dry airflow tends
452 to decrease the levels of IL-1α, IL-8, and RANTES compared to preconditioning by the
453 humid airflow. The situation is just the opposite for the induction of IL-1α, IL-8, and
454 RANTES by the two VOCs. Strikingly, both VOCs induce an almost identical response
455 despite their very different qualitative and quantitative compositions. This response is
456 similar to that of the negative control and suggests that, under the conditions used (low
457 doses and short exposure), the effect of preconditioning by the semi-dry airflow primes
458 over the response to VOCs. Taken together, these results suggest that the response of
459 the skin to RH is the main driver of the toxicological endpoint and that its outcome
460 depends mainly on the physical nature of the pollutant.
461 Several works studied the effect of dry conditions on the skin (reviewed in Engebretsen
462 et al., 2016; Goad and Gawkrodger, 2016). They show that human skin placed in dry
463 conditions presents an increased stratum corneum thickness, an increased TEWL, a
464 reduced elasticity, and an increased susceptibility to mechanical stress. In hairless mice,
465 dry condition (3 days at 10% RH) induces reduced desmosomal degradation that was
466 attributed to the reduced water content of the stratum corneum, leading to impaired
467 desquamation and a scaly surface (Sato et al., 1998). Furthermore, the amount of IL-1α

468 was found higher in mice kept under dry conditions (Ashida et al., 2001). RHE
469 produced in semi-dry conditions (30-50% RH) also show increased TEWL, a thicker
470 stratum corneum, and an increased level of epidermal protein deimination. These
471 alterations lead to the breakdown of filaggrin, a filament-associated protein that binds to
472 keratin fibers in epithelial cells (Cau et al., 2017). If all these works pinpoint at a barrier
473 deficit when the skin is subjected to dry conditions, one should be cautious in
474 extrapolating them to our study. Most of the works mentioned above were performed
475 comparing much longer incubation times in different RH conditions than the two hours
476 preconditioning time we used. In our case, preconditioning by the semi-dry airflow
477 might have only led to the very first step of the above-mentioned changes: the increased
478 water loss, the beginning of the dehydration of the upper layers of the stratum corneum
479 and, as we show it, the release of cytokines/chemokines. Therefore, for VOCs the effect
480 we observe would be driven by our dynamic experimental setup and would only reflect
481 the effect of the two hours preconditioning in semi-dry conditions.
482 The fate of PM would be different but can also be explained by the mechanical
483 characteristics of the skin under the different RH conditions. In that respect, the static
484 experimental design we used for TS reflects the particulate nature of TS rather than that
485 of its gaseous part since it induces a similar response than PM. Two mutually none
486 exclusive aspects could explain the lower cytokine/chemokine induction observed upon
487 preconditioning by the semi-dry airflow compared to the humid airflow. The first one is
488 that, as mentioned above, the semi-dry airflow rapidly lead to dehydration of the upper
489 layer of the stratum corneum and a scaly surface. These could have limited the contacts
490 between PM and RHE and/or limited the diffusion of the chemical components
491 absorbed to the surface of PM. On the other hand, preconditioning under humid

conditions might have favored these contacts. Using transmission and cryo-scanning electron microscopy, Warner et al. (2013) show that a four-hours treatment in an atmosphere saturated with water leads to a three-fold increase in the thickness of the stratum corneum with large pools of water accumulating in intercellular spaces where the lipid structures are disrupted. This study was performed *in vivo*, on the volar forearm of volunteers. With the weaker stratum corneum of RHE, we can assume that such an effect can occur when they are maintained under 90% RH. Not only would it explain better contact between PM and the upper cell layers, but the rupture of the stratum corneum barrier and the large pool of water might lead to an increased entry of PM. It would also explain a better diffusion of the metallic elements absorbed to the surface of PMm and, therefore, the stronger release of IL-1α, IL-8, and RANTES.

It is important to note that our conclusions are quite limited by the fact that the experiments were performed on a single batch of RHE which are known to show some inter-batch variability. A last limitation is due to the short period during which RHE can be used. This restricts the use of our system to the test of short exposure periods and precludes us from testing chronic like exposure. Still, the use of a 3D-skin model – a reconstructed human epidermis – enabled us to mimic the effects of close contact between pollutants and the skin. The experimental system we set up also allowed us to test realistic environmental pollutants. Not only are they realistic in their generation and, by extension, in their physicochemical composition but, also, in the way we delivered them to RHE, which reflects the real human dermal exposure route: an air-liquid exposure resulting in the topical application of pollutants. Finally, the use of the innovative humidification station has proven to be suitable, and the conditions we tested

515 are representative of real environmental conditions, since the 2 hours preconditioning is
516 sufficient to induce different reactions towards various environmental pollutants.

Conclusion

In the course of this study, we tested various pollutants with different chemical compositions, applying them to the apical side of RHE and being particularly interested in the effect relative humidity has on the reaction to pollutants. The negative effect of pollutants on the cellular functionality is aggravated when RHE are preconditioned for two hours by a semi-dry airflow. Investigating several cytokines and chemokines, we showed that IL-1α, IL-6, IL-8, and RANTES are the cytokines/chemokines almost systematically induced by most pollutants. When RHE are preconditioned by a semi-dry/humid airflow before being subjected to pollutants, the release of IL-1α, IL-8, and RANTES falls into two groups. For VOCs, preconditioning by a semi-dry airflow exacerbates IL-1α, IL-8, and RANTES release, similar to what happens in the absence of pollutants, indicating a response driven by RH rather than by VOCs. On the other hand, for PM and TS, preconditioning by a semi-dry airflow leads to a decrease in the release of IL-1α, IL-8, and RANTES. Our interpretation is that the effect of RH on the skin structure primes over the chemical composition of the pollutants. It is the changes induced by the different humidity conditions – and our experimental design – that determine the outcome of the toxicological endpoint of a pollutant depending on its physical nature.

Declaration of interest

The authors declare no conflict of interest.

CRediT authorship contribution statement

Emeline Seurat: Conceptualization, Methodology, Formal analysis, Writing-original draft. **Anthony Verdin:** Conceptualization, Methodology, Formal analysis, Investigation, Proof-reading. **Fabrice Cazier:** Conceptualization, Methodology, Formal analysis, Investigation, Proof-reading. **Dominique Courcot:** Supervision, Proof-reading. **Richard Fitoussi:** Funding acquisition, Supervision, Proof-reading. **Katell Vié:** Funding acquisition, Proof-reading. **Valérie Desauziers:** Methodology, Formal analysis, Supervision, Proof-reading. **Isabelle Momas:** Supervision, Proof-reading. **Nathalie Seta:** Supervision, Proof-reading. **Sophie Achard:** Conceptualization, Methodology, Formal analysis, Investigation, Writing-original draft, Visualization, Ressources, Data curation, Funding Acquisition, Supervision.

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741 # Tables

742

743 **Table 1**

744 Summary of the main metallic and organic components of PMm (Cotonou, Benin -

745 Cachon et al., 2014) and PMo (Dunkerque, France - Dergham et al., 2015).

Chemicals	**PMm** (ng/m^3)	**PMo** (ng/m^3)
Metallic elements (Al, Ba, Cr, Cu, Fe, Mn, Ni, Pb, Ti, V, Zn)	8,644	620
Water-soluble ions (Ca^{2+}, Cl^-, K^+, Mg^{2+}, Na^+, NO_3^-, SO_4^{2-})	6,225	2,437
Organic compounds (Benzene, Toluene, o,p-Xylene, Naphthalene…)	11	730

746

747 **Table 2**

748 Main elements of the qualitative profile of VOCs. Quantification was carried out using
749 SPME fibers and GC/MS/FID analysis on the elements released on the third day of
750 drying the paints. Concentrations determined as toluene equivalent from the FID signal.

Chemicals	**VOCwb** (mg/m^3)	**VOCsb** (mg/m^3)
Aromatic hydrocarbons (Toluene, Xylene, Ethylbenzene)	-	2,37
Aliphatic hydrocarbons (Decane, Nonane, Trimethyl cyclohexane…)	-	148.73
Substituted hydrocarbons (Propylene glycol, 2-butanone, Texanol…)	6.08	32.47

751

Legends of the figures

Fig. 1.

Experimental design for the exposure of RHE to pollutants

(A) Sequences of treatment for the exposure of RHE to pollutants. (B) Sequences of treatment event for the preconditioning of RHE by the semi-dry/humid airflow before exposure to pollutants. (C) Comparison of the different treatments and controls.

Fig. 2.

Cellular functionality of RHE after exposure to pollutants.

Results of MTT assays performed on RHE exposed to the different pollutants for 24h. n=3 independent experiments in duplicate, except for the negative control and SDS for which n=6 independent experiments with the MTT assay performed in duplicate for each. Conditions are compared to the negative control with * $p<0.05$, *** $p<0.001$.

Fig. 3.

Cellular functionality of RHE upon preconditioning by a semi-dry/humid airflow followed by exposure to pollutants.

Results of MTT assays performed on RHE preconditioned or not (RH control) by a humid (90% RH) or a semi-dry (45% RH) airflow before being incubated with the different pollutants. n=2 independent experiments with the MTT assay performed in duplicate for each; * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Fig. 4.

Inflammatory response of RHE after exposure to pollutants.

Profiling of cytokines/chemokines production in RHE exposed or not (negative control) to the different pollutants: (A) PMm, (B) PMo, and (C) VOCs and TS. n=1 experiment with the quantification performed in duplicate; * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

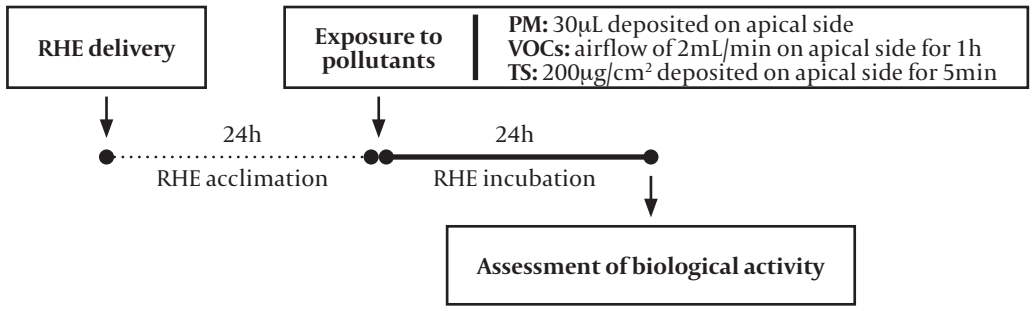
Fig. 5.

Inflammatory response of RHE upon preconditioning by a semi-dry/humid airflow followed by exposure to pollutants.

Quantification of (A) IL-1α, (B) IL-8, and (C) RANTES in RHE preconditioned or not (RH control) by the humid (90% RH) or the semi-dry (45% RH) airflow before being incubated with the different pollutants. n=2 independent experiments with the quantification performed in duplicate; * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Figure 1

A



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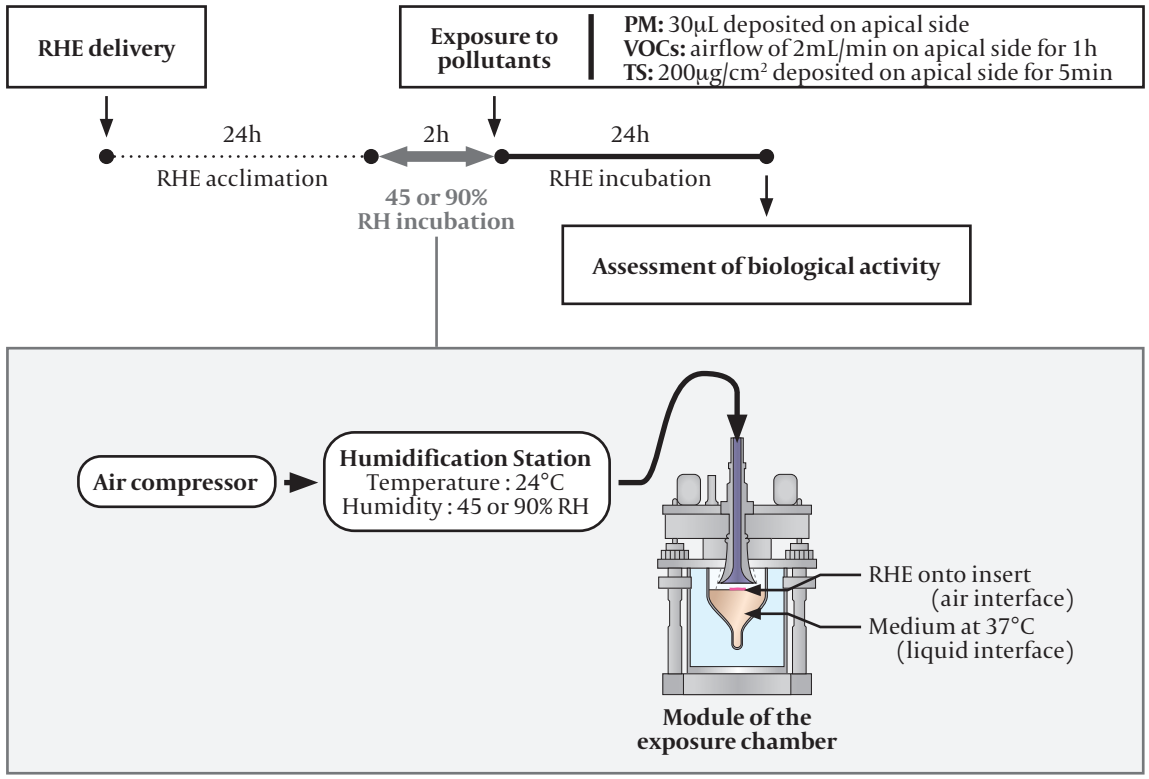

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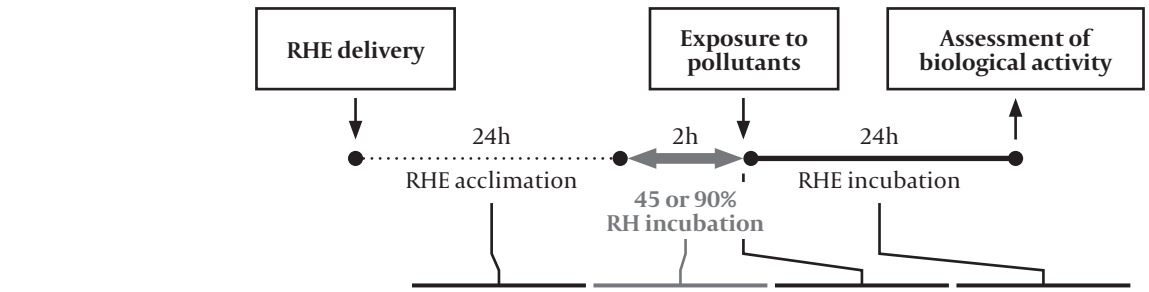


Negative control	X	no/yes	no	X
RH control (white bars)	X	no	X	X
Humid air: 90% RH (light grey bars)	X	X	X	X
Semi-dry air: 45% RH (dark grey bars)	X	X	X	X

Figure 2

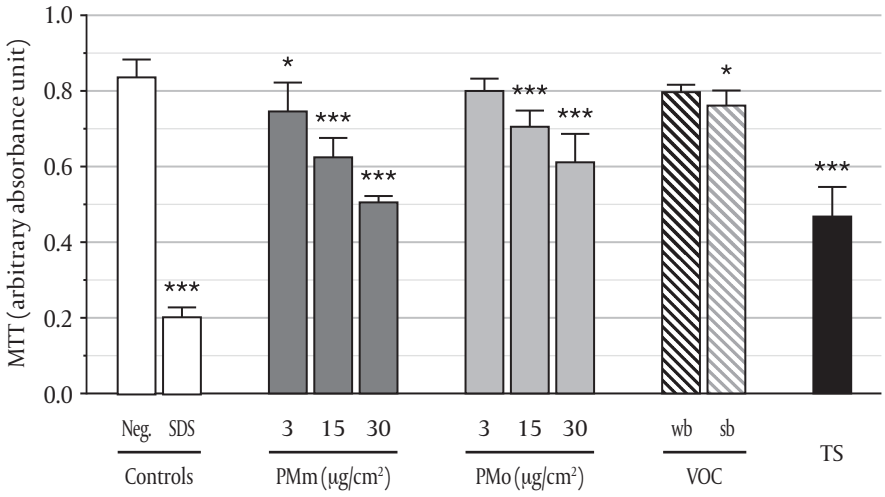

MTT (arbitrary absorbance unit)
1.0
0.8
0.6
0.4
0.2
0.0
*
***
***
***
***
***
*
***
Neg.
SDS
3
15
30
3
15
30
wb
sb
Controls
PMm (µg/cm²)
PMo (µg/cm²)
VOC
TS

Figure 3

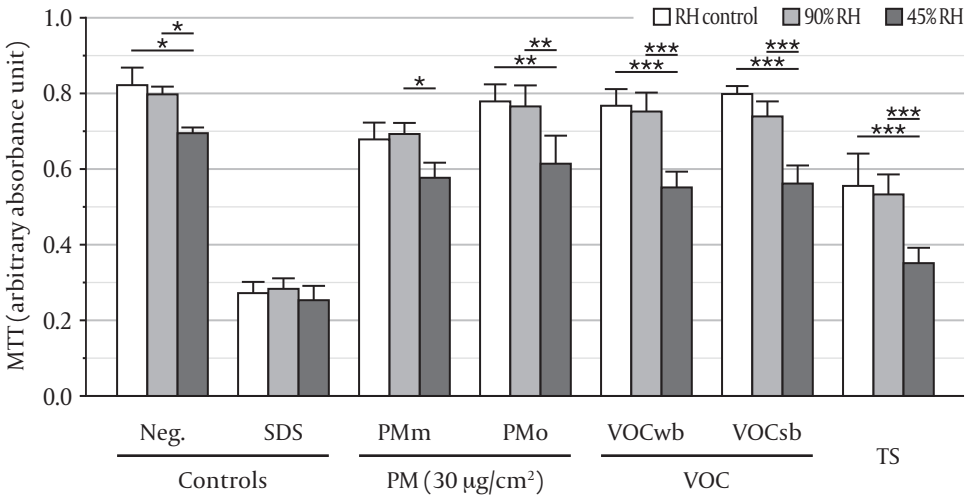

RH control
90% RH
45% RH
MTT (arbitrary absorbance unit)
1.0
0.8
0.6
0.4
0.2
0.0
*
*
*
**
**
***
***
***
***
***
Neg.
SDS
PMm
PMo
VOCwb
VOCsb
TS
Controls
PM (30 μg/cm²)
VOC

Figure 4

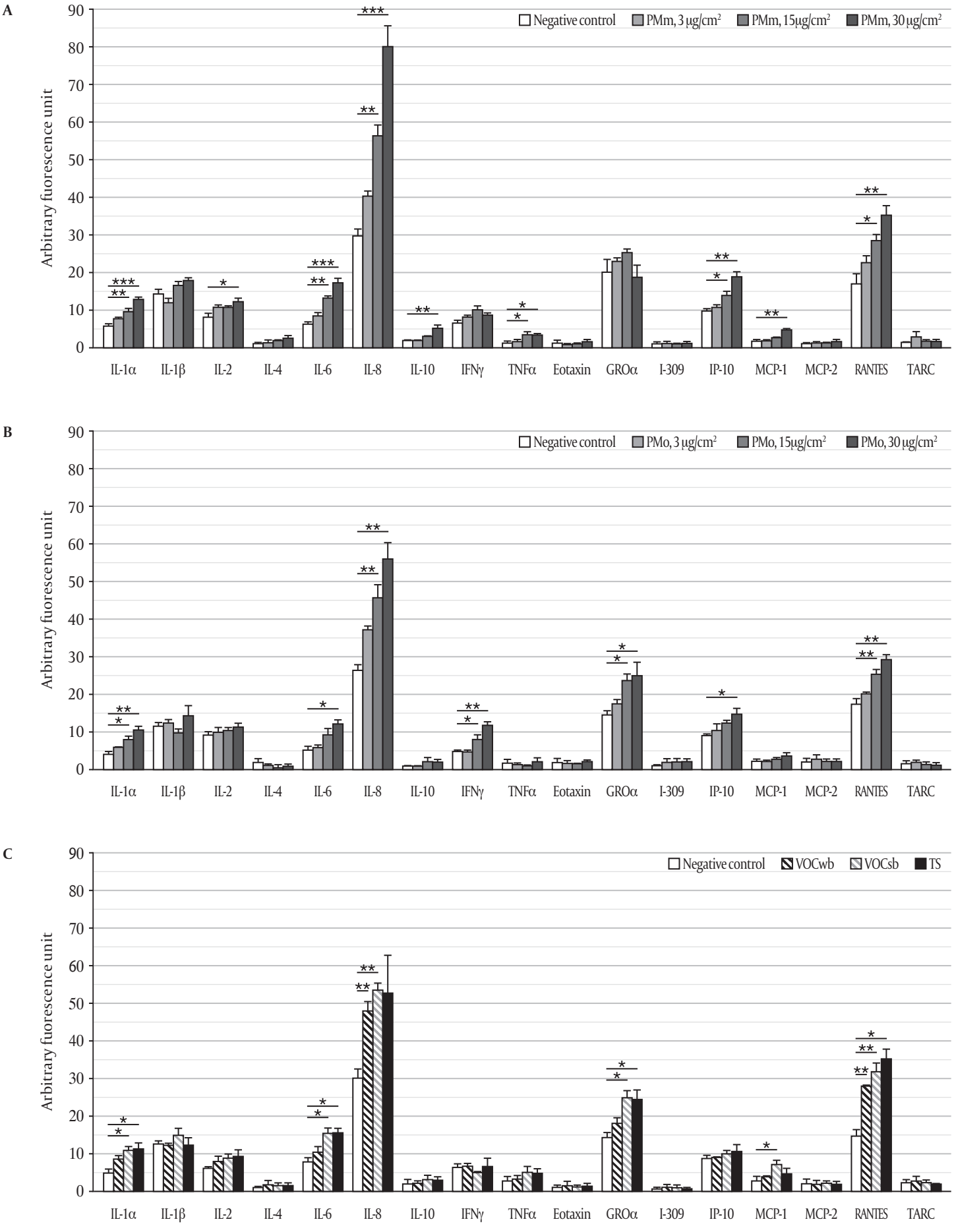
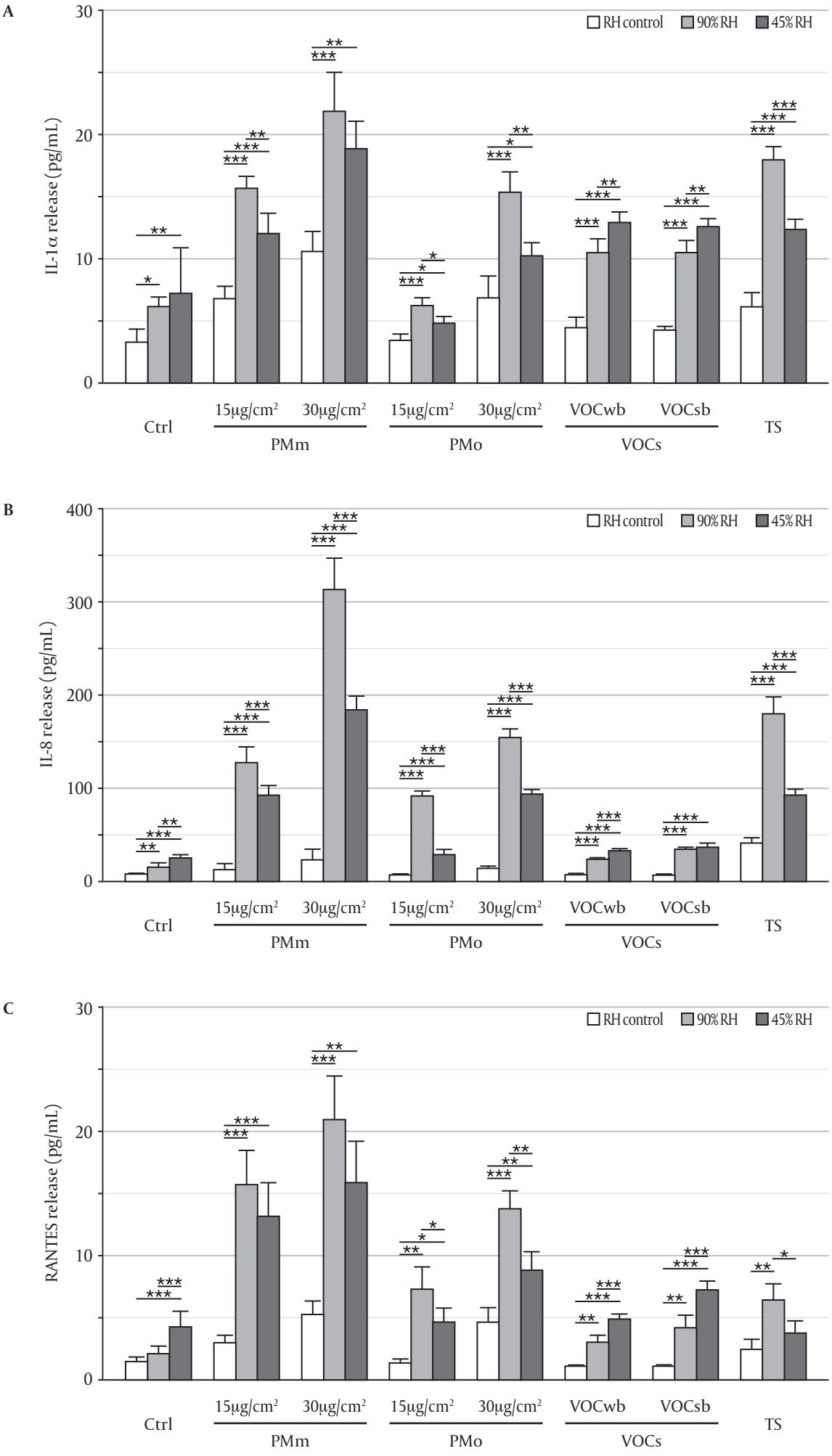
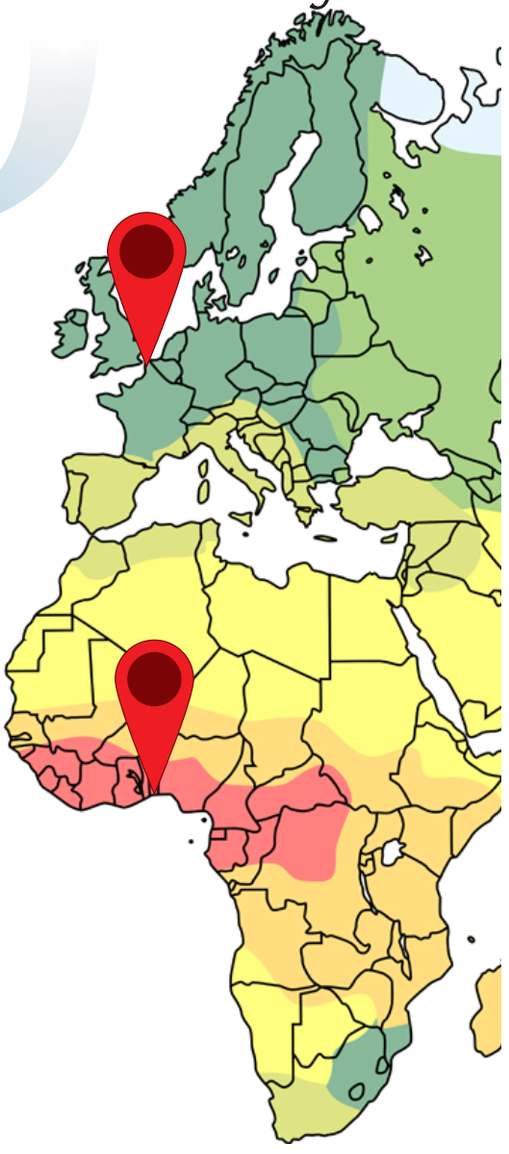

A
Negative control
PMm, 3 μg/cm²
PMm, 15μg/cm²
PMm, 30 μg/cm²
Arbitrary fuorescence unit
0
10
20
30
40
50
60
70
80
90
IL-1α
IL-1β
IL-2
IL-4
IL-6
IL-8
IL-10
IFNγ
TNFα
Eotaxin
GROα
I-309
IP-10
MCP-1
MCP-2
RANTES
TARC
B
Negative control
PMo, 3 μg/cm²
PMo, 15μg/cm²
PMo, 30 μg/cm²
Arbitrary fuorescence unit
IL-1α
IL-1β
IL-2
IL-4
IL-6
IL-8
IL-10
IFNγ
TNFα
Eotaxin
GROα
I-309
IP-10
MCP-1
MCP-2
RANTES
TARC
C
Negative control
VOCwb
VOCsb
TS
Arbitrary fuorescence unit
IL-1α
IL-1β
IL-2
IL-4
IL-6
IL-8
IL-10
IFNγ
TNFα
Eotaxin
GROα
I-309
IP-10
MCP-1
MCP-2
RANTES
TARC

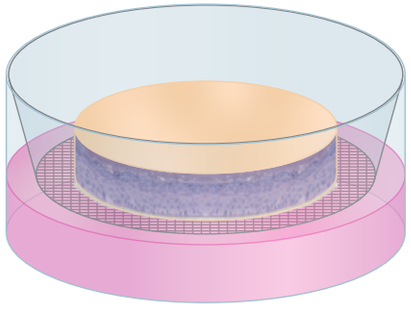
Figure 5


A
IL-1α release (pg/mL)
RH control
90% RH
45% RH
Ctrl
15μg/cm²
30μg/cm²
15μg/cm²
30μg/cm²
VOCwb
VOCsb
TS
PMm
PMo
VOCs
B
IL-8 release (pg/mL)
C
RANTES release (pg/mL)

Air humidity

Air pollutants





***In vitro* skin model**
Inflammation