Evaluating the effect of Ozone in UV induced skin damage

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Evaluating the effect of Ozone in UV induced skin damage

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Highlights

- Skin is one of the main organs directly exposed to pollution
- UV is the most aggressive outdoor stressors
- In our daily life we are exposed to more than one pollutant
- Ozone is another very noxious air contaminant
- Exposure to ozone and UV showed and additive skin damage compared to only UV

Abstract

Air pollution represents one of the main risks for both environment and human health. The rapid urbanization has been leading to a continuous release of harmful manmade substances into the atmosphere which are associated to the exacerbation of several pathologies. The skin is the main barrier of our body against the external environment and it is the main target for the outdoor stressors. Among the pollutants, Ozone (O_3) is one of the most toxic, able to initiate oxidative reactions and activate inflammatory response, leading to the onset of several skin conditions. Moreover, skin is daily subjected to the activity of Ultraviolet Radiation which are well known to induce harmful cutaneous effects including skin aging and sunburn. Even though both UV and O_3 are able to affect the skin homeostasis, very few studies have investigated their possible additive effect. Therefore, in this study we evaluated the effect of the combined exposure of O_3 and UV in inducing skin damage, by exposing human skin explants to UV alone or in combination with O_3 for 4-days. Markers related to inflammation, redox homeostasis and tissue structure were analyzed. Our results demonstrated that O_3 is able to amplify the UV induced skin oxinflammation markers.

Keywords: Oxidative stress, Pollution, Oxinflammation, Ahr, NF-kB, NRF2, Filaggrin,

1. Introduction

According to the World Health Organization (WHO), 4.2 million of premature deaths are now linked to ambient air pollution, which is therefore becoming a growing global concern in terms of both environment and human health (https://www.who.int/airpollution/ambient/health-impacts/en/, n.d.). The rapid urbanization and the industrial developments are leading to a continuous release of harmful manmade substances into the Earth's atmosphere such as Particulates Matters (PM), nitrous oxide (NOx), volatile organic compounds (VOCs) but also cigarette smoke (CS), which are all known to be involved in the exacerbation of several respiratory, cardiovascular and skin pathologies (Losacco and Perillo, 2018; Yang et al., 2017). Moreover some of these anthropogenic products present within the troposphere , such as NOx and VOCs, can interact with UV light, leading to the production of secondary dangerous products such as the ground level ozone (O_3), one of the most toxic pollutants contributing to photochemical smog (He et al., 2019; Wu et al., 2017). O_3 is an invisible gas with an unstable structure that makes it one of the strongest oxidation agents. Its harmful effects on human health were first associated to the development of respiratory and cardiovascular system complications such as asthma, Chronic Obstructive Pulmonary Disease (COPD), lung cancer and ischemia (Han et al., 2016; Mumby et al., 2019; Rosenthal et al., 2013; J. J. Zhang et al., 2019). Nevertheless, several growing evidences are now underlining the role of O_3 in modulating inflammatory and oxidative pathways within the skin contributing to skin aging (McDaniel et al., 2018; Pecorelli et al., 2019a; Valacchi et al., 2012). Indeed cutaneous tissue, due to its location, is the main protective barrier of our body against the environment and affecting its structure can seriously compromise the integrity of this organ (Proksch et al., 2008). The effects of pollutants such as PM, CS, but also O_3 and UV light on human skin have been long investigated in the past decades and linked to several skin inflammatory conditions (Araviiskaia et al., 2019; Rembiesa et al., 2018; Valacchi et al., 2012). For instance, even though O_3 is not a radical species per se, it can interact with the lipids and proteins present in the outermost layer of the skin, the stratum corneum, leading to the production of ROS and secondary mediators such as aldehydes (4-Hydroxy Nonenal,4-HNE). These mediators can then trigger and perpetuate the oxidative damage throughout the skin and initiate inflammatory processes (Valacchi et al., 2004; Packer and Valacchi, 2002; Pecorelli et al., 2019; Sticozzi and Valacchi, 2011; Valacchi et al., 2018, 2016). Similarly, the chronic exposure to UV is known to induce both an inflammatory and oxidative stress status within the skin, culminating in sunburn, skin photoaging, photo carcinogenesis, DNA damage and apoptosis (Young et al., 2017). UV light, and also O_3, have been shown to modulate the skin inflammatory response by activating the inflammasomes pathways, such as NLRP1, NLRP3, Absent in melanoma 2 (AIM2) (Beer et al., 2014; Ferrara et al., 2019; Hung et al., 2017; Rodríguez-Luna et al., 2019), as observed in several skin conditions (Ciążyńska et al., 2020; De Sá and Festa Neto, 2016; Gurung and Kanneganti, 2016; Zhong et al., 2016). Moreover, besides the alteration of the skin homeostasis, environmental pollutants have been found to compromise the physical and biochemical skin barrier properties, by altering the expression of the stratum corneum cornified envelope's (CE) components such as lipids (De Luca and Valacchi, 2010), proteins (Keratins, Filaggrin, Involucrin) and tight junctions (TJs) (Ferrara et al., 2020; J.J. Thiele, F. Dreher, H.I. Maibach, 2003; Moravcová et al., 2013). So far, several studies have shown how these noxious agents can affect skin homeostasis and today UV exposure is still considered the most harmful for skin. Of note, the ability of UV to photoactivate PM has been recently demonstrated, suggesting an additive effect when those pollutants are combined (Marrot, 2018)(Toyooka and Ibuki, 2007) (Bao et al., 2009)(Wang et al., 2007)(Wang et al., 2005)(Teranishi et al., 2010)(Murthy and Leslie, 2017). However, since environmental pollution is a broad and still not completely explored field, the role of other pollutants in UV-induced skin damage is still poorly understood. For instance, even though both UV and O_3 are able to affect the skin homeostasis when studied singularly, very few studies have investigated the interaction between these two pollutants (Boguniewicz and Leung, 2011; Murthy and Leslie, 2017; Sahle et al., 2015; Woodby et al., 2020). Therefore, the purpose of this study was to evaluate the ability of O_3 to implement UV induced skin damage in terms of oxinflammation (cross talk between oxidative stress and inflammations) (Valacchi et al., 2018) and skin barrier responses.

In the present study several markers of oxidative stress such as: 4-HNE, Nrf2, HO1, Ahr and the levels of protein related to skin structure and differentiation (Keratin 10, Involucrin, Filaggrin, Desmocollin-1, Claudin 1 and Aquaporin3) were measured. Furthermore, skin inflammatory condition was also determined the levels of the transcriptional factor NF-kB, Cyclooxygenase- 2 (COX2), and also by the activation of the NLRP1 inflammasome pathway. Of note, impairment of skin homeostasis was more evident after the combined exposure of O_3 and UV.

In conclusion, our results suggest that O_3 could exacerbate the loss of skin barrier functionality induced by UV exposure, and therefore it can contribute to the skin barrier impairment displayed in several skin pathologies already related to UV (atopic dermatitis, psoriasis etc).

2. Materials and Methods

2.1 Ozone Generator

Ozone was generated via electrical corona arc discharge from O_2 and combined with ambient air to flow into a plexiglass box (ECO3 model CUV-01, Italy, Model 306 Ozone Calibration Source, 2B Technologies, Ozone Solution), as previously described (Pambianchi et al., 2020) and constantly monitored by an ozone detector. The dose was based on our previous studies.

2.2 UV simulator

Skin biopsies were exposed to 200 mJ of UVA/UVB Newport, Oriel®, Sol1ATM, 1600W, Xenon Lamp, UVC & AM0 filters. UVA/UVB ratio: 21/1 as we used air mass filter AM 0) to correct the light output to closely match the solar spectrum when the sun is at a Zenith angle of 0. UV doses were carefully monitored by the radiometer ILT2400 Hand-Held Light Meter / Optometer (International Light Technologies, Inc., Peabody, MA, USA)

2.3 Skin explants culturing and exposures

Skin explants, obtained from elective abdominoplasties, have been purchased from HKB Surgery Hospital in Huntersville, NC. After trimming the subcutaneous fat, 12 mm punch biopsies were collected, rinse in PBS containing antibiotics/antimycotic and placed in 6 well plates pre filled with 1 ml of DMEM Media High Glucose containing 10% FBS, 100 U/ml Penicillin, 100 µg/ml Streptomycin and 0.25 µg/mL of Amphotericin B (Gibco). The skin explants were left in the incubator 37°C in 5% CO_2 for the overnight for recover. The experiment has been ran in triplicates for each conditions for each donor as previously described (Ferrara et al., 2020).

After 24 hours, the media was replaced with fresh one and the biopsies were exposed to 200 mJ UVA/UVB light alone as previously described or in combination with 0.25 ppm of O_3 for 2 hrs in an O_3 chamber as previously described (Ferrara et al., 2020). Samples have been exposed each day for a 4 days period and collected right after the first exposure (DAY 1) and after 4 days (DAY 4) for the future analysis. 324

2.4 Hematoxylin/Eosin staining

Briefly**,** tissues were fixed in 10% neutral-buffered formalin (NBF) and embedded in paraffin. For histological observation, 4 µm thickness sections were deparaffinized in xylene, rehydrated in a series of alcohol gradients and then stained with Mayer's hematoxylin solution for 10 minutes. After rinsing the sections in tap water, they were stained with aqueous Eosin Y solution (Sigma, USA) for 3 minutes and then dehydrated in 95%, 100% alcohol solutions. After the xylene step, the slides were mounted onto glass using a toluene based solution (Valacchi et al., 2017).

2.5 Immunohistochemistry

Human skin biopsies sections (4 µm) were deparaffinized in xylene and then rehydrated in decreasing alcohol gradients. For the Aentigen retrieval, the slides were put in a plastic jar containing a 10 mM sodium citrate buffer solution (AP-9003-500, Thermo Fisher Scientific, USA) (pH 6.0) and heatd in a microwave at a sub-boiling temperature (500 watt for 10 minutes). The slides were then cooled down by leaving them at room temperature for 20 minutes and then washed 2 times for 5 min in PBS. After blocking the slides with 5% BSA in PBS for 45 min at RT, the sections were incubated overnight at 4°C with primary antibodies supendend in 2% BSA/PBS at the following dilution: ASC (CAT NBP1-78977 NovusBio, USA) 1:100, NLRP1 (sc-166368 Santa Cruz, USA) at 1:50, 4-HNE (AB5605 Millipore Corp., USA) at 1:400, Desmocollin 1 (sc-398590, Santa Cruz, USA) 1:50, NFκB (8242, Cell Signaling) 1:400, AhR (83200, Cell Signaling) 1:100, Involucrin (sc-21748, Santa Cruz, USA) 1:50, Filaggrin (sc-66192, Santa Cruz, USA) 1:50, Claudin 1 (sc-166338, Santa Cruz, USA) 1:50, Aquaporin 3 (sc-518001, Santa Cruz, USA) 1:50. The next day, sections were washed 3 times

in PBS for 5 min and incubated with fluorochrome-conjugated secondary antibodies (A11004 Alexa Fluor 568, A11008 Alexa Fluor 488 and A11055 Alexa Fluor 488) at 1:500 dilutions in 2% BSA in PBS for 1 hr at RT. After washing the sections 3 times for 5 min in PBS, Nuclei were stained with DAPI (D1306, Invitrogen) for 1 min in PBS at RT. Sections were then washed in PBS and mounted onto glass slides using PermaFluor mounting media (ThermoFisher Scientific) and imaged via epifluorescence on a Zeiss LSM10 microscope equipped at 40x magnification. Images were quantified using ImageJ.

2.6 Protein extraction and Western blot

Human skin biopsies were harvested in T-PER™ Tissue Protein Extraction Reagent (Thermo Fisher Scientific, USA) with 1% of protease and phosphatase inhibitor cocktails (Sigma, USA) and then homogenized using a Precellys tissue homogenizer (Bertin instruments) at 10'000 rpm at 4°C for 20 sec with 30 sec breaks. This sequence was repeated 3 times and the protein content was measured using the Quick start Bradford protein assay (Biorad, USA). Equivalent amounts of proteins were loaded onto 4-12% polyacrylamide SDS gels and separated by molecular size. Gels were electroblotted onto nitrocellulose membranes and then were blocked for 1 hr in Tris-buffered saline, pH 7.5, containing 0.5% Tween 20 and 5% milk. Membranes were incubated overnight at 4°C with primary antibodies for COX2 (12282, Cell Signaling), HO-1 (PA00553, BioRad) and Ahr (83200, Cell Signaling) 1:1000 in TBS-T with 1% non-fat milk (BioRad,USA). The membranes were then incubated with horseradish peroxidase conjugated secondary antibodies (170-6515 or 170-6516, BioRad) for 1 hr at RT, and the bound antibodies were detected by chemiluminescence (BioRad, USA). β-actin (A3854 Sigma,USA) was used as loading control. Images of the bands were digitized, and densitometry analysis was performed using Image J software (Valacchi et al., 2015).

2.7 Statistical analysis

Statistics Statistical analyses were performed by using GraphPad Prism 6 software (GraphPad Software Inc., La Jolla CA). For comparisons between groups, analysis of variance (ANOVA) followed by Bonferroni's post-hoc test was conducted. All data were expressed as means ± standard deviations (SD). *$p \leq 0.05$ was considered as significant in UV or UV+O_3 vs Ctrl. $^{\#}p \leq 0.05$ was considered as significant in UV+O_3 vs UV.

3. Results

3.1 Effect of UV and O_3 exposure on skin morphology

The first step was to evaluate whether the experimental conditions could affect the skin morphology. As shown in Figure 1, H&E staining of human skin explants exposed to either UV or the combination of UV and O_3 do not show any sign of damage compared to the control tissues, suggesting that the experimental conditions were not over aggressive. Indeed, the skin shows clearly an healthy epidermis with the loss of nuclear structure from the basal layer to the stratum corneum (outermost layer). Stratum corneum is well visible and compact. No sign of inflammatory cells are present in the dermis.

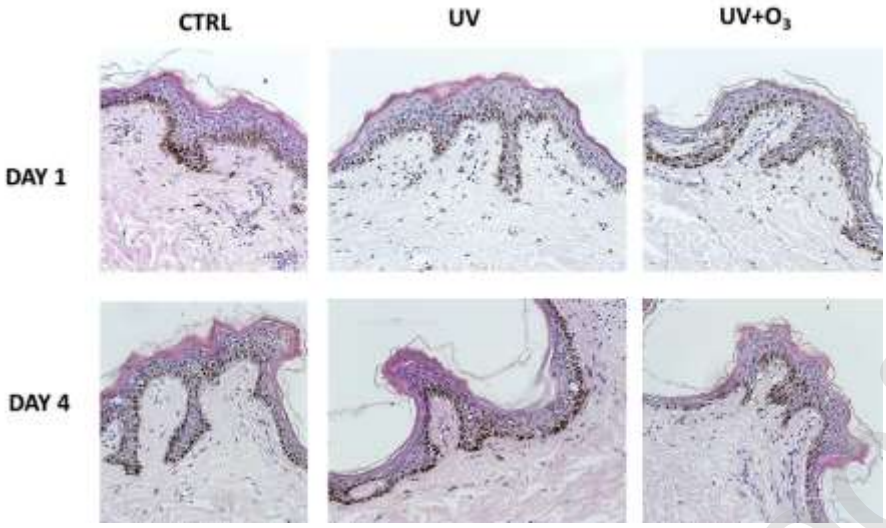

Figure 1. *H/E staining of human skin explants exposed to UV and UV+O_3 for 1 day and 4 days. The panel is representative of 1 donor.*

3.2 O_3 amplifies the UV-mediated skin structural damage in human skin biopsies

Although we did not observe any significant morphology changes, we still want to evaluate the eventual effect of the pollutants exposure on keratinocytes differentiation by measuring the main markers involved in this process. For this purpose, we first evaluated Keratin 10 (K10), a member of the keratins proteins family which are usually produced by keratinocytes and involved in the formation of the epidermis cytoskeleton, conferring structural resistance against mechanical trauma. Among them, Keratin 10 is the main keratin involved in the early keratinocytes differentiation within the spinous/suprabasal layer of the skin and it is therefore used as marker of early differentiation. As depicted in Fig.2, there was a decrease of K10 after the exposure to the combination of UV+O_3 at both DAY 1 (22.1%) and DAY 4 (37.7%). Interestingly, UV alone was not able to significantly affect K10 protein expression levels at both timepoints (Fig. 2a). In addition, similar trend was observed for the other 2 markers related to keratinocytes terminal differentiation, such as Involucrin (Fig. 2b) which is mainly present in the upper spinous/granular layer and Filaggrin (Fig. 2c), involved in the organization of the keratin filaments within the stratum corneum. Both of them are essential proteins part of the SC cornified envelope (CE), which confer the physical and biochemical barrier functions to the skin. As shown in Figure 2b-c, the exposure to UV and O_3 lead to a decrease of both Involucrin (28.1%)(Fig. 2b) and Filaggrin (73.4%) (Fig. 2c) levels at DAY 1 and at DAY 4 (Involucrin 65.0% and Filaggrin 64.4%) .

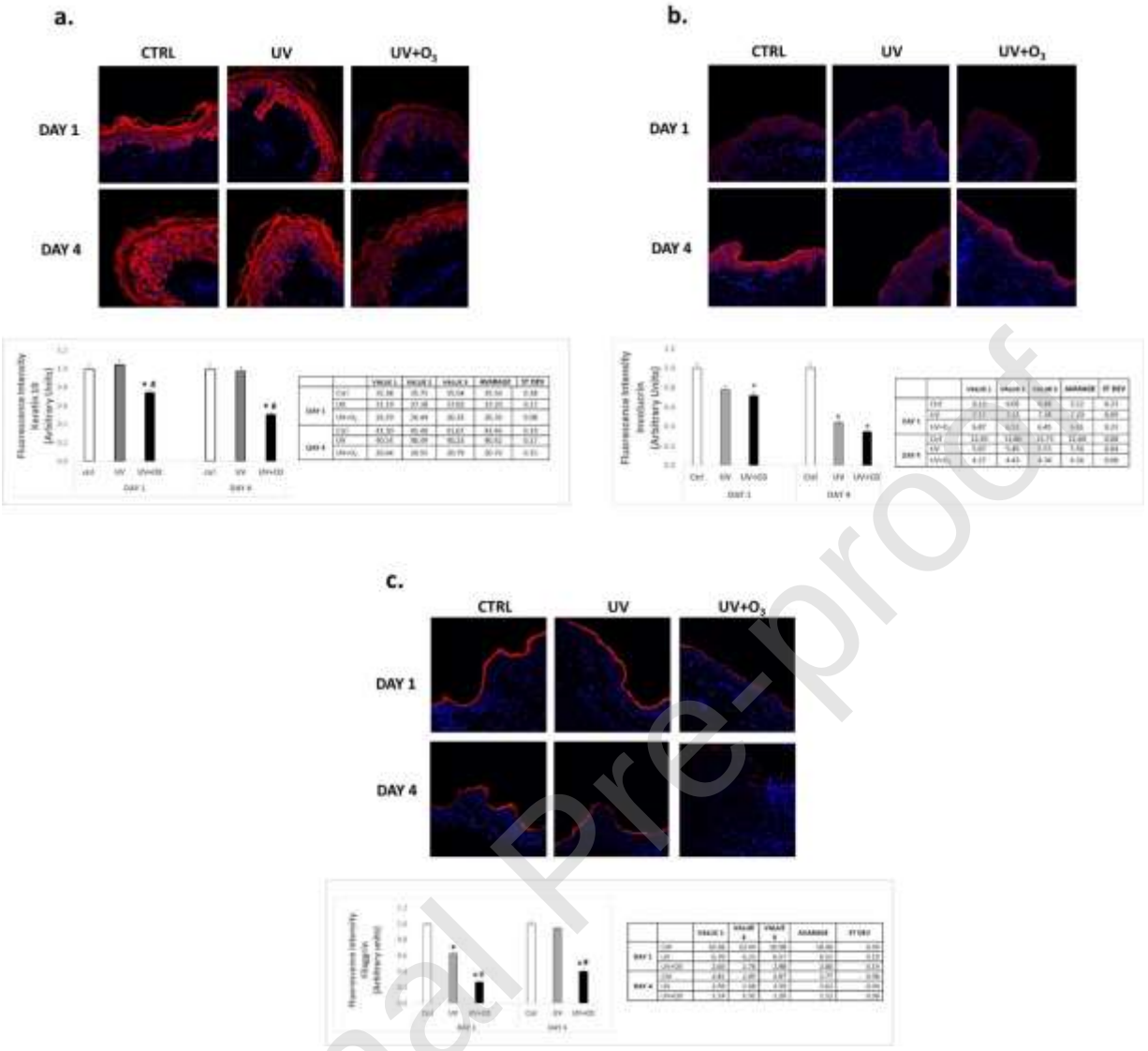

Figure 2. *Immunofluorescence (IF) staining for keratin 10* ***(a),*** *Involucrin* ***(b),*** *Fillagrin* ***(c),****), red staining, and DAPI (blue) in human skin explants exposed to UV and UV+O_3 for 1 day and 4 days at 20x of 40x magnification. Top panel is a representative of the immunofluorescence staining. Quantification was performed using ImageJ (Bottom panel).Values of the 3 different donors are shown in the bottom right table. Data are the results of the averages of at least three different experiments, *p ≤ 0.05 UV or UV+O_3 vs Ctrl by ANOVA and #p ≤ 0.05 UV+O_3 vs UV by ANOVA*

3.3 UV and O_3 exposure affect proteins related to cutaneous integrity and water channels

Tight junctions (TJs) along with the stratum corneum (SC) are the main components of the physical barrier of the skin, preventing the penetration of external antigens or leakage of internal constituents such as water and nutrients. Since both UV radiations and pollutants have been shown to affect the TJs distribution within the skin, leading to an impairment of the skin barrier function (Yuki et al., 2011), we wondered whether O_3 was able to enhance UV-induced skin damage by evaluating the protein levels of Claudin-1. As shown in Figure 3a, UV exposure induced a significant decrease (22.2 %) in Claudin-1 expression levels especially at DAY 1 and the combined exposure of UV and O_3 emphasized this effect at both DAY 1 (32.5%) and DAY 4 (45.7%).

As it concerns Desmocollin 1 (DCS1), one of the main components of the skin cell-cell desmosomes junctions, also in this case O_3 was able to further decrease the cutaneous levels of Desmocollin 1 induced by UV exposure alone, especially at DAY 1 (45.9%), and to a less extend also at DAY 4 (33.6%) (Fig. 3b). The loss of skin integrity in consequence of UV and O_3 exposure, suggests the ability of those pollutants to impair the skin barrier, one of which is the ability of the skin to retain water. This hypothesis was evaluated by the analysis of the protein levels for Aquaporin 3 which is involved in the cutaneous bidirectional water flux in the cells. As depicted in Fig. 3C, there was a clear loss of this protein when skin tissues were exposed to the combination of UV and O_3 at both timepoints DAY 1 (55.5%) and DAY 4 (43.4%).

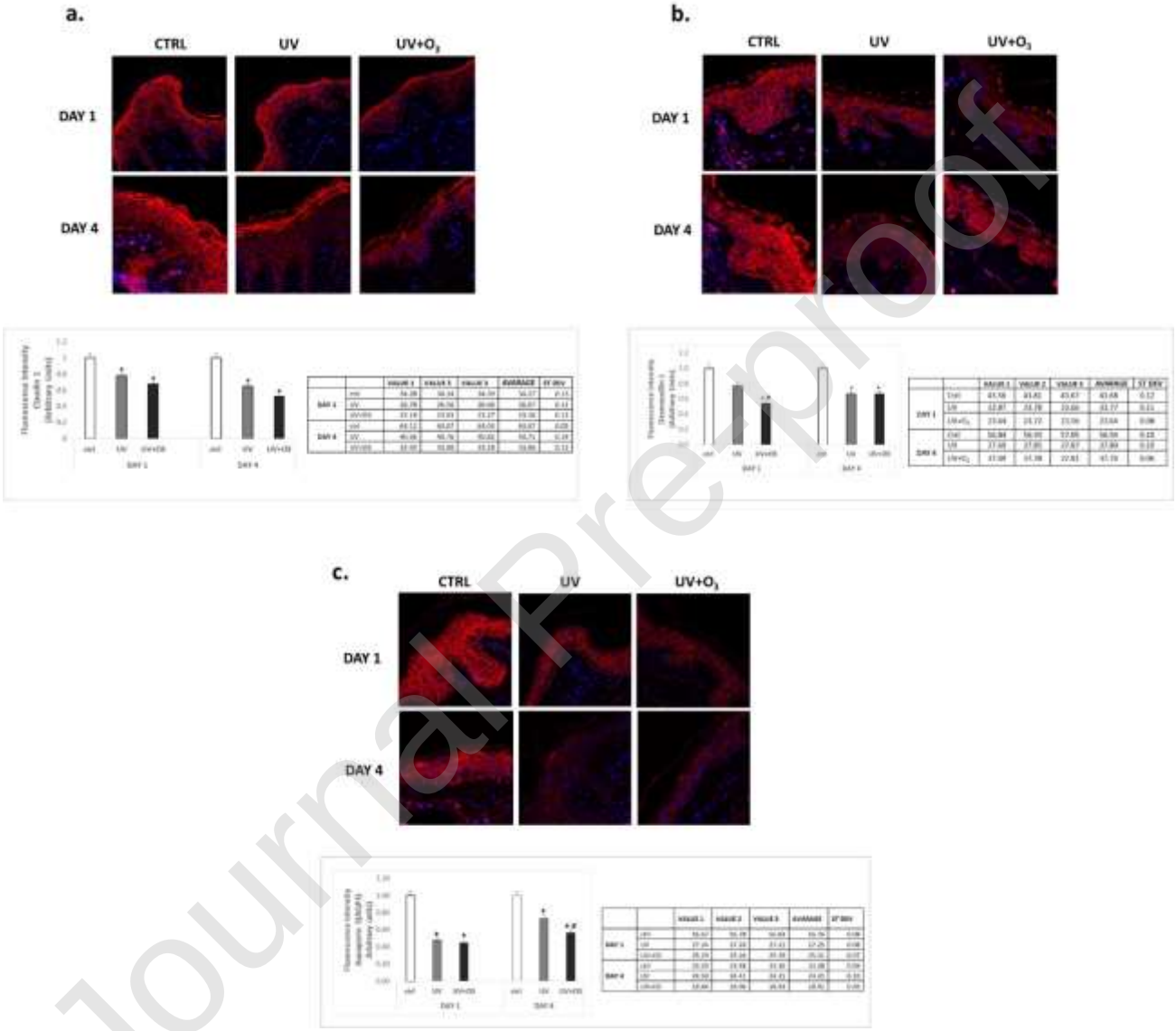

Figure 3. *Immunofluorescence (IF) staining for Claudin 1 (**a),** Desmocollin 1 **(b),** Aquaporin 3 **(c),**, red staining, and DAPI (blue) in human skin explants exposed to UV and UV+O_3 for 1 day and 4 days at 20x of 40x magnification. Top panel is a representative of the immunofluorescence staining. Quantification was performed using ImageJ (Bottom panel). Values of the 3 different donors are shown in the bottom right table. Data are the results of the averages of at least three different experiments, *$p \leq 0.05$ UV or UV+O_3 vs Ctrl by ANOVA and $^{\#}p \leq 0.05$ UV+O_3 vs UV by ANOVA*

3.4 The combination of UV and O3 affects cutaneous oxidative status levels

As shown in Fig. 4, UV exposure induced a significant formation of 4-HNE protein adducts at both DAY 1 (36.6%) and DAY 4 (96.9%). The combined exposure did not significantly differ from the UV alone although there was a slight increase at DAY 4 (105.5%). As a consequence of an altered redox status, the tissue can activate the defensive response represented by the Nuclear factor erythroid 2-related factor 2 (Nrf2) pathway. As shown in Figure 4b, both UV and UV+O_3 were able to induce Nrf2 expression at DAY 1 (UV vs ctrl 100%, UV+O_3 vs Ctrl 85,1%). As depicted in Figure 4c, we then decided to evaluate the protein levels of Hemeoxygenase-1 (HO-1), a stress-induced enzyme under the control of Nrf2 involved in the oxidative stress response. Our results showed increased HO-1 expression levels in response to UV+O_3 at DAY 1 (77.3%) and DAY 4 (99.6%) whereas UV alone was able to induce a significant increase only at the later time point (101.5%). Another important marker involved in cutaneous stress responses is the Aryl Hydrocarbon receptor (Ahr). This receptor has been shown to be able to affect not only the oxidative stress status, but also the adaptive-innate immune system response resulting in the activation of inflammatory processes. As showed in Fig.4d, UV and even more evident with the combination of UV and O_3, there was a clear induction of Ahr at DAY 1 (458.7%).

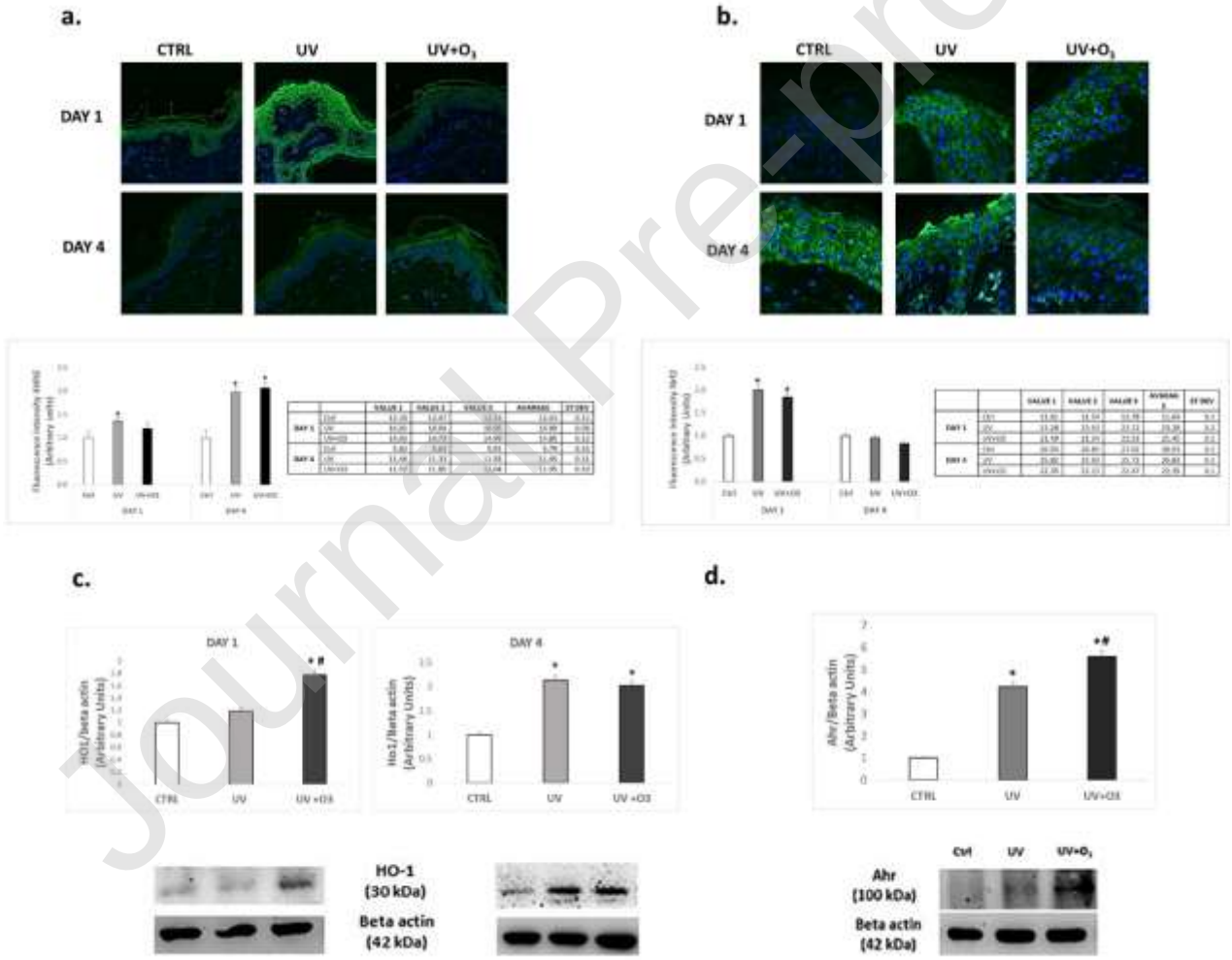

Figure 4. *Immunofluorescence staining for 4HNE (40x magnification) (**a**) and Nrf2 (60x magnification) (**b**), green, and DAPI (blue) in human skin biopsies exposed to UV and UV+O_3 for 1 day and 4 days. Top panel is a representative of the immunofluorescence staining. Quantification was performed using ImageJ (Bottom panel) (**c-d**). Values of the 3 different donors are shown in the bottom right table. Levels of Heme-Oxigenase (HO-1) (**c**) and ahr (**d**) and beta-actin protein in ex vivo human skin biopsies exposed to*

*UV and UV+O3 for 1 day (Left panel) and 4 days (right panel). Data are the results of the averages of at least three different experiments, *p ≤ 0.05 UV or UV+O_3 vs Ctrl by ANOVA and #p ≤ 0.05 UV+O_3 vs UV by ANOVA*

3.5 O_3 displays and additive effect to UV-induced inflammation

Considering the link between oxidative stress and inflammation, to better investigate the possible inflammatory status within the skin induced by UV and O_3 exposure, we performed an immunofluorescence staining for NF-kB, one of the main transcription factors involved in tissue inflammatory responses. As depicted in Figure 5a, pollutants exposure induced a significant expression levels of NF-kB at both time points DAY 1 (270%) and DAY 4 (165%),and this was more evident after the combination of UV and O_3. Moreover, Cyclooxygenase-2 (COX2) protein level, which is under the transcriptional control of NF-kB, was significantly up regulated by the combined pollutants exposure at both DAY 1 (168%) and DAY 4 (32%) timepoints, whereas UV alone did not significantly affect its levels when compared to un-exposed tissues (Fig. 5b). NF-kB has been involved also in the inflammasome activation, that has been shown to be one of the main regulatory pathways of the inflammatory response in tissues. Therefore, to better evaluate the possible colocalization of these two inflammasomes components under UV and O_3 exposure, we have performed an immunofluorescence staining for NLRP1 and ASC proteins. As shown in Figure 5c both UV and O_3 are able to induce the inflammasome activation by upregulating the expression of NLRP1 (67.7%) and ASC (31.1 %) at DAY 1 and DAY 4 (NLRP1 (46.1%) and ASC (85.1%). Moreover, the colocalization of the two proteins more likely occurred after the prolonged combined exposure (UV+O_3) at DAY 4, suggesting that O_3 is able to enhance the UV-induce inflammasome activation.

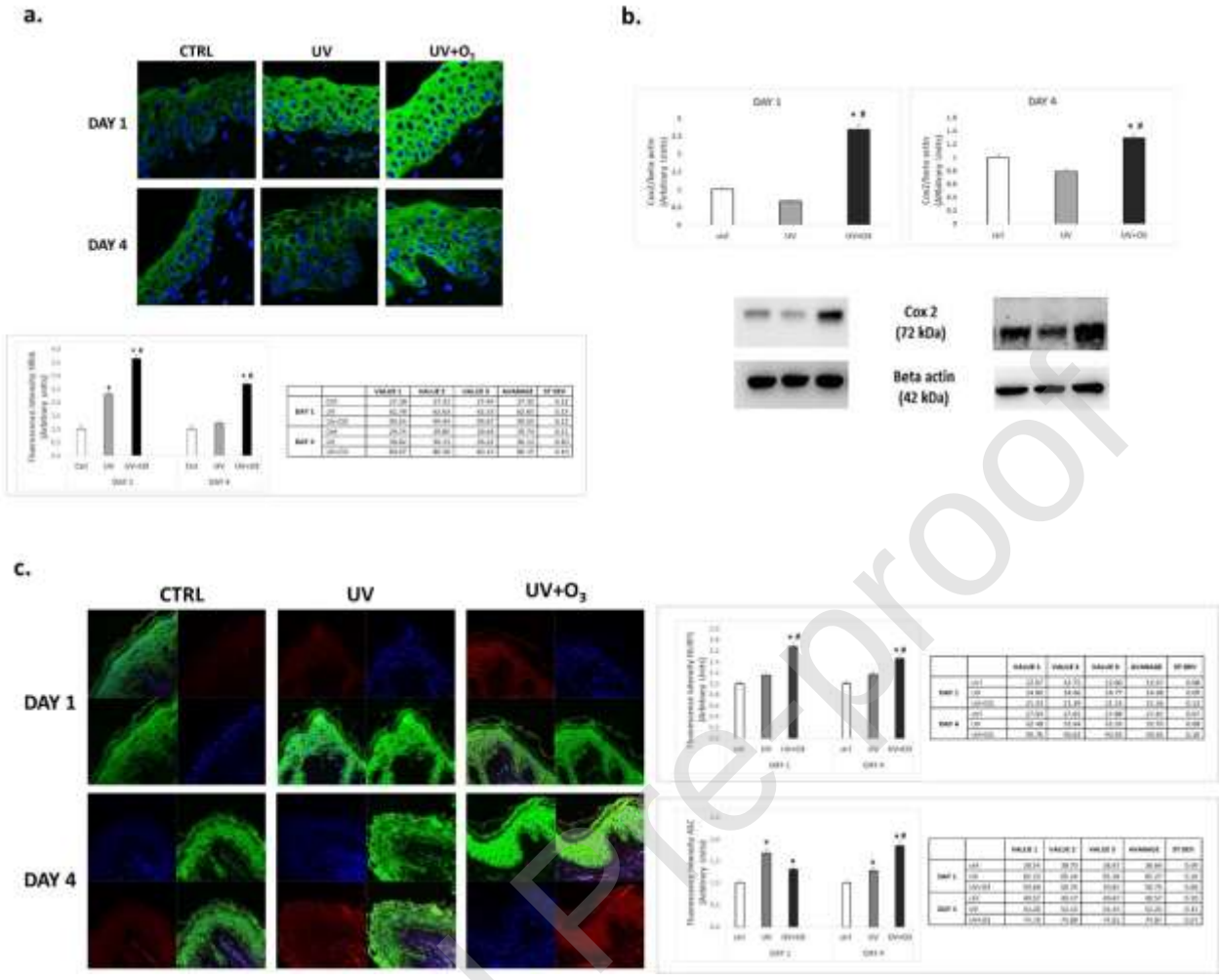

Figure 5. *(**a**)Immunofluorescence staining for NF-kB (green) and DAPI (blue) at 60x magnification in human skin biopsies exposed to UV and UV+O_3 for 1 day and 4 days. Top panel is a representative of the immunofluorescence staining. Quantification was performed using ImageJ (Bottom panel) (**b**) Values of the 3 different donors are shown in the bottom right table. Levels of COX2 and beta-actin protein in ex vivo human skin biopsies exposed to UV and UV+O3 for 1 day (Left panel) and 4 days (right panel). (**c**) Co-immunofluorescence staining for NLRP1 (red), ASC (green) and DAPI (blue) in human skin biopsies exposed to UV and UV+O_3 for 1 day and 4 days at 40x magnification. Top panel is representative of the immunofluorescence staining for 1 donor. Quantification was performed using ImageJ (Bottom panel) Data are the results of the averages of at least three different experiments, *p ≤ 0.05 UV or UV+O_3 vs Ctrl by ANOVA and #p ≤ 0.05 UV+O_3 vs UV by ANOVA*

4. Discussion

Environmental pollution is nowadays considered one of the most important risk factors for global human health and several studies have correlated the onset of several pathologies to pollutants exposure. The skin is the first barrier of our body against external stimuli and is therefore one of the main organs affected by environmental pollutants of which O_3 and UV light are among the most toxic (Valacchi et al., 2012). The deteriorating of the stratospheric O_3 layer, which normally act as a shield against UV light, is increasing our exposure to these dangerous radiations and therefore increasing the incidence in skin pathologies (D'Orazio et al., 2013). Even though the ability to induce cutaneous oxinflammation has been observed

for several pollutants (Valacchi et al., 2018)·(Mancebo and Wang, 2015), very few studies have investigated their possible synergistic effect in skin damage (Burke and Wei, 2009; Marrot, 2018). UV is still thought to be the most aggressive outdoor stressor, and in addition it is involved in the tropospheric O_3 formation. Indeed, photochemical O_3 is formed by reactions involving solar radiation and anthropogenic pollutants (methane, non-methane volatile organic compounds, carbon monoxide) in the presence of nitrogen oxides while in less polluted environments, O_3 is produced in the presence of sunlight (at wavelengths <424 nm), through the photolysis of NO_2 (Alvim-Ferraz et al., 2006). In addition, as recently reported, the levels of O_3 are still increasing making the effect of this pollutant to our health a real concern (Lin et al., 2020). Therefore, the aim of the present study was to investigate the possible additive effect of O_3 in UV induced cutaneous oxinflammation. As previously shown, O_3 is able to interact with biomolecules present within the skin, such as lipids and proteins, leading to oxidative reactions that then lead to the production of ROS and non-radical species such as the aldehyde, i.e. 4-hydroxy-nonenal (4- HNE) (Pecorelli et al., 2019a; Sticozzi and Valacchi, 2011). Our results confirmed the ability of both O_3 to further aggravate UV induced cutaneous damage. This result is in line with a previous work (Valacchi et al., 2000) where using a murine model the authors were able to evidence an additive effect of UV and O_3 in skin lipid peroxidation levels. It should be mentioned that SKH1 skin is circa 1:10 thinner than humans, and this needs to be taken into consideration when extrapolate such studies to real life (Benavides et al., 2009). In general, once the tissue is challenged with an oxidative insult, the cells activates a defensive response to cope with the altered redox homeostasis. The master regulator of such response is the transcription factor Nrf2. In our study, we have evidenced increased levels of Nrf2 and to prove its activation we have also analyzed the levels of the enzyme Heme oxygenase-1 (HO-1), which is under Nrf2 transcriptional control. Our results showed that the combination of O_3 and UV induced HO-1 level in a faster manner compared to UV alone (DAY 1), suggesting the ability of O_3 to augment UV effect. This is possible, as these two aggressors react differently with the skin. Indeed, while UV light, based on the wavelength can reacts with the deeper layers of the skin, O_3 is not able to penetrate the skin and its first reaction is mainly at the superficial levels and then leads to the formation of bioactive products that can further affect the epidermis and eventually even the dermis (Pecorelli et al., 2019). It should be also mentioned that the ability of O_3 and UV singularly to induce HO-1 has been already described (Valacchi et al.,2004) although never in combination.

Surprisingly, our data at DAY 1 showed a higher level of 4-HNE in tissues exposed to UV alone respect to the tissues exposed to the combination of UV plus ozone; this could be a consequence of an additive effect of the 2 pollutants that increase the levels of 4-HNE immediately after the exposure and when measured at DAY 1 several of the peroxidation products have been already metabolized by the tissues. The additive effect of O_3 on UV exposure in affecting the antioxidant response of the skin was already demonstrated in a study conducted on a murine model, where O_3 exposure was found to potentiate the UV-induced depletion of Vitamin E, one of the most recognized cutaneous antioxidant defense (Packer and Valacchi, 2002; Valacchi et al., 2000). Indeed both UV and O_3 are known to be able to deplete the antioxidants defense of the skin (Podda et al., 1998; Woodby et al., 2020) and several studies have demonstrated that the antioxidant damage induced by these pollutants can be prevented by the application of topical compounds containing vitamin E and C (Ferrara et al., 2020; Lin et al., 2003; Valacchi et al., 2016). Another important marker involved in the antioxidant response is the transcription factor Aryl hydrocarbon receptor (Ahr) and its expression levels have been found upregulated by UV plus O_3 in our human skin models right after the pollutants combination exposure. Indeed Ahr is known to be an important target for environmental stressors, especially PAHs found in PM, which can lead to its activation and consequent production of reactive oxygen species (ROS) and also to an inflammatory response (Vogel et al., 2020). O_3 has been shown to modulate Ahr receptor levels in human keratinocytes, leading to an increased expression of the cytochrome CYP1 isoforms genes which are known to be involved in the biotransformation of many environmental pollutants (Afaq et al., 2009; Nebert et al., 2004). The activation of Ahr results in its translocation into the nucleus where it can binds to specific sequences, the xenobiotic responsive elements (XREs) or dioxin responsive elements (DREs), leading to the transcription of genes encoding for phase I and II xenobiotic metabolizing enzymes (CYP1A1, CYP1A2, and CYP1B1), glutathione-S-transferases (GSTs), NADPH/quinone oxidoreductase (NQO1), and aldehyde dehydrogenase 3 (Dietrich, 2016), which all represent the Ahr canonical activation pathway. Nevertheless Ahr can also interact with other signaling pathways such as Nrf2, which also regulates the transcription of some of these detoxifying genes such as NQO1, GSTA2, and UGT1A6 (Yeager et al., 2009) and also the NF-kB pathway (Vogel et al., 2014). The activation of the Cytochrome P450 family enzymes by AhR results in the production of ROS which can promote not only the cross-talk between Ahr and Nrf2 pathway, as first described by *W. Miao et al* in 2005 (Albertolle and Peter Guengerich, 2018; Miao et al., 2005) , but also to modulate the NF-kB activation, suggesting that Ahr activation could be mediated by NF-kB via ROS

production (Morgan and Liu, 2011; Nakano et al., 2006). Nevertheless in our skin models, besides the oxidative stress status induced by pollutants exposure, we also determined an inflammatory response as showed by NF-kB and COX2 increased levels. Moreover we also demonstrated the activation of an important inflammatory pathway present within the skin, the NLRP1 inflammasome, which has already been shown to be upregulated in several skin conditions (Ekman et al., 2014; Grandemange et al., 2015; Rajendiran et al., 2019; Zhong et al., 2016) and being particularly susceptible to UV radiations (Fenini et al., 2018). NLRP1, as well as other inflammasomes, has been shown to be triggered by a variety of stimuli including UV and O_3 (Ferrara et al., 2019; Gruber and Holtz, 2019), leading to the formation of a scaffold resulting upon the recruitment of the Apoptosis-associated speck like protein containing a caspase recruitment domain (ASC) to the inflammasomes sensor NLRP1. The co-localization of the sensor NLRP1 with the speck like receptor ASC, indicated the activation and the assemble of the proteins complex in response to pollutants stimuli. Our results showed that O_3 is able to exacerbate the UV-induced skin inflammatory damage, by mediating the modulation of NLRP1 activation, confirming a previous work carried out in our lab in which we reported that NLRP1 inflammasome can be activated by O_3 exposure in different human skin models (keratinocytes, reconstructed human epidermis and also skin explants) and that this activation is redox regulated (Ferrara et al., 2019). A growing literature has been showing that pollutants such as Particulate Matters (PM), CS and also O_3 and UV can activate different Inflammasomes such as NLRP1, NLRP3 and AIM2 (Chen et al., 2016; Hasegawa et al., 2016; Palazon-Riquelme and Lopez-Castejon, 2018; Sollberger et al., 2015), which normally assemble in a proteins complex able to induce the maturation of caspase 1 and the release of cytokines such as Interleukins 1β (IL-1β) and Interleukin-18 (IL-18), that are also highly expressed in several skin conditions (Beer et al., 2014; Contassot et al., 2012). Nowadays there are solid scientific studies that correlates the IL-1β production with several skin inflammatory diseases such as psoriasis, atopic dermatitis to Inflammasomes activation (Beer et al., 2014; De Sá and Festa Neto, 2016). Moreover also the Ahr receptor has been shown to regulate the expression of inflammasomes such as NLRP3 (Huai et al., 2014) and to be able in modulate the immune response in macrophages after being upregulated in response to NF-kB activation via LPS, which is one of the most important triggers activating the inflammasome pathways (Vogel et al., 2014) (Latz et al., 2013). It is therefore possible that the inflammatory response detected in our skin models is the result of a crosstalk between ROS production and Ahr activation, mainly mediated by O_3, resulting in NLRP1 inflammasome activation and therefore in the exacerbation of the oxinflammatory damage induced by UV itself.

All these oxidative-inflammatory processes induced by pollutants exposure within the cutaneous tissue, can then culminate in the alteration of skin functionality by affecting the main components of the stratum corneum, which is the primary barrier of the skin (Rembiesa et al., 2018). Indeed this complex structure is mainly formed by proteins and lipids, known to be crucial targets for pollutants, and that confers biochemical properties to the skin by preventing water loss, the entrance of noxious compounds and conferring strength to the cutaneous tissues (Proksch et al., 2008). Even though the morphological analysis conducted on our human skin samples did not show any significant differences between exposed and un exposed tissues, we detected a decrease in the expression of the main skin protein markers such as Keratin 10, Involucrin and Filaggrin especially after the combined exposure of UV and O_3. These results demonstrated that these pollutants are actually able to modulate the main proteins involved in cutaneous cells differentiation and SC cornified envelope construction, suggesting a possible effect on skin barrier functionality. These results are in line with previous work that showed pollutants such as PM and UV are able to modulate these skin proteins (Bernerd et al., 2001; Hieda et al., 2020; Moravcová et al., 2013), which impairment is often associated with several skin disorders (Kubo et al., 2012). For instance, the aberrant expression of Filaggrin, which is essential for the regulation of epidermal homeostasis, has been associated to the development of skin pathologies such as ichthyosis vulgaris and atopic dermatitis, both in human (Irvine and McLean, 2006; Palmer et al., 2006; Smith et al., 2006) and mice (Presland et al., 2000). Also Involucrin is correlated to several keratinization disorders and impairment in barrier function (Kanitakis et al., 1987), as well as Keratins, which altered expression is known to lead to psoriasis, hyperkeratosis, inflammation, impaired wound healing and also to carcinogenesis (Coulombe and Lee, 2012; Elango et al., 2018; Fischer et al., 2014; Sequeira and Watt, 2019; Tsubota et al., 2007; X. Zhang et al., 2019). Even though the role of O_3 in affecting the skin barrier integrity is still unclear, our results suggest that O_3 might potentiate the loss of proteins expression visible already after UV radiations, exacerbating the skin structural damage.

Besides the proteins and lipids present in the SC, skin barrier homeostasis is maintained by other essential components such as tight junctions (TJs) and water channels. For instance the tightness of the skin is supported by desmosomes, TJs and gap junctions which mediate the cell-cell interactions

within the different cutaneous epidermal layers and maintain the skin homeostasis during epithelial turnover (Yokouchi and Kubo, 2018). Alteration of skin components such as Claudin 1, Zonula occludens-1 (ZO-1), Occludins, has been associated to the development of several inflammatory skin conditions such as atopic dermatitis and psoriasis (Balda and Matter, 2014). Moreover pollutants exposure, such as UV radiations, has been shown to compromise the distribution of TJs within human skin and keratinocytes, deteriorating the cutaneous tissue functionality (Yuki et al., 2011), as well as PM, which is able to decrease ZO-1 via an oxidative mechanism (Wang et al., 2012). A similar redox mechanism has been shown in lungs of mice exposed to O_3, where an alteration of claudins expression levels has been detected and associated to airways inflammation (Kim et al., 2018). However, whether O_3 is able or not to affect TJs functionality within the skin is still poorly understood. Of note, in our skin models we found an impairment in the expression of the main cutaneous junctions such as Claudin 1 and Desmocollin 1 after the combination of UV and O_3 exposure. Moreover, we also detected altered expression levels of the water channel Aquaporin 3 (AQP3) in skin tissues exposed to pollutants. The downregulation of AQP3 displayed after UV exposure was even further compromised by the addition of O_3, suggesting that these pollutants could compromise the ability of the skin to retain water. It should be mentioned that AQP3 is not only essential for the regulation of skin water permeability, but also for keratinocytes migration, proliferation, differentiation and its expression seems to be under the control of the NOTCH pathway signaling (Guo et al., 2013). Moreover, a recent study also to demonstrate that PM 2.5 can compromise the skin barrier functionality in 3D human skin model by altering the expression of several proteins such as AQP3 together with Filaggrin, Involucrin and Keratin 10, most likely through Ahr and NOTCH activation (Hieda et al., 2020). Furthermore, impairment of AQP3 due to different stress stimuli including UV radiations, has been associated to skin abnormalities (Bollag et al., 2020; Jeon et al., 2015; Shan et al., 2012). The maintenance of skin integrity and functionality is essential to prevent the entrance of pathogens and the activation of sensor of the innate immune system such as Pattern Recognition Receptors (PRRs), Toll-Like Receptors (TLRs) (Yuki et al., 2016). Nevertheless, an inflammatory response of the skin due to stress stimuli could affect the main skin barrier components, altering their function. For instance the downregulation of Claudin 1 found in atopic dermatitis patients, has been shown to promote the inflammatory response within the cutaneous tissue resulting in the release of IL-1β (Bergmann et al., 2020), whereas pro-inflammatory cytokines such as IL-1β and also IL-17 and TNF-a, which are highly expressed in patients affected by atopic dermatitis or psoriasis, are able to affect the expression of the main cutaneous TJs (Claudins, Occludins, ZO-1) (Kirschner et al., 2009; Yuki et al., 2016). It is therefore plausible that this relationship might exist also in our exposed skin models, where the skin barrier impairment induced by pollutants could also affect the activation of the cutaneous inflammatory response, resulting in NLRP1 and Ahr activation. Vice versa, oxinflammatory reactions triggered by UV and O_3 could have affected the skin functionality by altering lipids, proteins and components present within the cutaneous tissue. Taken together our results demonstrated that the combination of UV and O_3 can affect skin homeostasis mainly due to the interconnection between oxinflammatory reactions and skin structure/functionality impairment.

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Conflicts of Interest

The author declares no conflicts of interest.

Author Contributions

Investigation, Francesca Ferrara.; Erika Pambianchi; Brittany Woodby.; Nicolò Messano. Writing. Francesca Ferrara.; Alessandra Pecorelli; Giuseppe Valacchi.; Rita Canella; Jean-Philippe Therrien. Funding acquisition Giuseppe Valacchi.; Jean-Philippe Therrien. Data Analysis, Giuseppe Valacchi.; Francesca Ferrara. All authors have read and agreed to the published version of the manuscript.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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