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DIRECTOR Prof. Pinton Paolo

EXOGENOUS STRESSORS AS A SOURCE OF CUTANEOUS INFLAMMASOME ACTIVATION AND ALTERED TISSUE REDOX HOMEOSTASIS: THE OXINFLAMMATION PHENOMENA

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Candidate

Dott. Ferrara Francesca

Francesca Ferrara

(Signature)

Supervisor

Prof. Valacchi Giuseppe

(Signature)

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1.1 INTRODUCTION

1.1.1 Environmental pollutants

The rapid urbanization and the industrial developments are leading to a continuous release of harmful handmade substances into the Earth's atmosphere, contributing to the establishment of the well-known Environmental Air Pollution phenomena.
According to the World Health Organization (WHO), 4.2 millions of premature deaths are linked to ambient air pollution, which is therefore becoming one of the main environmental risk factors for the onset of several diseases.
Indeed the release of organic or inorganic particulates Particulate Matter (PM), gases (such carbon monoxide (CO), sulfur dioxide, nitrous oxides (NOx), chlorofluorocarbons CFCs, etc.) and other volatile bio molecules (VOCs) from industries, cars exhaust etc., are contributing to the incidence disease mortality and morbidity, becoming a global issue. Nowadays some of the most recognized air pollutants contributing to health issues are Particulates matters (PM), Ozone (O_3), and also cigarette smoke. The health risks associated to these environmental pollutants are not only due to their ability in being inhaled but also to the direct cutaneous contact affecting therefore the respiratory tract, the cardiovascular system and also the skin tissue. Indeed, once they get in contact with the different organs, they are able to induce oxidative and inflammatory responses within our body leading to the so called OxInflammation response [1]. Indeed the development of a continuous cross-talk between oxidative and inflammatory pathways can induce the onset of different conditions such as stroke, ischemic heart disease, lung cancer, obstructive pulmonary disease, but also gut diseases and the exacerbation of skin inflammatory pathologies [2–5]. Besides that, these noxious compounds are also contributing to the deteriorating of the stratospheric O_3 layer, which normally act as a shield against UV rays, increasing our exposure to these dangerous radiations. Indeed

the noxious effects of UV lights on human skin, such as sunburn, skin photoaging, photo carcinogenesis, DNA damage and apoptosis [6], are all well documented and they will be further discussed in the following chapters. However, even though environmental pollution has been associated to several pathologies, it is a very complex field to investigate in term of health issues, since its harmful effects on human health are strictly linked to the individuality of the human being. This concept was first described in 2005 by the American epidemiologist Christopher Wild[7] who coined the term "Exposome" to describe the "The totality of exposures to which an individual is subjected from conception to death" [8]. Indeed the susceptibility of all of us in developing certain pathologies is due to the interaction between internal factors (such as our genome, our genetic traits), which differ from person to person, and external factors, which comprehend our lifelong environmental exposures (our nutrition, routine and habits, where we live etc.). Moreover, growing evidences are now extending the concept of environmental pollution by comprehending not only the outdoor insults, but also the agents we are in contact with in an indoor environment. Indeed high levels of oxidants such as reactive nitrogen oxides (NO and NO_2), but also Volatile compounds (VOCs), O_3 bioproducts, formaldehyde etc.. have been found in indoor environments due to the use of gas stoves, or chlorine compounds(bleach), or even to the chemical composition of furniture [9]. Therefore, considering also the interaction between indoor and outdoor pollution it is clear how complex is to understand the effect of environmental pollution on human health. In addition, the variability of our response to the different environmental insults make hard to identify an unique way of action, especially if we consider that we can be exposed to more than one pollutants simultaneously.

In the present work we will focus on the main and most toxic air pollutants that are now to be present in urban area: PM, Cigarette Smoke and O_3.

I. Particulate matter (PM)

PM is the principal component of air pollution and it is a mixture of solid and liquid particles suspended in the air, such as poly-aromatic hydrocarbons, metals, inorganic and organic toxins, which may have both an anthropogenic and a natural biogenic origin. Indeed these particles can be originated naturally from volcanoes, fires in forest, living vegetation, dust

storms etc., but also by human activities, such as industrial process, burning of fossil fuels, coal combustion, in vehicles,[13] road dust and in cooling systems. Some of these particles can be also involved in oxidative reactions which rely on the oxidation of primary gases such as Nitrogen Oxides (NOx), Sulfur, but also of volatile organic compounds (VOCs). Moreover, the interaction of some of the particles components, such as NOx, CO, VOCs, mineral dust, black carbon and Sulfur dioxide (SO_2) with UV light, lead to the arise of the photochemical smog, which is actually visible. Particles have irregular shapes and they can be divided based on their aerodynamic diameter in different groups. The Coarse fraction is represented by particles with a size ranging from 2.5 to 10 μm ($PM_{2.5}$-PM_{10}), whereas the Fine fraction contains the particles with a size ranging from 0.1 and 2.5 μm. All the particles displaying a diameter less than 0.1 μm are then called Ultrafine particles (UFPs). Usually the fine and ultrafine particles are the most dangerous for human health since they can be inhaled, reach and then deposit in the deepest airways. Thus, exposure to PM has been primarily associated to the development/exacerbation of respiratory diseases such as asthma, COPD and respiratory diseases [10–12]. The adverse effect of PM is linked to the ability in trigger oxidative reactions, with the production of reactive oxygen species (ROS), and also an inflammatory response, by inducing important inflammatory pathways such as the nuclear transcription factor NF-κB, involved in the transcriptional regulation of inflammatory mediators as cytokines, chemokines etc.. For instance increased expression levels of key inflammatory mediators such as COX2 and PGE_2 and the pro-inflammatory cytokines TNF-α and IL-6, were found in human lung cells, underlying the involvement of NF-kB and MAPK inflammatory pathways in PM-Induced OxInflammation[13,14]. Moreover PM have been shown to stimulate the tissue antioxidant response via the activation of the transcription factor NRF2, which is the main player in cellular antioxidant response [15,16]. Moreover, both NRF2 and NF-kB have been found activated in murine lung exposed to Biodiesel PM (DB) along with higher levels of oxidative stress-induced enzymes such as Heme-oxygenase 1 (HO-1) and TNF-alpha [17].Since inhaled, PM are even able to reach the nervous system, where they display neurotoxic and neuroinflammatory effects [18]. Skin is another important target organ for PM which is able to interact and eventually penetrate

(concept still controversial) the human skin leading to increased oxidative stress and the activation of the inflammatory pathways, as well as DNA damage and skin aging [19–21]

II. Cigarette Smoke (CS)

CS represents one of the most dangerous indoor/outdoor environmental pollutants. It is a complex aerosol composed of a mix of several chemical substances, more than 4700, which are distributed in a gas phase and a particulate phase. The toxicity of cigarette smoke is mainly associated to the presence of a high level of pro-oxidants, such as free radicals, which can trigger oxidative stress reactions or can lead to secondary oxidative events mainly represented by lipid peroxidation [22–24]. Indeed CS has been estimated to contain 1014 low molecular-weight carbon- and oxygen-centered radicals within the gas phase [24,25],and also Nitric oxide (NO), up to 500 ppm, which can be oxidized into NO_2 and participates to oxidative events [26] . Beside the mainstream smoke represented by the combination of inhaled and exhaled smoke while taking a cigarette puff, there is also the second-hand smoke or side stream smoke, which is released into the air directly by a burning cigarette and has been demonstrated to be even more toxic than the mainstream CS [27]. The harmful effects of CS on human health have been widely investigated and among the main organs directly exposed to CS there are the respiratory tract and the cutaneous tissue although CS exposure has been also associated to several respiratory/cardiovascular conditions [28] [29], but also to cancer and neurotoxicity [30,31]. Besides the oxidative damage, CS has been shown to stimulates the release of pro-inflammatory cytokines and consequent epigenetic modifications [32]. High levels of IL-1beta, IL-6 and TNF-alpha have been found in mice exposed to CS for 120 min/day as well as high levels of MMP-1 [33,34]. A recent study also demonstrated that CS-induced inflammation in human lungs cells is autophagy mediated by involving the MAPK, JNK and p38 pathways and that blocking autophagy significantly reduced the released levels of IL-1β, IL-6 and IL-8 [35]. Regarding the skin, CS has been shown to be related to premature skin aging and wrinkling but also with several inflammatory pathologies. These effects are mainly due its ability in induce ROS production within the epidermal layers [36] and the consequent lipid peroxidation [37]. Moreover cigarette smoke has been found to be

able in upregulate the matrix metalloproteinases MPP-1 and -3 that are known to be involved in the degradation of collagen and other connective tissues [38,39]. Another study showed that human keratinocytes exposed to CS display decreased wound-healing capacity with an increased expression of NRF2 and MMP9 as well as an altered epithelial barrier integrity [40]**.** The development of psoriasis, a skin autoinflammatory pathology, has been associated to cigarette smoke exposure in several studies [41–43] as well as other skin pathologies**..**

III. Ozone (O_3)

Among the outdoor pollutants to which living organisms are daily exposed to, tropospheric O_3 is one of the most toxic compound in term of human health [44,45]. O_3 or trioxygen, is an unstable blue gas with a pungent smell (resembling the chlorine bleach) and it is already perceptible at a concentration of 0.01 ppb but it can reach the concentration of 0.8 ppm in polluted cities. Due to its unstable structure, O3 can exists in several different states (liquid or solid) and its toxic effect is mainly due to its strong oxidant ability which will be discussed more in deep in the following chapters. Indeed O_3 is able to initiate free radicals reaction by interacting with several biomolecules leading to the production of ROS, such as hydroxyl radical (HO-), superoxide anion (O2-) and Hydrogen peroxide (H2O2) or nonradical species such as aldehydes (4-hydroxy-nonenal, 4HNE). The oxidation of biomolecules, such as lipids, proteins or DNA, induced by O_3, cause the alteration of their structure and function [46,47] . It has been shown that actually the main target of O_3 is the cell membrane since it is enriched of lipids and proteins. For instance O_3 would not be able to penetrate the cells but it would interact with surfactant's polyunsaturated fatty acids (PUFA), leading to lipid peroxidation, and with amino acids, especially proline, histidine, cysteine, methionine and aromatic groups, altering their structure in an irreversible way. The harmful effect of tropospheric O_3 has been long investigated in the past decades. Indeed the anthropogenic emissions have led to an increase in tropospheric O_3 concentration (more than 1000 ppb) which in return has been associated to several respiratory system diseases, such as asthma, COPD, lung cancer but also heart diseases, inflammatory conditions and with the exacerbating of skin inflammatory

pathologies [45,48–50]. Since O_3 is a potent oxidizing gas able to induce an oxidative damage to the cells, it has been associated with several pathologies of the central nervous system in which oxidative stress plays an important role, such as Alxheimer's and Parkinson's diseases [51 51 52 53]. Moreover the O_3-induced oxidative damage has been shown to lead to an immune-inflammatory response within lungs and skin. For instance several studies reported high levels of IL-1beta, TNF-alpha, IL-6, and IL-8 in macrophages and epithelial cells of the lungs in response to O_3 exposure [54–56] and higher levels of inflammatory markers in human and mice skin [57,58]. Nevertheless, several studies have demonstrated that the additions of antioxidant compounds such as Vitamin E and C are able to prevent the damage induced by O_3, underlying the strictly link with oxidative stress [59,60].

1.1.2 *The cutaneous tissue and its structure*

The skin is the main barrier of our body against the external environment and it is therefore one of the main pollutants- targeted organs. It is the largest organs of our body covering an area of 1.7 m^2 and it weighs about 15% of the total body weight. Since the skin is the first organ in contact with the external environment, its main function is to protect us against an array of different chemical and infectious stimuli. Due to this function, the skin structure is very complex and well-organized and it consists of three main layers as shown in Figure 1.

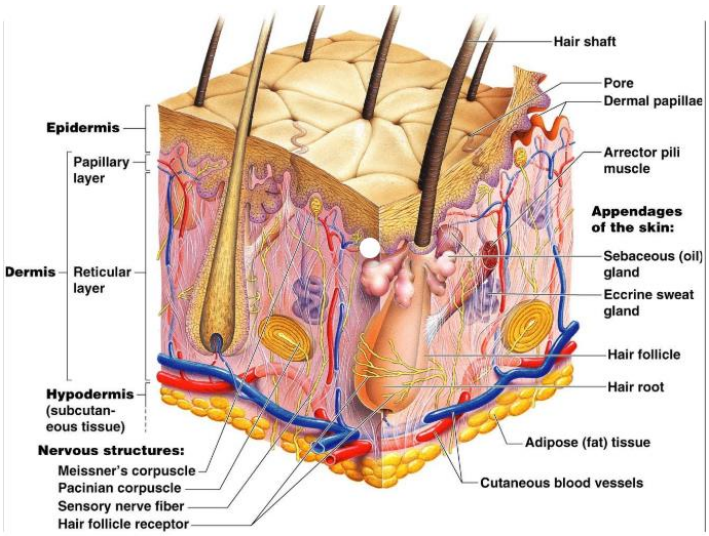


Figure 1. Skin structure: Epidermis, Dermis and the Hypodermis.

Beside the keratinocytes which compose the different epidermis layers that act as a physical barrier against the external insults, the skin displays also nervous structures able to sense the stimuli, immune cells, engaged for the inflammatory response against microorganisms infections and appendages such as hair follicles, sebaceous glands, nails etc.. to protect the skin itself. Moreover, the skin can display a different thickness, distribution of the appendages and pigmentation all over the body, depending upon the function and the needs of each area. The innermost skin layer is the Subcutaneous Tissue or Hypodermis, a layer of fat present just beneath the skin, under the dermis.

It is composed by adipocytes grouped in lobules which are separated by connective tissue and it also displays nerves and blood vessels. The main function of this layer is to act as an energy reserve and also to provide thermoregulation and body protection against a body trauma. Depending on the nutritional state of the body, the subcutaneous fat can vary its size and the collagen forming the connective tissue is continuous with the collagen of the dermis. Dermis is the fibrous layer of the skin, located between the subcutaneous fat and the epidermis. It consists of collagen fibers, elastic fibers and extrafibrillar matrix which confer elasticity and tensile strength to the skin, but also of ground substance (glucosaminoglycans), appendages, fibroblasts, dermal dendrocytes (dendritic cells with an immune function), mast cells, histiocytes, blood vessels, nerves, and lymphatics. The

dermis consists in an upper part, the papillary dermis and a lower part, the reticular dermis. Both the compartments present collagen fibers (collagen fibers 1 and 3) which confer the mechanical support to the skin. In the papillary dermis these collagen fibers interact with the epidermal rete ridges and they are thinner and loosely arranged, compared to the fibers of the reticular epidermis, which are thick and coarse. The elasticity of the skin is mainly due to the presence of elastic fibers within the dermis and usually the damage of this fibers by UV rays is responsible for the formation of wrinkles during skin aging [61]. Moreover the collagen and the elastic tissues is supported by the ground substance which also helps in the passage of hormones, nutrients through the dermis, the removal of waste products and it is able to retain the water In the reticular dermis are present the roots of the hair, sebaceous glands, receptors and also blood vessels. The blood vessels provide the nutriment for both dermis and epidermis and help in maintaining the temperature of the body. The nervous fibers present within the skin, such as the myelinated fibers, are able to provide the cutaneous sensation and can be divided in mechanoreceptors, such as Pacinian and Ruffini corpuscles, and thermoreceptors, which provide the sense of pain, pressure, heat, cold etc.. and therefore preventing our body from injury. Moreover the appendages present in the dermis, such as hair follicles, sweat glands, sebaceous glands (oil glands), apocrine glands are all derived from the epidermis and they display a protective activity for the skin, such as maintaining body temperature, moisturizing skin, protect from UV lights and excrete waste products. Since the main function of the dermis is to support the epidermis, in the papillary dermis can be found the so called dermal papillae (DP) which are extension of the dermis into the epidermis. Their role is to increase the surface area between dermis and epidermis, preventing their separation, and leading to a more functional exchange of nutrients, oxygen etc.. between the two layers [62,63]. The epidermis represents the first barrier of the skin and its main activities are related to its ability in be either a physical, a chemical / biochemical (antimicrobial, innate immunity) and an adaptive immunological barrier (Fig 2) [64]. The epidermis consists mainly of Keratinocytes (95%) whereas the left part is represented by melanocytes, Langerhans cells, and Merkle's cells. it obtains its nutrients from the blood vessels of the dermis diffusing through the dermo-epidermal junction. Keratinocytes are

arranged in several layers depending upon their differentiation phase and they produce keratin, which form the internal skeleton of the skin.

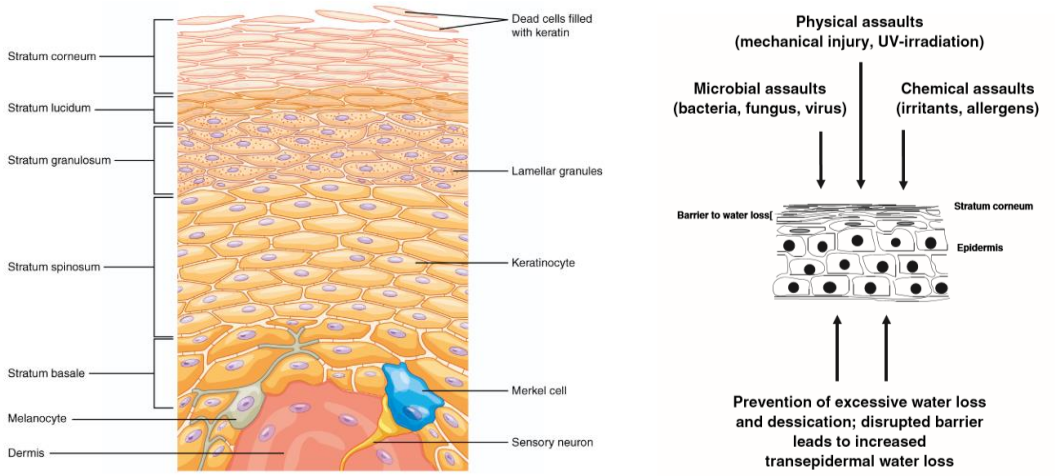


Figure 2. Skin structure

The innermost layer the *Basal layer (stratum germinatum)*, is mainly composed by basal cells which are the precursor of keratinocytes and they continuously undergo mitosis, producing new cells. Basal cells directly interact with the basal lamina through hemidesmosomes and therefore they allow the epidermis to be attached to the dermis.
Besides the basal cells, the basal layer present also other type of cells, such as Markel's cells which allow the perception of stimuli by stimulating the sensory nerves and also melanocytes which produce melanin, the pigment that confers color to the skin. Melanin is also essential for the skin protection against UV light [65].
Right after the basal layer, there is the *stratum spinosum* which is composed by 8-10 layers of keratinocytes resulting from the cell division of the basal cells. Here the keratinocytes interact to each other via a structure called a desmosome that strengthen the bond between the cells. Moreover, in this layer, the keratinocytes start to produce keratin and a glycolipids able to prevent water loss. Within the stratum spinosum there is a type of dendritic cell called the Langerhans cell. These cells are able to engulf bacteria, particles and damaged cells by acting as macrophages and therefore defending the skin.
Keratinocytes are then pushed from the stratum spinosum into the following layer, the *stratum granulosum,* where they are arranged in 3-5 layers. The grainy appearance of

this layer is due to the presence of lamellar granules, resulting by the production of great amounts of keratin and keratohyalin from keratinocytes. During the final steps of differentiation, keratinocytes undergo profound changes in their structure, losing their nucleus and cytosolic organelles and therefore becoming corneocytes, which compose the 15-30 layers of the *stratum corneum* (SC), the most external layer of the skin. The stratum corneum is enriched in keratin filaments, proteins and lipids which respectively form a cross-linked proteins envelope and a covalently bound lipid envelope (hydrophobic matrix), forming a protein/lipid polymer structure called Cornified cell envelope (CE) that surround corneocytes [66]. This envelope confers impermeability to the skin and also its biochemical properties. The entire keratinocytes differentiation process takes a long time, for a total of 52-72 days. Cells from the basal layer need about 26–42 days to migrate to the top of the granular layer, and then 13–14 days to cross the stratum corneum. Moreover the SC layer is replaced during a period of about 4 weeks.

I. Skin components and function

The complex structure of the skin and the presence of several different components, all defined with specific roles, allow the skin to be either a physical and an antimicrobial/ adaptive immunological barrier and a connection with the external stimuli. The presence of receptors such as Merkel cells, Ruffini corpuscles, nociceptors and thermoreceptors allow the skin to sense the stimuli and transfer them to the central nervous system [67,68]. Moreover the skin contain several elements of the innate and adaptive immune system such as Langerhans cells in epidermis and dendritic cells, T cells, B cells, Natural killer (NK cells), Mast cells within the dermis, which are able to sense antigens and therefore protect from infection [69,70]. The activation of the immune response leads to the production of pro-inflammatory mediators (TNF-α), cytokines such as Interleukins (IL-1beta, Il.18, Il6 etc.) and the dysregulation of these mechanisms is associated to the development of several skin inflammatory diseases [71]. Moreover, skin microbiota plays an important role in tissue homeostasis and help in the regulation of the inflammatory response. For instance Antimicrobial peptides (AMPs) such as Cathelicidins, alarmins etc., are involved in the first defense of the skin and their aberrant expression is linked to the development

of inflammatory skin diseases (atopic dermatitis, psoriasis) and to microbial infections susceptibility [72,73].

Another function of the skin is to protect from the radiation of the sun and this property is due to the presence of melanocytes within the basal layer of the skin. In response to UV rays. Melanocytes are stimulated to produce the pigment melanin which is able to absorb UV radiation and therefore protect the skin. Indeed UV radiation are known to be dangerous for the skin health, and excessive exposure to the sun is associated to the development of melanoma, skin aging etc. [6]. The physical barrier property of the skin is mainly due to the presence of the cornified envelope (CE) within the SC. Indeed, this complex structure confers biochemical properties to the skin by preventing water loss and the entrance of noxious compounds and conferring the skin strength.

CE is mainly composed by proteins, lipids and keratin filaments that surround corneocytes (Fig. 3).

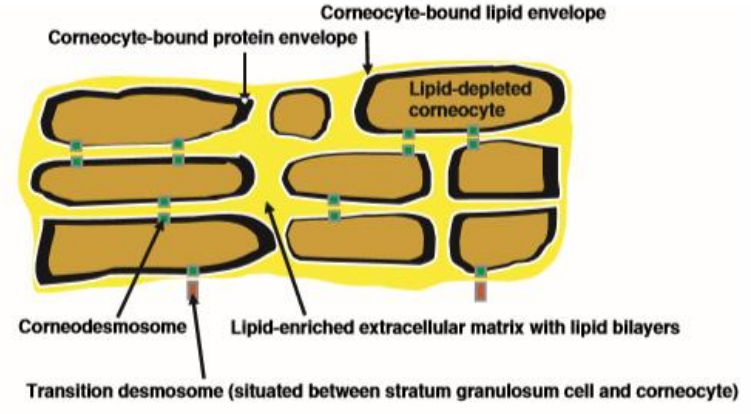


Figure 3. Cornified envelope (CE)

The keratin filaments are aligned and ordered into tight bundles thank to interactions with the matrix protein filaggrin. Filaggrin is essential for the regulation of epidermal homeostasis and its aberrant expression has been associated to the development of skin pathologies such as ichthyosis vulgaris and atopic dermatitis, both in human [74–76] and mice [77]. The presence of this protein with the SC makes Filaggrin a marker of terminal keratinocytes differentiation. The formation of the protein envelope is mainly due to the activity of enzymes such as transglutaminase (TG 1,3,5) which catalyze the covalent cross-linkage between the main proteins composing the envelope, such as Involucrin, Loricrin, trichohyalin and the class of small proline-rich proteins [78,79]. Transglutaminases

and these proteins are usually used as marker of epidermal differentiation and they are good hallmarks for better understand the status of the skin [80–82]. For instance both Involucrin and loricrin are markers of terminal keratinocytes differentiation and altered distribution and expression of these proteins have been associated to several keratinization disorders and an impairment in barrier function [83–86]. Involucrin is synthesized by human squamous epithelial cells and is mainly present in the upper spinous layer and granular layer [87], whereas Loricrin is normally crosslinked to Involucrin and present within the granules of the stratum granulosum. Loricrin can also interact with keratin filaments, providing flexibility to CE and it protects against mechanical stress [88]. Keratins represent the 30-80% of the total protein and they are involved in the formation of the epidermis cytoskeleton, conferring structural resistance against mechanical trauma. There are 54 types of keratins in humans and, within the skin, they are crucial for keratinocytes proliferation and differentiation processes. For instance, K14/5 are mainly expressed in the basal layer during the stratification phase leading to the formation of the suprabasal cells whereas K1/10 are the main keratins involved in the early keratinocytes differentiation within the spinous/suprabasal layer. When keratinocytes differentiate into the granular layer, K1/10 will be then replaced by late/terminal differentiation markers [89] Alteration in Keratins function and expression are known to lead to several skin pathologies, such as Epidermolysis bullosa simplex, psoriasis, hyperkeratosis, inflammation and also to impaired wound healing [90–94]. The SC lipid composition and organization are fundamental for prevent excessive water loss through the epidermis and avoid the entrance of noxious compounds into the epidermis [95]. The main lipids present within the CE envelope can be divided in sebaceous lipids, such as primarily non polar lipids (triglycerides, wax esters and squalene) and epidermal lipids which are represented by ceramides, free fatty acids and cholesterol [96,97]. All These lipids can interact with the cornified envelope proteins to improve the skin barrier function; for instance, Ceramides A and B are covalently bound mainly to Involucrin.

The impairment of SC lipid envelope has been associated to altered skin barrier function and also to a more susceptibility in bacterial infection, impaired wound healing and skin pathologies[98].To support the tightness of the skin there also other important components, such as desmosomes, Tight Junction (TJs) and gap junctions, which mediate the cell-cell

interactions within the different cutaneous epidermal layers. Desmosomes are network of linker proteins, keratin intermediate filaments and cadherin proteins, such as desmocollin and desmoglein, which provide adhesion between the cells. They can also be involved in the regulation of the availability of signalling molecules or in several processes such as proliferation, differentiation and morphogenesis [99]. When They are present on corneocytes, they can be called corneodesmosomes, whereas when they connect the keratinocytes of the granular layer to the corneocytes of the SC transition desmosomes. TJs, such as occluding, claudin-1, ZO-1, are normally present within the interfollicular epidermis as well as in the skin appendages [100] . Different skin diseases present altered expression of TJs, confirming their role in skin burrier function [101,102].

1.1.3 Molecular mechanisms involved in pollution toxicity in skin tissue

The skin, due to its composition, has been identified as one of the main target tissue for environmental pollutants. The effect of pollutants on the cutaneous tissues mainly results in the induction of an oxidative stress/inflammatory status[103], which is even more exacerbated when pollutants act synergistically [104,105]. The pollutants mechanism of action can vary based on their chemical and physical properties, which allow them to penetrate the skin layers or not. For instance O_3 is not able to penetrate the skin but, even though it is not a radical species per se, it can interact with biomolecules present within the stratum corneum, such as proteins and lipids, leading to oxidative reactions that result in Reactive oxygen species (ROS) and aldehydes production such as 4-hydroxy-2-nonenal (4HNE) [97,106–109] . These secondary mediators can perpetuate the pollutant damage throughout the cutaneous tissues by reaching live cells such as keratinocytes, fibroblasts etc. and inducing the activation of pro-oxidative and pro-inflammatory pathways, such as NRF2 ,NF-kb, and heat shock proteins (HSPs), which regulate the cutaneous antioxidant and inflammatory response [58,110–112]. Moreover 4HNE is able to interact with proteins leading to the formation of HNE protein adducts (PAs) [113,114], resulting in modification of protein conformation and thereby into the alteration of their function which can lead to several metabolic, autoinflammatory and neurological diseases or even to cell death [115–118]. Also other pollutants such as PM and cigarette smoke can alter skin redox homeostasis but, despite O_3, some of their components such

as PAHs can penetrate the skin by moving transdermally or through air follicles. For instance PM PHAs can be absorbed through the skin and trigger the production of ROS and lipid peroxidation (4HNE), leading to apoptosis, DNA and mitochondria damage, activation of pro-inflammatory pathways such as NF-kb, AP1 and the antioxidant response (NRF2)[19,119,120]. Moreover water-soluble PHAs of cigarette smoke have been shown to induce and oxidative imbalance and increased NADPH oxidase activity within the skin [36,121] and also to modulate the activation of Metalloproteinase (MMPs) in the connective tissues, which are essential for tissue remodeling and whom dysregulation has been associated to skin aging [122]. Also Oxides of nitrogen display an oxidizing effect in skin tissue[123] and their effect on human skin are strictly related to ultrafine PM and black carbon since they are all emitted during traffic and industries emission [124,125]. The pollutants-induced oxidative damage and inflammation has been shown to lead to the depletion of the antioxidant defense of the skin (tocopherol, ascorbate, GSH etc.) [126–129], which can be restored by the application of antioxidant compounds [60,111,130–132] and to lead to the alteration of the skin barrier function , contributing to the extrinsic skin aging [21,133,134]. Indeed pollutants have been shown to be involved in the skin aging process mainly activating the transcription factor Aryl hydrocarbon receptor (Ahr). Ahr is able to sense several ligands such as aromatic hydrocarbons and its activation can modulate the antioxidant/oxidant response of our body. For instance Ahr regulates the transcription of genes encoding for phase I and II xenobiotic metabolizing enzymes (CYP1A1, CYP1A2, and CYP1B1), which are involved in the detoxification of polycyclic aromatic compounds, and also the activation of antioxidant pathways such as NRF2. The activation of the antioxidant response regulates the transcription of some detoxifying genes such as the glutathione-S-transferases (GSTs), NADPH/quinone oxidoreductase (NQO1), ,aldehyde dehydrogenase 3 able to prevent the oxidative damage [135,136]. However Ahr can act as a master switch for oxidation and antioxidation depending on the ligand. For instance the modulation of the Ahr receptor by pollutants, such as PAHs in PM , but also UV, has been shown to mediate the upregulation of CYP1 enzymes whom overexpression is known to generate mutagenic metabolites and reactive oxygen species (ROS) [137] [138] . ROS production can then lead to DNA damage and the activation of Inflammatory pathways such as Nf-kB, resulting in inflammatory cytokines release and the development

of an inflammatory status, activating the immune response. The ox-inflammatory response induced by pollutants via Ahr activation can result in the exacerbation of skin pathologies such as acne or atopic dermatitis [139–141] and also skin aging, carcinogenesis and skin barrier impairment[139,142–149] , which are all conditions already related to UV exposure. Indeed Ahr is largely expressed in all cutaneous subpopulation and is involved in several skin functions such as epidermal barrier function, keratinocyte differentiation and also melanogenesis [150,151]. Therefore its modulation is essential for skin homeostasis. Of note also O_3 has been shown to modulate the activation of Ahr receptor 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 [145,152]. Thus, it is not a surprise that both inflammation and oxidative stress are displayed in several skin conditions [153–155], and that the cross-talk between these two conditions results in skin inflammaging [156–160].

I. OxInflammation phenomena: the cross-talk between inflammation and oxidative stress

As previously described, the harmful effect of Environmental pollutants on human skin are mainly due to their ability to trigger Inflammatory and oxidative stress reactions within the cutaneous tissues, by mainly affecting skin lipids and proteins, and therefore altering skin functionality and homeostasis [103]. Indeed Inflammation and oxidative stress are the most important responses of our body against dangerous stimuli and they are displayed in several pathologies included skin pathologies. Interestingly these two processes constantly affect each other and the prolonged crosstalk between pro-oxidative and inflammatory mediators can lead to a local/systemic damage that is now referred to as OxInflammation phenomena [1]. For instance Inflammation is the primary response of our body against a variety of noxious stimuli able to alter the tissue homeostasis and it is therefore an essential mechanism to eliminate the injury or infectious insults [161,162]. The innate immune system is the key regulator of the inflammatory response and it relies on particular sensors expressed on immune cells, called Pattern Recognition receptors (PRRs), able to sense endogenous molecules such as Pathogen-associated molecular

patterns (PAMPs) belonging to infectious pathogens or danger associated molecular patterns (DAMPs) released by dead cells [163]. Normally the activation of these receptors, such as Toll-like receptors (TLRs), retinoic acid-inducible gene (RIG)-I-like receptors (RLRs), or NOD-like receptors (NLRs), induce the release of several inflammatory mediators (cytokines IL-1beta, IL-18, TNF-α, INF-γ etc..) upon the activation of transcription factors such as nuclear factor κ B (NF-κB), activator protein-1 (AP-1) and signal transducer and activator of transcription-3 (STAT3) [164]. The inflammatory mediators are then able to induce the recruitment and the infiltration of immune cells (leukocytes) to the infection sites, which can help in resolve the acute inflammatory process by repairing the tissue damage and restoring the tissue homeostasis. During the inflammatory process Reactive oxygen species (ROS), such as hydroxyl radical (HO•) , superoxide anion (O_2-) and hydrogen peroxide (H_2O_2), and reactive nitrogen species (RNS) can also be released together with inflammatory mediators either by leukocytes themselves, to potentiate the recruitment of macrophages and solve the injury, or by the activity of lipoxygenases, cyclooxygenases, xanthine oxidase or NADPH oxidases (NOXs) normally induced by stressors [165,166]. Indeed ROS, which are normally generated during the metabolism of oxygen in mitochondria or in response to infectious pathogens or cytokines, are key mediators of cellular signaling pathways involved in proliferation (MAPK kinases), apoptosis, DNA damage, oxidant genes transcription (NRF2), aging, and their metabolism is normally under the control of several detoxifying enzymes [167,168]. However when there is an imbalance between ROS production and the antioxidant response, an excessive amount of reactive species can occur, leading to an altered redox homeostasis called oxidative stress [169], often associated to several pathologies [170,171]. For instance during a prolonged and unresolved inflammation process defined as chronic inflammation and displayed in a variety of inflammatory pathologies [172], the continuous production of reactive oxygen species can also occur. In this scenario, antioxidants enzymes, such as Glutathione peroxidase (GPx), Catalase (CAT), Superoxide dismutase (SOD), which normally act as scavenger of ROS under the control of antioxidant pathways [173], are no more able to cope the excessive ROS production resulting in oxidative stress [169]. Vice versa the perturbation of the oxidative stress equilibrium induced by noxious stimuli can lead to a prolonged inflammatory response within the damaged

tissue, leading to the development of inflammatory conditions. Indeed the continuous crosstalk between oxidative and inflammatory mediators is nowadays a common figure displayed in several pathologies [1,174,175] and the interplay between important regulatory pathways, such as NRF2 for the antioxidant response and NF-kb for inflammation, is now well documented [176–179]. For instance ROS have been shown to modulate the activity of NF-kB pathway, whom dysregulation results in an impaired ability in the transcription of the main inflammatory mediators leading to the exacerbation of several autoinflammatory diseases [180–182], as well for the transcription factor Aryl Hydrocarbon receptor which is known to regulate both the antioxidant and inflammatory response of our organism [136,183–185]. Moreover, both inflammation and oxidative stress are common figure displayed in several skin conditions [153–155] and their cross-talk results in the inflammaging process which is involved in several age-related diseases, in skin pathologies correlated to pollutants exposure and also in the exacerbation of the skin aging process [156–160].

2. *GENERAL RATIONAL AND AIM*

According to the World Health Organization (WHO), exposure to air pollution leads to approximately 4.2 million deaths each year (https:// www.who.int/airpollution/en/) and impacts the function of multiple organs, including the heart, lungs, gut, and brain [186–189]. However, recent literature has shown that cutaneous tissue is also susceptible to pollution. Indeed, development and exacerbation of a variety of skin conditions such as premature aging, psoriasis, acne, eczema, and atopic dermatitis are now linked to pollution exposure [4,5,8,190,191]. Several reports have highlighted the mechanisms of action of single pollutants/stressors, which all induce oxidative and inflammatory reactions [37,105,119,192,193]. In particular exposure of skin to UV light has been identified as the main risk factor contributing to the so-called “extrinsic skin aging” (photoaging) [193]. While UVB typically comprises less than 10% of UVR, it is a high energy component that is mainly absorbed by epidermal cells, while UVA, the more prevalent component, is weaker but penetrates into the dermis [194]. UVR absorbed from solar radiation can induce extensive skin damage through a variety of mechanisms, ranging from direct DNA damage to those resulting from oxidative stress caused by UVR-induced reactive oxygen species (ROS). When UVR-induced ROS overwhelms the skin's natural defensive mechanisms, this results in DNA damage, lipid and protein peroxidation, immune dysregulation and inflammatory reactions. In addition, it has now been accepted that other pollutants, such as O_3 and PM (PM), can play key roles in pollution-induced skin damage and possibly exacerbate UV-induced skin conditions [5,8]. However very few study so far have investigated the possible synergistic harmful effects of environmental pollutants on skin tissue damage. For instance it has been shown that UV can act synergistically with PM by exciting polycyclic aromatic hydrocarbons (PAHs) in the core structure of particulates [105,195] and with O_3 by increasing tissue peroxidation and decreasing cutaneous a-tocopherol [127]. Indeed pollutants have been shown to disrupt the skin antioxidant defense system by affecting the main antioxidant micronutrients present within the skin such as Vitamin E and C and also to affect the expression of endogenous defensive enzymes such as Superoxide dismutase (SOD), Catalase (CAT) or Glutathione peroxidase (GPx) [196]. All these events induced by pollutants exposure, result in an ox-inflammatory condition of the skin tissue which can then modulate the integrity and the functionality of

the cutaneous tissue, by affecting the main skin barrier components and leading to the onset of skin pathologies. Indeed the interconnection between oxidative and inflammatory markers has now been accepted to represent one of the main event in the development of several pathologies [1]. Besides the canonical inflammatory pathway activation, fairly recently a new inflammatory mechanism has been discovered. In 2002, *Martinon et al*. identified a new multiprotein signaling platform, called Inflammasomes, involved in the activation of proinflammatory caspase cleavage and the consequent productions of inflammatory mediators such as cytokines interleukin-1β (IL-1β) and IL18, that will evoke an inflammatory status[197–200] . Several studies so far have correlated Inflammasomes activation to several inflammatory pathologies such us neurodegenerative diseases (Alzheimer's disease (AD), Parkinson's disease (PD), atherosclerosis, type 2 diabetes[201–203], autoinflammatory diseases (arthritis and dyskeratosis) [204] and also skin diseases [205,206]. Indeed, although inflammasomes are mainly expressed in immune cells, NLRP1 and NLRP3 are the main complexes present within the skin and they have been shown to be involved in autoinflammatory skin diseases such as vitiligo, atopic dermatitis, psoriasis, acne, and carcinogenesis[206–208]. Of note, reactive oxygen species (ROS) seem to have great relevance in regulating the inflammasome activity, playing a dual role as a trigger and effector in the activation of the complex [209], and air pollutants, including O_3 are known to generate free radicals and promote inflammation[197]. However the role of pollutants in induce inflammasomes activation within skin is still an unexplored field.

The purpose of the present study was to investigate the role of pollutants in skin OxInflammation and a possible mechanism behind this phenomena, mainly assessing analysis of western blotting, rt-PCR, Immunofluorescence, Immunoprecipitation and ELISA assays. Therefore two different projects have been conducted in parallel:

- *The first project focuses on the investigation of the possible involvement of the Inflammasome pathway NLRP1 in the pollutants- Induced skin damage.* Therefore we evaluated the activation of the NLRP1 inflammasome within the skin, in response to one of the main toxic environmental pollutants, such as O_3, and the regulatory mechanism mediated by the pollutant behind NLRP1 triggering. In order to conduct this study, several skin models have been used, ranging from 2D *in*

vitro model to RHE 3D models (Reconstructed human epidermis) and *ex vivo* human skin explants.

- *The second project investigates the role of pollutants (UV, O_3 and Diesel Exhaust), alone or in combination, in induce an ox-inflammatory status within human skin, which is a common figure displayed in several skin pathologies.* For this purpose human skin explants have been used to investigate the cross-talk between oxidative and inflammatory markers in response to a short and long terms pollutants exposure and whether the pollutants combination could display a synergistic effect in modulating the skin damage. Moreover a topical application of an antioxidant cosmeceutical compound (CF Mix) containing 15% vitamin C (L-ascorbic acid), 1% vitamin E (α-tocopherol), and 0.5% ferulic acid has been used as protective agent against the pollutants-induced skin tissue damage, alone or in combination with the antioxidant Iron chelator Deferoxamine (DFO).

3. *GENERAL MATERIALS AND METHODS*

3.1 Culture of human skin models

3.1.1 Cell culture

HaCaT cells were cultured in high glucose Dulbecco's Modified Eagle's Medium (Corning, USA) supplemented with 10% FBS (Sigma, USA), 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco, USA). All cell cultures were performed at 37°C in 5% CO_2 and 95% air. For all experiments, keratinocytes were grown in 6 cm^2 petri dishes (Corning, USA) at a density of $1.5x10^6$ cells in 3 ml of media.

3.1.2 Culture of EpiDerm 3D skin models

EpiDerm skin model samples were obtained from MatTek corporation (EpiDerm, EPI-200). Briefly, under sterile conditions and using sterile forceps, 24 inserts containing tissues were transferred into 6-well plates pre-filled with 1 ml of MatTek Assay medium, according to the manufacturer's instructions. The plates were placed in the incubator overnight (5% CO2, 37 °C) for recovery.

3.1.3 Collection and culture of E*x vivo* human biopsies

Healthy human skin was obtained from elective abdominoplasties, as approved by the IBC at NC State University, USA. After removing the subcutaneous fat, 12mm punch biopsies were obtained and rinsed with PBS containing antibiotics/antimycotic. Next, the human biopsies were immediately transferred to 6-well dishes and cultured in DMEM containing 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin (Gibco) at 37°C in 5% CO_2 humidified atmosphere.

3.2 Immunocytochemistry

HaCaT cells were grown on coverslips at a density of $1x10^5$ cells/ml. Cells were fixed in 4% paraformaldehyde (PFA) in PBS for 30 min at 4°C and then permeabilized with 0.25%

of Triton X-100 in PBS and then blocked in PBS containing 1% BSA at room temperature for 1 h. Coverslips were then incubated with primary antibodies overnight at 4°C. The day after, samples were incubated 1 hour at RT with fluorochrome-conjugated secondary antibodies and Nuclei were stained with DAPI (D1306 Invitrogen, USA) after removal of secondary antibody. Coverslips were mounted onto glass slides using PermaFluor Aqueous Mounting Medium (TA-006-FM ThermoFisher Scientific), and examined using a Zeiss Z1 AxioObserver LSM10 confocal microscope equipped at 40x and 60x magnification. Images were quantified using ImageJ [210].

3.3 Skin tissues collection and paraffin embedding

Skin tissues were collected for each timepoints by rinsing them in fresh PBS for 3 times. The samples for protein and RNA extraction were frozen in nitrogen for few seconds and then stored at -80°C for future extractions, whereas the histology samples were placed in cassettes and fixed in Formalin for 48 hours at 4°C.

After 2 days, the samples were dehydrated in a series of Alchol gradients and after xylene, they were left in paraffin overnight. The day after the tissues were embebbed in paraffin and the paraffin blocks were stored at 4°C. 4 µm paraffin sections of human skin biopsies were cut using a microtome (Leyca, USA) and left drying for 24 hours at RT before continue with the histological analysis.

3.4 Immunohistochemistry

Paraffin sections of human skin biopsies (4 µm) were deparaffinized in xylene and rehydrated in decreasing alcohol gradients. Antigen retrieval was achieved using heat-based epitope retrieval with 10 mM sodium citrate buffer (AP-9003-500, Thermo Fisher Scientific, USA) (pH 6.0) at a sub-boiling temperature in a 500 watt microwave for 10 min. After cooling for 20 min, sections were washed 2 times for 5 min in PBS, blocked with 5% BSA in PBS at RT for 45 min, and incubated overnight at 4°C with primary antibodies. The next day, sections were washed 3 times in PBS for 5 min, followed by a 1 hr incubation with fluorochrome-conjugated secondary antibodies at RT and then washed

with PBS 3 times for 5 min. Nuclei were stained with DAPI (D1306, Invitrogen) for 1 min in PBS at RT, and sections were then washed with PBS. The sections were 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 [211].

3.5 Protein extraction

Cell lysates were extracted in ice-cold buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl, 10% glycerol, 1% Nonidet P-40, 1 mM EDTA, 0.1% SDS, 5 mM N-ethylmaleamide (Sigma) and protease and phosphatase inhibitor cocktails (Sigma). Lysates were cleared by centrifugation (12700 rpm) for 15 min at 4°C, and protein concentration was measured by Bradford method (BioRad, USA). For 3D skin models and ex vivo human skin biopsies, the tissues were harvested in T-PER™ Tissue Protein Extraction Reagent (Thermo Fisher Scientific, USA) with 1% of protease and phosphatase inhibitor cocktails (Sigma, USA). Once 3D skin model were subjected to 3 cycles freezing/thawing by moving from liquid nitrogen to 37°C, and centrifuged at 12'700 g for 15 min at 4 °C, the supernatant was collected. Instead, *Ex vivo* human skin biopsies were 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. Protein content for all lysates was measured using the Quick start Bradford protein assay (Biorad, USA).

3.6 Western blotting

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 1hr 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 and Then incubated with horseradish peroxidase conjugated secondary antibodies for 90 min at RT. 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.

4. PROJECT 1: O_3 AS A NEW TRIGGER FOR INFLAMMASOME ACTIVATION IN CUTANEOUS TISSUES

4.1 INTRODUCTION

4.1.1 O_3 and its formation

Among the outdoor pollutants to which living organisms are daily exposed to, tropospheric O_3 is one of the most toxic, having not only a great impact on climate changes but also on human health [45,212]. O_3 or trioxygen, is an unstable blue gas with a pungent smell (resembling the chlorine bleach) and it is already perceptible at a concentration of 0.01 ppb but it can reach the concentration of 0.8 ppm in polluted cities.
It is composed by three oxygen atoms, for a final molecular weight of 48 kDa and it has a very short half-life, resulting in its ability to fall back into its original form, following the reaction:

$$2O_3 \rightarrow 3O_2$$

Due to its unstable structure, O_3 can exists in several different states (liquid or solid) which are in equilibrium one with another. It is the result of the addiction of an oxygen atom to an oxygen molecule and this reaction is promoted by the presence of an electric high voltage. For instance, O_3 is normally present within the atmosphere where it arises from photochemical reactions that require the interaction between an oxygen molecule and an energy source, such as UV light.
Depending in which layer of the atmosphere O_3 is present, it can display a dual role and be considered “good” (Stratospheric O_3) or “bad” (Tropospheric O_3). Indeed, within the Stratosphere (10-50 km from the earth surface), O_3 forms a filtering layer called O_3 layer, which act as a barrier against the radiation of the sun, protecting us from DNA damage and skin cancer. Here most of the oxygen is able to absorb short-wave ultraviolet rays between 240 and 160 nm, converting the UV radiations into heat and leading to O_3 formation. This continuous O_3-oxygen cycle within the stratosphere is called Chapman

cycle and is fundamental for the O_3 layer formation. The first step of this process is the creation of two oxygen atoms resulting in the interactions between an oxygen molecule and UV rays with wavelength less than 240 nm**.** Then, the formed Oxygen atoms can combine with an oxygen molecule leading to the formation of O_3 **(1).** The O3 molecule is now able to interacts with UV rays with an higher wavelengths (240- 310 nm). This reaction leads to O_3 photolysis and to the formation of an oxygen molecule and an oxygen atom. These products can now react again with other oxygen atoms and molecules to form O_3 and extra kinetic energy (E_k) **(2).** The final phase, the removal, promotes the formation of oxygen molecules by the interaction between the O_3 molecule with an oxygen atom **(3).**

1) **Creation**

$$O_2 + h\nu\ (<240\ nm) \rightarrow 2\ O\bullet$$

$$O\bullet + O_2 \rightarrow O_3$$

2) **O_3-oxygen Cycle**

$$O_3 + h\nu\ (240\text{- }310\ nm) \rightarrow O_2 + O\bullet$$

$$O\bullet + O_2 \rightarrow O_3 + E_K$$

3) **Removal**

$$O_3 + O\bullet \rightarrow 2\ O_2$$

Normally within the stratosphere, the oxygen atoms and molecules produced after O_3 photolysis, can interact just with each other to keep a balance between O_3 generation and removal. This balance determines the total amount of O_3 within the stratosphere, allowing the formation of the protecting filtering layer against UV radiations. However with the industrialization of the 20th century, other organic molecules and compounds emitted from man-made activities and able to interact with O_3 were found in the stratosphere,

resulting in the depletion of the O_3 layer. Moreover the combination of these anthropogenic sources within the lowest atmosphere layer, the troposphere (10 km from the ground), led to the formation of the "bad" ground-level O_3 which is hazardous for the terrestrial health. Indeed the tropospheric O_3 results from the combination between different man-made emissions such as nitrogen oxide (NOx) and volatile organic compounds (VOCs) in presence of UV light, leading to the well-known photochemical smog.

$$VOCs + NOx \xrightarrow{sunlight} O_3$$

$$O_3 + VOCs + NOx \longrightarrow \text{Photochemical Smog}$$

NOx, CO and VOCs are considered O_3 precursors and are normally produced by Motor vehicle exhaust, chemical solvents and industrial emissions. The peculiarity of Nitric oxide (NOx) is that it is able to both form and destroy O_3. Indeed, it can react with UV radiations, leading to the formation of nitric oxide (NO) and an oxygen atom which can in turn react with a molecule of oxygen resulting in the formation of an O_3 molecule.

$$NO_2 \xrightarrow{\text{UV lights (<310nm)}} NO + O$$

$$O + O_2 \longrightarrow O_3$$

NO can also directly react with O_3 to form NO_2 and O_2

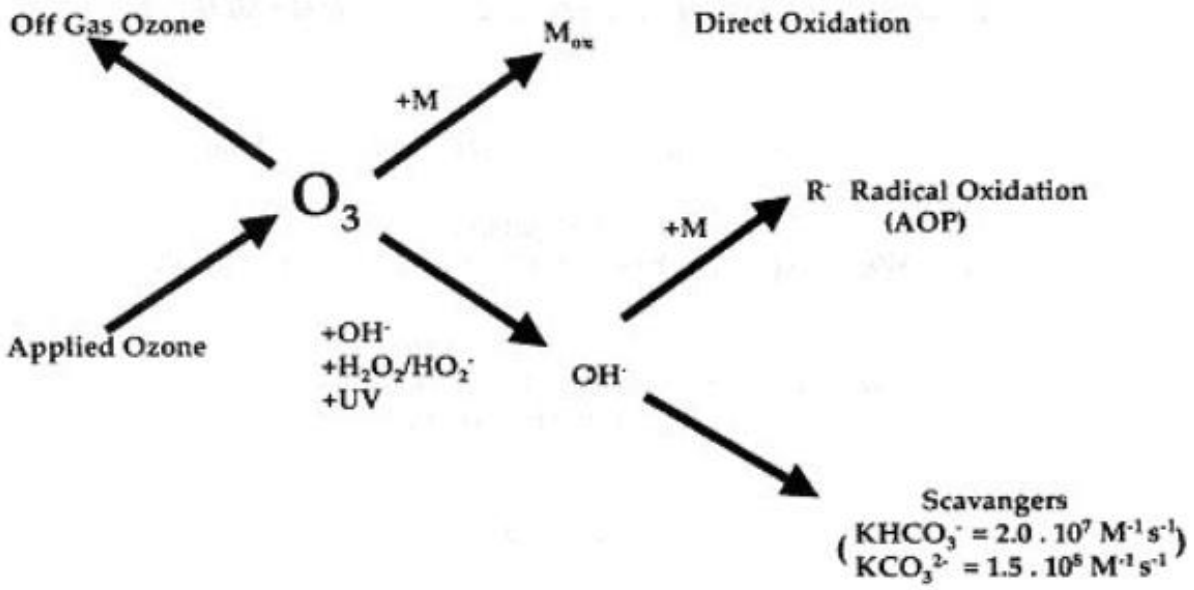
$$O_3 + NO \longrightarrow NO_2 + O_2$$

I. The O_3 reactivity

The harmful effects of O_3 on human health are mainly due to its unstable structure which make O_3 one of the strongest oxidation agents. Indeed, even though it is not a radical species per se, O_3 represents one of the major oxidants arises during photochemical smog, leading to direct and indirect oxidative reactions. (https://www.lenntech.com/library/ozone/reaction/ozone-reaction-mechanisms.htm).

Once formed, O_3 can decompose in water with the consequent formation of free radicals such as hydrogen peroxy (HO2) and hydroxyl radicals (OH-). These radicals have a great oxidizing capacity, even more than O_3 itself. The O_3 oxidation reactions can be divided in Direct reactions, which requires the direct interaction between O_3 and different compounds, and Indirect reactions, which instead require the presence of secondary oxidative agents such as the free OH-radicals.

Several factors such as temperature, chemical composition and pH of the water, will determine the type of reaction that will take place.



During direct reactions, O_3 is able to act as an electrophilic agent by reacting mainly with aromatic compounds containing -NH2 and OH groups in high electronic density solution,

as an neutrophilic agent reacting with carbons compounds containing -COOH and -NO_2 groups, or by undergoing to a dipolar Cyclo addiction with saturated compounds that lead to the formation of a compound called “ozonide”.
Despite the direct O_3 reactions, the indirect reactions are more complex and they require 3 steps: initiation, radical chain reaction and termination.

1) Normally an initiator can be an HO-radical but also Hydrogen peroxide (H_2O_2) and $Fe2^+$. In particular HO-radicals can interact with an O_3 molecule leading to the formation of $O2^-$ and $HO2^{\cdot}$.

$O_3 + OH^- \rightarrow O_2\bullet^- + HO_2\bullet$

$HO_2\bullet \rightarrow O_2\bullet^- + H^+$

2) During the radical chain reaction, HO-radicals are formed by the following reactions:

$O_3 + O_2\bullet^- \longrightarrow O_3\bullet^- + O_2$

$O_3\bullet^- + H^+ \longrightarrow HO_3\bullet$

3) The HO-radicals can now react with O_3 leading to the formation of $HO_2\bullet$ which can start all over again the reactions:

$OH\bullet + O_3 \longrightarrow HO_4\bullet$

$HO_4\bullet \longrightarrow O_2 + HO_2\bullet$

The radical chain reaction is maintained by substances called Promotors, such as Aryl-R, humic acid or primary and secondary alcohols, which are able to transform OH-radicals to superoxide radicals $O_2\bullet^-$

The harmful effect of tropospheric O_3 is therefore mainly due to its strong oxidant ability and its instability since It is able to initiate free radicals reaction altering structure and function of lipids, proteins and DNA [46,47,213,214]. As previously described in the general overview, the main tissues affected by O_3-induced oxidative stress are lungs, the nervous system but also skin, leading to several pathologies which also display an altered inflammatory response. The ability of O_3 to induce both oxidative and inflammatory responses (OxInflammation) in its target tissues is mainly regulated by different oxidative stress mediators whose role in skin homeostasis will be further investigated in the following chapters, .

4.1.2 O_3 mediators in skin homeostasis: hydrogen peroxide and 4-hydroxinonenal in O_3 induced- skin oxidative damage

The skin, along with the lungs, has been identified as one of the main target tissue for O_3. In particular, the stratum corneum, which is the first layer of the skin in contact with the external environment and its harmful pollutants, was identified as the main affected target, due to its structure and composition. As previously mentioned, O_3 is not able to penetrate the cells but it displays its oxidant activity by oxidizing components of the cell membrane, such as lipids and proteins, resulting in the production of reactive oxygen species, such as H_2O_2 able to then propagate the oxidative damage throughout the skin tissue. The production of ROS is strictly linked to another oxidative process called lipid peroxidation, which normally occurs in response to oxidative stress leading to the degradation of lipids. Indeed, free radicals such as HO-radicals, are able to interact with lipids present within the cell membrane, triggering a free radical chain reaction mechanism. Due to the presence of several double bonds and hydrogen atoms, polyunsaturated fatty acids are the main targets of lipid peroxidation and the chemical products of this process are called lipid peroxides or lipid oxidation products, such as 4-Hydroxy-Noneal and Malondialdehyde [107]. In 1994 Pryor et al for the first time, suggested the possibility that O_3 was able to interact with constituents of the surface of epithelial cells within the lungs, leading to radical bioproducts formation [215] and a similar mechanism was then hypothesized for the skin. Indeed, the presence in the outermost stratum of the skin of un-nucleated cells embedded within an intracellular matrix rich in lipids (free fatty acids,

triglycerides, cholesterol and ceramides), makes easy for O_3 to interact with the SC, triggering oxidative reactions with the consequent production of secondary oxidative messengers such as ROS and 4-Hydroxy-nonenal. Several studies have shown that actually O_3 is able to induce a depletion in the antioxidant defense of the skin (tocopherol, ascorbate, GSH etc.), but also trigger lipid peroxidation and protein oxidation both *in vivo* and *in vitro* [216,217]*.* Moreover O_3 has been found to be correlated to the alteration of the skin barrier function, contributing to the extrinsic skin aging [21]. Of note, even though O_3 is not able to penetrate the skin, its secondary products such as ROS and 4HNE, are able to reach the deeper layers of epidermis, propagating the oxidative damage throughout the whole skin. For instance, once they reach live cells such as keratinocytes, fibroblasts etc., they can induce several cellular responses to counteract the oxidative damage, resulting in the activation of prooxidative and proinflammatory pathways, such as NRF2 ,Nf-kB, and heat shock proteins (HSPs) [37,58,111,129,218,219]. Since both ROS and Lipid are able to modulate important defensive responses of our organism, in the past decades they have been considered important markers in the investigation of skin pathologies development. Indeed their activity leads to an oxidative-inflammatory status of the skin (OxInflammation) which is common in several skin pathologies, suggesting a role for O_3 in exacerbate the development of these skin conditions throughout its mediators [190].

I. Hydrogen peroxide and oxidative damage

Among the secondary oxidative messengers of O_3, H_2O_2 has been considered one of the most suitable molecule for redox signaling and it has been shown to be able in modulate several signaling and transcription factors, as well as lipid peroxidation [107,220–222]. For instance H_2O_2 has been correlated to the regulation of several transcription factors activity such as NRF2, Nf-kB, AP1, NOTCH, via a redox regulation mechanism, largely discuss in several reviews and articles [221,223]. This redox-regulation mechanism is mediated mainly by the reactivity of H_2O_2 with Thiols groups, which are largely present in several biomolecules, and that leads to its ability in modulate the synthesis, stability, translocation of these transcription factors, their degradation via the proteasome and also their affinity in binding the DNA. For the first time In the 90's, was proposed that H_2O_2 was able to

regulate the Transcription factor Nf-kB via a redox mechanism [224,225]. Indeed, In several further studies, H_2O_2 has been reported to either activate or inhibit the inflammatory activity of this transcription factor via different mechanisms which involved the IKKb protein. For instance the phosphorylation of the IKKb serine residue by H2O2 results in the activation of Nf-kB whereas the oxidation of IKKb cysteine residues is involved in Nf-kB inhibition [226–229]. Another mechanism involved in the Nf-kB pathway regulation by H_2O_2 is the inhibition of the proteasome which in turn is not able to lead to the degradation of ikkb, resulting in the inhibition of Nf-kB [230]. The dual role of H_2O_2 on Nf-kB activity has been longed investigated. Indeed, It is known that Nf-kB activation is very sensitive to oxidative events within the environment since it requires an oxidative environment for its translocation in the nucleus and a reductive environment for its binding with the DNA. Thus in 2007 De Olivera-marques et al. demonstrated that the inhibition or activation of Nf-kB depends on H_2O_2 concentration levels and therefore that H_2O_2 is a fine regulator of pro-inflammatory and anti-inflammatory events within the cells [224,231,232].

Upon several stressor stimuli, it is known that living cells respond with the activation of specific stress response factors such as NRF2, which then induce the synthesis of antioxidant proteins that protect against the oxidative damage. H_2O_2 has been shown to have a role in the activation of the antioxidant response of our organism by modulating the activity of NRF2. For instance H_2O_2 has been reported to lead to oxidative events within the ITAF, IRES trans-acting factors, which normally interact with sequences present in the 5_untranslated region of human NRF2 (IRES) leading to the translation of NRF2 [233–236]. Moreover NRF2 has been shown to be modulated by H_2O_2 through several events which include NRF2 phosphorylation and its consequent interaction with KEAP1 [237–241]. It is now evident that H_2O_2 is able to regulate both the antioxidant and inflammatory response of our organism and therefore be a key mediator for O_3 in skin damage. Indeed the oxidative stress status induced by ROS production has been shown to be an important figure in several skin pathologies, cancer and skin aging and also in skin lipid peroxidation [196] .

II. 4-hydroxynonenal and lipid peroxidation

To protect the cell membranes from the oxidative damage, Living organisms display anti-oxidant defense, such as Vitamin C, Vitamin E, superoxide dismutase, peroxidase and catalase which are able to neutralize free radicals. If these antioxidant defense are not sufficient to cope the oxidative stress induced damage, we could have a propagation of the reactions and an oxidative stress imbalanced will occur.

Indeed, free radicals such as HO-radicals, are able to interact with lipids present within the cell membrane, triggering a free radical chain reaction mechanism called lipid peroxidation. Due to the presence of several double bonds and hydrogen atoms, polyunsaturated fatty acids are the main targets of lipid peroxidation and the chemical products of this process are called lipid peroxides or lipid oxidation products, such as 4-Hydroxy-noneal and Malondialdehyde [107,242]. Two of the most presented w-6 polyunsaturated fatty acids (PUFAs) within the bio-membranes are the essentially arachidonic and linoleic acid, which represent important target for lipid peroxidation. Indeed their oxidation lead to the production of 4-hydroxy-nonenal (4HNE) which has been shown to be one of the main critical target in oxidative stress [106,108].

4HNE is an α,β-unsaturated hydroxyalkenal, characterized for the first time from Esterbauer, et al. in 1991 [243,244] and the reactivity of 4HNE relies on its chemical structure which display 3 reactive functions: a C2 =C3 double bond, a C1 = O carbonyl group and a hydroxyl group on C4 and makes 4HNE an electrophilic molecule. Therefore 4HNE is highly reactive toward nucleophilic thiol and amino groups, resulting in different reactions (Michael addition and the formation of Schiff bases) with a large number of macromolecules such as proteins, mainly containing histidine, cysteine and lysine residues, lipids containing an amino group and with nucleic acids, as show in figure 4 [243,245,246].

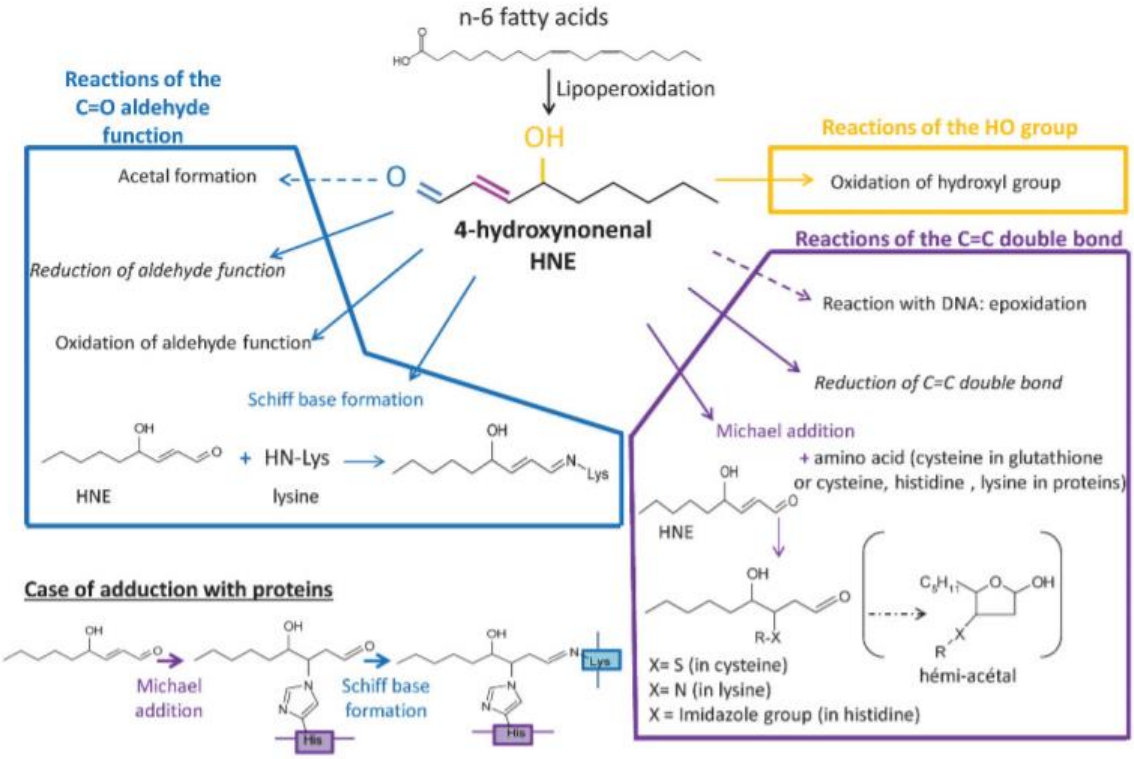


Figure 4. Reactivity of 4HNE [245]

In particular the interaction between 4HNE and proteins, results in the formation of cross-links within or between proteins called HNE protein adducts (PAs) [113,114,247,248] which lead to the changing of the protein conformation and thereby the alteration of their function or even to cell death [109,115]. HNE PAs aggregates have been associated with several metabolic, autoinflammatory and neurological diseases [117,118,249] which shows the importance of their degradation and their utility as oxidative stress related diseases biomarkers. One of the possible fate of 4HNE PAs is the degradation through the 20S proteasomal pathway, which is normally involved in the degradation of Oxidized proteins. However 4HNE itself has been reported to modulate the proteasomal activity [250,251] and higher levels of 4HNE have been associated to an impairment in the proteasome activity. Indeed, 4HNE is able to interact with the proteasome subunits such as 20s and 26s resulting in post-translational modifications which can compromise the proteasomal activity itself [252–254]Normally 4HNE is under the control of several enzymes, including glutathione S-transferases (GSTs), aldehyde dehydrogenase, and alcohol

dehydrogenase [255] However, if it escapes to its detoxifying processes, it can interact with several targets due to its amphiphilic properties and its electrophilic nature and therefore be involved in the modulation of several mechanisms such as gene expression, enzymes activity and signal transduction [256,257]g. Indeed several studies in literature have demonstrated that 4HNE is able to modulate the expression of several genes and the activation of inflammatory responses such as nuclear factor-kappa B (Nf-kB), but also detoxification mechanisms (NRF2) and MAPKs [258–262] . A prolonged exposure to oxidative stress can lead to an accumulation of 4HNE which can results in some pathological disorders [263]. Indeed 4HNE PAs has been associated with various neurodegenerative diseases such as Parkinson's and Alzheimer's disease, contributing to neurodegeneration due to proteasome inhibition. For instance, 4HNE-modified amyloid b-peptides have been found to be able in inhibit the proteasome system in Alzheimer's patients, whereas in Parkinson's patients 4HNE interacts directly with proteins belonging to the proteasome leading to neuronal cell death [249,264,265]. Within the vascular cells, high levels of 4HNE PAs have been found in atherosclerotic lesions since ROS are able to oxidize LDLs leading to the formation of reactive aldehydes [266] . Moreover some studies have demonstrated the presence of high levels of 4HNE in cancer tissues and their correlation with the malignancy of the cancer [267–269], as well as in metabolic diseases, such as diabetes or non-alcoholic fatty liver disease (NAFLD) [247,270,271].

4HNE has been shown to be also a key regulator of important pathways such as apoptosis, necrosis, proteasome and autophagy which are all fundamental in the regulation of cell death [245]. It is therefore clear that 4HNE could play an important role in O_3-induced damage and new evidences highlight the role of 4HNE as a possible trigger factor in pollution-induced skin OxInflammation [37].

III. 4-hydroxynonenal in skin conditions

Since the skin is one of the main target organ for pollutants which interact with the stratum corneum leading to the production of ROS and consequent lipid peroxidation, in the past years 4HNE has been used as an important oxidative stress marker in skin inflammatory conditions. For instance, several studies have shown the presence of higher 4HNE levels

after O_3 exposure in several skin models, in *vitro* but also in *ex vivo* human explants and *in vivo* hairless mice [60,111,130,272]. Also other pollutants such as Cigarette smoke (CS) and PM have been associate to increased 4HNE PAs levels in human skin [210,273]. Moreover the formation of these 4HNE proteins aggregates has being found to be strictly linked with the loss of function of important skin proteins, such as SRB1, both in keratinocytes and sebocytes [36,274] and also with the exacerbation of skin aging and other skin conditions. Indeed several studies have associated higher 4HNE levels to an alteration in skin colors, skin elastosis, but also to psoriasis and Atopic dermatitis [275–280]. All these evidences suggest that the involvement of environmental pollutants in several skin inflammatory conditions is mediated by the activity of 4HNE itself. Indeed, the ability of 4HNE in modulate several pathways , such as Nf-kB or NRF2, which normally regulate the antioxidant and inflammatory response of our organism and that have been found to be altered in skin conditions, make 4HNE a key regulator in pollution-induced skin homeostasis.

4.1.3 O_3 and the ox-inflammatory response of the skin

It is evident how O_3 is able to induce stress and inflammatory responses within the skin, most likely by indirect mechanism since it is not able to penetrate the stratum corneum [60]. Skin exposure to high levels of O_3 has been shown to be associated with a depletion of antioxidant levels and the activation of several pathways [58]. For instance, O_3 seem to have a role in the activation of the heat shock proteins (HSPs), a family of proteins induced in response to several stress stimuli and involved in apoptosis, proliferation but also in inflammatory response. Indeed high levels of 4HNE and an up regulation of the HSPs 27,32 and 70 have been detected in skin of mice exposed to O_3 0.8 ppm for 6 days with a different time-corse response [111]. In particular HSP32, also known as Heme-oxygenase 1 (HO-1), is an oxidative stress response under the control of the NRF2 pathway, which modulate the antioxidant response of our body. Therefore this study suggested the ability of O_3 in induce an oxidative response within the skin leading to an alteration of the skin physiology. O_3 has been reported to induce the activation of metalloproteinases MMPs , such as MMP2 and MPP9, which are important targets

involved in tissue remodeling, wound healing, but also in skin aging, and in skin lesions associated with psoriasis and atopic dermatitis [281]. In literature It is known that MMPs can be activated by reactive oxygen species [282] and this would suggest an oxidative-stress regulation by O_3 of these targets. In particular MPP9 and MPP2 are the only members able to degrade Type IV collagen of the basal membrane, resulting in the control of extracellular matrix degradation and remodeling, which are important events of the wound healing process. Wound healing is a very critical process, also modulated by oxidative stress and related to skin aging. Indeed it has been shown that Hydrogen peroxide H2O2 is able to induce VEGF which can in turn stimulate wound healing [283]. Moreover the entire wound healing process and the induction of MMP9 is strictly associated with the activation of inflammatory pathways such as Nf-kB another key molecular target for O_3 [284] .

O_3 is therefore able to induce an oxidative stress response within the skin which can be strictly linked to a an inflammatory status resulting in the OxInflammation phenomena [1]. Indeed the exposure of the skin to O_3 has been found in up regulate not only the Nf-kB pathway but also the pro-inflammatory citokynes such as IL-8, Aryl hydrocarbon receptor (Ahr), and other pro-inflammatory markers such as TGFβ COX 2 and iNOS [58,129,285]. Since the relevant O_3-induced cytotoxicity within the skin has been demonstrated to be prevented by the topical application of antioxidants compounds, it is evident that the O_3 effect is likely mediated by ROS generation [60]. Indeed, as previously described, The role of reactive oxygen species such as H_2O_2 but also non radical species, such as 4HNE, have been resulted to be fundamental in the modulation of the antioxidant response but also for the activation of inflammatory pathways. Therefore It is clear how the onset of several inflammatory skin pathologies associated with the upregulation of pro-inflammatory markers can be easily exacerbate from O_3 exposure. In the last few decades the exposure to pollutants such as PM, CS and O_3 has received great attention in the onset of skin inflammatory due to their ability in induce an inflammatory response. Moreover the inflammatory status displayed in these skin pathologies has been shown to be related to the activation of new cytosolic multiprotein complexes pathways of the innate immune system, called Inflammasomes [286]. Of note some pollutants, such as PM and CS, have already been discovered to be involved in the activation of different inflammasomes complexes, exacerbating several pathologies. However O_3, which

normally contributes to the development of an oxidative stress damage and inflammatory status especially in lungs and skin, it is one of the few pollutants still unexplored in inflammasomes activation. Therefore, all these findings suggest an interesting and uninvestigated role for O_3 in inflammasome activation in the onset of inflammatory skin conditions.

4.1.4 The inflammasome pathway: a new inflammatory target for pollutants induced skin damage

The Immune system is the first host defense against harmful stimuli involved in maintaining the normal tissue homeostasis and protecting us from infections and diseases. The innate immune system is able to recognize foreign molecular structures belonging to pathogens, such as *virus* and *bacteria*, as well as self-molecules that are altered, providing an immediate but non-specific response and activating the adaptive immune system for a more specific and long-lasting response. For instance, the Innate immune system relies on pattern recognition receptors (PRRs) which recognize components deriving from invading pathogen called pathogen-associated molecular patterns (PAMPs), and Danger-associated molecular patterns (DAMPs) that are normally released from host cells during cell damage or death [287]. Activation of PRRs leads to downstream signaling cascades, resulting in the activation of pro-Inflammatory pathways and the production of type 1 Interferons (INF-ɒ and INF-β) and pro-inflammatory cytokines. PRRs sensors can be divided in Membrane-bound Toll-like receptors (TLRs), which are transmembrane proteins able to recognize PAMPs on cell surface, and nucleotide-binding oligomerization domain-like receptors (NOD-like receptors, NLRs), RIG (retinoic acid-inducible gene)-like receptors (RLNs), and cytosolic DNA sensors which detect infection or cell damage within the cytosol.

In 2002, *Martinon et al*, identified for the first time a new cytosol multiprotein oligomers complex of the innate immune system, the Inflammasome [197]. Inflammasomes are normally expressed in immune cells such as monocytes, macrophages, dendritic cells, neutrophils but they can be found also in other cells type, such as keratinocytes, and they are involved in the inflammatory response against several stimuli [203].

The assemble of this Intracellular multiprotein signaling complexes relies on the interaction of different domains, CARD-CARD or PYD-PYD domains, belonging to the different co-receptor proteins forming the inflammasome complex: the cytosolic sensor molecule, the adaptor protein ASC and Caspase 1. These interactions lead to the oligomerization and inflammasome assembly [288–290]. Indeed, upon PAMPs and DAMPs recognition, the inflammasomes can assemble around a cytosolic PPRs receptor such as NLRs as well as other cytosolic and nuclear DNA sensors such as AIM2 (absent in melanoma 2) belonging to the ALRs family, IFI16 (IFN-inducible protein 16) and pyrin, promoting the cleavage of Caspase 1 and the maturation and consequent release of inflammatory cytokines such us Interleukin 1-β (IL-1β) and Interleukin 18 (IL-18). The activation of Caspase 1 requires the recruitment and oligomerization of the Apoptosis-associated speck like protein (ASC or Pycard) [291,292].

ASC is first recruited by the sensor molecule via the ASC pyrin domain, leading to the formation of a large protein consisting mainly of multimers of ASC dimers. After this scaffold formation, The CARD domain of ASC is now able to interact with the CARD domain of pro-caspase 1 monomers, leading to the auto-cleavage and the formation of active caspase 1. Caspase 1 then leads to the proteolytically cleavage of the pro-inflammatory cytokines interleukin-1β (IL-1β) and IL-18, that will induce an inflammatory status[198,200,293].

In contrast to the canonical inflammasomes, there are non-canonical inflammasome complexes whose activation is independent of Caspase 1. Indeed, these inflammasome complexes can rely on other inflammatory caspase such as Caspase 4 and 5 that directly bind Lipopolysaccharides (LPS), found in the outer membrane of Gram-negative bacteria [294 295 296]. Both canonical and non-canonical inflammasomes can lead to a type of programmed cell death distinct form Apoptosis named Pyroptosis, responsible for the secretion of DAMPs, ATP, DNA, ASC oligomers and cytokines in consequence of pores formation in plasma membrane due to Gasdermin-D [297–300]. Indeed, the Pyroptosis mechanism relies on the substrate Gasdermin D, belonging to the Gasdermin family, which is cleaved from caspase 1 upon inflammasomes activation and assemble. The cleavage generates a 31 kDa Gasdermin D N-terminal fragment which translocate on the cell membrane where it presumably binds to cardiolipin and oligomerize to form pores on

the bacterial cell membrane, leading to release of pro-inflammatory mediators [301–303]. The release of these mediators recruits more immune cells leading to the perpetuation of the inflammatory cascade in the tissue and even to cell death [294,304,305]. However not all cell types undergo pyroptosis and consequent cell death upon inflammasome activation. The most studied inflammasome complexes in the past years belong to the NLRs and ALRs family. Of note, the NLRs Family includes 22 human genes and it is characterized by the presence of different domains: A central nucleotide-binding and oligomerization domain (NATCH), which is common to all NLR family member and enables the activation of the signaling complex, a C-terminal Leucine-rich repeats (LRRs) domain involved in the ligand sensing and autoregulation and The N-terminal Caspase recruitment (CARD) or Pyrin (PYD) domains necessary for the down streaming signaling. The NATCH domain can be divided in 3 subfamilies: the NODs, The NLRPs and IPAFs [306]. In particular the NLRPs subfamily have been largely studied in the past years, highlighting a role for the Inflammasome NLRP1 and NLRP3 in several inflammatory pathologies such as neurodegenerative diseases (Alzheimer's disease (AD), Parkinson's disease (PD), atherosclerosis, type 2 diabetes autoinflammatory diseases (arthritis and dyskeratosis) and also skin diseases [201–203,205,307,308].

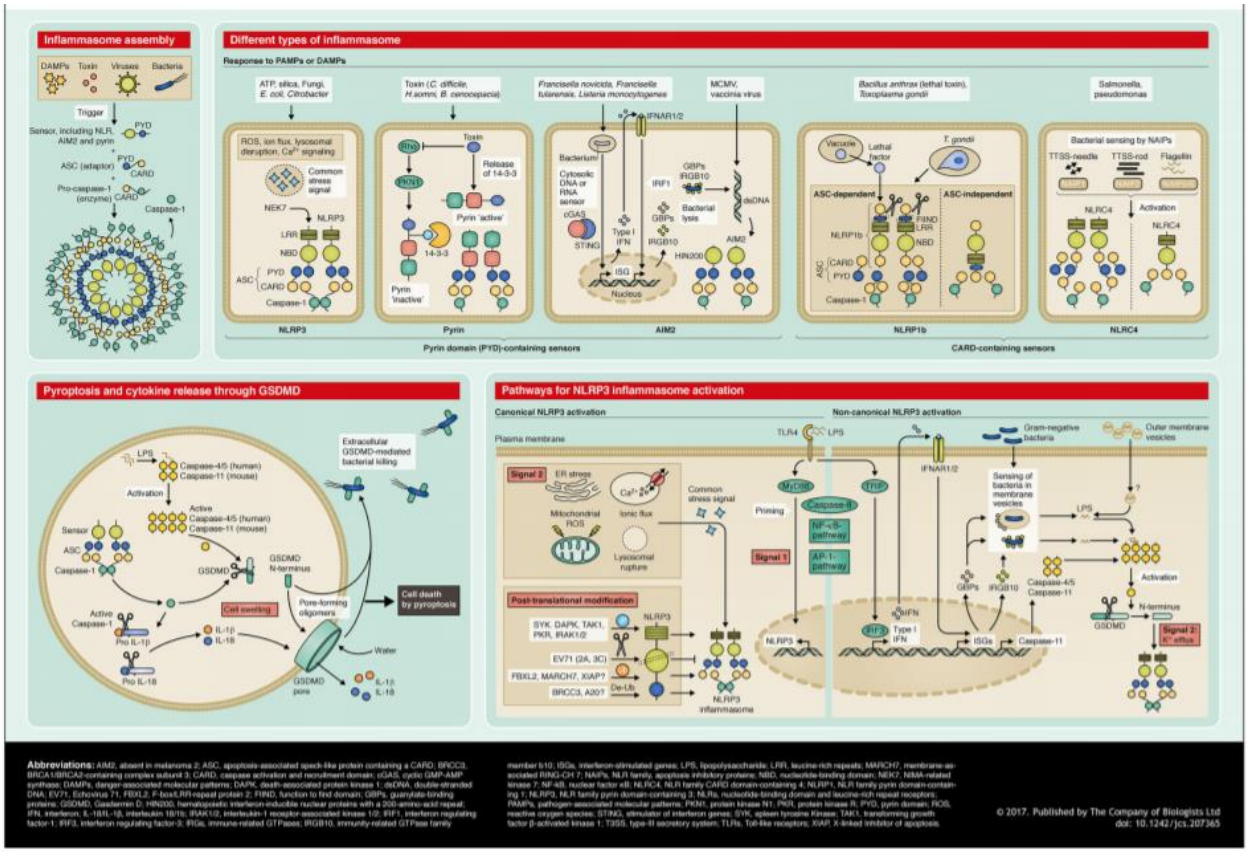


Figure 5: Inflammasome activation and pyroptosis [309]

I. Inflammasomes and their activation

So far 22 NLRs and 4 ALRs have been identified in humans and , of those, NLRP3, NLRC4, NLRP1 as well as AIM2, represent the best-characterized studied inflammasomes. Generally Inflammasomes can be activated by a variety of stimuli such as pathogens, PAMPs, DAMPs. Several studies have investigated the mechanism of their activation which predominantly require the protein adaptor ASC for caspase 1 activation and IL 1beta/IL18 release [203,310–312]. Upon inflammasome activation, ASC normally translocate from the nucleus to the cytosol where it can form a large aggregate [312,313]. Several studies showed that ASC specks formation is regulated by several post-translational modification in different Inflammasome complexes. For Instance ubiquitination of ASC seems to be required for NLRP3 and AIM2 inflammasomes activation [314,315], as well as Phosphorylation [316,317].

Thus, It is clear that ASC is a crucial component in inflammasome assemble and that its post-translational regulation is critical for specks formation and the propagation of inflammation from cell to cell [313,318,319]. For some inflammasome such as NLRC4, the role of ASC is still unclear and its recruitment it is not always required. Indeed, despite the other NLR inflammasomes, NLRC4 do not display the PYD domain and It is possible that it can directly interact with Caspase 1 [320]. Nevertheless other studies demonstrated that NLRC4 can collaborate with other proteins containing a PYD domain such as NLRP, forming a NAIP/NLRC4 inflammasome able to recruit ASC [321–324]. NLRC4/IPAF inflammasome recognizes several intracellular gram-negative bacteria and its activation seems to be dependent on the PAMPs flagellin recognition and the bacterial type III secretion system (T3SS) components [325 322 326 327 328].

Among the NLR inflammasome one of the most well characterized sensor protein is NLRP3, belonging to the NLRs family. This sensor can be activated by a large number of stimuli including pathogens (*Candida albicans, Staphilococcus aureus*, adenovirus, influenza virus, *Saccharomyces cerevisiae* etc.), environmental irritants such as PM or UVB, pore-forming toxins, PAMPs or DAMPs, and synthetic substances that can induce Lysosomal damage, K+ efflux, ROS generation and [329–336]. NRP3 activation relies on a two- steps mechanism: the priming (*signal 1*), which promotes the expressions of Inflammasome components, and the activation (*signal 2*) that induce NLRP3 activation and scaffold formation [337]. One of the best known priming signal activating NLRP3 is LPS combined with ATP even though other signals were identified such as TLR agonists (e.g. R848), cytokines (e.g. TNF-α or IL-1β) and NLR ligands (MDP). While LPS induces the transcription of pro IL-1β and NLRP3 , ATP activates NLRP3 by inducing potassium efflux via the P2X7 receptor [338]. Indeed several studies demonstrated that NLRP3, along with pro IL-1β, needs to be first transcribed by transcriptional factors such as Nf-kb. Once transcribed, NLRP3 can be activated from an array of stimuli including the translocation of NLRP3 to the mitochondria, the release of mitochondrial DNA or cardiolipin, potassium efflux out of the cell, the release of cathepsins into the cytosol after lysosomal destabilization and the generation of mitochondrial reactive oxygen species (ROS) [203,311,312,339].

Another important inflammasome complex belonging to the ALRs family is AIM2, a HIN-200 family member able to recognize cytosolic dsDNA such as synthetic DNA, plasmid DNA, bacterial or virus DNA of different pathogens. Even though AIM2 does not display a central oligomerization domain, such as NATCH, it can form a scaffold by binding specific binding sites on its ligand, via its C-terminus HIN domain, and recruits ASC for caspase 1 activation [340–344]. Finally another non-NLR inflammasome, Pyrin, is a member of the PYD family able to the detect bacterial toxin-induced Rho guanosine triphosphatase (Rho GTPase)-inactivation. Upon the recognition, it interacts with ASC through its N-terminal PYD domain and induce the activation of caspase 1 [345,346] .

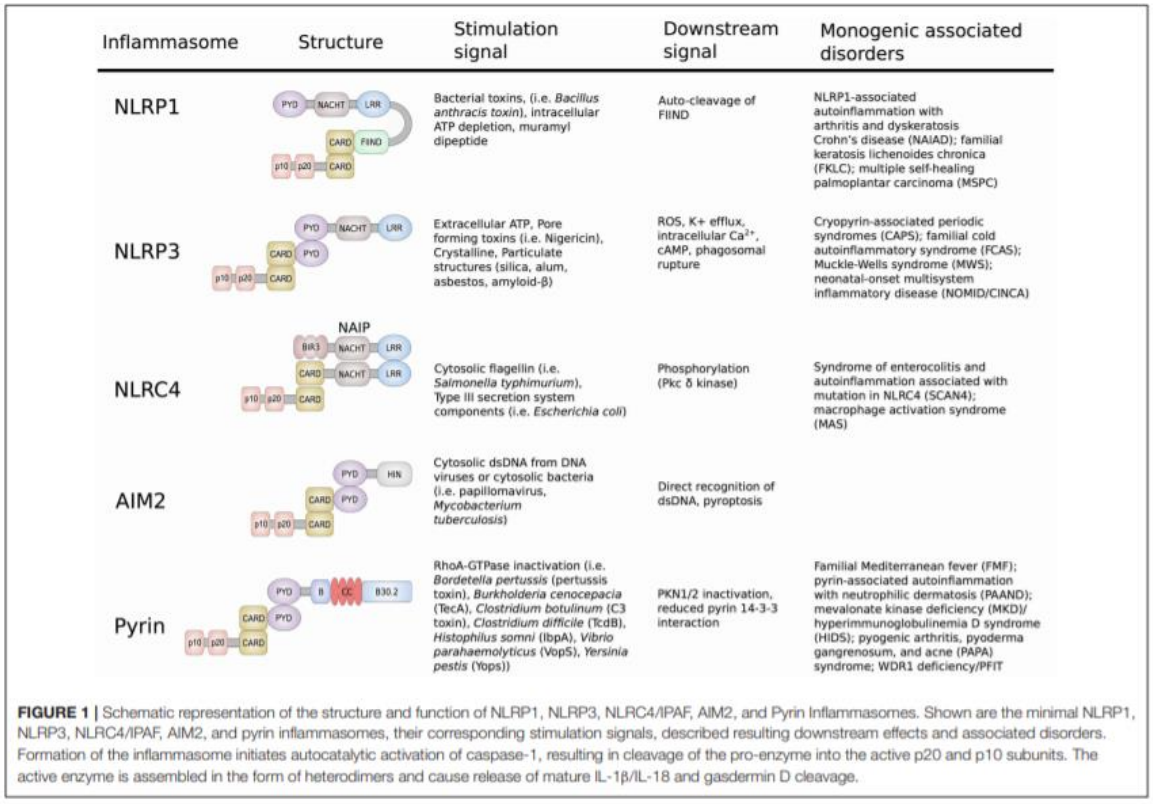


FIGURE 1 | Schematic representation of the structure and function of NLRP1, NLRP3, NLRC4/IPAF, AIM2, and Pyrin Inflammasomes. Shown are the minimal NLRP1, NLRP3, NLRC4/IPAF, AIM2, and pyrin inflammasomes, their corresponding stimulation signals, described resulting downstream effects and associated disorders. Formation of the inflammasome initiates autocatalytic activation of caspase-1, resulting in cleavage of the pro-enzyme into the active p20 and p10 subunits. The active enzyme is assembled in the form of heterodimers and cause release of mature IL-1β/IL-18 and gasdermin D cleavage.

Figure 6. Structure and function of Inflammasomes [345]

II. Non canonical inflammasomes

In 2011 *Kayagaki et al*.[294], showed a new pathway able to trigger Pyroptosis and immune defenses called non-canonical inflammasomes. Despite the canonical inflammasomes which are able to assemble in a multiprotein scaffold complex, leading to caspase 1 and IL-1beta release, the non-canonical inflammasome do not need a PRR sensor [347]. Indeed, they relies on the direct activation of other inflammatory caspases, such as caspase 11/4/5 upon infectious stimuli and the consequent pyroptosis event. The non-canonical initiation requires a priming step in which the transcription of mouse Caspase 11, along with its human counterparts caspase 4 and 5, is induced by lipopolysaccharide (LPS), IFN-α/β, or IFN-γ. [295,348]. Extracellular LPS (also known as endotoxin) is able to access the cytosol through TLR4/MD2/CD14 receptor complex by its agonist receptor Lipid A. Lipid A is also responsible for caspase 11 dependent pyroptosis and it is able to directly bind caspase 11 as well caspase 4 and 5 through their CARD domain, leading to their activation [295,296]. Since LPS is largely present in Gram-negative bacteria, the noncanonical inflammasome plays an important role in host defense against various bacterial infections such as Salmonella and other pathogens [349–351] . Once activated, Caspase 11/4/5, despite of Caspase 1, do not process Interleukins, but they only induce pyroptosis by cleaving the substrate Gasdermin D (GSDMD). The cleavage of GSDMD leads to the release of the autoinhibition N-terminus domain which can translocate on the plasma membrane, leading to pore formation and causing cell swelling and osmotic lysis [301,302,352]. Moreover recent studies investigated the possible cross talk between non-canonical inflammasome and other canonical inflammasomes such as NLRP3, revealing a possible cooperation in the context of inflammatory response [353,354].

4.1.5 NLRP1 Inflammasome

NLRP1 was the first protein sensor described by *Martinon et al, 2002* able to form the inflammasome and has been discovered to be correlated with several skin autoinflammatory pathologies such as Vitiligo, atopic dermatitis, psoriasis, acne and carcinogenesis, melanoma [206,208,355], but also to diabetes, rheumatoid arthritis, Crohn disease and other autoinflammatory diseases [356,357].

As previously described, the NLRs family members have a similar domain architecture, consisting of a NATCH , LRR and PYD domains able to interact through CARD-CARD or PYD-PYD interactions and forming the Inflammasome complex. NLRP1 however, is different from the other NLRs family members for several reason. First of all it encodes for 2 additional domains at its C-Terminus: FIIND domain (function to fiind domain) and CARD. The presence of these two domains had led to several questions about the mechanism undergo the activation of NLRP1 and the role of ASC in the scaffold formation. Second, the protein structure of NLRP1 is not conserved across species. For example mice have tree paralogs of the Nlrp1 gene such as Nlrp1a, Nlrp1b and Nlrp1c all of which lack the PYD and differ in the response to the different stimuli [358] For several years the role of the FIIND domain had been unknown and the only other protein known to possess this domain was the Inflammasome CARD8. Finally in 2011, *D'Osualdo et al* [359], demonstrated that CARD8 and NLRP1 undergo autoproteolytic cleavage at a conserved SF/S motif within the FIIND domain, find a similarity between the FIIND and the ZU5-UPA domain present in another autoproteolytic protein PIDD[360]. In the following years, several studied demonstrated that the autolytic proteolysis within the FIIND was essential for the NLRP1 inflammasome activity [361] , and that following this cleavage, the two fragments remained associated, leading to stabilization of NLRP1 and the consequent recruitment of ASC [362] . However, the role of ASC in NLRP1 inflammasome assemble has been long discussed. Indeed, the presence of a CARD domain at the C-terminus of NLRP1 suggested the possibility that ASC wasn't necessary for the maturation of the pro-caspase 1 and that NLRP1 was able to recruit the pro-caspase 1 via a CARD-CARD domain interaction [363]. Indeed several PRRs can directly interact with pro-caspase 1 without recruiting ASC but directly interact with pro-caspase1 such as NLRC4 or CARD8 [323,364,365]. Even though several studies demonstrated that ASC is necessary for human NLRP1 inflammasome activation [362,366–368]others showed the ability of NLRP1 in directly activate Caspase 1 as well as another inflammatory caspase, the caspase 5 [197]. Moreover in mouse, NLRP1a and NLRP1b inflammasomes seem not require the speck like receptor ASC [369,370].

I. NLRP1 activation

Despite the other NLRP inflammasome, the mechanism of human NLRP1 activation is still not clear. However several studies have demonstrated that the mouse variant NLRP1b is activated by the *Lethal factor* (LF), a component of the anthrax lethal toxin produced by the *Bacillus Anthracis,* that induce the cleavage within the FIIND domain of NLRP1b [361,362,371–373]. Several studies have shown that LF is able to cleave the N-terminus both in rat and mouse NLRP1 and that this site is degraded by proteasome via the N-end rule pathway, leading to caspase 1 activation and cell death [374,375]. This degradation would allow the release of the active C-terminal fragment containing the CARD domain which can now interact with the CARD domain of Caspase 1, resulting in the activation of the Inflammasome [368,376].Moreover *Joseph Chavarrı´a-Smith et al, 2013,* demonstrated that the cleavage of NLRP1b is not only necessary but also sufficient for Inflammasome activation by LF and that this cleavage occurs even in the absence of LF. This data suggested the possibility that NLRP1b acts just as a sensor of protease activity and therefore would be able to detect non only *B. anthracis* but also other pathogens and stimuli. For instance, In the last few years it has been reported that chemical inhibitors of dipeptidyl peptidases DPP8 and 9 are able to activate both the murine Nlrp1b inflammasome and the human NLRP1 [365,367]. Indeed, DPP9 and 8 seem to display an important role in regulate the inflammasome activation involved in human autoinflammatory diseases, even though the mechanism is still unclear. Moreover this peptidase inhibitor have already been demonstrated to induce GasderminD (GSDMD)- and caspase 1–dependent pyroptosis in human macrophages [377].

II. Inflammasomes regulation by posttranslational modifications

The inflammatory response is a very delicate mechanism which is normally self-limited and it should be resolved after the removal of the noxious stimuli. If this response is prolonged or excessive, it could lead to the alteration of tissue homeostasis resulting in the development of autoinflammatory syndromes and other diseases or even to cell death [378]. Therefore the regulation of the immune system mediators in the inflammatory process, is crucial to limit the collateral damage. For instance, Inflammasomes, which

represent important mediators for the innate immune system, have being found to be regulated at different levels by Post-translation modifications (PTMs), which mainly involve phosphorylation and ubiquitination of inflammasomes components [379–382] . These PTMs regulate the activation or the inhibition of inflammasomes upon different stimuli, providing the preservation of the balance of the inflammatory signaling. The main inflammasomes components subjected to PTMs are the sensors such as NLRP3, NLRP1, AIM2, NLRC4 but also the protein adapter ASC.

Indeed the phosphorylation of the CARD domain of ASC by Syk has been found to be fundamental in NLRP3 and AIM2 inflammasomes activation both in mouse and human macrophages [316,317], promoting the formation of ASC specks. The protein adapter ASC is normally negatively regulated by the IkB kinase IKKa which phosphorylates specific serine residues within the CARD domain of ASC, inhibiting its translocation from the nucleus to the cytoplasm and therefore the inflammasomes activation. Dephosphorylation of IKKa by serine/threonine phosphatase PP2A have been associated to the activation of NLRP3 inflammasome but not AIM2 whereas another IKK-related kinase IKKi have been found to phosphorylate ASC at Ser5, leading to its translocation to the nuclei and consequent inflammasome activation [383]. Moreover, phosphorylation induced by syk have been found also in NLRP3 itself upon several infection stimuli, such as the fungal pathogen *Candida Albicans*. This phosphorylation has been reported to be fundamental in NLRP3 inflammasome activation, by inducing the release of ROS and potassium efflux [384,385]. Other phosphorylation events due to different kinase have been correlated to the activity of several inflammasomes such as NLRP1, NLRP3, NLRC4 and AIM2 suggesting an important role for this PTM in regulate the inflammatory response [386–390].

Another important post-translational regulatory mechanism, Ubiquitination, has been found to be fundamental for the modulations of the different inflammasomes components. Indeed, ubiquitination normally regulates the faith of several proteins by leading them to be degraded in the lysosome or proteasome. For instance, linear ubiquitination of ASC have been associated with the activation of certain inflammasomes such as NLRP3 [314,391], whereas the K63 ubiquitination of ASC can be associated to both the activation or inhibition of ASC-dependent inflammasomes such as AIM2 and NLRP3 and also to autophagy [315,392].

Several E3 ubiquitin ligase has been reported to lead to the ubiquitination and degradation of NLRP3 inflammasomes [393,394], whereas the ubiquitination of NLRC4 due to the ligase activity of the regulatory protein associated with the 26S proteasome, Sug1, has been linked to NLRC4 activation with consequent Caspase 8 release [395]. De-ubiquitination of NLRP3 inflammasome due to the blockage of the activity of DUB inhibitors has been shown to induce NLRP3 activation [396,397].

Phosphorylation and ubiquitination are just two of the several post-translational modifications to which inflammasomes can undergo [382], which control the turnover, the location, distribution and protein-protein interaction of the main inflammasomes components. The cross-talk between the different PTMs, especially between ubiquitination and phosphorylation, is fundamental in inflammasomes assembly cause they can negatively or positively regulate each other [398,399] . Therefore understand the mechanism of these PTMs in inflammasomes regulation is essential for the development of new therapeutic approach for inflammasomes-related pathologies such as neurodegenerative, metabolic, cardiovascular disorders but also for skin conditions.

4.1.6 Inflammasomes and related diseases

The production of IL-1β and IL-18, as well as other interleukins, have been always linked to a variety of autoinflammatory and autoimmune diseases, revealing their role as crucial mediators in inflammation [400,401]. Since IL-1β and IL-18 are both final mediators of Inflammasomes, several studies so far demonstrated that the activity and regulation of these multiprotein complexes are strictly involved not only in the onset of these autoinflammatory diseases, but also in neurologic and metabolic disorders, which often display an inflammatory status.

In the Central nervous System (CNS), PRRs are expressed in several cells type such as microglia, astrocytes, and macrophages, but also oligodendrocytes, neurons, and endothelial cells are fundamental for the host defense [402,403]. Upon CNS infection, brain injury, and neurodegenerative diseases, such as multiple sclerosis, amyotrophic lateral sclerosis, Parkinson's disease, and Alzheimer's disease , high levels of IL-1β and IL-18

are displayed leading to neuroinflammation and neuronal degeneration [404–406]. For instance IL-1β and IL18 have already been shown to be involved in autoimmune demyelination, the development of autoimmune encephalomyelitis and in the induction of IFN- γ within the central nervous system, which are all common figures displayed in Multiple Sclerosis [407–411]. Later studies revealed that, NLRP3 or Pyrin-deficient mice displayed a reduced severity of the disease, with a delay in oligodendrocyte loss, demyelination and decreased IL-18/ IL-1β levels [412–416].

Other neurodegenerative diseases such as Alzheimer's and Parkinson's diseases are now known to be strictly linked to inflammasomes activation, such as NLRP1, NLRP3 and NLRC4 [202,417–420]. For instance The accumulation of Amyloid- β peptide within the cerebrum induces inflammation of the central nervous system and the release of pro-inflammatory cytokines and chemokines such as IL-1β and IL-18, which play an important role in Alzheimer's disease pathogenesis. Amyloid-Beta was found to be involved in triggering NLRP3 and NLRP1 inflammasomes, resulting in IL-1beta production [336,421,422]. Moreover the formation of a-Synuclein aggregates in the substantia nigra, which are the pathological hallmarks of Parkinson's diseases (PD), seems to be linked to the release of high levels in caspase 1 and IL-1β in serum of PD patients [423]. Further studies show that a-synuclein aggregates are able to trigger NLRP3 inflammasome in human monocytes and microglia cells [424] [425] [426].

In the past decades several evidenced linked the progression of obesity associated diseases, such as insulin resistance, gout, atherosclerosis, Type 2 diabetes, cardiovascular diseases and nonalcoholic fatty liver disease (NAFLD) to the onset of an inflammatory status named metabolic inflammation [427–430] . In particular Inflammasomes, seem to be able to recognize the presence of abnormal metabolic conditions within tissues and, once activated, induce the release of IL-1β and IL-18, resulting in the development of metabolic pathologies [431].

For instance, NLRP3 components such as ASC, Caspase 1 and Interleukins were overexpressed in obese mice and human livers and fatty tissue, whereas NLRP3 deficient mice showed improved glucose tolerance and insulin sensitivity[432–434] .

Several evidences have been suggested that IL-1β and IL-18 induced by Inflammasomes activation, have a crucial role in the progression of Atherosclerosis. Indeed, human

atherosclerotic plaques display elevated Il1beta and IL-18 receptors and elevated IL-18 levels have been linked to the instability of these plaques and vascular inflammation [435–438]. In particular NLRP3 inflammasome has been demonstrated to be activated by Cholesterol crystal both in mouse and human cells, following cathepsin B release and resulting in atherosclerotic plaques rupture [439]. Similarly, the accumulation of uric acid crystal displays in Gout, are able to trigger NLRP3 inflammasome in LPS-primed macrophages [440] and the severity of the diseases has been shown to be reduce by blocking IL-1β production [441].

I. Inflammasomes activation in skin pathologies: environmental pollutants as possible trigger stimuli

Although Inflammasomes are mainly present in immune cells such as macrophages and dendritic cells, also keratinocytes express some of these complexes, in particular NLRP1, NLRP3 and AIM2 inflammasomes [442,443]. For instance, It has been shown that keratinocytes are able to respond to DAMPs [444,445] and that the consequent aberrant production of IL-1 cytokines would contribute to the onset of several autoinflammatory diseases within the cutaneous tissue such as autoinflammatory syndrome (FCAS), Muckle–Wells syndrome (MWS), neonatal-onset multisystem inflammatory disease (NOMID) but also atopic dermatitis, vitiligo, psoriasis, acne and carcinogenesis [206,207,286,446]. Moreover Several studies have demonstrated that high levels of both IL-1 beta and inflammatory caspases are found in psoriatic and dermatitis murine models [447,448] , as well as in human psoriatic skin explants [449,450], which are known to be modulated by inflammasomes [451]. Indeed several studies have shown that actually mutations affecting inflammasomes components are associated to the development of these skin conditions [366,452–458] and that the blockage of aberrant IL-1 productions is able to ameliorate the related symptoms [459]. Since the skin is the main barrier of our body against environment, is continuously exposed to environmental stressors, resulting in an inflammatory and oxidative stress status (OxInflammation) within the cutaneous tissues and In the past years, several inflammatory skin pathologies have been associated to air pollutants exposure [5] . Actually, the role of environmental pollutants such

as PMs, UV, Cigarette smoke and also O_3 in inflammasomes activation have been longed studied in the past decades. In particular, reactive oxygen species (ROS) produced by these pollutants, have been identified as crucial figures in triggering inflammasomes activation in several pathologies. For instance PM, due to its composition, has been shown to be able in trigger NLRP3 inflammasome in several cardiovascular [330,460] and lung/ pulmonary diseases such as Malignant mesothelioma, Fibrosis, lung cancer, Asthma and chronic obstructive pulmonary diseases (COPD) [270–272], demonstrating that the NLRP3 inflammasomes activation in these pathologies were associated to ROS production as a result of both PM and O_3 exposure [329,330,464,465]. However, The role of ROS as effectors or triggers factors in Inflammasomes activation is still under investigation [466]. Higher levels of the AIM 2 inflammasome, induced by cytosolic DNA, has been found in human keratinocytes of psoriatic lesions, and the antimicrobial peptide LL-37 has been shown to prevent this activation by inhibiting cytosolic DNA itself [467,468]. Also, mutations of the NLRP1 inflammasome have been associated with vitiligo, atopic dermatitis, psoriasis, cancer and photoaging [307,357,446,469–473].For instance, a recent study demonstrated that the high levels of IL-1beta associated with the development of a Th17 micro-milieu, displayed in several autoinflammatory diseases, such as psoriasis or atopic dermatitis, was induced by activation of the NLRP1 inflammasome via caspase-5 maturation [474]. Genetic variations of NLRP1 have also been associated with a higher susceptibility to psoriasis [473] and non-segmental vitiligo in human patients [472]. Other studies have investigated the role of NLRP3 in different skin pathologies and have found that altered expression of NLRP3 is associated with psoriasis and atopic dermatitis in human patients and mice [475,476]. NLRP3 activation in human skin has been also correlated to ROS production via the release of inflammatory cytokines such as IL-17 and IL-22 which are normally involved in the onset of skin inflammatory conditions[477] In addition, inhibition of NLRP3 inflammasome via metformin in human keratinocytes can actually prevent caspase 1 maturation and consequent IL-1β production, ameliorating psoriatic symptoms [478]. Moreover, *Propionibacterium acnes*, which plays a pivotal role in acne development, has been shown to activate NLRP3 in acne lesions [479–481]. Several studies have demonstrated that higher levels of NLRP3-induced- caspase 1 and IL-1beta can be prevented by several compounds in different models of acne [482,483]. Even though several

inflammatory skin conditions have already been associated with environmental pollutants exposure , the regulation of the inflammasome in skin inflammation related to pollutants is complex and still not completely understood [5,484]. Indeed very few studies have investigated the association between these stressors and Inflammasomes activation in the development of skin pathologies, revealing just a role for UVB radiation in human keratinocytes [485–490]. Moreover since skin aging is associated with systemic inflammation and oxidative stress, mainly due to the activities of environmental stressors [491,492], different studies have demonstrated that excessive inflammasome activity can lead to the onset of premature aging, particularly in the case of UV exposure [493,446], which can also cause photodamage and skin cancer [487,488] . For instance it has been shown that Inflammasomes inhibition and consequent interleukins production blockage may represent a possible therapeutic approach for the treatment of Inflammatory Skin Diseases [494]. Therefore, since pollution is strictly linked to the onset of skin pathologies and to the activation of inflammasomes by ROS production, a new fascinating field of investigation has been opened, giving space to oxidative stress as a possible key regulator in the activation of inflammasomes in pollutants-induced skin conditions

4.2 *RATIONAL AND AIM*

Among the outdoor pollutants to which living organisms are daily exposed to, tropospheric O_3 is one of the most toxic [212] and can reach the concentration of 0.8 ppm in polluted cities[191]. O_3 formation in the troposphere (ground level) is mainly due to the interaction between car exhausts and UV light (photochemical smog). Since the late '50s, the consequences of O_3 exposure have been linked to the development of respiratory diseases [495–497] and only in the last two decades the cutaneous effects of O_3 exposure have been investigated [58,103,111,219,498] [8]. Recently, it was confirmed that long-term exposure to O_3 is associated with the development/exacerbation of premature skin aging and skin inflammatory conditions [191,499–501]. Indeed O_3 can rapidly react with the lipids present in the outermost layer of the skin, the stratum corneum (SC), generating bioactive molecules such as free radicals (including hydrogen peroxide (H_2O_2) and lipid peroxidation products (such as 4-hydroxynonenal), oxidation of functional groups, leading to the alteration of membrane permeability and induction of inflammatory responses[60,110,130,502,503]. One of the main inflammatory pathways involved in tissue inflammatory response, are Inflammasomes, multiprotein complexes able to induce the release of inflammatory cytokines. Inflammasomes can be triggered by several stimuli, including oxidative stress, and their activation has been related to several pathologies including skin conditions[205–208]. Besides the canonical inflammasome triggering stimuli, the role of reactive oxygen species (ROS) in oxidative stress-related inflammasomes activation has gained great attention in the past years. For instance, recent studies have demonstrated that exposure to air pollutants, such as PM, increased the levels of proteins involved in the NLRP3 inflammasome by triggering the production of ROS, leading to pulmonary and cardiovascular injury [329,330] , although neither of these studies actually demonstrated the formation of the inflammasome scaffold. In addition to PM, O_3 exposure has also been demonstrated to regulate the NLRP3 inflammasome in the lungs via generating mitochondrial mt-ROS [465,504]. However, no previous studies have investigated the effects of pollutant exposure in inducing cutaneous inflammasome activation, which is one of the main organs exposed to environmental pollutants. Therefore, since O_3 exposure as well as inflammasome activation is related to the development/exacerbation

of inflammatory skin conditions[205,206], often associated to excessive ROS production (OxInflammation), *the aim of this study was to investigate whether O_3 exposure was able to activate the cutaneous inflammasome and if this activation could me modulated through a redox-dependent mechanism, contributing to inflammatory skin conditions.* For this purpose the first step was to evaluate the modulation of the main inflammasome components (i.e ASC, NLRP1 and final mediators Caspase 1, IL-1β , IL-18) and the possible NLRP1 inflammasome assemble in response to O_3 exposure in a variety of skin models, ranging from *in vitro* 2D and 3D models and *ex vivo* human skin biopsies. We also hypothesized that O_3 exposure, through ROS and/or 4HNE, was able to induce post-translational modifications in NLRP1 that results in the inflammasome assembly. For instance several studies have already demonstrated that inflammasomes sensors are subjected to post-translational modifications (PTMs) such as phosphorylation and ubiquitination [386–390,393,394], which are also commonly induced by pollutants exposure [505]. For this purpose we investigated the role of H_2O_2 and 4HNE in HaCaT cells as possible O_3 mediators in modulating the NLRP1 inflammasome activation via ubiquitination. The results of this study bring new insight in the inflammasome activation and modulation by environmental pollutants, underlying the role and the connection between the oxidative and the inflammatory responses within the skin in response to stress stimuli. Indeed the OxInflammation phenomena has been demonstrated to be a common figure displayed in several pathologies[1] and NLRP1 could represent a relevant key target for a possible therapeutic approach against pollutants- induced skin OxInflammation and related skin conditions.

4.3 MATERIALS AND METHODS

4.3.1 O_3 Generator

O_3 was generated via electrical corona arc discharge from O_2 and combined with ambient air to flow into a plexiglass box (ECO_3 model CUV-01, Torino, Italy Model 306 O_3 Calibration Source, 2B Technologies, O_3 Solution), as previously described [506]. The concentration of O_3 in the chamber was adjusted to 0.4 ppm and continuously monitored by an O_3 detector.

4.3.2 Cell culture treatments and O_3 exposure

HaCaT cells were pre-treated either with 20 μM of 4HNE (CAS 75899-68-2 Chem Cruz, Santa Cruz Biotechnology, USA) or 50 μM of H2O2 (CAS 7722-84-1 Sigma, USA) and placed in the incubator at 37°C and 5% CO2. After 30 min of pre-treatment, the untreated cells petri dishes were placed in the plexiglass box connected to the O_3 generator, where they were exposed for 1 hour to 0.4 ppm of O_3. For the catalase experiments, keratinocytes were pre-treated with 1000 U/ml of catalase (Sigma, cat. C4963) for 2 hours and then exposed to 0.4 ppm of O_3 for 1 hr. As proteasome inhibitor, MG-132 (Millipore, Cat 474787) has been used at a concentration of 20 μM for 3 hours. The caspase- 1 inhibitor Z-YVAD- fmk (Biovision, cat 1012) has been used at a concentration of 2 and 10 μM for 1 hour. After the different treatments/exposure, RNA or protein samples were collected at the indicated timepoints [507].

4.3.3 EpiDerm 3D skin models and Human skin biopsies O_3 exposure

After the overnight recovery, 1 ml of fresh new media was added to each well of the 24 wells plate containing the EpiDerm 3D skin models and to the 6 wells plates containing the Human skin biopsies. The tissues were exposed to 0.4ppm of O_3 for 4 hrs into the

plexiglass box connected to the O_3 generator. At the end of Exposure, the tissues were placed into the incubator and protein, RNA, histological samples and media were collected at the indicated timepoints [508].

4.3.4 ASC Oligomerization Assay

HaCaT cells were grown in 6 cm^2 petri dishes at a density of $1.5x10^6$ cells in 3 ml of media. After 24 hours, cells were exposed to 0.4 ppm O_3 for 1 hour and then collected right after the exposure. Samples were then washed in cold PBS and centrifuged for 5 min at 1500 g. The resulting cell pellet was resuspended in 0.5 ml of ice cold lysis Buffer containing Hepes KOH 20 mM (pH 7.5), KCl 150 mM, NP-40 1%, 1% protease inhibitor cocktails (Sigma), and PMSF 0.1 mM. Cell lysates were then centrifuged again for 8 min at 1800 g at 4°C to remove bulk nuclei, and 30 ul of the lysates were collected as input for Western blot analysis. The remaining volume of the cell lysates were then centrifuged for 10 min at 5000 g at 4 °C and then resuspended in 0.5 ml of cold PBS containing Disuccinimidyl Suberate, DSS (CAS 68528-80-3 Alfa Aesar, Thermo Fisher scientific, USA) for crosslinking oligomers and incubated at RT for 30 min on a rotator. After 30 min, the samples were centrifuged for 10 min at 2500 rpm at 4°C, and the crosslinked pellets were then resuspended in 1x Laemmli buffer. The input and crosslinked samples were boiled for 10 min at 95°C and then analyzed by running samples on a 4-12% SDS-PAGE gel.

4.3.5 Immunocytochemistry

ICH on HaCaT cells was carried out as previously described in the general methods. Briefly after the blocking step, coverslips were incubated with primary antibody ASC (CAT NBP1-78977 NovusBio, USA) 1:100, NLRP1 (sc-166368 Santa Cruz, USA) 1:50, 4HNE (Abcam ab46545) 1:400 in 0.25% BSA/PBS overnight at 4°C. The Alexa Fluor Fluorochrome-conjugated secondary antibodies (A11004 Alexa Fluor 568, A11008 Alexa Fluor 488 Invitrogen, ThermoFisher USA) were used at a dilution of 1:500 in PBS-BSA 2% for 1 hr at RT. After staining the Nuclei with DAPI 1µg/ml for 1 min at RT, coverslips were mounted onto glass slides. The images were taken at 40x and 60x magnification.

4.3.6 Immunohistochemistry

The tissue section were incubated overnight at 4°C with primary antibodies for ASC (CAT NBP1-78977 NovusBio, USA) 1:100 in 2% BSA in PBS, NLRP1 (sc-166368 Santa Cruz, USA) at 1:50 dilution in 2% BSA in PBS and 4HNE (AB5605 Millipore Corp., USA) at 1:400 dilution in 2% BSA in PBS. The fluorochrome-conjugated secondary antibodies (A11004 Alexa Fluor 568, A11008 Alexa Fluor 488 and A11055 Alexa Fluor 488, Invitrogen, Thermofisher USA) were diluted 1:500 in 2% BSA in PBS After nuclei staining with DAPI 1 µg/ml for 1 min at RT, the sections were mounted onto glass slides.

4.3.7 Western blotting

The Western blot was performed as described in general methods and the following primary antibodies were used to incubate the Nitrocellulose membranes, obtained as previously described: ASC (CAT NBP1-78977 NovusBio, USA) 1:1000, Caspase 1 (2225S cell signaling, USA) 1:1000, NLRP1 (sc-166368 Santa Cruz, USA) 1:500, Ubiquitin (Abcam, ab7780) 1:2000 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) 1:10'000 in TBS-T with 1% non-fat milk for 90 min at RT. β-actin (A3854 Sigma,USA) was used as loading control at 1:50'000 dilution in TBS-T with 1% of non-fat milk. HDAC1 (Cell signaling) was used as loading control for nuclear proteins extraction at 1:1000 in TBS-T with 1% no- fat milk.

4.3.8 Nuclei-cytosol proteins extraction

HaCaT cells in 10 cm petri dishes at 70% confluence have been pre-treated with MG-132 20 uM for 3 hours and exposed to O_3 0.4 ppm for 1 hour. After the end of O_3 exposure, protein samples have been collected by washing the cells 2 times with fresh PBS and the cell pellets have been processed by using the Nuclear and cytoplasmic extraction G Biosciences kit (Cat. 786-182), following the manufacture procedure. After measuring protein content by Bradford assay, protein samples have been prepared and western

blotting has been performed as previously described in general methods by loading the same protein amount.

4.3.9 RNA extraction and quantitative Real Time PCR

For RNA extraction of 3D skin models and human biopsies, total RNA was extracted using the Aurum Total RNA Mini Kit with DNase digestion (Bio-Rad,USA), according to the manufacturer's recommended procedure. Briefly, after adding 700 µl of Lysis buffer provided by the Kit, the tissues were homogenized using the Precellys tissue homogenizer using 9 cycles of 30 sec with a 30 sec break at 8000 rpm at 4°C. For RNA extraction of HaCaT samples, total RNA was extracted using the same kit. Next, cDNA was generated from 1 µg of total RNA, using the iScript cDNA Synthesis Kit (Bio-Rad). To investigate the mRNA levels of ASC, CASPASE 1 and IL- 18 genes, quantitative real-time PCR was performed using SYBR® Green Master Mix (BioRad, USA) on a LightCycler® 480 Real-Time PCR system (Roche), according to the manufacturer's protocol. Gene expression was quantified by obtaining the number of cycles to reach a predetermined threshold value in the intensity of the PCR signal (C_T value). Beta Actin was employed as the reference gene, and the samples were compared using the relative cycle threshold (CT). After normalization, the fold change was determined using the 2^-ΔΔCT method. The primers used are listed in the table below:

Gene	Forward	Reverse
β-actin	ATTGCCGACAGGATGCAGA	AGTACTTGCGCTCAGGAGGA
NLRP1	ACCCTCTTAACTCCGGGACA	GAGTGCGCTTTATTGGCGAG
ASC	ATGCGCTGGAGAACCTGA	TCTCCAGGTAGAAGCTGACCA
CASPASE1	CCGTTCCATGGGTGAAGGTA	TGCCCCTTTCGGAATAACGG
IL-18	TGCAGTCTACACAGCTTCG	ACTGGTTCAGCAGCCATCTT

4.3.10 Amplex Red Assay

H_2O_2 production rate was evaluated using the Amplex Red–horseradish peroxidase (HRP) method, as described by Chen *et al*. (2003) [509] in media of epidermal samples. After exposure to 0.4 ppm of O_3 for 4 hours, media was collected directly after exposure and 6 hours post exposure. 10 µl of media for each sample were added to the reaction mixture and resorfurin formation, resulting from Amplex Red oxidation by HRP bound to H_2O_2, which was measured in SYNERGY H1 microplate reader (BioTek,USA) at 563 nm (excitation) and 587 nm (emission). Gen5 software (BioTek,USA) was used for the detection. The calibration curve was performed using H_2O_2 solutions as a standard, and the H_2O_2 production was expressed in µM.

4.3.11 Immunoprecipitation

HaCaT cells have been seeded in 10 cm petri dishes and once reached the 70% of confluence, cells have been treated with catalase 1000 units/ml for 2 hours and exposed to O_3 0.4 ppm for 1 hour. Samples have been collected right after O_3 exposure by washing 2 times in PBS and centrifuged 250 g for 5 min. proteins have been extracted as previously described in general methods and the content has been measured by Bradford assy. The immunoprecipitation assay for NLRP1 has been performed using the Dynabeads™ Protein G (Invitrogen , cat 10003D) as following: 4 ug of NLRP1 antibody (Santacruz sc-) has been diluted in 200 µL PBS-T and incubated with 50 ul of Dynabeads Protein G for 3.5 hours at RT using a rotator. After washing the antibody-protein G complex 3 times with PBS-T, the antibody has been crosslinked to the beads by using the crosslinker Disuccinimidyl Suberate, DSS (CAS 68528-80-3 Alfa Aesar, Thermo Fisher scientific, USA) at a concentration of 5mM. The beads have been incubated with 250 ul of a 5 mM DSS Conjugation Buffer solution (20 mM Sodium Phosphate, 0.15M NaCl (pH 7-9) for 1 hr at RT on the rotator. After quenching the crosslinking reaction by adding 12.5 µL of Quenching Buffer (1M Tris HCl (pH 7.5) for 30 min at RT, the cross-linked Dynabeads have been washed three times with 200 µL PBST and then incubated with 250 ug of sample proteins overnight at 4°C using a rotator. The magnetic bead-Ab-Ag complex has been washed in PBS-t for 3 times and then 30 ul of Laemmli buffer 1x

containing 10% beta-mercaptoethanol has been added. The samples have been denatured at 95 °C for 10 min and the westernblot has been performed as previously described for both immunoprecipitated and input samples.

4.3.12 Detection of IL-1β using ELISA assays

IL-1β levels were measured in the media and cell lysates of *ex vivo* human skin biopsies, using the IL-1β ELISA kit (Proteintech, USA), according to the manufacturer's instructions. Media and cell lysates were harvested 24 hours after the end of O_3 exposure 0.4 ppm for 4 hours as previously described. IL-1β levels were adjusted for protein (pg/mg protein) and for media (pg/ml). Gen5 software (BioTek,USA) was used for the detection.

4.3.13 Statistical analysis

For each of the variables tested, analysis of variance (ANOVA), followed by Bonferroni post-hoc test, was used. Statistical significance was considered at $p<0.05$. Data are expressed as mean ± SD of duplicate determinations obtained in two independent experiments.

4.4 RESULTS

4.4.1 O_3 exposure induces activation of keratinocytes inflammasome

The first step of our study was to evaluate the levels of proteins involved in the inflammasome pathway, in response to O_3 exposure in a 2D skin model, represented by cultured keratinocyte (HaCaT). After exposing the cells for 1 hour to O_3 0.4 ppm, we assessed transcript levels of ASC, Caspase 1, and IL-18 at various time points. Our results showed increased mRNA levels for ASC (Fig. 7A) as well for caspase 1 and IL-18 (Fig. 7B-C) 24 hours after exposure. Next we also wanted to determine the eventual formation of the inflammasome complex, which can be assessed by analyzing ASC oligomerization [510]. Normally, upon stimuli, the canonical activation of inflammasome complex results in the interaction between the Pyrin domain (PYD) or Caspase activation and recruitment domain (CARD) of the NLR sensor and the CARD/PYD domain of the speck-like receptor ASC, which can then oligomerize and interact itself with the CARD domain of pro-caspase1. This interaction leads to the autocleavage of pro-caspase 1 in active caspase1, which then process cytokine pro-forms such as IL-1beta and IL-18 [306]. We observed a striking increase of 50% in the levels of ASC oligomers right after O_3 exposure (Fig. 7D). To further validate these findings, we used immunofluorescence by assessing NLRP1 and ASC colocalization. As shown in Figure 7E, we observed increased colocalization (yellow color) of ASC (green color) and NLRP1 (red color) at 0, 3, 6 and 24 hours post-exposure. Furthermore, speck like receptors ASC oligomers formation was detected right after O_3 exposure (0h), with the evident colocalization of ASC (green color) and NLRP1 (red color) represented by the yellow dots (Fig. 7F). The activation of NLRP1 inflammasomes was also confirmed by high levels of IL-1β and IL-18 found in media of HaCaT cells exposed to O_3 (Fig.7G). To prove that O_3 was actually able to induce the interleukins release via inflammasome activation, we treated HaCaT cells with the caspase 1 inhibitor Z-YVAD-fmk and we measured the protein levels of Caspase 1. As shown in figure 7H, we found that O_3 was able to induce the activation of Caspase 1 and that Z-YVAD- fmk pre-treatment was able to inhibit this activation.

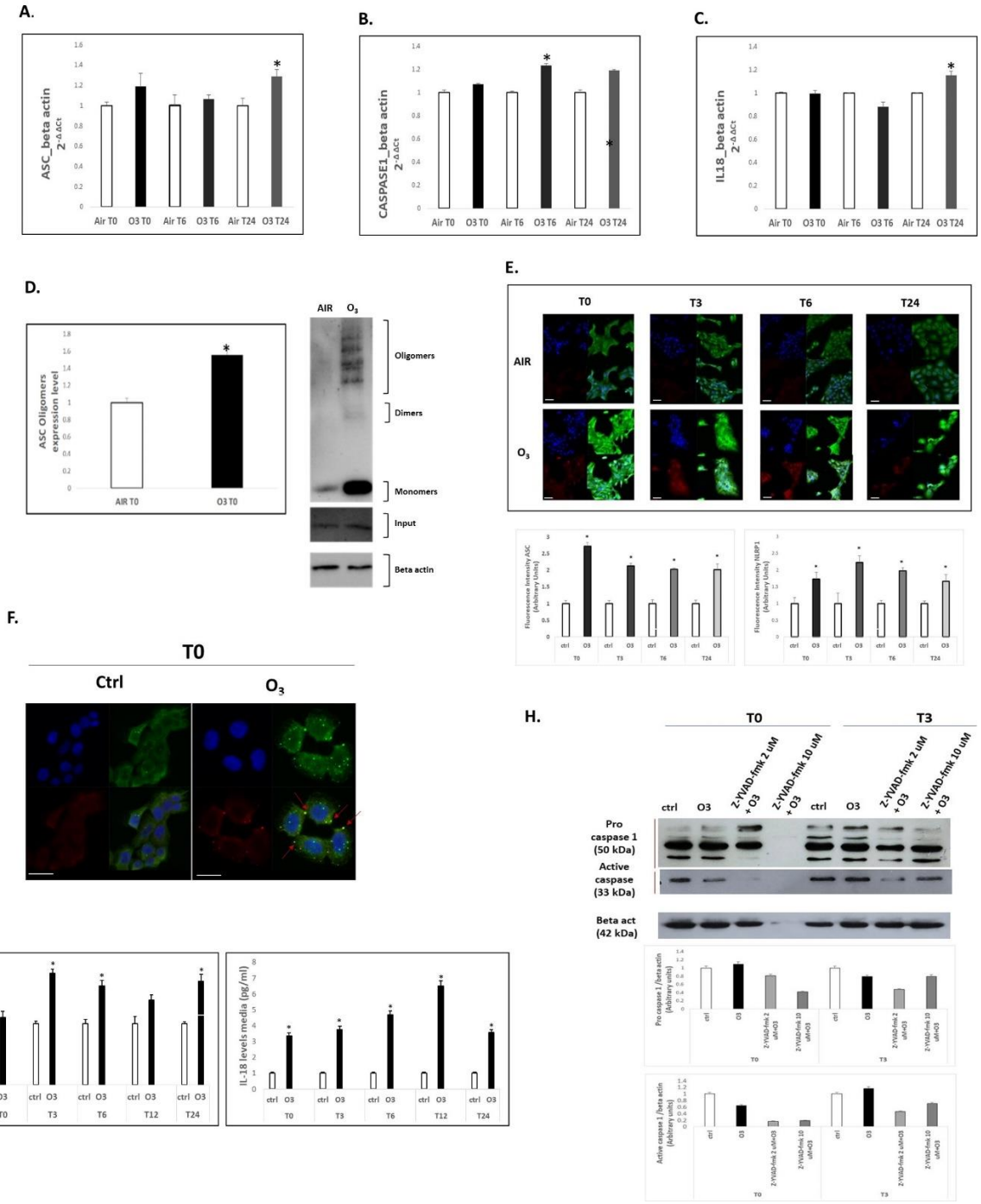


Figure 7. O_3 exposure induces activation of the inflammasome in HaCaT cells. *HacaT cells were exposed to 0.2 ppm of O_3 for 1 hr. Transcript levels of (**A**) ASC, (**B**) caspase 1, and (**C**) IL-18 were measured using qRT-PCR at 0, 6, and 24 hours post-exposure. (**D**) Levels of ASC oligomer levels and beta-actin in HaCaT exposed to 0.2ppm of O_3 for 1 hr. (**E**) Immunofluorescence staining of DAPI (blue), ASC (green), and NLRP1 (red) in HaCaTs exposed to 0.4 ppm of O_3 for 1 hr at 0, 3,6 hours and 24 post-exposure at 40x magnification (Scale bar 20 µm). Immunofluorescent signal was semi quantified by using ImageJ software (National Institutes of Health, Bethesda, MD). (**F**) Immunofluorescence staining of speck like receptor ASC oligomers*

*formation and co-localization with NLRP1 in HaCaTs exposed to 0.4 ppm of O_3 for 1 hour at 0hrs post-exposure at 60x magnification (Scale bar 20 μm). Green staining represents ASC, red staining NLRP1 and blue the DAPI. (**G**) Levels of IL-1β (Left panel) and IL-18 (Right Panel) in media of HaCaT cells exposed to O_3 0.4 ppm for1 hours at 0,3,6,12 and 24 hours post-exposure. **(H)** Levels of Caspase 1 and beta-actin in HaCaT cells pre-treated with Caspase 1 inhibitor Z-YVAD-fmk 2 and 10 μM for 1 hour and exposed to 0.4 ppm of O_3 for 1 hr. Samples collection 0 and 3 hours after O_3 exposure. Data are the results of the averages of at least three different experiments, *p < 0.05 by ANOVA.*

4.4.2 O_3 exposure increases levels of inflammasome components in 3D Reconstructed Human Epidermal tissues (RHEs)

To validate our previous results, we move to a 3D skin model, Reconstructed Human Epidermal tissues (RHEs) which is a more complex in vitro model that better resembles the human skin as all the epidermis layers are present including the stratum corneum [511]. First, we wanted to confirm the ability of O_3 exposure to alter the redox status in these tissues measuring the levels of hydrogen peroxide (H_2O_2) levels. Due to the different complexity of the skin model utilized (presence or not of *stratum corneum)*, we exposed RHEs to O_3 at 0.4 ppm for 4 hours while, as previously described, HaCaT cells were exposed for 1 hour. As shown in Figure 8A, we observed a significant increase in released H_2O_2 levels in media at 0 and 6 hours post-exposure. Next, we examined whether O_3 exposure affected the levels of inflammasome components using qRT-PCR and immunoblotting. Our results demonstrated that O_3 exposure was able, also in this model, to increase the transcript levels for caspase 1 (Fig. 8A) and IL-18 (Fig. 8B). We then evaluated the protein levels of ASC and Caspase 1 by immunoblotting and observed increased levels of ASC and active caspase 1 after and 6 hours post-exposure (Fig. 8C-D), confirming our previous findings in HaCaT cells.

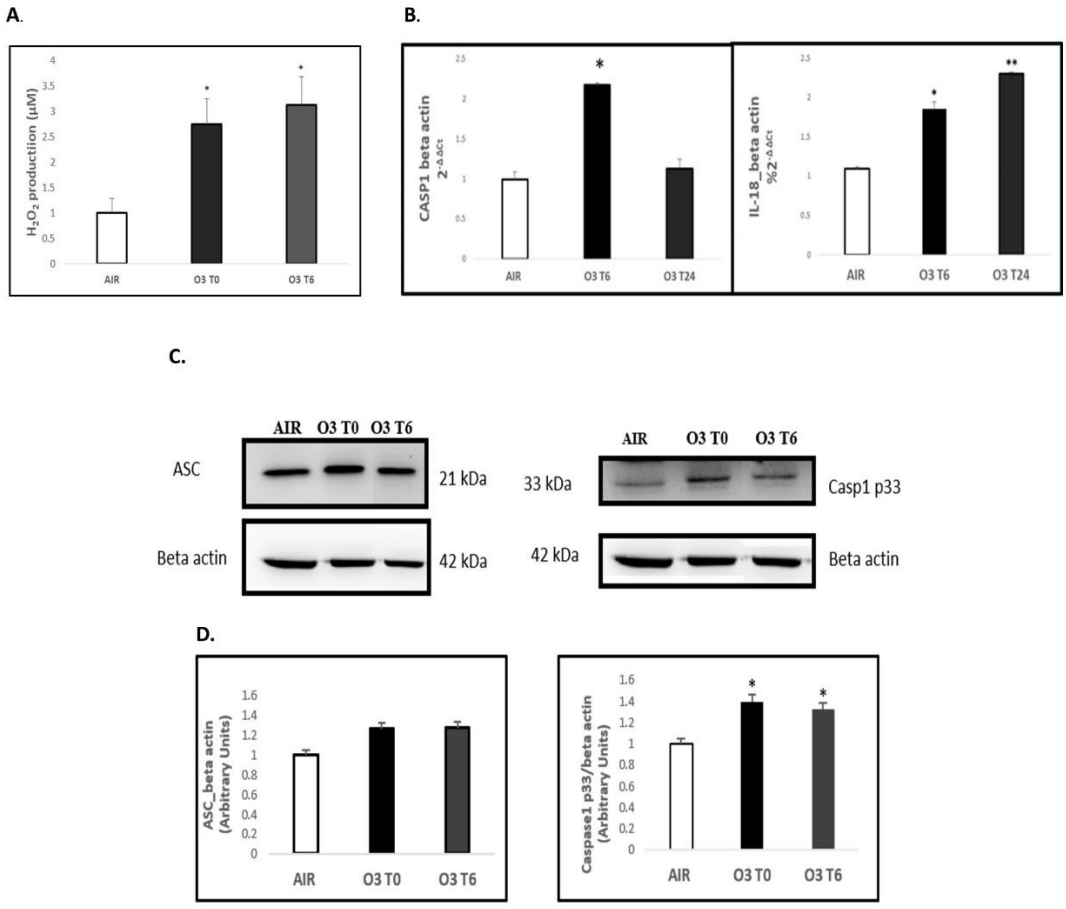


Figure 8. ***O_3 exposure increases levels of inflammasome components in 3D Reconstructed Human Epidermal tissues (RHEs).*** ***(A)*** H_2O_2 levels (µM) in media *and cell lysates in RHEs exposed to 0.4ppm of O_3 for 4 hours directly after exposure and 6 hours post-exposure.* ***(B)*** *mRNA levels of IL-18 and caspase 1 6 hrs and 24 hors post-exposure. (**C**) Protein levels of ASC, active caspase 1, and beta-actin in RHEs exposed to 0.4ppm of O_3 for 4 hours at different time points;(**D**) quantification of ASC and caspase 1 levels via analysis in imageJ. Data are the results of the averages of at least three different experiments, *p < 0.05 by ANOVA.*

4.4.3 Inflammasome activation in ex vivo human skin explants exposed to O_3

In addition to 2D and 3D *in vitro* human skin models, we also wanted to confirm our data in a model even closer to real life, represented by *ex vivo* human skin explants. We first evaluated the ability of O_3 to affect tissue redox homeostasis by measuring the levels of 4-hydroxy-nonenal levels (4HNE). As depicted in (Fig. 9A) there was a clear increase of 4HNE right after the O_3 exposure and 6 hours post exposure while the signal slightly

decrease at 24 hr. We next evaluated the levels of inflammasome components by qRT-PCR and observed higher transcript levels for NLRP1, ASC, and IL-18, at 6 hours post-exposure (Fig. 9B). We also observed increased protein levels of ASC and active caspase 1, at 6 hours post-exposure (Fig. 9C), confirming what we observed in the previous skin models.

To confirm that O_3 exposure induces inflammasome activation, as we observed in the 9D model, we investigated the co-localization of NLRP1 and ASC in human skin biopsies. As It is shown in Fig. 9D, we observed that O_3 exposure induced co-localization of NLRP1 at 0, 6, and 24 hours post-exposure. In addition, we also assessed whether O_3 exposure induced IL-1β release, which is considered the final step for the inflammasome activation [197]. As shown in Fig.9E, there was a striking increase in the levels of IL-1β, 24 hours post-exposure either in media and tissue lysate, confirming the presence of an inflammatory status in the human biopsies exposed to O_3.

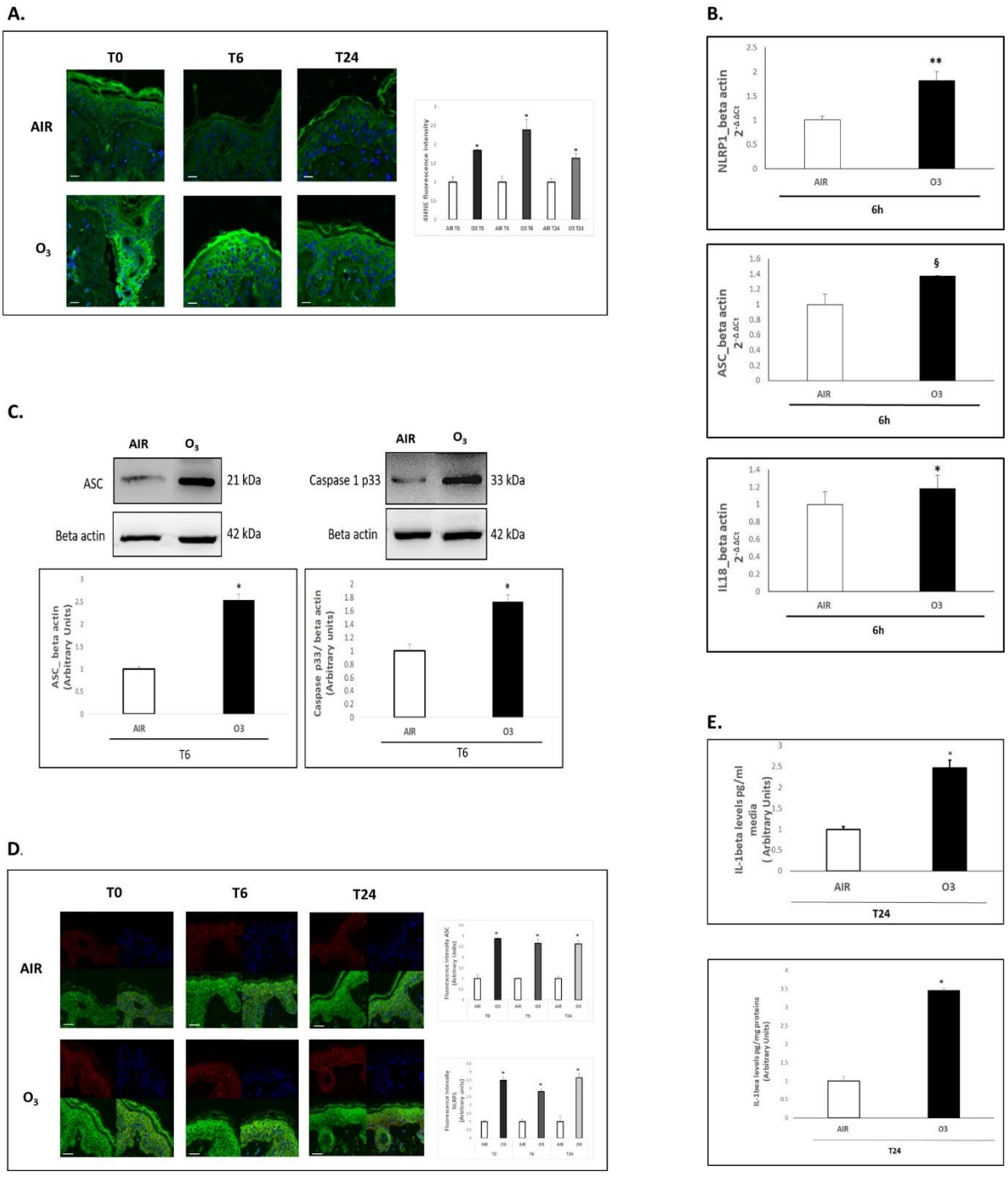


Figure 9. Inflammasome activation in ex vivo human skin biopsies exposed to O_3. (A) *Immunofluorescence (IF) staining for 4HNE (green) and DAPI (blue) in ex vivo human skin biopsies exposed to 0.4 ppm of O_3 for 4 hours directly after exposure, 6 hours, and 24 hours post-exposure at 40x magnification (scale bar 20 µm). Immunofluorescent signal was semi quantified by using ImageJ software (National Institutes of Health, Bethesda, MD).* ***(B)*** *Transcript levels of NLRP1, ASC, and IL-18 in ex vivo human skin biopsies exposed to 0.4 ppm of O_3 6 hours post-*

exposure. (**C**) Levels of *ASC, active caspase 1 p33, and beta-actin protein in ex vivo human skin biopsies exposed to 0.4 ppm of O_3 6hrs post-exposure. (**D**) IF staining for ASC (green), NLRP1 (red), and DAPI (blue) in ex vivo human skin biopsies exposed to 0.4 ppm of O_3 6hrs and 24 hours post-exposure at 40x magnification (scale bar 20 μm). (**E**) Levels of IL-1β (pg/mL) in the media and cell lysate of ex vivo human skin biopsies exposed to O3 0.4 ppm for 4 hours exposed to 0.4 ppm of O_3 6 hours and 24 hours post-exposure using an IL-1β ELISA kit. Data are the results of the averages of at least three different experiments, *p < 0.05 by ANOVA.*

4.4.4 *H_2O_2 and 4HNE as mediators of O_3-induced inflammasome activation*

Our previous results demonstrated that O_3 exposure within skin increased transcript and protein levels of the inflammasome components, and, most importantly, stimulated activation of the inflammasome. Since we also observed increased levels of 4HNE and H_2O_2 in response to O_3 exposure, we wanted to understand whether these molecules could be responsible for O_3 induced inflammasome activation. For this purpose, we treated HaCaT cells with 4HNE and/or H_2O_2, and evaluated the levels of ASC oligomers. Our results demonstrated that both 4HNE and H_2O_2 induced ASC oligomerization 6 hours post-treatment, compared to the untreated control (Fig.10A). Furthermore, we also observed increased protein levels of ASC directly after treatment and active caspase 1 at 6 hours post-treatment (Fig 10B). To further validate the idea that the inflammasome can be activated by O_3-induced H_2O_2 and 4HNE, we pretreated HaCaT cells for 2 hours with catalase and then exposed the cells to O_3 for 1 hour. As shown in Figure 10C, we observed a clear decrease in ASC oligomers when the cells were pretreated with catalase. We then assessed protein levels of active caspase 1 in these samples, and we observed that pretreatment with catalase reduced also the levels of active caspase 1 induced by O_3 exposure (Fig.10D).

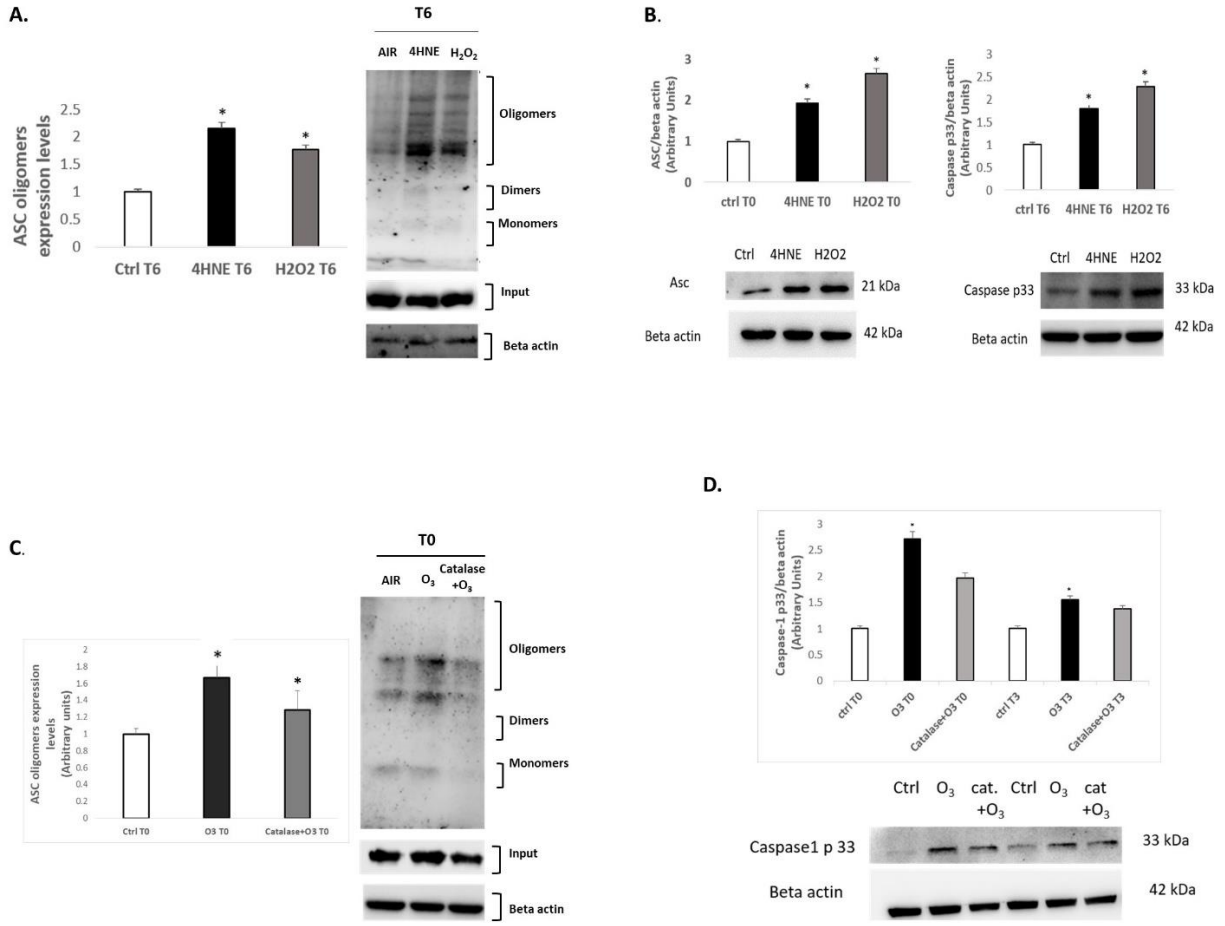


Figure 10. H_2O_2 and 4HNE as mediators of O_3-induced inflammasome activation. ***(A)*** *ASC oligomer levels and quantitation of these levels in HaCaTs treated with either 20 μM of 4HNE or 40 μM of* H_2O_2 *for 30 min 6 hrs post-treatment.* ***(B)*** Protein levels of *ASC, active caspas -1, and beta-actin in HaCaTs treated with either 20 μM of 4HNE or 40 μM of* H_2O_2 *for 30 min directly after treatment and 6 hours post-treatment.* Levels of ASC oligomers **(C)**, active caspase 1 *β-actin* (**D**) in *HaCaT that were pre-treated with 1000 U/mL of catalase for 2 hours then exposed to 0.4 ppm of O_3 for 1 hour directly after exposure and 3 hours post-exposure. . Data are the results of the averages of at least three different experiments, *p < 0.05 by ANOVA.*

4.4.5 Post-translational modification induced by O_3-related oxidative stress as possible key regulator event in NLRP1 inflammasome activation

Since 4HNE is able to induce adducts by interacting with biomolecules such as proteins, we wanted to investigate whether 4HNE induced by O_3 exposure was able to interact with inflammasomes proteins components such as NLRP1 and ASC. Therefore, we performed a double immunofluorescence staining for NLRP1/4HNE and ASC/4HNE. As shown in figure 11A a clear colocalization between NLRP1 and 4HNE occurred especially 0 and 3 hours after the end of O_3 exposure whereas for ASC and 4HNE (Fig. 11B), the colocalization was detected 0 and 6 hours after the end of O_3. To prove that cutaneous inflammasome activation by O_3 was redox regulated, we pre-treated HaCaT cells with Catalase before O_3 exposure and we performed a double IF staining for NLRP1/ASC and NLRP1/ 4HNE. Our resulted confirmed the formation of the inflammasome complex after O_3 exposure (Fig. 11C, left panels) as depicted by the yellowish color resulted by the colocalization of NLRP1/ASC and that catalase was actually able to prevent the complex formation and therefore the inflammasome activation after O_3 exposure at all timepoints. Moreover also the colocalization of NLRP1 and 4HNE was prevented by catalase pre-treatment (Fig. 11C, right panel) suggesting that the O_3 redox regulation of the inflammasome NLRP1 activation was mediated by 4HNE induced by O_3 itself.

One of the major fates of modified proteins such as 4HNE protein adducts (PAs) is to be degraded via the ubiquitin-proteasome pathway. Since inflammasome protein components such as NLRP1 and ASC turned out to be target proteins of 4HNE, we wondered if also these proteins could be ubiquitinated. The first step was to measure the levels of ubiquitinated proteins in cytosol and nuclei of HaCaT cells after O_3 exposure to evaluate in which compartment the ubiquitinated proteins were more present. Therefore we pre-treated with the proteasome inhibitor MG-132 and exposed the cells to O_3 0.4 ppm for 1 hour (timepoints 0,3 and 6 hours). The Western blot result showed that O_3 was able to induce an increase in ubiquitinated proteins levels and that this increase was more evident in the cytosol fraction compared to nuclei, especially at timepoints 0 and 3 hours (Fig 11D). Since NLRP1 is mainly present in cytosol and it is the first protein directly involved in inflammasome activation, we decided to evaluate whether O_3 was able to

induce its ubiquitination by performing an immunoprecipitation for NLRP1 and probing for ubiquitin. As shown in figure 11E, O_3 exposure was able to induce an increase in ubiquitinated- NLRP1 protein levels right after the end of exposure and interestingly, catalase pre-treatment was able to prevent this increase, suggesting that NLRP1 ubiquitination is a redox regulated mechanism induced by O_3 exposure.

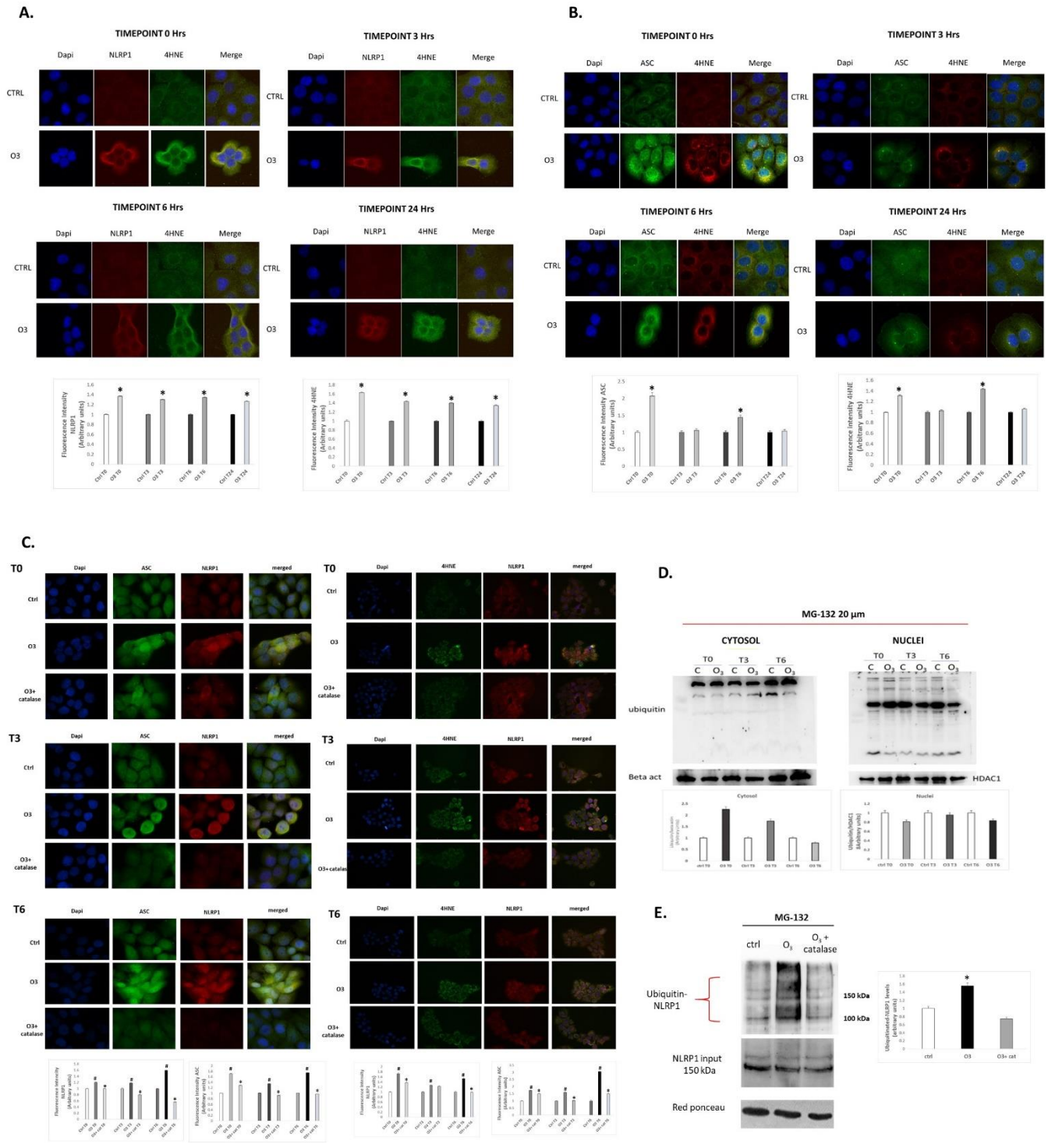


Figure 11. ***(A)*** *Double Immunofluorescence staining for NLRP1 (Red), 4HNE (green) and Dapi (blue) in HaCaT cells exposed to O_3 0.4 ppm for 1 hour (60x magnification). Samples collection 0,3,6 and 24 hours after O_3 exposure. (**B**)) Double Immunofluorescence staining for ASC (green), 4HNE (Red) and Dapi (blue) in HaCaT cells pre-treated with Catalase1000 units/ml for 2 hours and exposed to O_3 0.4 ppm for 1 hour (60x magnification). Samples collection 0,3,6 and 24 hours*

*after O_3 exposure. (**C**) Double Immunofluorescence staining for NLRP1 (Red), ASC (green) (**left panel**) and NLRP1 (red), 4HNE (green) (**right panel**) in HaCaT cells pre-treated with Catalase1000 units/ml for 2 hours and exposed to O_3 0.4 ppm for 1 hr. Dapi staining (blue) for nuclei, Samples collection 0,3 and 6 hours after O_3 exposure. (**D**) Levels of ubiquitinated proteins in nuclei and cytosol of HaCat cells pre-treated with proteasome inhibitor MG-132 20 μM for 3 hours and exposed to O_3 0.4 ppm for 1 hour. Samples collection at 0,3 and 6 hours after O_3 exposure. β-actin has been used as loading control for cytosol and HDAC for nuclei. (**E**) Immunoprecipitation assay for NLRP1 and probed with Ubiquitin in HacaT cells pre-treated with proteasome inhibitor MG-132 20 μM for 3 hours and exposed to O_3 0.4 ppm for 1 hour. β-actin has been used as loading control. Data are the results of the averages of at least three different experiments, *$p < 0.05$ by ANOVA.*

4.5 DISCUSSION AND CONCLUSIONS

O_3 is commonly recognized as one of the most noxious air pollutants and its noxious effects have been well demonstrated not only in the respiratory tract but also more recently in the cutaneous tissues [191 58512 513]. In general, O_3 is able to alter the skin redox homeostasis and induce tissue inflammation processes that have been linked to the development and exacerbation of several skin conditions. *Xu et al* were able to demonstrate a direct association between O_3 and emergency hospital visits for total and specific skin conditions (such as urticaria, eczema, contact dermatitis, rash/eruption, and infection) in Shanghai, China [499]. Interestingly, other air pollutants (PM_{10}, sulfur dioxide (SO_2) and NO_2) were uncorrelated with skin conditions (Xu et al. 2011). In addition, O_3 concentrations were associated with physician home visits for skin rash and conjunctivitis in a study from 22 cities in France; similarly, authors found no associations with PM_{10} and NO_2 [500]. More recently, *Szyszkowicz et al*. have found positive associations of short-term O_3 concentrations with hospital admissions for skin conditions (such as dermatitis, urticaria, cellulitis) in multiple areas in Canada [514501]. In addition a recent epidemiological study from *Fuks et al*. [190] clearly showed the correlation between O_3 levels and skin aging. Although the link between O_3 exposure and skin inflammatory processes has been hypothesized, the exact mechanism involved in this process needs further investigation. In the last two decades inflammatory process regulation has been related also to the inflammasome activation. Indeed, inflammasome machinery is characterized by intracellular multiprotein complexes that, once activated, can play a key role in the development of several inflammatory pathologies including skin conditions[446,452,469,515]. Few studies have suggested the ability of air pollutants, such as cigarette smoke, PM and O_3 to increase the levels of inflammasome components through ROS production, in both the respiratory tract and vascular system but to date no data on the role of pollution on the cutaneous inflammasome activation has been studied [516464 329]. In addition, in the previous studies the activation of the inflammasome, either via ASC oligomerization or scaffold formation, was not analyzed. To understand whether O_3 is able to affect the inflammasome components in cutaneous tissues we perform the first set of experiments in human keratinocytes *(*HaCaT). We found that O_3 exposure increased transcript levels

of ASC and caspase 1 (directly after and 6 hours post-exposure), and IL-18 (24 hours post-exposure) and this timeline is representative of the inflammasome activation as IL-18 release is the last step of the process. We also observed colocalization of NLRP1 and ASC in the 2D model exposed to O_3 data that suggests the formation of the scaffold and therefore its activation.

Even though we obtained encouraging results in cell culture, this model fails to represent the complexity of skin tissues, where keratinocytes undergo a differentiation process, called keratinization, to form the different layers of the epidermis. Therefore we decided to focus our attention on a 3D skin model, Reconstructed Human Epidermal tissues (RHEs), which reproduces the different skin layers, including the stratum corneum, which is the first barrier with which O_3 interacts [517] [97]. Indeed, it has been suggested that O_3 is too reactive to penetrate the skin and its interaction with the stratum corneous lipids will generate reactive molecules such as H_2O_2 and unsaturated aldehydes (i.e. 4HNE). Therefore, to evaluate the reaction between the O_3 and SC, we first evaluated the levels of hydrogen peroxide (H_2O_2) after O_3 exposure in RHEs. We clearly observed higher levels of H_2O_2 in exposed tissues, in both the media and cell lysates, confirming the ability of O_3 to induce an imbalanced redox status. Also in this model, we observed higher transcript levels of caspase 1, as well as increased protein levels of caspase 1 and ASC, confirming the data obtained in HaCaT cells and that O_3 is able to modulate the inflammasome machinery. Considering that human skin is even more complex that RHE, we want to confirm our data in human skin explants. Indeed, skin biopsies exhibit normal skin barrier function, a mature stratum corneum, a functional basal layer, and all the cell types and skin appendages as *in vivo* human skin [518]. Also in this set of experiments we want to first understand whether O_3 exposure was able to affect the redox status by detecting increased levels of 4HNE in skin tissues. qRT-PCR and immunoblotting results confirmed again ASC, IL-18 and caspase 1 mRNA and protein levels increase as well, as we had observed in the other skin models. Moreover, NLRP1, one of the main inflammasome complex scaffolding proteins expressed within the skin, seemed to be modulated by O_3, showing significantly increased transcript levels after exposure. Of note is that we were able to confirm the assembly of the inflammasome scaffold after exposure via immunofluorescence staining. Importantly, we also observed that exposure increased

the level of IL-1β in both media and cell lysates, indicating the effective activation of the pathway.

Since we also observed increased expression levels of 4HNE and H_2O_2 in the different skin models after O_3 exposure, we wondered whether the production of these mediators was responsible for O_3-induced inflammasome activation. We observed increased levels of ASC oligomers levels after 4HNE and H_2O_2 treatment as well as higher protein levels for both ASC and active caspase 1. Moreover, by inhibiting H_2O_2 production using catalase, we observed a decrease of ASC oligomer levels right after O_3 exposure and also less active caspase 1 protein levels. These data suggest that H_2O_2 and lipid peroxidation products, produced by the interaction between O_3 and skin lipids, are most likely the responsible molecules for the inflammasome activation in our models. Previous studies from our and other labs have already shown that O_3 exposure increases levels of 4HNE protein abducts both in human skin and in murine models [58,111,519]. It should be mentioned that the interaction between O_3 and cutaneous tissues is very similar to the one with the respiratory tract. Indeed, it has been showed by Pryor [215] that when O_3 is inhaled, reacts with the lung lining fluid leading to the formation of 4HNE and H_2O_2. We can therefore suppose that O_3, also in the lung, is able to activate the inflammasome pathways in similar manner. In addition, a previous study from our group, was able to demonstrated “in vivo”, that lung and skin tissues respond similarly in terms of inflammatory and oxidative markers when exposed to O_3 [58]. Furthermore, 4HNE adducts formation and ROS production are linked to the onset of several diseases which also display an inflammatory status mediated by inflammasome activation [116,520,521]. However, there are fundamental differences between the different types of inflammasomes and the assembly of the scaffold depends on several stimuli. For instance ROS generation has been demonstrated to be an important trigger signal in the activation of certain inflammasomes such as NLRP3, which normally requires two-step activation.

The two-step activation includes a priming phase, stimulated by microbial or endogenous molecules, that involves the activation of NF-κB and subsequent expression of pro-IL-1beta upon an external stimulus as well as post-translational modifications (i.e. phosphorylation etc..) to NLRP3 and ASC [522]. Interestingly, we did not observe changes in the transcript levels of inflammasome components until after we detected ASC

oligomerization. The second signal in inflammasome activation induces conformational changes in NLRP3 and binding with ASC (although the exact sequence of events is under debate) and subsequent assembly of the complex [522]. This signal can be triggered by ATP, pore-forming toxins, and viral RNA due to the formation of mitochondrial reactive oxygen species (ROS), release of cathepsins from lysosomes, potassium efflux, and increase in intracellular calcium [293] [523]. In contrast to NLRP3, which is the best-studied NLRP inflammasome and is generally activated from a wide array of stimuli [524,525], the causes leading to NLRP1 inflammasome activation are still under investigation [372] [367] [357] [371]. However, we know that activation of the NLRP1 inflammasome, which we have detected in this study in *ex vivo* human skin biopsies, requires cleavage of the FIIND domain (not present in NLRP3). This idea is supported by studies demonstrating that mutations in the FIIND domain can lead to the activation of the complex [359,469,526] and that the presence of FIIND domain co-factors/ agonists can keep the complex inactivated [367]. Cleavage of the FIIND domain results in ubiquitination and degradation of the N-terminus, leading to release of the active C-terminal fragment [376] . Thus, we hypothesize that O_3 exposure, through ROS and/or 4HNE, induces post-translational modifications in NLRP1 that results in the inflammasome assembly. Indeed, Pollutants-oxidative stress mediators such as ROS and 4HNE have already been demonstrated to induce several modifications in their molecular targets [527,528] mainly due to phosphorylation and ubiquitination mechanisms, leading to an altered skin homeostasis [37]. Interestingly we found higher co-localization levels of inflammasomes components such as NLRP1 and ASC with 4HNE in HaCaT cells exposed to O_3, and pre-treatment with Catalase, an antioxidant enzyme quenching excessive H_2O_2, was able to prevent the NLRP1 inflammasome assemble and the colocalization of 4HNE to both NLRP1 and ASC, in response to O_3 exposure. Since our results suggested that H_2O_2 and 4HNE are the main bioproducts able to activate the inflammasome NLRP1, we wondered a possible role of H_2O_2 and 4HNE in regulating this activation by inducing NLRP1 ubiquitination and consequent degradation of the N-Terminus fragment via the ubiquitin-proteasome pathway. Higher levels of ubiquitinated proteins was found in HaCaT cells exposed to O_3, confirming the ability of the pollutant in induce this post translational modification. In addition, catalase treatment was able to decrease the levels of ubiquitinated- NLRP1 found in HaCaT cells in response to O_3

exposure, suggesting that NLRP1 ubiquitination is mediated by oxidative stress, more likely as a consequence of its modification by 4HNE. Therefore, we suggested that O_3, interacting with skin tissue biomolecules, induces the production of ROS such as H_2O_2 which then lead to lipid peroxidation and 4HNE formation. 4HNE is then able to interact with proteins, including NLRP1, inducing the N-Terminus fragment degradation via the proteasome pathway through ubiquitination. Indeed It is possible that pollutants such as O_3 can modulate the activation of inflammasomes by inducing PTMs of inflammasome proteins components throughout their oxidative stress mediators. Moreover previous reports have indicated that photochemical smog (of which O_3 is a major component) is able to induce alterations in the skin microbiome [529] [530]. The consequences of this alteration can contribute to the development of skin pathologies such as atopic dermatitis, which are also linked to O_3 exposure [531] [532] [533]. In light of our findings, it would be interesting to see whether O_3 exposure results in alteration of the skin microbiome, due to increased inflammasome activity, since this complex is not activated in resting keratinocytes, as we have shown in this study. In conclusion, for the first time, we have demonstrated that O_3 induces activation of the inflammasome complex within the skin using a variety of models, from 2D in vitro cell culture to 3D *ex vivo* human skin biopsies linking O_3 exposure to the inflammatory skin conditions already suggested in epidemiological studies. For instance, many inflammasome inhibitors have been investigated as treatments for inflammatory diseases by targeting either Inflammasomes sensors such as NLRP3 or inflammasome-associated proteins, as caspase 1 or ASC, or the non-canonical inflammasome pathway[534–543]. However, most of the existing data on inflammasome inhibition comes from *in vitro* or *in vivo* experiments in animal models, and these studies have primarily only focused on targeting the NLRP3 inflammasome. Future studies should focus also on whether targeting other types of skin-associated inflammasomes, such as NLRP1 or Aim2, can prevent the development/exacerbation of inflammatory skin disorders also associated to environmental pollutants[470,494,544,545], since it is likely that multiple types of inflammasomes are activated in these skin disorders, simultaneously[546].

5. PROJECT 2: INDUCTION OF CUTANEOUS OXINFLAMMATION BY COMBINED POLLUTANTS EXPOSURE

5.1 INTRODUCTION

5.1.1 Ox-Inflammatory effect of combined pollutants exposure on human skin

Environmental pollution is a very complex field to investigate, considering both the individuality of human being in term of genome and habits [7,134] and the ability of toxic compounds to be simultaneously released within the lower atmosphere, interacting to each other. Moreover, since the deteriorating of the O_3 layer is favoring our exposure to UV radiations, in the past decades the interaction between toxic compounds such as Particulates Matter, ground level O_3, volatile compounds VOx and NOx etc. with UV light has becoming more common, possibly resulting in a combined and synergistic noxious effect on tissues, in particular on skin [104,105]. Indeed pollutants and UV radiations are all able to induce oxidative and inflammatory reactions within the cutaneous tissue, altering the skin homeostasis and exacerbating skin conditions [547,548]. The increasing incidence of skin pathologies, sunburn, cancer and photoaging due to UV radiations exposure could be actually exacerbated by the activity of environmental pollutants [65]. However, even though pollutants and UV radiations are all able to induce the same skin ox-inflammatory status, also resulting in an altered skin barrier functionality , very few studies so far have investigated their possible synergistic effect in enhance skin damage [104,105,549,550].

I. UV lights

Ultraviolet radiations (UV lights) represent the 10% of the total electromagnetic radiation emitted from the sun [551]. Based on the wavelengths, sun radiations can be divided in different subtypes: long wavelengths such as UVA (400-315 nm), medium wave lengths

such as UVB (315- 280 nm) and the short wavelengths UVC (280-100 nm) [65]. The O_3 layer within the atmosphere acts as a natural shield protecting us against a great part of these dangerous radiations. For instance, even though the O_3 layer is not able to block UVA, it is able to absorbs UVC and part of UVB. Therefore the most predominately and harmful sunlight radiations present in the environment are UVA (90-95%) and the fraction of UVB radiations able to pass through the atmosphere (5-10%), which are known to affect especially skin and eyes health, whereas UVC is able to interact with oxygen in the atmosphere to form the O_3 composing the O_3 layer itself. In the past decades the developing urbanization had led to the continuous release of toxic substances into the atmosphere, such as Chlorofluorocarbons (CFCs), able to dissociate in the presence of UV light and deplete the O_3 layer via a catalytic reaction[552,553]. The lack of O_3 layer has been correlated to several important climate changes and also to an increase in the onset of different skin pathologies due to the prolonged exposure to sun radiations. Indeed even though a short sun exposure (5-15 min) is known to be good for our health since it induces the production of vitamin D [554], a longer exposure, especially during midday, is extremely dangerous for skin health and can cause sunburn, skin cancer, collagen depletion, skin aging etc.[555]. Moreover UVB can affect eyes by inducing cataracts, pterygium, pinguecula formation and damage the cornea, retina and lens [556–558]. Both UVA and UVB are able to penetrate the skin causing inflammation, oxidative stress and DNA damage via a different mechanism. For instance UVB radiations, which are normally absorbed by the epidermis, lead to the formation of pyrimidine dimers in DNA and consequent mutations (direct mechanism), whereas UVA which are able to penetrate more in deep the skin up to the dermis, lead to the production of reactive oxygen species able to damage the DNA (indirect mechanism) [559] as well as other biomolecules such as protein and lipids. UV-induced DNA damage is the primary cause of melanoma, [560]. Melanocytes, which are a type of skin cells mainly present within the Stratum Basale of the epidermis, are normally involved in the production of a pigment, melanin, which represent a natural sunscreen to protect the skin against sun radiations. These cells can be found in the basal layer but also in hair follicles and are also involved in keeping skin homeostasis by protecting from oxidative stress induced by UV light and also from microbes injury [561–564]. Indeed UV radiations are able to induce the activation of

inflammatory pathways within the skin such as NF-kb [565,566], which lead to the release of cytokines or other inflammatory mediators whom effects culminate in the onset of sunburn[555,567] and also in the activation of the Aryl hydrocarbon receptor (Ahr).

Ahr is a transcription factor that modulates the expression of genes involved in both the antioxidant and the inflammatory responses of our body[144]. Within the skin, Ahr is also involved in the modulation of keratinocyte proliferation, differentiation, skin immunity, epidermal barrier function and also melanogenesis [150]. Activation of Ahr by UV radiations has been associated to inflammation, altered pigmentation, DNA damage and apoptosis leading to the onset of skin aging and carcinogenesis [142]. Moreover UV radiations can also activate other pathways such as the tumor suppressor p53, which is known to modulate the cell cycle, apoptosis, DNA repair [568,569] and to induce an iper-proliferation of keratinocytes which can lead to a form of skin thickness called hyperkeratosis or epidermal hyperplasia [570,571]. UV photons, especially UVA, are able to interact with oxygen inducing oxidative stress reactions which lead to the production of ROS able to interact with biomolecules within the skin, such as lipids, proteins and DNA, altering their structure and function and also leading to the activation of oxidative stress-related pathways such as NRF2 [572,573]. All these UV-induced ox- inflammatory events and DNA damage can cause skin cancer, aging and also exacerbate a variety of skin conditions such as dermatosis [574–576]. To counteract the UV-induced skin damage in term of sunburn, DNA damage and free radicals production, there are several products such as sunscreen which contain molecules able to absorb UVA and UVB radiations[6,577]. However the increasing presence of other pollutants in the atmosphere, such as Particulates Matters, O_3, cigarette smoke etc. are now leading to an increase in skin conditions by contributing to the development of oxidative and inflammatory reactions within the cutaneous tissues, suggesting their possible additive/synergistic effect in enhance the UV induced skin damage.

5.1.2 Effect of Combined pollutants exposure on skin redox-homeostasis and functionality

Even though pollutants and UV lights are all able, singularly, to induce an Oxinflammatory status, altering the skin homeostasis and exacerbating skin conditions [547,548] , very few studies have investigated their possible additive/synergistic [105,549,550]. Poly aromatic carbons (PAHs) which mainly form PM but also cigarette smoke, have been shown to be able to absorb UVA photons and therefore be photoactivated by UV lights , exacerbating the UV-skin damage. Indeed Photoactivate PAHs can transfer energy and electrons to oxygen (singlet oxygen) initiating oxidative reactions which lead to the production of ROS and DNA damage [578–582], leading to an altered skin redox homeostasis [583]. For instance squalene, one of the main lipid present within the skin, can be oxidized by singlet oxygen [584,585], suggesting that the combination of UV and PAHs could exacerbate the skin damage and the inflammatory response induced by oxidation of squalene already demonstrated in keratinocytes exposed to UV light [586].Pollutants induced photo-oxidative stress can lead to the depletion of skin surface antioxidants, especially vitamin E, vitamin C and glutathione resulting in a structural skin damage and an impairment of the barrier function and skin aging, which can be prevented by the application of topical compounds containing vitamin E and C [130,587],[574]. For instance O_3 in combination with UV lights has been demonstrated to potentiate the UV-induced depletion of Vitamin E, one of the most recognized cutaneous antioxidant defense [127,129]. 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, the cornified envelope (CE), which is the primary barrier of the skin [588]. For instance several studies showed that pollutants such as Particulates Matter and UV are able to modulate skin proteins such as Involucrin, Filaggrin, keratin which are essential for skin differentiation and proper barrier function [589–591] and whom impairment is often associated to an altered skin homeostasis which can lead to skin disorders such as atopic dermatitis and psoriasis, carcinogenesis and impaired wound healing etc.. [74,75,83,90,92,101,592]. Moreover, pollutants have been shown to affect also other essential skin barrier components, such as TJs (claudin-1, ZO-1, Occludins) and water channels that are involved in maintaining the skin barrier homeostasis [102] and whom alteration has

been associated to the development of several inflammatory skin conditions such as atopic dermatitis, psoriasis [593]. For instance 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 [594], as well as PM, which is able to induce TJ ZO-1 downregulation via ROS generation [595]. All these evidences suggest that the combination of pollutants can actually enhance the skin damage by acting synergistically to activate inflammatory pathways and to induce oxidative stress reactions, exacerbating skin disorders, carcinogenesis[596] including the skin aging process.

5.1.3 Antioxidants compounds as therapeutic approach to prevent the Ox-inflammatory damage within the skin

As previously described, skin is one of the first tissue targeted by environmental pollutants exposure and it therefore represents the main barrier for our body against harmful stimuli. Thus, cutaneous tissue is equipped with different defensive systems to counteract the oxidative damage induced by pollutants exposure. For instance, skin synthetizes several antioxidant enzymes such as Superoxide dismutase (SOD), Glutathione peroxidase (GPx) and it also relies on micronutrients such as Vitamin C, A, E that display antioxidant properties. Indeed, excessive amount of ROS induced by the interaction between pollutants and skin biomolecules can lead to the activation of inflammatory pathways, apoptosis, DNA, proteins, lipids damage etc.. All these events are correlated to premature skin aging, and skin conditions such as dermatitis, acne, psoriasis etc.[112].

To counteract the skin oxidative damage, several topical antioxidant formulations have been developed in the past decades. Moreover a correct diet, which introduce vitamins and micronutrients so to also restore the gut microbiome, is often associated to the application of topical antioxidant compounds to preserve the skin health maintenance[597]

I. Endogenous defensive enzymes

To counteract the oxidative damage induced by pollutants exposure, cells disposes of endogenous enzymes able to scavenge ROS, such as SOD, CAT or GPx [598,599]. SOD, which exists in three different isoforms and it is primarily present within the epidermis in the skin [600], catalyzes the dismutation of the superoxide radical O_2^- into oxygen and H_2O_2 by using metal co-factors such as Zn, Cu, Mn [601]. Catalase, which is mainly present within the epidermis in the stratum corneum [602], and also GPXs are instead the main enzymes able to convert H_2O_2 in water and therefore prevent the production of hydroxyl radicals ($^{\bullet}HO$) which is known to trigger lipid peroxidation and other free radicals reactions. Indeed, even though H_2O_2 is not a radical species per se, it can interact with free copper or iron leading to the production of $^{\bullet}HO$ via the Fenton's reaction, first described in 1984[603]. The GPXs family encodes for 8 different isoforms of GPxs in humans which almost all of them use selenium as cofactor [604] and they are essential to prevent lipid peroxidation.

An altered activity or mutation of these antioxidant enzymes have been associated with several pathologies such as diabetes, obesity, Atherosclerosis, chronic obstructive pulmonary disease (COPD) and ages associated neurodegenerative diseases such as Parkinson and Alzheimer [605–608] as well as to the skin aging process and carcinogenesis [609–611], which are often associated to pollutants exposure. Indeed pollutants are known to be able to alter the skin redox homeostasis by modulating the activity of cutaneous antioxidant enzymes [210]. For instance PM has been associated with high levels of SOD and GPx in human keratinocytes [612] and higher level of oxidative stress and a reduction in catalase activity have been found in mice skin exposed to UVB radiation [613]: Furthermore, environmental pollutants have been found to increase levels of SOD and oxidized stratum corneum proteins in cutaneous tissues of patient affecting from atopic dermatitis [280] suggesting that they are involved in the exacerbation of skin pathologies by modulating the antioxidant response. Indeed altered levels of SOD, CAT and GPx 6 as well as increased lipid peroxidation have already been associated to the onset of several skin inflammatory diseases such as psoriasis[614–616], confirming that the existing cross-talk between the oxidative and inflammatory response can be exacerbated by pollutants exposure in the onset of these skin condition [95].

II. Skin micronutrients and topical antioxidants application

Besides endogenous enzymes, skin present other micronutrients able to prevent oxidative stress reactions and restore the skin redox homeostasis which can be often altered by the action of pollutants and other external factors.

For instance the most abundant antioxidant components within the cutaneous tissue are Tocopherol (Vitamin E) and Ascorbic Acid (Vitamin C), which represent the primary antioxidant skin defense, but other components are also present such as carotenoids, Uric acid and Co-enzyme Q_{10} ($CO\text{-}Q_{10}$)[112]. Great part of these components are synthesize by plants and they can be taken up by the diet and their main activity is to quench ROS production and therefore stop lipid peroxidation, protecting the skin from oxidative stress [611,618–620]. Moreover, they can even promote keratinocyte differentiation and skin barrier function. For instance, the detoxifying process of α-tocopherol, the most abundant form of Vitamin E in human skin, resided in its ability to lose protons quenching the reactivity of ROS, such as the singlet oxygen [621] but also superoxide anion radical, perhydroxyl radical and hydroxyl radical [622], and be transformed in a less reactive radical, whereas Vitamin C can donate electrons to free radicals and be transformed in semi dehydro ascorbic acid [618,623]. However, pollutants can affect the normal antioxidant defense by inducing oxidative reactions and reducing the levels of these main antioxidants. For instance O_3 has been shown to deplete Vitamin E and C in mice skin, leading to lipid peroxidation [128,624,625]. Therefore nowadays, the protective effects of Vitamins and other antioxidants compounds have been led to the development of topical formulations containing antioxidant compounds able to restore the skin redox homeostasis and prevent pollutants-induced skin damage. For instance a recent study has demonstrated the protective effect of caffeic acid against cigarette smoke in human skin explants [626] and the topical application of Vitamin E has been showed to reduce photoaging, DNA damage and reduce free radicals production induced by UV exposure [620,627] as well for Vitamin C. Indeed the photoprotection properties of Vitamin C has been demonstrated to reside in its ability to inhibit the UV-induced activation of protein-1 AP-1 or NF-kb which in turn up regulate MPPs leading to collagen degradation and consequent wrinkles formation, photoaging and elastin accumulation[628]. Moreover Vitamin C displays and antiaging effect

by helping in the cross-linking of collagen fibers and helping in the biosynthesis of collagen [618] and it is also able to reduce oxidized vitamin E [629]. Moreover several studies showed that topical application of Vitamin C and E are able to protect from O_3 skin damage [130] [60,218] and are also involved in photo protection [587], suggesting that these two antioxidants display better antioxidant capacity when acting together. However, L-ascorbic acid, which is the form of Vitamin C found in the skin, is an hydrophilic molecule and it therefore displays poor penetration properties through the cutaneous stratum corneum [630]. To improve L-ascorbic acid stabilization and permeability new formulations containing other compounds, such as Ferulic acid, or by using more stable esterified forms of vitamin C have been established [631,632]. Indeed Ferulic acid has been demonstrated to stabilize Vitamin C solutions by adjusting the Ph and favoring skin permeability [633]. For instance several new formulations containing these compounds have been shown to protect from photodamage induced by UV radiations [634,635] by improving the protecting effect of UV filters [636] and also to prevent O_3 damage [60]. Another important skin micronutrients is B-carotene which belong to the carotenoids family and it is a precursor of Vitamin A. B-carotene needs to be taken up through the diet and it is able to inhibit the enzymes lipoxygenases which are responsible for the production of ROS. Moreover β-carotene is able to quench singlet oxygen and peroxyl radicals and it has been shown to protect from sunlight and photodamage together with Vitamin E [637,638]. Indeed the chlorophyll present within carotenoids is able to absorb UVA through a porphyrin related molecular structure called Chlorin[638]. Coenzyme Q10 (CoQ10) or Ubiquinone is a co-enzyme known to be involved in metabolic cells processes such as the production of energy within mitochondria, but also for its antioxidant properties[223,639]. For instance the topical application of CoQ10, as well as the dietary intake [640], has been demonstrated to improve the antioxidant defense of the skin [641] and preventing from wrinkles due to skin aging [642]. It is clear that the long-term exposure to pollutants is leading to an increase development and exacerbation of skin pathologies such as psoriasis, dermatitis, due to their ability to induce oxidative stress reaction and therefore inducing the depletion of the natural antioxidant skin defense. The topical application of antioxidant compounds is therefore a good strategy to protect skin from pollutants induced-oxidative

insult and from the onset of related skin pathologies and their beneficial effect are often associated to a good diet [112,643].

5.1.4 Pollutants exposure and altered cell iron homeostasis: a possible correlation in cutaneous lipid peroxidation and OxInflammation

As previously mentioned, pollutants exposure can trig oxidative stress reactions within target tissues through the formation of reactive oxygen species (ROS) that are able to damage biomolecules, altering their function and thus resulting in tissue injury[644].

Skin is enriched of proteins and lipids, which are a good substrate for the ROS reactions. In particular the interaction of ROS especially HO· with the cutaneous lipids can lead to a lipid peroxidation cascade [103].

This process results with the release of a variety of reactive bioproducts such as Lipid hydroperoxides (LOOH), but also very reactive aldehydes, i.e. 4HNE, able to perpetuate the tissue damage[64537]. Lipid peroxidation is now a common event correlated to many pathologies related to oxidative stress[646157] , including cell death[647], and consists of 3 steps: initiation, propagation and termination. In the initial step, radical species, among which hydroxyl radical (HO·) and hydroperoxyl (HO· 2) are the most common, attacks the carbon-carbon double bond present within lipids forming a lipid radical able to react with O_2 to form the lipid peroxyl radical (LOO·). In the propagation phase, LOO· continues to react with other lipids, subtracting an hydrogen and generating new lipid peroxyl radical, perpetuating the reaction. The final step relies on antioxidant enzymes able to stop the reaction by donating an hydrogen and forming non radical lipid products, as described in Figure 12.

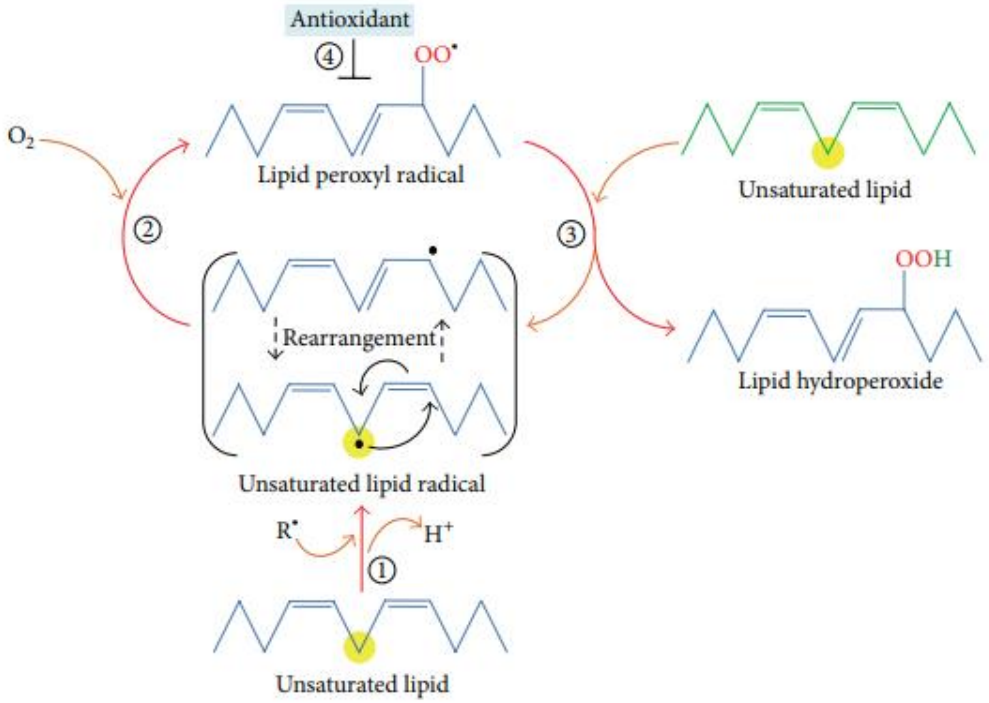


Figure 12. Lipid peroxidation process[107]

It has been well demonstrated that one of the main way of pollutants to damage cells and tissues is via the generation and a lipid peroxidation cascade thanks to the production of radicals species, as hydroxyl radical and hydroperoxyl and also by depleting the antioxidant defensive system, (i.e. GPx, glutathione reductase GRx, SOD,CAT), which can results overwhelmed but the oxidative challenges[112]. It should be mentioned that the continuous production of Hydroxyl radical (HO·) and hydroperoxyl (HO·2), is maintained alive by a redox cycle involving two main reactions called Fenton and Haber-Weiss reaction. As shown in Figure 13 these two reactions are catalyzed by the presence of a transition metal, normally iron (Fe), that can react with H_2O_2 in its free form (Fe2+) leading to the production of HO·. Fe2+ is obtained by the interaction of ferric iron (Fe3+) with anion superoxide ($O_2^{\bullet -}$), providing the maintenance of the redox cycle (Fig 13)[645]. Thus, iron represents a key regulator of this process, allowing the continuous production of radicals species that can initiate the chain oxidation of polyunsaturated phospholipids and lead to the impairment of membrane function[648] .

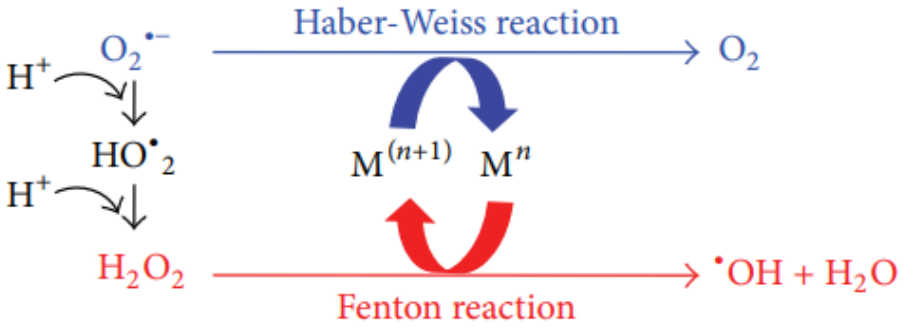


Figure 13. Fenton and Haber-Weiss reaction[107]

Iron is an essential micronutrient involved in several biological and metabolic functions such as transport of oxygen, DNA synthesis, electron transport where it is often utilized as cofactor. For instance Iron is an essential transition metal involved in mitochondria functionality and it participates in several metabolic processes[649]. Mitochondria represents one of the principal sources of endogenous ROS within cells since radical species such as $O_2^{\bullet-}$,are normally produced during the oxidative phosphorylation process of the electron transport chain, as result of the transfer of electrons from NADH or $FADH_2$ to O_2 [650]. Excessive amount of iron within cells has been shown to fuel mitochondrial ROS production, by interacting with $O_2^{\bullet-}$, and therefore triggering oxidative reactions which lead to oxidation of biomolecules such as lipids, DNA and proteins [651], resulting in several oxidative stress-related pathologies [57,646,647652].

Therefore, to prevent ROS production and the impairment of cell processes, iron metabolism needs to be fined regulated and its homeostasis relies on the balance between the uptake, storage and utilization of this metal [653,654] . Normally iron can be introduced by the diet in several forms such as heme, ferritin or inorganic iron and its recycle is regulated by several proteins such as the divalent metal transporter 1 importer (DMT1), the transferrin receptor 1 (TfR1) and the storage protein Ferritin which are under the control of specific iron-regulatory proteins (IRPs)[652,655,656] A perturbation of iron homeostasis can lead to an altered activity of these proteins, resulting in the accumulation of iron amounts within cells which can be used as catalyzing agent in Fenton's reaction to produce ROS.

The oxidative damage induced by altered iron homeostasis has been associated to several pathologies such as neurodegenerative diseases, anemia, carcinogenesis, stroke, ischemia-reperfusion injury, kidney degeneration and cancer as well [652,657–660], and also to a type of cell death called ferroptosis.

Of note, all these events are common figures displayed in tissues exposed to air pollutants and several studies so far have been demonstrated that also air pollutants or their catabolic products, are able to disrupt iron homeostasis. Indeed air pollutants displaying double bound or electronegative functional groups containing sulfur atom, oxygens, nitrogen etc., can complex/chelate the available iron and therefore alter the metabolic cell functions [661,662]. As a consequence of iron chelation by pollutants, cells will increase the import of iron by inducing the activation of IRPs and therefore increasing the expression of metal receptors such as DMT1, transferrin and Ferritin. This event culminates in the accumulation of iron in cells which is now available to react with oxygen as a Fenton's reagent, catalyzing the production of reactive oxygen species such as O_2^-. Iron can also interact with Hydrogen peroxide to generate hydroxyl and lipid radicals able to interact with macromolecules and altering their functions [660]. Moreover in response to iron loss due to complexation of the metal with pollutants, superoxide can be generated as a ferriredutanct able to reduce Fe^{3+} to Fe^{2+} and therefore to require more Iron [663]. Indeed Inorganic iron need to be reduced from the oxidized form (Fe^{3+}) to the reduced form Fe^{2+} by ferrireductases present within the intestine before being up taken [652]. Oxidative stress induced by pollutants exposure has been demonstrated to be followed by activation of kinases, phosphatases, transcription factors which will induce inflammation and also apoptosis, resulting in OxInflammation[103,652]. PM (PM), as well cigarette smoke, due to its composition is one of the main air pollutants able to alter iron homeostasis, especially in the respiratory tract [664]. In particular it has been demonstrated that organic compounds present within particles, such as Humic like substances or HULIS**,** display functional groups able to chelate iron in lungs [665–669]Also O_3 and its bioproducts such as aldehydes, ketones, peroxides etc.. have been shown to alter iron homeostasis and induce elevated concentration of non-heme iron and increased expression of Transferrin receptor and ferritin in lungs [670]. Moreover, the interaction between O_3 and particles has been shown to enhance the biological effects and disturbance of iron homeostasis. For instance the

concomitant exposure to particulates and O_3 can increase inflammation in lungs by inducing inflammatory cytokines and NF-kB [671–673] and particulates can also activate MAP kinase increasing the concentration of Iron [674]. Thus It is plausible that pollutants-induced oxidative stress, lipid peroxidation and related inflammation within the cutaneous tissue could be mediated by the ability of pollutants to alter iron homeostasis and therefore leading to increased Iron concentrations able to induce production of reactive oxygen species and consequent inflammation responses. However the possible correlation between iron and pollutants induced OxInflammation in skin tissue has been poorly investigated.

I. Deferoxamine (DFO) as a new topical approach to prevent pollutants induced OxInflammation in skin tissue

The detrimental effect of iron-induced oxidative stress which can culminate in skin lipid peroxidation, can be prevented by the utilization of drugs able to prevent the excessive accumulation of iron within cells [675–677]. For instance chelating agents represent a good approach to prevent ROS generation by occupying all the 6 binding sites present within iron ions, preventing iron to participate in Fenton's reactions [677] and to attenuate iron overloaded related diseases [678,679].Deferoxamine (DFO) is one of the most potent iron chelator with an high affinity for iron [680] and it has been used since 1986 to treat iron overdose, hemochromatosis, blood transfusion etc.[681]. Due to its angiogenic and antioxidant properties, DFO represent an important medical treatment in several tissue injuries such as ischemia, wound healing, bone rigeneration[682,683], and it has also been used to counteract ferroptosis (cell death linked to iron overload and peroxidation) [684]. It is usually administered by injection, either muscle, vein or subcutaneous. For instance several studies demonstrated that DFO can help in skin wound healing and diabetic ulcers regeneration by upregulating the hypoxia-inducible factor-1 alpha (HIF-1a) which is involved in angiogenesis and vascularization and it regulates other important mediators such as the vascular endothelial growth factor (VEGF), helping to bring nutrients and other factors essential for tissue regeneration [685–688]. Beside the angiogenesis properties, DFO display also antioxidant properties which reside in its ability to directly quench ROS such as $^{\cdot}HO$ and O_{2^-} and forming the deferoxamine nitroxide radical (DfNO) [689,690]. For

instance a study demonstrated that DFO is able to stop the iron mediated propagation of lipid peroxidation by quenching and reducing the levels of alkoxyl and peroxyl radicals in erythrocyte membranes [691]. Moreover the combination of DFO as iron chelator with other antioxidant compounds has been demonstrated to be useful in iron-overloaded rats [692,693]. Since oxidative and inflammatory events within the skin are correlated to onset and exacerbation of different skin pathologies including skin cancer, it is likely that accumulation of Iron induced by pollutants exposure can play a crucial role in initiate the skin oxidative damage and exacerbate skin conditions. For instance several oxidative insults such as UVA have been demonstrated to induce the release of iron from the iron-binding proteins within the skin and the consequent generation of ROS [677,694] as well as other pollutants such as PM and O_3 [662,664,695]. So far several studies have been demonstrated the ability of iron chelators to counteract skin photodamage, also in combination with other antioxidants compounds [696,697]. Therefore the presence of an iron chelator such as DFO could represent a good approach to enhance the protective antioxidant effect of formulations containing antioxidant compounds to prevent the pollution induced- skin OxInflammation phenomena.

5.2 *RATIONAL AND AIM*

To counteract the oxidative and inflammatory damage induced by pollutants exposure and to restore the skin antioxidant defensive system, several topical formulations containing antioxidant compounds such as Vitamin E, Vitamin C, co-Q10 etc. are now largely used [698]. As of today, only a few studies have investigated the possible additive effect of multiple pollutants on cutaneous tissues, although this paradigm represents the realistic, everyday urban environment. Therefore the first step of the he present study aimed to investigate whether exposure of human skin explants to different pollutants, such as O_3 and PM, for different period of time (1 and 4 days) could further exacerbate cutaneous OxInflammation induced by UV exposure. In addition, we also evaluated the possible long-term protective effect of a commercially available antioxidant cosmeceutical formulation mixture (CF Mix) containing 15% vitamin C (L-ascorbic acid), 1% vitamin E (α-tocopherol), and 0.5% ferulic acid in prevent this Oxinflammatory skin damage. Since Iron is an essential micronutrient involved in several biological processes and alteration of its homeostasis has been correlated to the production of Reactive oxygen species (ROS), but also to inflammation, apoptosis and cell death [699], we hypostasized a possible involvement of the metal in pollutants- induced skin oxidative damage. Indeed altered iron homeostasis has already been shown to be involved in the onset of several pathologies such as genetic disorders, cardiotoxicity, neurodegeneration but also aging and cancer [699,700] and air pollutants have been found to be able to modulate iron balance [660]. For instance PM has been demonstrated to chelate the metal, leading to an upregulation of proteins involved in iron uptake, resulting in accumulation of iron within cells and the consequent production of ROS [662]. Moreover altered iron homeostasis and consequent oxidative and inflammatory reactions have been associated to carcinogenesis [701], promoting angiogenesis and tumor growth.

Nowadays Iron chelators agent, able to quench iron, has been longed investigated as therapeutic approach against iron overload and iron-related diseases [678,679]. For instance Deferoxamine (DFO) is one of the most studied iron chelators, particularly used in wound healing and skin regeneration processes [686,687]. Even though altered iron homeostasis mainly results in oxidative stress and inflammation events, which are also common figures in pollutants induced tissue damage and skin carcinogenesis[103], the possible involvement

of iron in pollution induced skin damage is poorly investigated. Since Particular matter has been shown to be one of the main pollutant able to chelate iron and alter Iron homeostasis [664], the second step of the study was to investigate whether the ox-inflammatory damage induced by Diesel Exhaust (DEE) in human skin tissues (1 and 4 days exposure), could be mediated by iron and if the chelating agent DFO was able to enhance the antioxidant properties of the cosmeceutical formulation mixture (CF Mix), opening new insights behind the mechanism regulating the pollutants induced skin OxInflammation.

5.3 MATERIALS AND METHODS

5.3.1 Culture and exposure of e*x vivo* human biopsies

Human skin biopsies were collected and culture as previously described in general methods and recovered overnight in the incubator at 37°C, 5% CO_2. The day after media was changed, and an antioxidant mixture containing 15% vitamin C (L-ascorbic acid), 1% vitamin E (α-tocopherol), and 0.5% ferulic acid (CE Ferulic, SkinCeuticals Inc., New York, NY) was topically applied. After 24 hours of pre-treatment, biopsies were exposed to 200 mJ UVA/UVB light alone as in [127] , UV light and then 0.25 ppm of O_3 for 2 hours in an O_3 chamber as in [506], UV light then 30 min of DEE, or UV light, O_3, and then DEE. Samples were collected after the first exposure (DAY1) or after four days of treatment and exposures (DAY 4). DEE was generated by a Kubota RTV-X900 diesel engine (3-cylinder, 4-cycle diesel with overhead valves, 1123 cc that has 24.8 HP at 3000 rpm). For Deferoxamine experiments, a solution of DFO 100 μM was applied on human skin biopsies with or without an antioxidant mixture containing 15% vitamin C (L-ascorbic acid), 1% vitamin E (α-tocopherol), and 0.5% ferulic acid (CE Ferulic, SkinCeuticals Inc., New York, NY). After 24 hours of pre-treatment skin tissues were exposed to Diesel exhaust for 30 min and then collected after the first exposure (DAY 1) or after 4 days of treatment and exposure (DAY 4).

5.3.2 Hematoxylin & Eosin staining

The 4 μm paraffin tissues sections were deparaffinized in xylene and then rehydrated in a series of alcohol gradients. Sections were then stained for 10 min with Mayer's hematoxylin solution (Sigma, USA), washed with tap water, stained 3 minutes in Aqueous Eosin Y solution (Sigma, USA) and then immersed in 95% EtOH, 100% EtOH, and xylene. The sections were mounted onto slides using a toluene mounting solution.

5.3.3 Immunohistochemistry

The immunofluorescence staining was carried out as described above in general methods. The primary antibodies used were 4HNE (AB5605, Millipore) 1:400 in PBS-BSA 0.5%, NF-κB (8242, Cell Signaling) 1:400 in PBS-BSA 0.5%, Ahr (83200, Cell Signaling) 1:100, Involucrin (sc-21748, Santa Cruz) 1:50, Filaggrin (sc-66192) 1:50 or pro-MPP9 (Santa Cruz) in 2% BSA in PBS. The secondary antibodies were fluorochrome-conjugated secondary antibodies (Alexa Fluor 568 A11004 or Alexa Fluor 488 A11055 Invitrogen, Thermofisher USA) diluted 1:500 in 2% BSA in PBS. After DAPI staining, sections were mounted and imaged at 20x and 40x magnifications.

5.3.4 Protein extraction and Western blotting

Proteins from Skin explants were extracted using the Qiagen TissueLyser in T-PER tissue protein extraction reagent (Thermo Scientific) containing Halt protease inhibitor cocktail (Thermo Scientific) as previously described. After performing the Western blotting, membranes were incubated with primary antibodies for COX2 (12282, Cell Signaling) or HO-1 (PA00553, BioRad) both diluted 1:1000 in TBS-T with 1% non-fat milk (BioRad,USA). The horseradish peroxidase-conjugated secondary antibodies (170-6515 or 170-6516, BioRad) were diluted 1:10'000 in TBS-T with 1% of non-fat milk and incubated for 90 min at RT. β-actin (A3854, Sigma) was used as loading control at a concentration of 1:50'000 in TBS-T with 1% non-fat milk. Densitometry analysis was performed using Image J software.

5.3.5 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 all cases.

5.4 *RESULTS*

5.4.1 Effect of combined stressors on skin morphology before and after CF Mix topical application

First, we wanted to determine whether the dosage/time of exposure altered skin structure. As shown in Fig. 14A and B, we did not observe any morphological alterations in tissues exposed to UV light in combination with O_3 and DEE both at DAY 1 and DAY 4, suggesting that the doses used are not overly aggressive and can represent real-life pollution exposure.

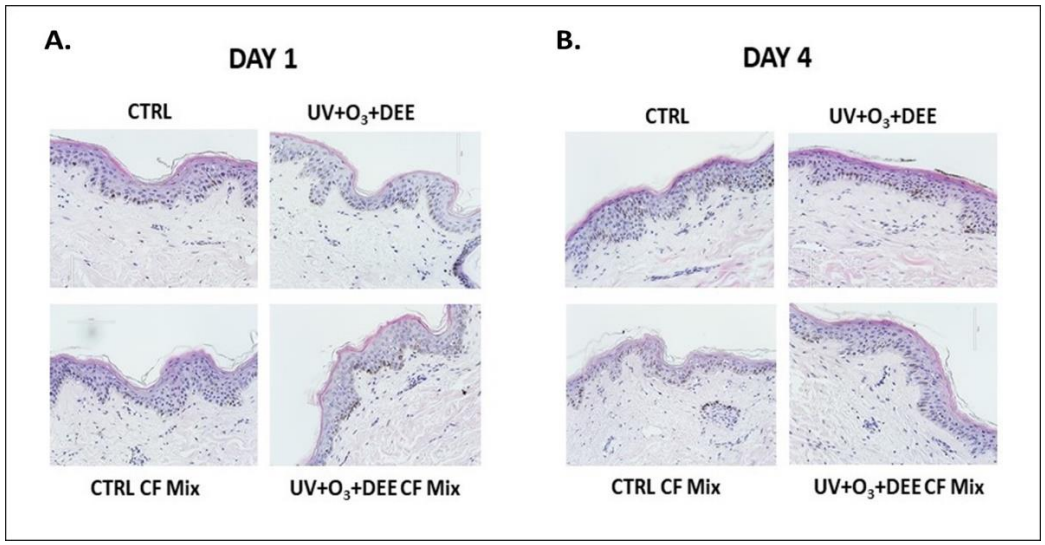
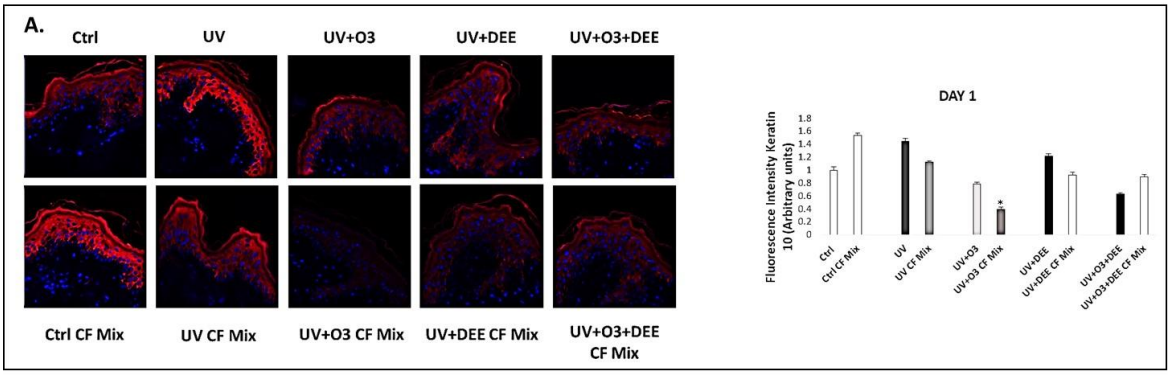


Figure 14. H/E staining of human skin explants pre-treated or not with CF Mix and then exposed to UV in combination with O_3 and DEE for 1 day (left panel) and 4 days (right panel). All data are expressed as the mean ± standard deviation (n = 3). The panel is representative of 1 donor.

5.4.2 Combined pollutants exposure is able to enhance the UV-induced skin barrier structure impairment

Prolonged exposure to pollutants can affect skin health and barrier functionality that are also displayed in several skin conditions [5,702]. Therefore we investigated the levels of the main skin proteins involved in epidermis keratinocytes differentiation and in forming the cornified envelope of the stratum corneum in response to pollutants exposure. 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. 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 shown in figure 15A-B, we observed a decrease in Keratin 10 expression levels especially after the different pollutants combination exposure at both DAY 1 (Fig 15A) and DAY 4 (Fig. 15B). Furthermore, the pre-treatment with CF Mix was able to significantly restore the loss of Keratin 10 levels induced by pollutants exposure after 4 days, while no significant protective effect was shown at day 1. We then examined two markers related to keratinocytes terminal differentiation, such as Filaggrin (Fig. 15C-D) involved in the organization of the keratin filaments within the stratum corneum and Involucrin (Fig 15E-F), which is mainly present in the upper spinous/granular layer. Indeed 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 depicted in Figure 15C-D, the combined stressors exposure induced a decrease in Filaggrin expression levels at DAY 1 (Fig. 15C) and DAY 4 (Fig. 15D) and the pre-treatment with the CF Mix was able to prevent this effect at both timepoints. Moreover, Involucrin expression levels were affected by the different pollutants combinations after 1 day of exposure (Fig 15E) and the pre-treatment with the CF Mix prevented this effect especially against UV in combination with O_3 alone and together with O_3 and DEE. However, after 4 days of exposure, we did not observe a significant effect on Involucrin levels (Fig. 15F), suggesting that Involucrin modulation is an early event under our experimental conditions.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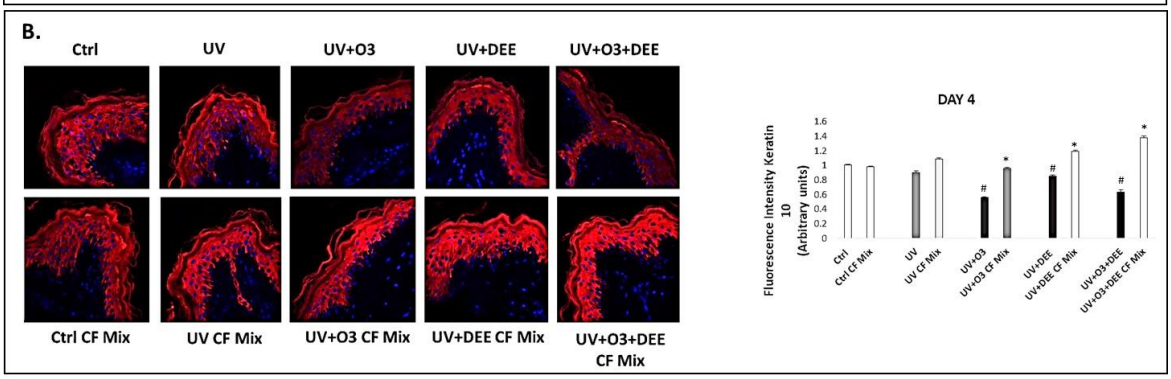
DAY 1
Fluorescence Intensity Keratin 10 (Arbitrary units)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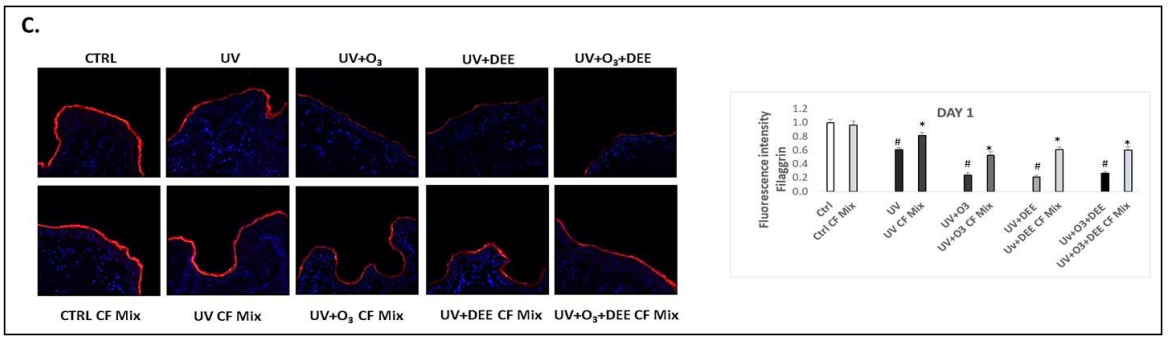
DAY 4
Fluorescence Intensity Keratin 10 (Arbitrary units)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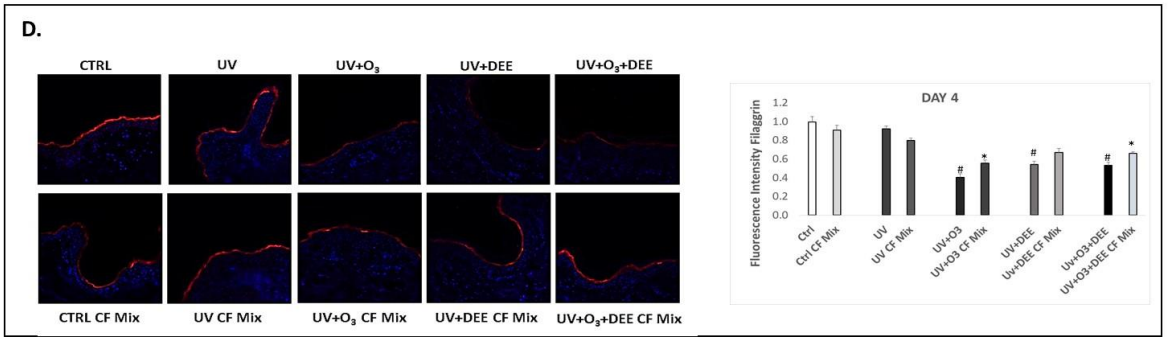
DAY 1
Fluorescence intensity Filaggrin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DAY 4
Fluorescence Intensity Filaggrin

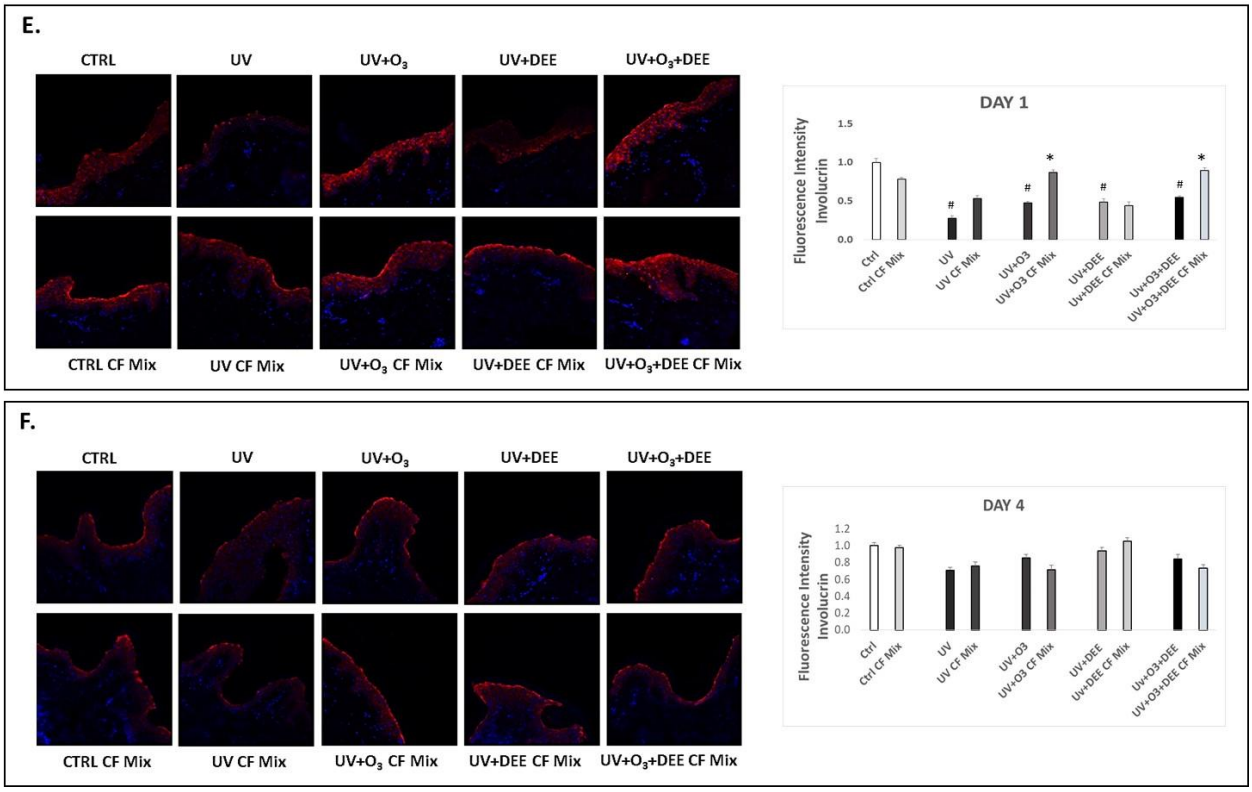
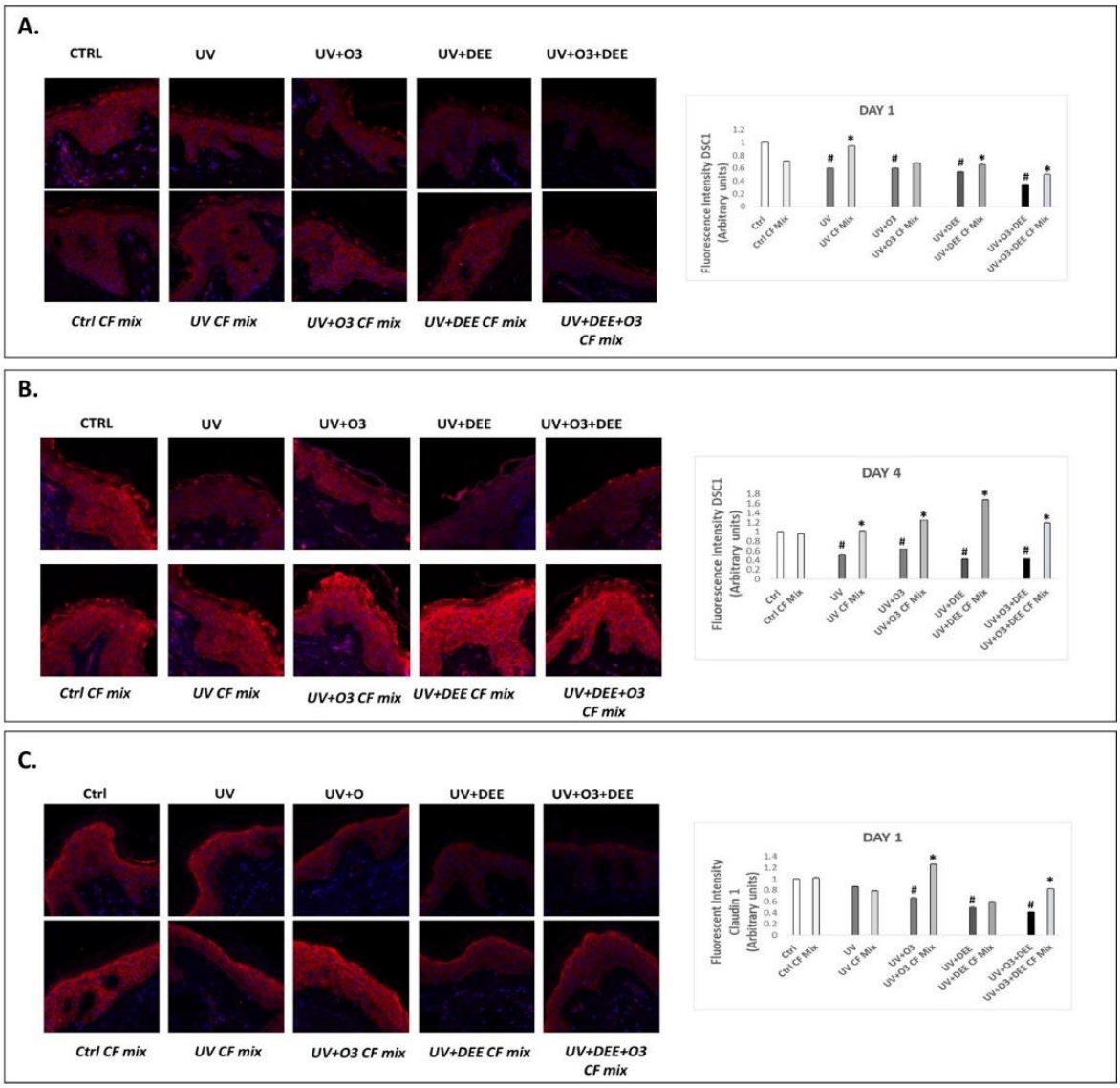


Figure 15. *Immunofluorescence (IF) staining for skin-barrier associated proteins Keratin 10 (A,B) Filaggrin (C,D) and Involucrin (E,F) expression levels on ex vivo human skin biopsies exposed to different combinations of pollutants for 1 day (A,C,E) and 4 days (B,D,F) and pretreated with the cosmeceutical formulation mixture (CF Mix). Red staining represents proteins keratin 10 (A,B) Filaggrin (C,D) and Involucrin (E,F) and the blue staining (DAPI) represents nuclei; original magnificent 40X. Quantification (right panels) of proteins levels was performed using ImageJ. Data are expressed as arbitrary units (averages of three different experiments), *p < 0.05 CF Mix vs pollutant, #p < 0.05 pollutants vs Ctrl by ANOVA.*

5.4.3 Combined pollutants 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 burrier function [594] , we wondered whether O_3 and DEE were able to enhance UV-induced skin damage by evaluating the protein levels of Desmocollin 1, one of the main components of the skin cell-cell desmosomes junctions, and Claudin-1.

As shown in Figure 16A-B, skin biopsies exposed to the different pollutant's combination displayed decreased levels of Desmocollin 1 compared to un exposed tissues at DAY 1 (Fig 16A) and even more evidently at DAY 4 (Fig 16B). Interestingly O_3 and DEE seemed to exacerbate the UV-induced skin damage by enhancing the loss of Desmocollin 1 at both timepoints. Moreover the treatment with the CF Mix was able to prevent Desmocollin 1 decrease already at DAY 1 (Fig 16A), displaying an even more evident effect at DAY 4 by completely restoring the protein loss (Fig 16B). A similar trend was shown for Claudin 1, whom expression levels were impaired at both DAY 1 (Fig 16C) and DAY 4 (Fig 16D), especially after the combination of pollutants, and the CF Mix treatment was able to prevent this loss at both timepoints, especially at DAY 4. Skin integrity and barrier functionality are essential features to prevent water loss and retain water, allowing the skin to be hydrated and maintain the exchange of water and micronutrients between cells. Since pollutants exposure has been shown to impair the skin barrier structure, we wondered if this impairment could affect the ability of the skin to retain water by evaluating the protein levels of a protein channel involved in the cutaneous bidirectional water flux in the cells, Aquaporin 3 (AQP3). As depicted in Figure 16E-F, pollutants exposure enhanced the UV-induced decrease of Aquaporin 3 (AQP3) at both timepoints ad the prolonged CF Mix treatment prevented this loss at DAY 4 (Fig 16F), already showing an effect ad DAY 1 against UV+ O_3 and UV+O_3+DEE (Fig 16E).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DAY 1
Fluorescence Intensity DSC1 (Arbitrary units)
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UV+DEE+O3 CF mix
DAY 4
Fluorescence Intensity DSC1 (Arbitrary units)
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UV+O
UV+DEE
UV+O3+DEE
Ctrl CF mix
UV CF mix
UV+O3 CF mix
UV+DEE CF mix
UV+DEE+O3 CF mix
DAY 1
Fluorescent Intensity Claudin 1 (Arbitrary units)

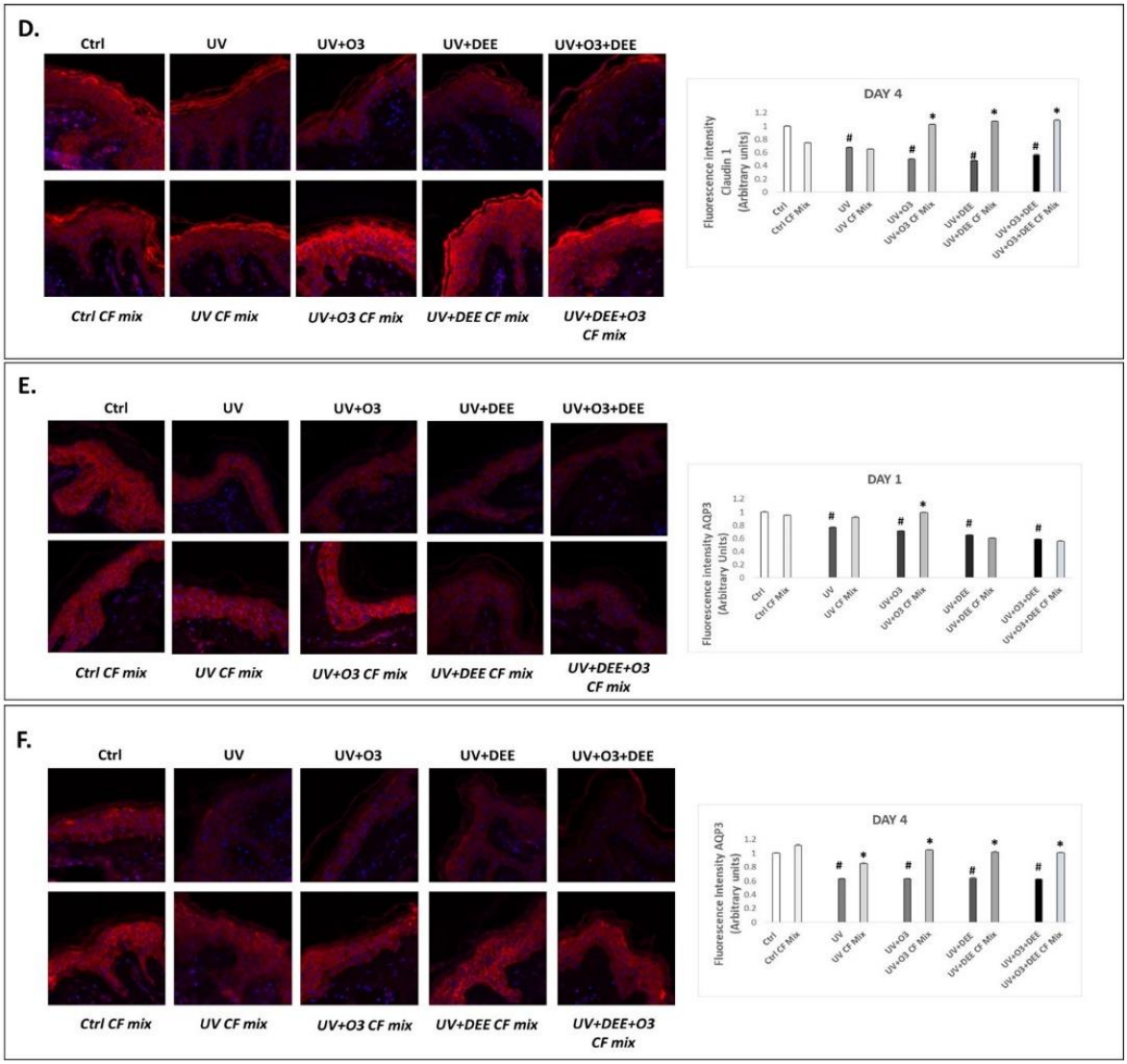


Figure 16. *Immunofluorescence (IF) staining for Desmocollin 1 (DSC1) (A,B), Claudin 1 (C,D) and Aquaporin 3 (AQP3) (E,F) expression levels on ex vivo human skin biopsies exposed to different combinations of pollutants for 1 day (A,C,E) and 4 days (B,D,F) and pretreated with the cosmeceutical formulation mixture (CF Mix). Red staining represents proteins Desmocollin 1 (A,B) Claudin-1 (C,D) and Aquaporin 3 (E,F) and the blue staining (DAPI) represents nuclei; original magnificent 40X. Quantification (right panels) of proteins levels was performed using ImageJ. Data are expressed as arbitrary units (averages of three different experiments), *p < 0.05 CF Mix vs pollutant, #p < 0.05 pollutants vs Ctrl by ANOVA.*

5.4.4 The cutaneous oxidative status levels is affected by combination of UV, O_3 and DEE

Next, we wanted to determine whether exposure to the combination of outdoor stressors can have an additive effect in terms of cutaneous oxidative damage. As depicted in Figure 17A, 4-hydroxynonenal (4HNE) levels, a marker of lipid peroxidation [37], were clearly increased in response to individual UV light exposure and in combination with O_3 and DEE at day 1. No additive effect of the pollutants was noticed at DAY 4 (Fig. 17B), confirming that the oxidation pathway is an early event of pollution-induced skin damage. Topical application of the CF Mix counteracted this effect, particularly at DAY 1, when the tissues were exposed to all three stressors (Fig. 17A). Interestingly, pre-treatment with the CF Mix still decreased 4HNE levels at DAY 4 as well (Fig. 17B). To confirm the ability of the pollutants to affect tissue redox homeostasis, we also evaluated levels of hemeoxygenase-1 (HO-1), an enzyme involved in response to oxidative challenges. As depicted in Fig. 17C, the combination exposure of all three stressors synergistically increased levels of HO-1 4 days after the challenges. We also observed that UV light in combination with either O_3 or DEE increased levels of HO-1, compared to exposure to UV light alone. Furthermore, CF Mix topical application counteracted increase of HO-1 levels. Collectively, this data suggests that UV light, O_3, and DEE can act synergistically to increase oxidative damage in the skin.

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.17D the combination of UV, O_3 and DEE has been found to be able in induce the activation of the Aryl hydrocarbon receptor at the early timepoint (DAY 1).

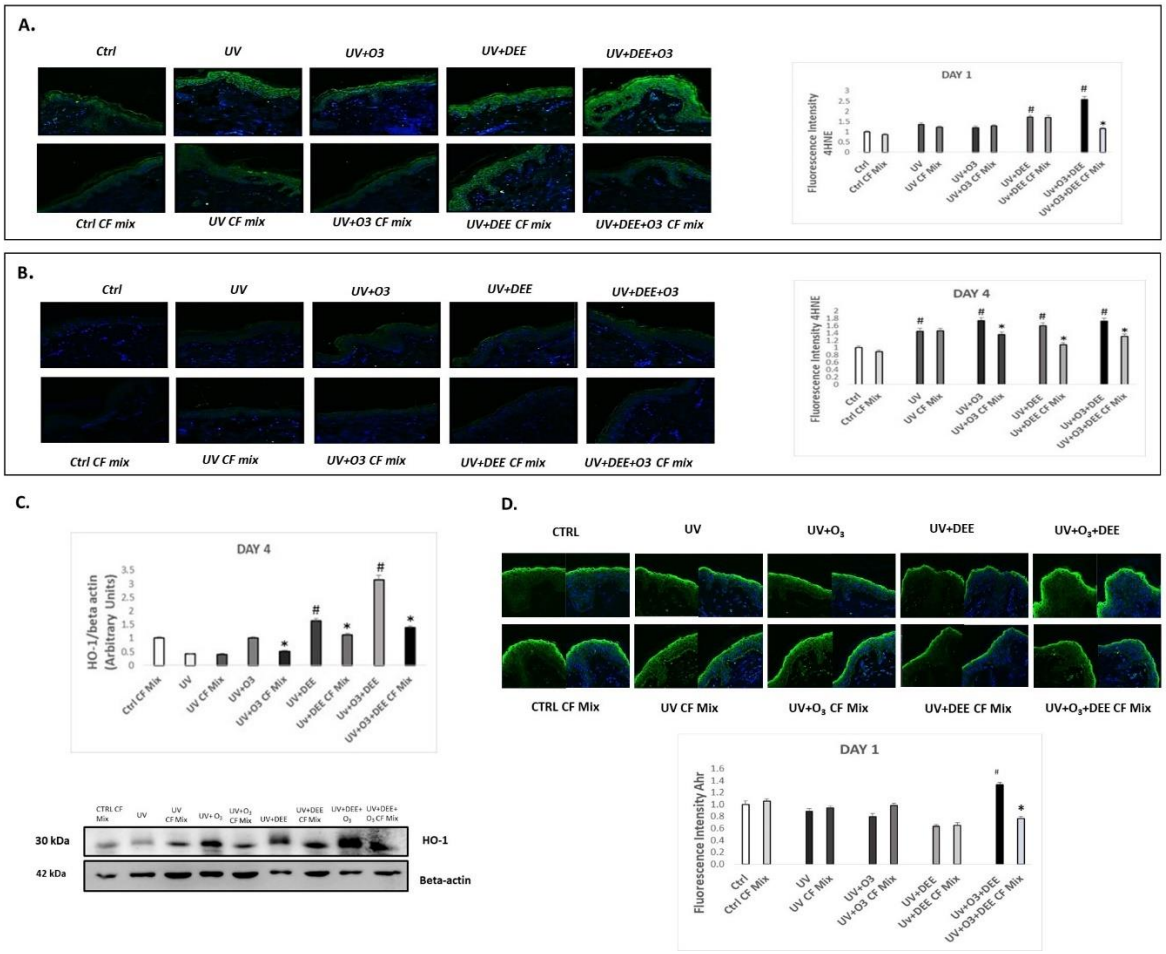


Figure 17. *Levels of 4- HNE, a marker of lipid peroxidation, in ex vivo human skin biopsies exposed to UV light alone or in combination with O3 and DEE and treated with the cosmeceuticals formulation mixture for 1 day (A) an 4 days (B). Green staining represents 4HNE, and the blue staining (DAPI) for nuclei; original magnification 40x. Quantification (right panels) of proteins levels was performed using ImageJ. (C) Levels of Heme-Oxigenase (HO-1) and beta-actin protein in ex vivo human skin biopsies exposed to combined pollutants exposure for 4 days (DAY 4). Quantification (top panel) of proteins levels was performed using ImageJ and β-actin was used as loading control. (D) Immunofluorescence (IF) staining for Ahr expression levels on ex vivo human skin biopsies exposed to different combinations of pollutants for 1 day and pretreated with the cosmeceutical formulation mixture (CF Mix). Green staining represents Ahr and the blue staining (DAPI) represents nuclei; original magnificent 20X. Quantification (bottom panel) of proteins levels was performed using ImageJ. Data are expressed as arbitrary units (averages of three independent experiments), *p < 0.05 CF Mix vs pollutant, #p < 0.05 pollutants vs Ctrl by ANOVA).*

5.4.5 O_3 and DEE display an additive effect to UV-induced inflammation in human skin.

Considering the link between oxidative stress and inflammation, to better investigate the possible inflammatory status within the skin induced by pollutants exposure, we evaluated the levels of the transcription factor NF-κB, which is a key factor involved in the regulation of tissue inflammatory responses [703,704]. Activation of NF-κB in keratinocytes upon exposure to UV, O_3 and PM individually has been well-documented [58,60,273,705]. As a consequence of the oxidative challenge, we observed increased levels of NF-κB in response to exposure to UV light individually and in combination with O_3 and DEE after 24 h and 4 days of exposure, although we did not observe any additive effects of combined exposure (Fig. 18A and B). We also observed that topical application of the CF Mix prevented stressor-induced increases in NF-κB levels after 24 h and 4 days of exposure (Fig. 18A and B). Since activation of NF-κB results in the transcription of several inflammatory genes, including cyclooxygenase 2 (COX2) [706,707], we examined the levels of COX2 in tissues exposed to the pollutants. Similarly, the combination of pollutants increased COX2 levels after 4 days of exposure (Fig. 18C). In addition, we found that exposure to UV light and DEE in combination resulted in a dramatic increase in COX2 levels. We also observed that the CF Mix prevented stressor-mediated increases in COX2 levels (Fig. 18C). One of the main inflammatory pathways involved in the inflammatory responses are Inflammasomes, whom transcription can be also regulated by NF-kB [708]. Since NLRP1 is one of the main inflammasome expressed in human skin tissue, we wanted to evaluate its possible activation induced by pollutants exposure and weather CF Mix treatment was able to prevent this activation. For this purpose, we assessed a double immunofluorescence staining for Inflammasomes components NLRP1 and ASC, whom colocalization normally occur under the multiprotein complex activation. As shown in Figure 18 D-E pollutants exposure was able to induce the inflammasome activation by upregulating the expression of NLRP1 and ASC. Moreover, the colocalization of the two proteins more likely occurred after the prolonged combined exposure at DAY 4 (Fig 18E) and the topical formulation CF Mix was able to prevent this colocalization, suggesting its protective effect against an inflammatory insult.

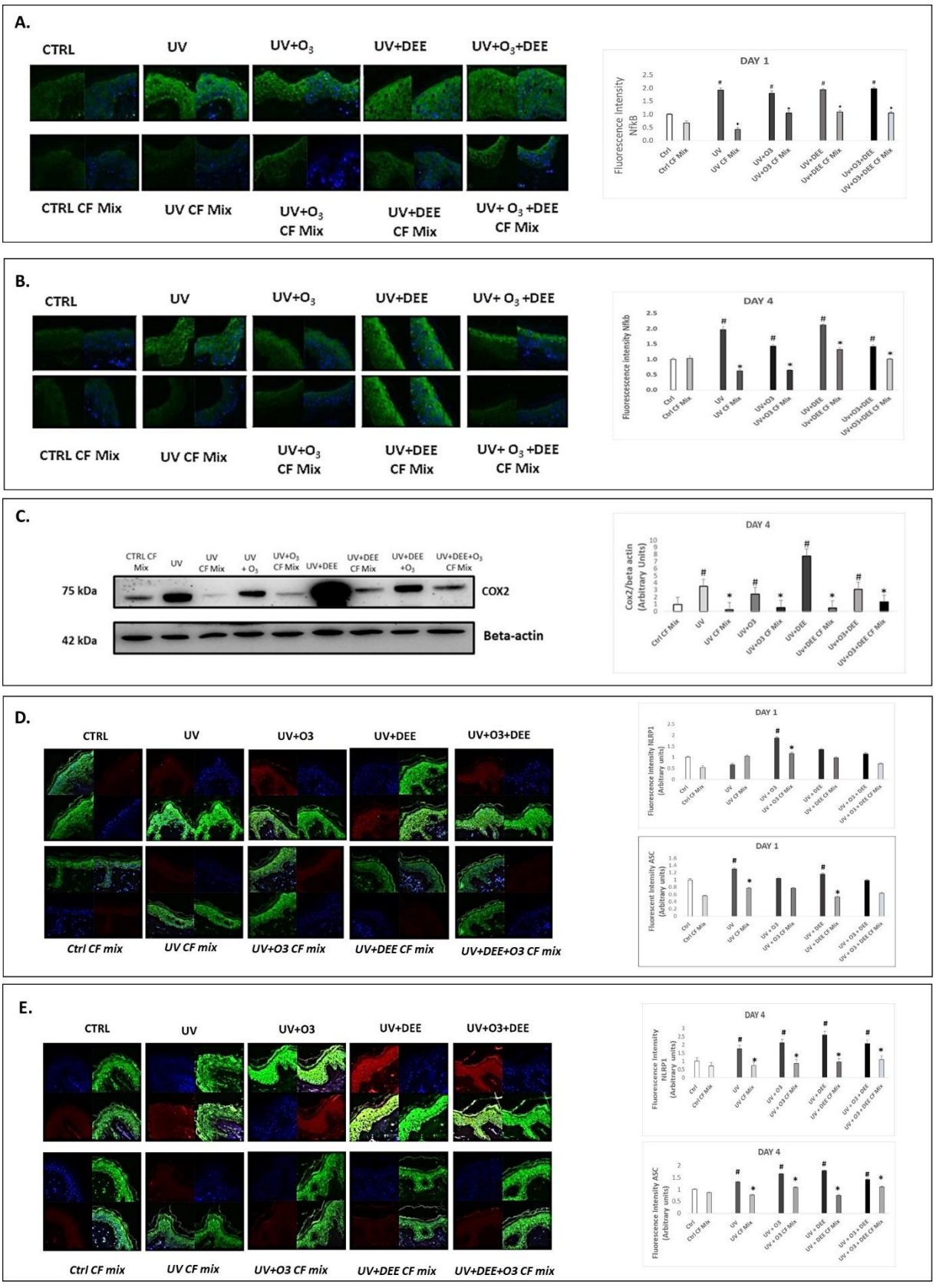

A.
CTRL
UV
UV+O3
UV+DEE
UV+O3+DEE
CTRL CF Mix
UV CF Mix
UV+O3 CF Mix
UV+DEE CF Mix
UV+ O3 +DEE CF Mix
DAY 1
Fluorescence Intensity NfkB
B.
DAY 4
Fluorescence Intensity NRkb
C.
CTRL CF Mix
UV
UV CF Mix
UV + O3
UV+O3 CF Mix
UV+DEE
UV+DEE CF Mix
UV+DEE +O3
UV+DEE+O3 CF Mix
75 kDa
COX2
42 kDa
Beta-actin
Cox2/beta actin (Arbitrary Units)
D.
Ctrl CF mix
UV CF mix
UV+O3 CF mix
UV+DEE CF mix
UV+DEE+O3 CF mix
Fluorescence Intensity NLRP1 (Arbitrary units)
Fluorescent Intensity ASC (Arbitrary units)
E.
Fluorescence Intensity NLRP1 (Arbitrary units)
Fluorescence Intensity ASC (Arbitrary units)

Figure 18. Topical application of a cosmeceuticals formulation mixture inhibits outdoor stressor induced inflammation. *Levels of NF-κB in ex vivo human skin biopsies exposed to different combinations of pollutants (UV, DEE and O_3) at day 1 (A) and day 4 (B) post-exposure was evaluated using immunofluorescence. Green staining represents NF-κB, and the blue staining (DAPI) represents nuclei; Original magnification 40x. Quantification was performed using ImageJ (right panels). (C) Protein levels of COX2 were measured by immunoblotting and βactin was used as loading control. (D-E) Immunofluorescence staining for NLRP1 and ASC in human skin explants exposed to different combinations of pollutants (UV, DEE and O3) at day 1 (D) and day 4 (E). Red staining represents NLRP1, green staining represents ASC and blue staining (DAPI) represents nuclei. Original magnification 40x. Quantification was performed using ImageJ (right panels). All Data are expressed as arbitrary units (averages of three different experiments), *p < 0.05 CF Mix vs pollutant, #p < 0.05 pollutants vs Ctrl by ANOVA.*

5.4.6 Iron-chelator Deferoxamine (DFO) is able to enhance the protective effect of CE Ferulic against DEE-induced ox inflammation

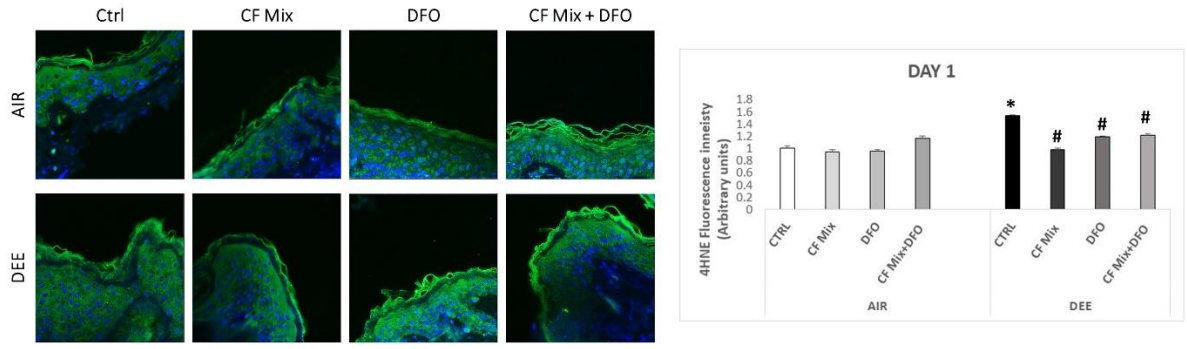
As previously demonstrated the combined exposure to different pollutants including ultraviolet light, diesel engine particles and O_3 showed to induce a strong oxidative-mediated damage in skin and that the antioxidant compound CF Mix is able to prevent this damage [104]. Since one of the main pathways involved in pollution toxicity is represented by increased peroxidation, that can derive from the generation of hydroxyl radical via Fenton reaction, our interest was to evaluate the eventual protective effect of an iron chelator, DFO, alone and in combination with CF Mix.

In order to explore the potential and synergistic protective activity of the combined CF Mix / DFO topical application against the harmful effects of DEE, in this study, we first evaluated the levels of 4-hydroxynonenal protein adducts (4HNE PA)[37,247]. As shown in Fig. 19A, exposure of untreated skin explants to DEE for 30 min slightly increased the levels of 4HNE PA compared to the tissues exposed to air at DAY 1. Moreover, this effect was more pronounced at DAY 4, indicating the accumulation of 4HNE PA after repeated DEE exposures (Fig. 19B). Topical application of CE Mix significantly counteracted the increase of 4HNE PA levels in DEE exposed tissues at DAY1 and DAY 4 (Fig. 19A and 19B). Instead, DFO alone showed to prevent 4HNE PA formation at DAY 4. Interestingly, the combined treatment with CF Mix and DFO seemed to exert a slight additive effect in preventing 4HNE PA formation after DEE exposure, as noticeable from the decreasing trend compared to the individual treatments, at both time points (Fig. 19A and 19B).

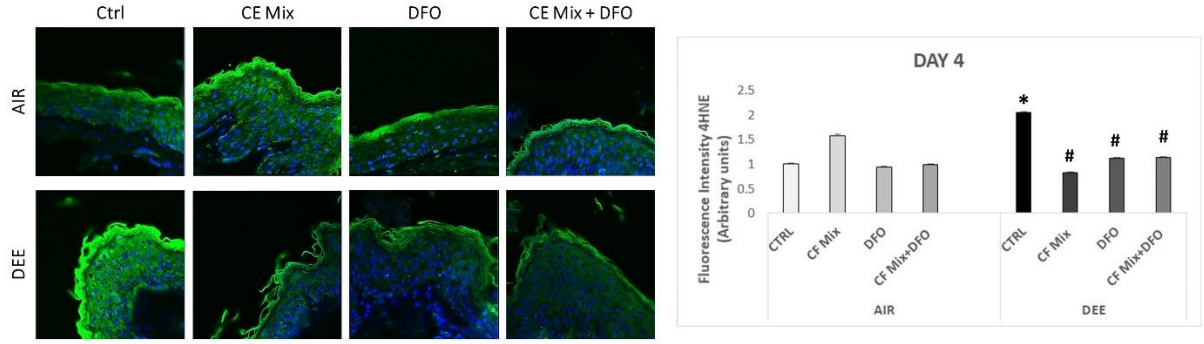
Taken together, these results confirm the induction of a lipid peroxidation-mediated damage on the cutaneous tissue following DEE exposure and suggest a potential additive effect of the combined topical application of a cosmeceutical mixture formulation with an iron chelator, i.e. DFO, in counteracting this harmful mechanism.

In a positive feedback loop called OxInflammation, pollution-induced oxidative stress sustains and, at the same time, is fueled by aberrant inflammatory responses [104,612]. In particular, pollution-related oxinflammatory phenomena are associated with increased expression and activation of matrix metalloproteinases (MMPs), enzymes involved in the turnover and degradation of extracellular matrix membrane (ECM) proteins such as collagens and involved in wound healing, angiogenesis, neovascularization, photoaging and skin carcinogenesis[281,709,710] which has been also been related to high levels of iron[711]. Furthermore, upon pollution exposure, aberrant expression of MMPs can compromise skin structure and integrity [218,219,712]. Here, we investigated the levels of the pro-form MMP-9 without proteolytic cleavage of the pro-domain. MMPs are commonly present in the tissues as inactive zymogens (pro-forms), which require processing of a pro-domain by other MMPs or serine proteinases to attain full activity. As depicted in Fig. 19C, we observed a slight decrease in pro-MMP-9 levels in the untreated skin explants after exposure to DEE at DAY 1. After 4 days, this decrease in pro-MMP-9 levels in untreated and DEE exposed skin tissues was significantly lower than in the untreated and air-exposed tissues (Fig. 19D). Furthermore, the combined treatment with CF Mix and DFO showed an additive effect in counteracting the decrease of pro-MMP-9 levels after DEE exposure at DAY 1 (Fig. 19C). At DAY 4, both individual treatments and the combined application of CF Mix and DFO effectively prevented this decrease (Fig. 19D). Also, in this case, the combined application showed an additive effects compared to the individual treatment (Fig. 19D). Based on the mechanism of MMPs activation, we hypothesize that DEE exposure could promote pro-MMP-9 cleavage and activation in untreated and exposed skin tissues mediating iron induction, while CF Mix and DFO could inhibit this harmful effect.

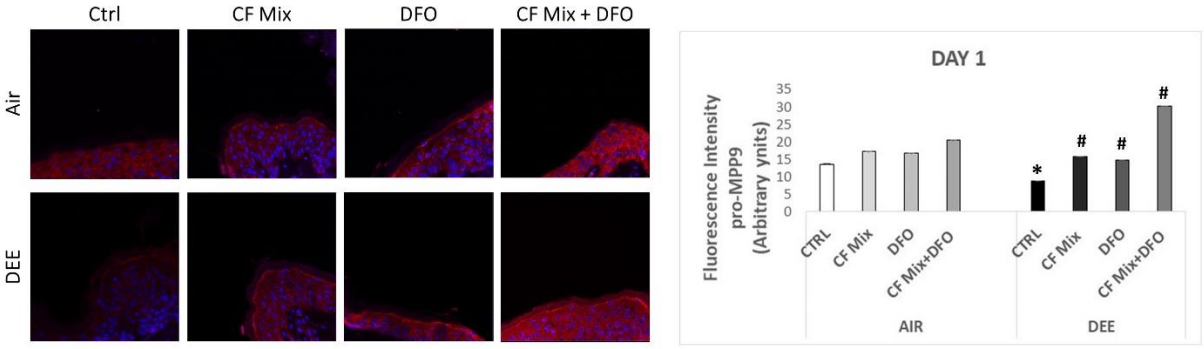
A.


Ctrl
CF Mix
DFO
CF Mix + DFO
AIR
DEE
DAY 1
4HNE Fluorescence inneisty (Arbitrary units)
CTRL
CF Mix
DFO
CF Mix+DFO
AIR
DEE

B.


Ctrl
CE Mix
DFO
CE Mix + DFO
AIR
DEE
DAY 4
Fluorescence Intensity 4HNE (Arbitrary units)
CTRL
CF Mix
DFO
CF Mix+DFO
AIR
DEE

C.


Ctrl
CF Mix
DFO
CF Mix + DFO
Air
DEE
DAY 1
Fluorescence Intensity pro-MPP9 (Arbitrary ynits)
CTRL
CF Mix
DFO
CF Mix+DFO
AIR
DEE

D.

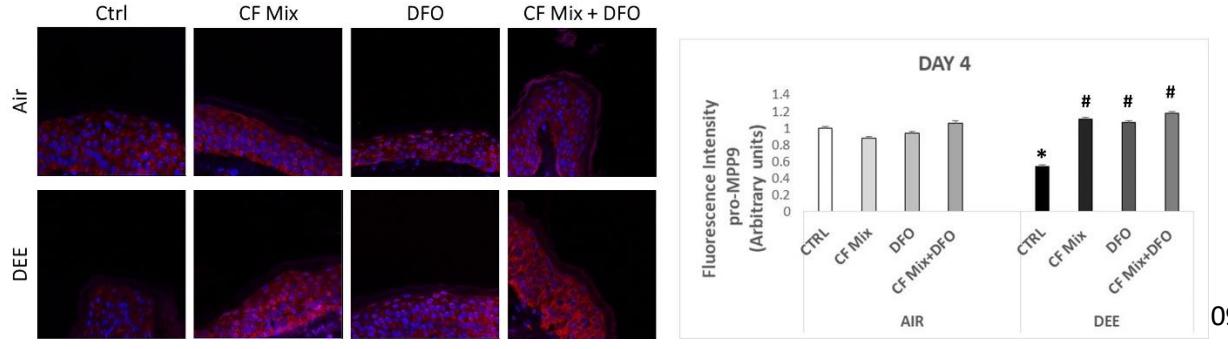
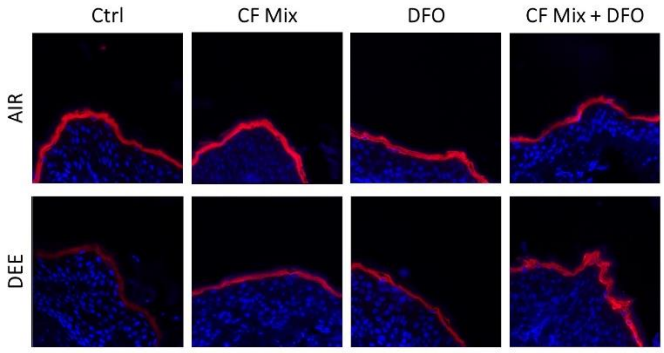

Ctrl
CF Mix
DFO
CF Mix + DFO
Air
DEE
DAY 4
Fluorescence Intensity pro-MPP9 (Arbitrary units)
CTRL
CF Mix
DFO
CF Mix+DFO
AIR
DEE

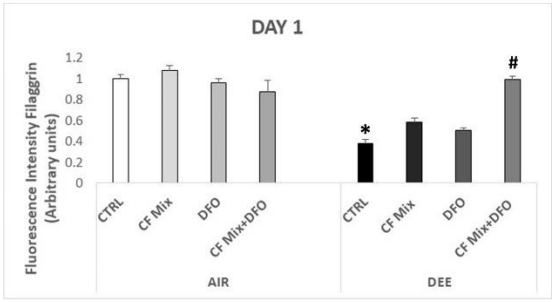
Figure 19. *Expression levels of 4HNE (A-B) and MMP-9 (C-D) in human skin tissues exposed to DEE for 30 min and pre-treated with CE Ferulic and/or DFO (alone or in combination) for 1 day and 4 days. Green staining represents 4HNE , Red staining represents MMP-9 and blue staining (DAPI) represents nuclei. Original magnification 40x. Quantification was performed using ImageJ (right panels). All Data are expressed as arbitrary units (averages of three different experiments), *p < 0.05 DEE ctrl vs Air Ctrl, #p < 0.05 DEE exposed and treated tissues vs DEE exposed and untreated tissues by ANOVA.*

5.4.7 DEE-induced skin barrier impairment is counteracted by the combined application of CE Ferulic and DFO

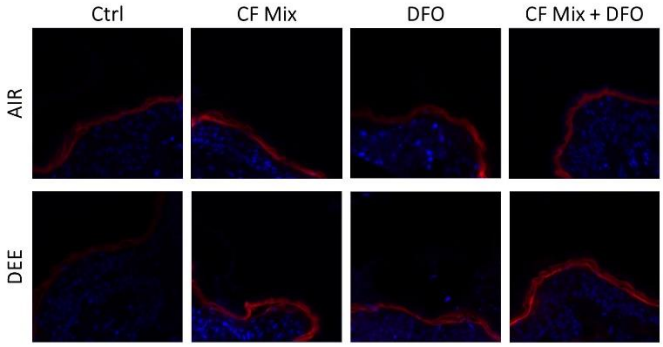
As previously shown, multipollutant exposures can affect skin barrier-associated proteins such as Filaggrin and Involucrin[104]. Indeed, cutaneous oxinflammatory responses activated by the exposure to environmental pollution can affect the skin barrier functions, contributing to cutaneous premature aging. Moreover skin aging has been demonstrated to be related to Iron-catalyzed ROS generation induced by sun exposure and that it can be prevented by the application of iron chelators [677] [676]. Here, after both short and long exposure to DEE (DAY 1 and DAY 4), we noticed a significant decrease in Filaggrin levels in untreated skin explants compared to air-exposed tissues (Fig. 20A and 20B), confirming the negative impact of airborne pollution on a structural protein critical for epidermal hydration and barrier function. However, the topical application of CF Mix or DFO prevented the loss of Filaggrin in DEE exposed tissues at DAY 4 (Fig. 20B). Interestingly, the combined topical application of CF Mix and DFO has proven particularly effective in increasing Filaggrin expression after DEE exposure at both time points, showing therefore a strong additive action (Fig. 20A and 20B). Similarly, DEE at both short and long exposures (DAY 1 and DAY 4) significantly downregulated the cutaneous levels of Involucrin as compared to untreated and air-exposed skin explants (Fig. 20C and 20D). This effect was prevented by topical application of CF Mix and DFO, alone or in combination. Of note, the combined treatment was particularly effective in counteracting the DEE-induced loss of Involucrin at DAY 4 (Fig. 20D).Collectively, these data corroborate the damaging effects of DEE on skin and indicate that combined topical application of CF Mix and DFO can strongly improve skin barrier functions and moisturizing by increasing Involucrin and Filaggrin levels in DEE-exposed skin.

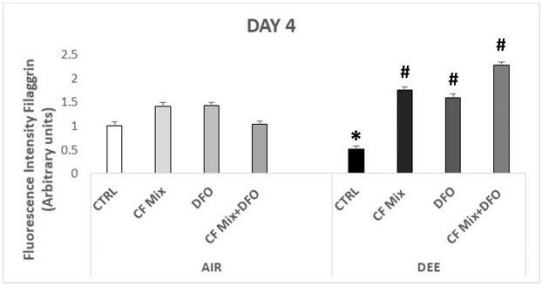
A.


Ctrl
CF Mix
DFO
CF Mix + DFO
AIR
DEE

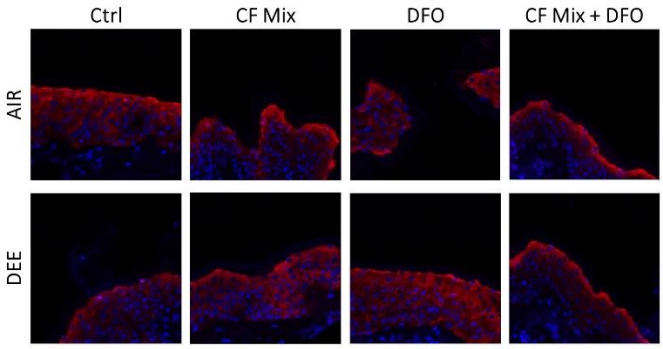

DAY 1
Fluorescence Intensity Filaggrin (Arbitrary units)
1.2
1
0.8
0.6
0.4
0.2
0
*
#
CTRL
CF Mix
DFO
CF Mix+DFO
AIR
DEE

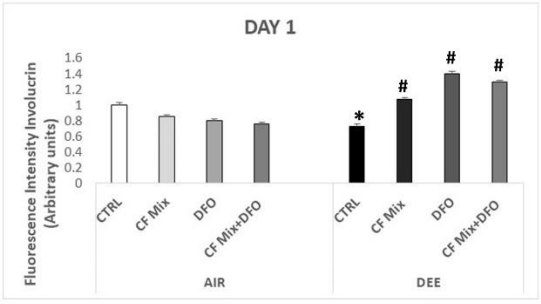
B.


Ctrl
CF Mix
DFO
CF Mix + DFO
AIR
DEE

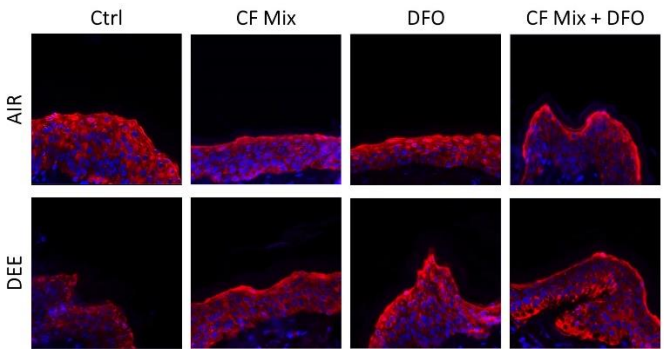

DAY 4
Fluorescence Intensity Filaggrin (Arbitrary units)
2.5
2
1.5
1
0.5
0
*
#
CTRL
CF Mix
DFO
CF Mix+DFO
AIR
DEE

C.


Ctrl
CF Mix
DFO
CF Mix + DFO
AIR
DEE


DAY 1
Fluorescence Intensity Involucrin (Arbitrary units)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
#
CTRL
CF Mix
DFO
CF Mix+DFO
AIR
DEE

D.


Ctrl
CF Mix
DFO
CF Mix + DFO
AIR
DEE

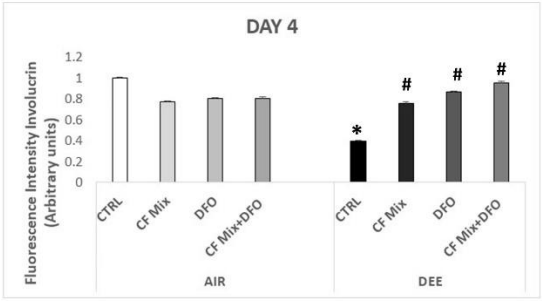

DAY 4
Fluorescence Intensity Involucrin (Arbitrary units)
1.2
1
0.8
0.6
0.4
0.2
0
*
#
CTRL
CF Mix
DFO
CF Mix+DFO
AIR
DEE

Figure 20. *Expression levels of Filaggrin (A-B) and Involucrin (C-D) in human skin tissues exposed to DEE for 30 min and pre-treated with CE Ferulic and/or DFO (alone or in combination) for 1 day and 4 days. Red staining represents Filaggrin or Involucrin and blue staining (DAPI) represents nuclei. Original magnification 40x. Quantification was performed using ImageJ (right panels). All Data are expressed as arbitrary units (averages of three different experiments), *p < 0.05 DEE ctrl vs Air Ctrl, #p < 0.05 DEE exposed and treated tissues vs DEE exposed and untreated tissues by ANOVA.*

5.5 DISCUSSION AND CONCLUSIONS

Due to the lack of studies evaluating the effects of combined exposure to UV light, O_3, and PM, the present work aimed to investigate whether these outdoor stressors can act synergistically in inducing skin damage. UV light is one of the strongest outdoor stressors; therefore, we focused our efforts in understanding the synergy between this outdoor stressor and the other 2 most toxic anthropogenic environmental air pollutants, PM and O_3. We confirmed that the combination of outdoor stressors induced oxidative damage by measuring levels of 4HNE, a marker of lipid peroxidation, and HO-1, a defensive enzyme under the control of NRF2, which is a redox sensitive transcription factor. As a consequence of altered redox homeostasis, we also assessed levels of inflammatory markers, NF-κB and COX2. In response to exposure to the combination of stressors, we clearly noticed an additive effect on both inflammatory and oxidative markers, especially in tissues exposed to all three stressors. We believe that these additive effects are due to different ways of interaction of the stressors with the skin. Although it has been suggested that all of these stressors are able to induce oxidative damage, this damage possibly derives from different pathways [37]. For instance, O_3 does not penetrate the skin but does interact with the polyunsaturated fatty acids (PUFAs) and squalene found in the stratum corneum. Thus, it generates a cascade of bioactive molecules (among them 4HNE and H_2O_2) that affect the deeper layers of the tissue [713]. A recent study, using an ex-vivo approach, was able to mimic squalene oxidation by O_3 and demonstrated the ability of the derived mono- and di-carbonyls to penetrate the skin epidermis [714]. This recent study confirms the idea that the skin is also a gateway for certain pollutants, as previously suggested by Weschler et al. [715]. More unclear is the mechanism by which particulates can affect cutaneous tissues. The toxicological properties of diesel-derived PM, especially for ultrafine particles (UFP) is mainly attributed to the presence of PAHs that are present in the particle structure, although their ability to penetrate healthy skin is still under debate. Filon et al. have suggested that only UFP can penetrate damaged skin and that the larger particles will interact with the outermost layer of the skin [716]. The theory that PM can enter the skin via the hair follicle is now less accepted, as this eventual passive penetration would be almost negligible [717]. Our group was able to show that, in a 3D in vitro model, several particles could reach the epidermis [273]. However, this study

was limited by the fact that skin produced using this model consists of a thinner stratum corneum and an overall more permeable structure [273]. Kammer et al. demonstrated that PAHs topically applied to the skin of human volunteers were found in the deep stratum layers using the tape stripping technique [718]. On the other hand, the ability of PM to enter skin cells in vitro has been well documented, which results in localization to the mitochondria, thereby inducing damage and ROS production [719–721]. Therefore, it is possible that, although PM particles are not able to deeply penetrate skin, they can enter the stratum corneum and possibly induce a cascade of bioactive molecules, similar to the mechanism by which O_3 induces skin damage, by reacting with lipids and squalene, which could result in the production of different end products. Moreover, the presence of transition metals in the particles could also be a source of oxidative damage [210,612]. In contrast, UV light can easily penetrate the epithelium (UVB), reach the dermis (UVA), and can be absorbed by proteins, lipids, and DNA, eventually inducing the production of ROS [103,193]. This was also confirmed in our study, where we observed increased levels of 4HNE and proinflammatory mediators in tissues exposed to UV, as proof of oxidative damage. As a result of the aforementioned cascade of effects, exposure to these outdoor stressors also alters barrier function of the skin, amplifying the effects of further exposure while facilitating the interaction of pollutants with the tissue. It may be possible, that, although PM itself can barely penetrate the skin, in the presence of other pollutants, the effects of multiple stressors on the skin barrier could make the skin more accessible to further penetration by outdoor stressors and/or their derived bioactive products. In support of this idea, we observed that Filaggrin and Involucrin, which are important for differentiation and maintaining the proper skin barrier function [722], are decreased in response to combined exposure. In a fairly recent work, researchers demonstrated that PAH exposure decreases Filaggrin levels in pig skin, making this effect a possible consequence of PM-induced oxidative damage [723]. In addition, Jin et al. observed that barrier disruption via tape-stripping resulted in increased penetration of PM in murine skin[721]. Thus, exposure to UV light, O_3, and PM in combination, which reflects the everyday urban environment, could result in increased penetration of single pollutants and/or their resulting bioactive molecules, such as 4HNE. In addition to Filaggrin and Involucrin, we observed that Ahr, which is involved in xenobiotic responses and also skin barrier function [724,725], is increased

in response to combined exposure, suggesting that these stressors are indeed recognized as xenobiotics. 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 [139]. 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 [145,152]. 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 [135], 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 [136] and also the NF-kB pathway [183]. Actually the continuous crosstalk between oxidative and inflammatory markers is nowadays a common figure displayed in several tissues damaging conditions, defined with the term OxInflammation [1]. For instance the activation of the Cytochrome P450 family enzymes by Ahr results in the production of ROS which are known to be able in promote not only the cross-talk between Ahr and NRF2 pathway, as first described by *W. Miao et al* in 2005 [184,185] , but also to modulate the NF-kB activation, suggesting that Ahr activation could be mediated by NF-kB via ROS production [180,182]. Nevertheless, the inflammatory response found in our skin model in response to pollutants stimuli was ulteriorly confirmed by 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 [307,469,472,473] and being particularly susceptible to UV radiations [485]. Several evidences so far have been shown that pollutants such as PM, cigarette smoke but also O_3 and UV can activate different Inflammasomes such as NLRP1, NLRP3 and AIM2 [484,486,726,727], which normally assemble in a protein 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 [453,728] [729,730], 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 protein complex in response to pollutants stimuli. Our results showed that PM and O_3 are able to exacerbate the UV-induced skin inflammatory damage, most likely mediating the modulation of NLRP1 activation, confirming previous data of this thesis 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 [729]. Nowadays there are strong evidences that correlates the IL-1β production with several skin inflammatory diseases such as psoriasis, atopic dermatitis to Inflammasomes activation [205,728]. Moreover also the Ahr receptor has been shown to regulate the expression of inflammasomes such as NLRP3 [731] 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 trigger factors activating several inflammasome pathways [183] [200]. This is the first study showing that exposure to all of these outdoor stressors can be correlated to Ahr activation. 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 pollutants, resulting in NLRP1 inflammasome activation and therefore in the exacerbation of the ox-inflammatory damage induced by UV itself. Indeed activation of Ahr could be a consequence of the bioactive products derived from the oxidation of squalene or fatty acids in the stratum corneum that are then recognized as xenobiotic by Ahr. Besides the proteins and lipids of the SC, skin barrier homeostasis is maintained by other essential components such as 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 [102]. 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 [593]. 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 [594], as well as PM, which is able to decrease ZO-1 via an oxidative mechanism [595]. 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 [732]. 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 pollutants exposure. Our results demonstrated that both O_3 and PM are not only able to compromise the expression of the main cutaneous junctions but they seem also to exacerbate the UV-induced skin damage. 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 addiction of O_3 and PM, suggesting that these pollutants could compromise the ability of the skin in 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 [733]. Moreover, a recent study also 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 [591]. Furthermore, impairment of Aquaporin 3 due to different stress stimuli including UV radiations, has been associated to skin abnormalities [734–736]. 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) [737]. 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 [738], whereas pro-inflammatory cytokines such as IL-1β and also IL-17 and TNF-ɒ, 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) [737,739]. 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, ox-inflammatory reactions triggered by pollutants 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 pollutants such as UV, O_3 and PM can affect skin homeostasis mainly due to the interconnection between ox-inflammatory reactions and skin structure/functionality impairment. Finally, although skin health can be improved by diet [112], the daily use of topical applications to prevent pollution-induced skin damage is still strongly recommended. This has been confirmed by the use of specific combinations of a Cosmeceuticals formulation mixture that has been previously shown to also protect against single pollutants in preclinical and clinical studies [60,130,211]. The formulation that has been applied in this study is commercially available and composed of 15% ascorbic acid, 1% alpha tocopherol and 0.5% ferulic acid. Previous studies have shown the ability of this composition, not only to penetrate the skin, but also to have an additive effect, compared to protection mediated by single components [633 587740]. We suggest that adding Ferulic acid to the formula, an hysroxycinnamic acid, probably protects L-ascorbic and a-tocopherol, by serving as a sacrificial substance [633]. In addition, Murray et al. demonstrated that this formulation was able to prevent UV-induced erythema, sunburn cells, p53 activation, and DNA damage[634]. Furthermore, the ability of this formula to prevent pollution-induced damage is likely due to the activation of skin defensive mechanisms, as a consequence of its demonstrated percutaneous absorption [633] and not to its UV absorption properties, as the same authors have shown its inability to act as a sunscreen [740]. Besides the current work, only a few studies have shown the possible interaction between different pollutants. For example, the *Marrot* group has demonstrated the “photo-pollution” effect of PAH and UVR in both 2D and 3D skin models [105,583]. In addition, our group was also able to demonstrate the ability of O_3 in vivo to enhance the damaging cutaneous effects of UV by decreasing levels of endogenous antioxidant micronutrients, thus potentiating the inflammatory impact of UV [741]. Another promoter

and catalyzing agent of oxidative stress reactions within our body is iron, a transition metal able to transfer electrons and therefore participating to different biological processes [652] Therefore Iron chelators agents together with antioxidants compounds represent a good approach to possibly prevent iron induce tissue peroxidation. For instance, the application of a cosmeceutical formulation mixture of antioxidant compounds (CF Mix) and Deferoxamine (DFO), a potent iron chelator, has been shown to prevent the OxInflammation status within our skin tissues model by counteracting the activation of 4HNE against Diesel exhausts particles (DEE). Oxidative stress and inflammation are common figures in several skin pathologies and they are known to affect skin barrier integrity and functionality. Indeed Inflammatory markers such as TNF-alpha, interleukins, cytokines, transcription factors such as NF-kb and also oxidative stress [742] are able to induce the activation of Metalloproteinases (MMPs), zinc-containing endopeptidases essential for tissue homeostasis, involved In the degradation of components of the extracellular matrix (ECM), such as collagen, fibronectin, elastin etc.. [743]. ECM remodeling by MMPs is an essential step in wound healing, regeneration processes but also in tumorigenesis, favoring malignant tumor growth, invasion and metastasis [281,744,745]. Up regulation of MMPs has been demonstrated to be associated to impaired skin barrier integrity, keratinization process and skin photoaging and carcinogenesis which are all events correlated to oxidative stress [746–749]. Moreover air pollutants exposure has been shown to modulate the inflammatory response via modulation of metalloproteinases [750] and this modulation related to pollutants- induced Oxidative Stress [219,712] and UV exposure has been found to induce MMPs activation via ROS production followed by ECM alteration, promoting angiogenesis, tumor growth and metastasis resulting in skin cancer [176 198] . Indeed MMPs are normally secreted as inactive pro-forms (zymogens) which need to be activated by different mechanism, including oxidative stress [752,753]. For instance in our skin models the possible activation of MMP9 induced by Diesel exhausts (DEE) exposure was counteracted by the application of the CF Mix alone and more evidently in combination with DFO, suggesting that DEE induced MMP9 activation might be mediated by Oxidative Stress and that iron could play a role in the oxidative damage. For instance Iron has been shown to modulate MPP9 expression in squamous carcinoma cells [711], also in ulcer lesions [754] and to trigger the activation of the Hypoxia-inducible

factor system (HIF system) [755–757] which is usually activated in hypoxia condition favoring angiogenesis, neovascularization, inflammation, and oxidative stress [758–760]. Indeed HIF-1α activation has been shown to induce the activation of MMPs such as MMP2, MMP9, MPP13 allowing the degradation of ECM and tumor progression [761–766], to induce the release of Inflammatory mediators such as IL-1β, IL-17, TNF etc.. [767] and to modulate the production of ROS, favoring the oxidation of biomolecules such as DNA, proteins and lipids[768]. It is therefore plausible that DEE exposure can induces the upregulation of iron, which in turn is able to modulate the ox-inflammatory reactions within the skin by inducing oxidative stress, 4HNE production and inflammatory mediators that can then affect the expression of Metalloproteinases such as MMP9. All these events can culminate in a structural skin damage by modulating the expression of proteins involved in skin barrier integrity.

For instance, the loss of skin barrier proteins such as Filaggrin and Involucrin found in skin tissues after DEE exposure, was counteracted by the application of the antioxidant formulation (CF Mix). Moreover, the concomitant application of the iron chelator DFO was even able to enhance this effect, confirming a possible iron-mediated mechanism.

In conclusion, the current study brings new insights on the consequences of skin exposure to multiple pollutants and how daily topical application of specific cosmeceutical formulations can protect cutaneous tissues against outdoor stressors. In addition we have demonstrated that the oxinflammatory and structure skin damage induced by pollutants, in particular by Diesel exhaust (DEE), as already demonstrated in other studies [104], could be iron mediated. Indeed, the application of an iron chelating agent such as Deferoxamine (DFO) was able to enhance the antioxidant properties of CF Mixture revealing a new therapeutic approach to potentiate antioxidants topical compound in counteract the pollutants- induced skin damage.

6. FINAL CONCLUSION

Overall, the present study has investigated and brought new insight in understanding the effect and the mechanism of environmental pollutants in induce skin Ox-inflammatory and structural damage. For the first time we demonstrated that the activation of inflammasome NLRP1 within the skin can be triggered by environmental stressors, most likely mediating oxidative stress reactions. In addition, we showed that the concomitant exposure to environmental pollutants such as UV, O_3 and PM can enhance and potentiate the skin damage induced by the single stressors, leading to a cascade of pathways able to interact to each other and promote skin damage (OxInflammation). Moreover, the daily application of an antioxidant topical compound, CE ferulic, was able to prevent this Oxinflammatory damage and the addition of the iron chelator deferoxamine (DFO) improved the protective effect of CF Mix opening a new path of investigation for iron in mediate the onset of skin conditions related to pollutants exposure. We suggest that CF Mix and also DFO, are able to prevent the production of reactive oxygen species (ROS), resulted from the interaction between pollutants and the skin, and therefore inhibit the activation of inflammatory mediators able to initiate inflammatory reactions within the cutaneous tissue. For instance we found that the topical application of CF Mix was able to inhibit the activation of inflammatory pathways such as NF-kB and Ahr, but also inflammasome NLRP1, corroborating the hypothesis of a redox-regulated mechanism of pollutants in triggering the inflammatory platform, already suggested for O_3. Indeed we hypostasize that pollutants oxidative mediators such as H_2O_2 and 4HNE could induce post-translation modification PTMs in proteins forming the Inflammasome complex and therefore modulate the activation of the inflammatory pathway. The activation of Inflammasome NLRP1 by pollutants stressors open a new fascinating field of investigation to better understand the etiopathogenesis behind several pollutants related skin pathologies. Indeed, these multiprotein platforms should be investigated as a target to prevent the development/exacerbation of stressor-associated skin conditions, including atopic dermatitis, psoriasis, acne, and premature aging. Although numerous strategies to target the inflammasome have been explored, none of these studies have investigated the efficacy of preventing stressor-induced skin damage and inflammation by inhibiting

inflammasome activation, therefore opening a new therapeutic strategy to prevent skin damage.

7. NOTES

Parts of the data presented in this project have been published and discussed in the following papers:

- **Ferrara F**, Pambianchi E, Pecorelli A, Woodby B, Messano N, Therrien JP, Lila MA, Valacchi G. Redox regulation of cutaneous inflammasome by ozone exposure. Free Radic Biol Med. 2020 May 20;152:561-570. doi: 10.1016/j.freeradbiomed.2019.11.031. Epub 2019 Nov 26. PMID: 31778733. (IF 6.17)
- **Ferrara F**, Woodby B, Pecorelli A, Schiavone ML, Pambianchi E, Messano N, Therrien JP, Choudhary H, Valacchi G. Additive effect of combined pollutants to UV induced skin OxInflammation damage. Evaluating the protective topical application of a cosmeceutical mixture formulation. Redox Biol. 2020 Jul;34:101481. doi: 10.1016/j.redox.2020.101481. Epub 2020 Apr 18. PMID: 32336667; PMCID: PMC7327990. (IF 9.986)

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