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Oxidative contribution of air pollution to extrinsic skin ageing

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Introduction

The skin is a mechanically protective and flexible barrier organ. Standing at the interface with the outside world, it is subject to lifelong exposure to a several environmental factors and in doing so, provides the body with overall protection. It consists of two main layers. The dermis (inner layer) is composed primarily of fibroblasts that produce an interconnected extracellular matrix of collagenous and elastic fibres. Whilst the latter provide some elasticity to the skin, collagen fibers provide structure and tensile strength and keep the skin hydrated. The dermis also contains blood and lymph vessels, nerves, hair follicles and sweat glands. The avascular epidermis (outer layer) consists of a basal layer of living epithelial cells called keratinocytes that manufacture keratin, a fibrous protein that gives hair, nails, and skin their hardness and water-resistant properties. As keratinocytes migrate to the skin surface, they progressively differentiate into non-nucleated (dead) epithelial cells (corneocytes) that are enriched by sebaceous and intercellular lipids including squalene, triglycerides, ceramides, free fatty acids, wax monoesters, and cholesterol [1, 2]. This lipid-enriched intercellular matrix comprises the stratum corneum (SC). Constituting the outermost skin barrier, the SC has important functions, limiting transepidermal water loss and posing a mechanical barrier to penetration by exogenous chemicals and pathogens. The shedding of old, worn out corneocytes is matched by replacement with younger cells from the basal epidermis, in the orderly and often imperceptible process known as desquamation [3].

The lipid rich components of the SC serve as targets of environmental stressors. Squalene is highly sensitive to oxidization owing to the presence of six carbon double bonds. Indeed, squalene is the major source of lipid peroxides at the skin surface and its oxidization is a reliable marker of various sources of pollution-induced skin assaults [4]. The anatomical organistion of the skin presents an analogy with that of the respiratory tract that is of relevance to the toxic effects of environmental stressors [5]. That is, in much the same way as the lung epithelial cells are not directly exposed to the ambient environment owing to being coated by the lipophilic respiratory tract lining fluid, air pollutants do not interact directly with keratinocytes but react with the lipophilic matrices of the outermost layers of the epidermis. The skin is well equipped with an enzymatic (glutathione peroxidase [GPx], superoxide dismutase [SOD], catalase) and non-enzymatic low-molecular antioxidant defense system (isoforms of vitamin E, vitamin C, glutathione [GSH], uric acid and ubiquinol) [6]. Its distribution follows a gradient with higher concentrations in deeper layers that lessen in concentrations towards the outer layers [7], probably owing to physiological turnover of skin cells.

Skin ageing

The skin is the most visible organ of the body and as a consequence, its ageing has medical, psychological and social repercussions that impact on an individual's self-esteem [8]. Skin ageing can be categorised into intrinsic - due entirely to the passage of time - and extrinsic, which is under the influence of environmental factors. The intrinsic deterioration of the skin is a multifactorial phenomenon, influenced for example by genetics and metabolism. It is an oxidative process in that it is associated with progressive, age-related decline in antioxidant capacity coupled with an increased production of reactive oxygen species ([ROS]; eg carbonyl species) from oxidative metabolism (eg lipid peroxidation) in cells of the skin [9, 10]. Clinical hallmarks of chronological skin ageing are thinning of the epidermal and dermal layers, leading to a loss of elasticity and the appearance of fine wrinkles. Extrinsic skin ageing is defined as an enhanced process of degradation of skin structural integrity and functionality as a consequence of being continuously and simultaneously exposed following to many environmental factors such as severe physical and psychological stress [11], tobacco smoke, alcohol intake, poor nutrition, overeating, exposure to ultra-violet radiation (UVR) and air pollution. [12]. Often characterised by coarse wrinkles, pigmented spots (lentigines) and uneven skin tone, it is phenotypically distinct from intrinsic ageing [13]. In translating the concept of the exposome to human skin, by collating existing knowledge about interactions between the skin and the environment, Krutmann et al. recently defined the skin ageing exposome as consisting "of external and internal factors and their interactions, affecting a human individual from conception to death as well as the response of the human body to these factors that lead to biological and clinical signs of skin ageing [14] (Figure 1).

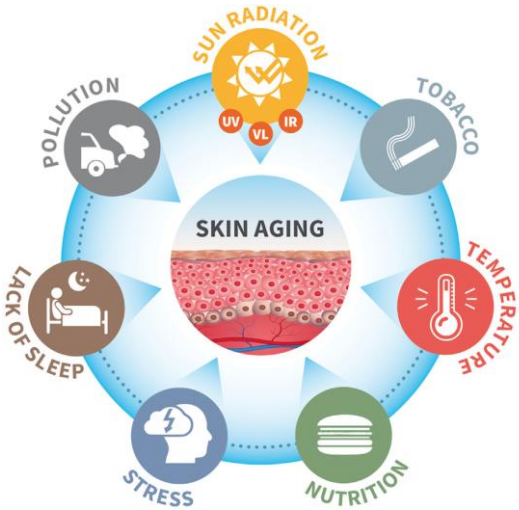

Figure 1: Exposome factors identified to potentiate skin ageing. Reproduced from Krutmann et al. [14].

Indeed, skin that is aged only by intrinsic factors is not generally believed to exist. Instead, an individuals' skin will reflect several stages of extrinsic ageing as a result of additive, if not synergistic effects of several environmental factors, superimposed on the degree of intrinsic ageing.

Air pollution and skin ageing

Exposure of cutaneous tissue to UVR is by far the most understood and researched environmental risk factor, and its detrimental effects on promoting skin cancer and ageing are well understood [15-17]. Indeed, terms such as extrinsic ageing/photoageing and lentigines/solar lentigo are often used synonymously but despite this, evidence is accumulating that other factors such as cigarette smoking and exposure to air pollution may have an additional impact. This has led to the introduction of the term 'environment-induced lentigo' to refer to the development and persistence of pigment spots in human skin in response to a UVR and a range of environmental toxicants [18]. The growing number of epidemiological and mechanistic studies that have investigated the association of particulate and gaseous air pollution with skin ageing are summarized below (Table 1). It should be noted that although there is also mounting evidence that exposure to air pollution is a risk factor for skin disorders including urticaria, eczema, dermatitis and rash [19-21] this is beyond the scope of this review.

Epidemiological evidence

Ozone

Whether a long-term exposure to ground level (tropospheric; up to 18 km high) O_3 is associated with traits of skin ageing has been addressed in two elderly cohorts of men and women living in densely urbanized areas of Germany: the SALIA study (806 women aged 66–79 years from the highly industrialized Ruhr area) and the Berlin ageing study II (BASE-II; 1207 men and women aged 60–84 years residing in Berlin) [22]. Five-year mean residential exposure to O_3 was modeled as the number of days O_3 concentrations exceeded European Union (EU) regulatory limits ($\geq$120 $\mu g/m^3$). Skin ageing was clinically accessed by a validated "score of intrinsic and extrinsic skin ageing" (SCINEXA: (SCore for INtrinsic and EXtrinsic skin Ageing) that differentiates the two ageing determinants. Positive associations (in each cohort as well as in the combined sample of both cohorts) between O_3 exceedances and coarse facial wrinkles were found, but not for pigment spots. Wrinkles were observed on the forehead in the pooled sample and SALIA cohort, under the eyes in the SALIA cohort and in the crow's feet area and on the upper lip in the BASE-II cohort (Table 1). The associations were independent of chronic UVR exposure as the most obvious confounder, and also of co-pollutants PM and nitrogen dioxide (NO_2).

Study	Location	Study population	Pollutant	Findings
Fuks et al 2019	Ruhr & Berlin, Germany	Subsets of 2 cohorts: SALIA (n=806 women; 66-79 y) BASE-II (n=1207 men & women; 60-84 y)	Ambient ozone	Coarse facial wrinkles No association with lentignes
Huls et al 2016	Ruhr, Germany Taizhou, China	Subsets of 2 cohorts: SALIA (n=806 women; 66-79 y) Taizhou Longitudinal Study (n=743 men & women; 28-90 y)	Ambient nitrogen dioxide	Cheek lentignes
Vierkotter et al 2010	Ruhr & Berken, Germany	Subset (n=400 women; 70-80 y) of SALIA cohort: n=189 from Berken (low exposure) n=211 from Ruhr (high exposure)	Traffic related air pollution	Forehead and cheek lentignes Slight association with smile lines none with wrinkles on forehead, upper lip, under eyes or crow's feet.
Peng et al 2017	Yanqing County & Xuanwumen, China	N=400 woman (40-90 y): n= 210 women from Yanqing County (low exposure) n=190 women from Xuanwumen (high exposure)	Ambient $PM_{2.5}$	Senile lentigo on cheeks and back of hands No association with seborrheic keratosis
Li et al 2015	Pingding and Taizhou, China	2 cohorts: Independent cohort from Pingding (n=405 women; 33-82 y) Subset of Taizhou Longitudinal Study (n=857 women; 28-90 y)	Cooking with solid fuels	Severe facial wrinkles and fine wrinkles on back of hands No association with lentignes
Ding et al 2017	Taizhou, China	Subset of Taizhou Longitudinal Study (n=1877 men and women; 35-89 y)	Indoor $PM_{2.5}$	Forehead lentignes and upper lip wrinkles

Table 1. Epidemiological studies evaluating the association between air pollution and skin aging.

Nitrogen dioxide

A link between chronic exposure to NO_2 and lentigo development has also been investigated using data from an extended SALIA population (mean NO_2 exposure: 28.8 $\mu g/m^3$) and an independent study of Han Chinese from a Chinese Taizhou cohort (mean NO_2 exposure: 24.1 $\mu g/m^3$) [23]. Exposure to NO_2 was significantly associated with more lentigines on the cheeks in the two populations, in both studies, in individuals over 50 years old (Table 1). In SALIA, an increase of 10 $\mu g/m^3$ in NO_2 was associated with 25% more cheek lentigines. In Chinese women, an increase of 10 $\mu g/m^3$ in NO_2 was associated with 24% more lentigines on the cheeks. In a two-pollutant model analysis of the SALIA study, including NO_2 and PM_{10}, the effects of neither pollutant were significant anymore, showing that their effects cannot be disentangled owing to high correlation between these measures.

Particulate matter

A relationship between traffic-related air pollution (TRAP) and clinical signs of skin ageing, including pigment spots, coarse wrinkles, solar elastosis and telangiectasias, was first investigated in 400 70-80 year old Caucasian women who were participating in the SALIA (Study on the Influence of Air Pollution on Lung function, Inflammation and Ageing) cohort [24]. The women resided in areas of either higher (urban Ruhr region, n = 211) or lower (villages and farms of rural Berken, n = 189) concentrations of TRAP. Exposure reflected a long-term one, for the following reasons: SALIA study participants were mainly housewives, almost all remained at the same address for the preceding 30 years and the pattern of pollution in the investigated cities remained the same over previous decades. Traits associated with facial skin ageing were assessed by means of SCINEXA. Traffic-related exposure at the place of residence was determined by traffic particle emissions and by estimation of soot in fine dust, whilst exposure to background particle concentration was determined by measurements of ambient particles at fixed monitoring sites. Controlling for potential confounding variables such as age, body mass index, use of hormone replacement therapy, smoking history, Fitzpatrick skin type, history of sunburns, and sun-bed usage, the investigators found that air pollution exposure was significantly correlated to extrinsic skin ageing signs, this was strongest for pigment spots and less pronounced for wrinkles (Table 1). An interquartile range (IQR) increase in $PM_{2.5}$ (particulate matter less than 10 μm in diameter) absorbance (soot; 0.5×10^{-5} per m) and traffic particles (475 kg per year per km^2) was associated with more pigment spots on the forehead (22% and 16% respectively) and cheeks (20% and 16% respectively). Background particle pollution, not directly attributable to traffic but rather to other sources of particles, was also positively correlated to pigment spots on the face. Soot, particles from traffic and to a lesser extent PM_{10} (particulate matter less than 10 μm in diameter) background concentrations were also associated with a slightly more pronounced nasolabial fold (smile lines). Although

UV exposure was significantly associated with a more pronounced occurrence of pigment spots, the researchers commented on unlikely confounding by sun exposure as all investigated cities lay next to each other where the general climate and UVR flux was essentially identical.

To investigate the association between fine particulate matter ($PM_{2.5}$) and skin ageing, Peng et al enrolled 400 Chinese women aged 40-90 years, including 210 from the Yanqing county in Beijing (low $PM_{2.5}$ exposure group) and 190 from the Xuanwumen in Beijing (high $PM_{2.5}$ exposure group) [25]. Using the SCINEXA score, senile lentigo on cheeks and back of hands in the Xuanwumen study population was 1.48 and 2.8 times higher respectively, compared with the Yanqing county residents (table 1). No association was found between $PM_{2.5}$ and seborrheic keratosis.

Indoor air pollution from cooking with solid fuels

A detrimental effect of indoor air pollution on skin ageing has also been investigated in a pooled analysis of two independent cross-sectional studies assessing the impact of cooking with solid fuels on women (30-90 years old) living in two geographically different areas of China: Pingding, to the north (n = 405) and Taizhou, to the south (n = 857) [26]. In China, an estimated 450 million people still rely heavily on solid fuels such as biomass (eg, wood, charcoal, and dung) and coal (International Energy Agency 2016), which when combusted indoors for heating and cooking purpose, can generate a substantial amount of PM, PAHs and carbon monoxide [27]. The analysis, evaluated by the SCINEXA score, indicated that independent of age and other influencers of skin ageing, solid fuel use was associated with a 5-8% increase in severe wrinkling on the face, and a 74% greater risk of having more fine wrinkles on the back of the hands in both studies combined (Table 1). Additional signs of skin ageing included more pronounced laxity of the eyelids and cheeks. The absence of an effect on pigment spot formation, in contrast to the German study population [24], might be explained by differences between outdoor and indoor pollutant sources and constituents, the higher baseline risk for pigment spot development in Asian populations [28], and/or other genetic differences between Chinese and Caucasians. Additional evidence that indoor $PM_{2.5}$ exposure is associated with skin ageing in a Chinese population has emerged from a study using a combination of direct measurement (in 30 households in Taizhou, China) and indirect modeling of indoor $PM_{2.5}$ exposure [29]. In an initial examination group (n=874), indoor $PM_{2.5}$ exposure was positively associated with pigment spots on forehead (12.5% per IQR increase) and wrinkle on upper lip (7.7% per IQR). The results were replicated in a second examination group (n=1003) as well as in the pooled dataset (Table 1).

Mechanistic evidence

Mechanistic explanations, involving the generation of reactive oxygen species (ROS) and cellular stress through recognised signaling pathways, for the epidemiological

associations between air pollution and skin ageing have arisen from experimental studies examining the effects of O_3 and different types of particulate matter in human clinical, animal and a variety of in vitro systems.

Ozone

Ground level O_3 is one of the most reactive environmental oxidant pollutants to which skin is exposed. It is a secondary air pollutant, formed by complex photochemistry (photoactivation, photodecomposition, and free radical chain reaction) that is dependent upon the presence of precursor gases such as oxides of nitrogen (NO_x) and volatile organic compounds (VOCs), temperature and UVR [30]. Alongside globally rising ambient temperatures, concentrations of O_3 are predicted to rise. In contrast to other modern day air pollutants, rapid scavenging of O_3 by nitric oxide emitted from motor vehicles, results in lower concentrations of O_3 in traffic dense city centres compared to suburban and rural locations. The World Health Organization (WHO) air quality guideline (AQG) is 100 $\mu g/m^3$ (~51 ppb; 0.05 ppm) for a daily maximum 8-hour mean however this was exceeded by 96% of the European urban population in 2017 [31].

As a consequence of its high reactivity and low aqueous solubility within the cutaneous tissue, O_3 is not able to gain access and directly damage live epidermal and dermal cells. Instead, it is consumed in entirety via indiscriminate reactions with the antioxidants, unsaturated fatty acids and lipids of the SC layer, leading to diffusion and detrimental effects by bioreactive mediators in the deeper, viable layers [32]. Indeed, although O_3 is not a radical species per se, it's toxicity is mediated through free radical reactions, either directly by the oxidation of cell membrane components that generate classical radical species (eg hydroxyl radicals) or by the latter subsequently driving the production of a heterogeneous mixture of cytotoxic, non radical species (eg reactive aldehydes such as 4-hydroxynoneal [4-HNE]) [33].

The recent epidemiological research that reported an association between O_3 and facial wrinkles is consistent with well established mechanistic studies dating back to the late 1990s, describing an oxidative stress response in human and mouse skin following short-term exposure to O_3. Early studies using SKH-1 hairless mice focused on the disturbance by O_3 of antioxidants and lipids in the SC, showing that a single high dose (1 to 10 ppm for 2h) significantly depleted both water soluble (vitamin C, GSH and uric acid) and lipophilic (vitamins E) antioxidants as well as producing the lipid peroxidation product malondialdehyde (MDA) [7, 34, 35]. Importantly, repetitive and longer O_3 exposure periods (single exposure to 0, 1,5,10 ppm for 2h or daily 0 or 1 ppm exposures for 6 days) have demonstrated a dose dependent vitamin E depletion and MDA formation [36]. That these effects in the dead cells of the SC could conceivably induce a cascade of cellular processes and in doing so, modulate pathological and physiological pathways in the deeper layers of the skin has also been demonstrated, again using SKH-1 hairless mice [32, 37, 38]. For example, animals

exposed to toxic concentrations of O_3 (8.0 ppm for 2h) exhibited not only a significant increase in protein carbonyls and 4-HNE-protein adducts (HNE-PAs) in the SC, but also a stress response in the cellular skin layers in the form of an induction of nitric oxide synthase (iNOS) protein expression and up-regulation of heat shock proteins HSP27 (20-fold), HSP70 (2.8-fold) and HSP32 (better known as heme oxygenase-1 [HO-1], an enzyme system that is induced by oxidative stress) [32].

The role of 4-hydroxynoneal

4-hydroxynonenal is a main product of oxidative stress following exposure of skin to O_3 (in addition to cigarette smoke and PM) and is derived from the oxidation of ω-6 polyunsaturated fatty acids (Figure 2). 4-hydroxynonenal-protein adducts are formed when the highly reactive, amphiphilic and electrophilic 4-HNE escapes detoxifying processes to migrate to intracellular sites where it reacts with biomolecules, especially target proteins [39]. Oxidative modification ensues and with that, the loss of important cellular proteins and corruption of the normal cell structure and function.

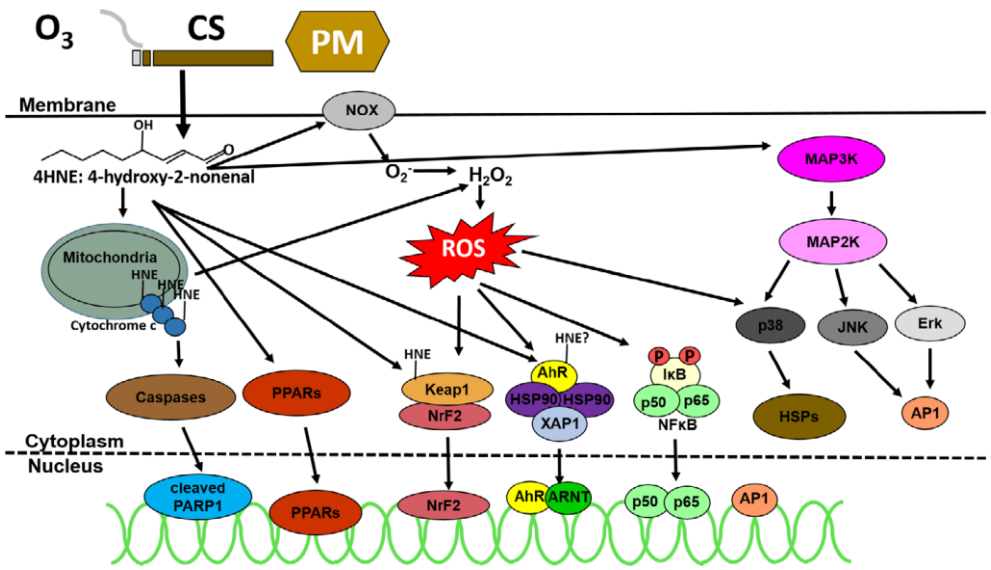

Figure 2. 4-Hydroxynonenal as a pollutant-induced signaling mediator. Reprinted with permission from Pecorelli et al. [40].

Of note, HNE-PAs contribute to the pool of damaged enzymes that increases during ageing and in several pathological states [41]. 4-hydroxynonenal-protein adducts are not only a marker of oxidative stress but also regulate the activity of critical redox transcription factors such as nuclear factor erythroid 2-related factor 2 (Nrf2; responsible for both constitutive and inducible expression of antioxidant response element [ARE]-regulated genes), peroxisome-proliferator activated receptors (PPARs), activator protein 1 (AP1) and nuclear factor kappa-light-chain-enhancer of

activated B cells (NFκB; promotes transcription of genes involved in the inflammatory response) [42-44]. It is surmised therefore that HNE is causally involved in many pathophysiological effects associated with a disturbed redox balance [45]. This is highly relevant within the skin owing to its rich concentration of omega-6 fatty acids. In line with the aforementioned increase in HNE-PAs in the epidermis of hairless mice exposed to O_3 [32], concentrations were increased after O_3 exposure in both 2D (0.1-0.5 ppm for 30 min or 1h) and 3D (0.4-0.8 ppm for 3h) human epidermal models [46, 47] and in human skin biopsies after 5 days of O_3 exposure (0.8 ppm for 3 h/day) [48].

The role of matrix metalloproteases

Mechanistic studies have also focused on cutaneous matrix metalloproteinases (MMPs) following O_3 exposure [37, 49, 50]. This group of enzymes regulate extracellular matrix metabolism by cleaving various molecules such as collagen and elastin [51] and are involved in various skin pathologies including tumor development, cutaneous lesions as well as skin ageing [52]. Pertubations in the synthesis and/or activity of cutaneous MMPs following environmental insults has the potential to degrade extracellular matrix fibers, diminish the elasticity of the skin and promote wrinkle formation [53]. Within the MMP family, MMP-9, whose role in human skin ageing has been demonstrated [54], is activated and upregulated in SKH1 hairless mice in response to O_3 concentrations occasionally experienced in heavily polluted cities (0.8 ppm [~1600 $\mu g/m^3$] for 6h) [37]. This was accompanied by increases in lipid peroxidation, as quantitated by 4-HNE-PAs and induction of HSP27 and HO-1. Age-related differences in the expression of MMPs and tissue inhibitors of metalloproteinases (TIMPs) between young and old hairless SKH-1 mice following exposure to O_3 (0.25 ppm 6h/d for 4 days) has also been observed [49]. The oxidant gas increased MMP-2 in both young (8 weeks) and old (18 months) animals, while MMP-9 was strongly induced only in old mice. In further exploring the relationship between ageing and response to O_3 using the same murine model and exposure regimen, the same research group observed a clear increase in skin carbonyls and 4HNE-PAs formation in younger mice exposed to O_3, an effect that was less evident in the aged animals, presumably owing to higher constitutive adduct concentrations in this group [50]. On evaluating the effect of O_3 on players involved in the generation of cellular ROS, increased expression in the skin of p22phox, p47phox (sensitive measures of nicotinamide adenine dinucleotide phosphate [NADPH] oxidase activity) and p66Shc were found in both young and old animals. It has been reported that the life span can be controlled by p66Shc protein concentrations as a consequence of way in which cells are regulated by oxidative stress [55]. In line with this, aged animals showed a higher basal level of p66Shc.

The role of the aryl hydrocarbon receptor

Toxicological metabolism following exposure of skin to O_3 has been investigated in

normal human epidermal keratinocytes (NHEKs) [56], by studying effects on the aryl hydrocarbon receptor (AhR) and the family of cytochrome P450 (CYP) isoforms. The latter plays a critical role in the oxidative metabolism of xenobiotics and endogenous compounds and hence excretion from the body but in some cases metabolism may lead to bioactivation and enhanced toxicity [57]. The aryl hydrocarbon receptor is a cytosolic, ligand-activated transcription receptor, expressed in keratinocytes, melanocytes and fibroblasts, that binds various endogenous and exogenous ligands including polycyclic aromatic pollutants, cigarette smoke, benzo[a]-pyrene (BaP), dioxin and food metabolites [58, 59]. Its major function in skin is therefore to sense environmental changes, facilitate adaptive responses and maintain skin integrity [58]. In its inactive state in the cytosol, AHR forms a multiprotein. Upon ligand binding, the complex dissociates and AhR translocates to the nucleus where it heterodimerizes with the AhR nuclear translocator and regulates the expression of many genes [60]. Exposure of NHEKs to O_3 (0.3 ppm) resulted in an increase in protein and messenger RNA (mRNA) expression of CYP1A1, CYP1A2, and CYP1B1, nuclear translocation of the AhR and phosphorylation of epidermal growth factor receptor (EGFR) [56]. Effects downstream of EGFR were activation of phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) and phosphorylation of mitogen-activated protein kinases (MAPKs) - signaling pathways that are activated in response to oxidant injury and play an important role in cell growth, proliferation, and survival [61, 62]. In that AhR knockdown by AhR small interfering RNA abolished CYP1 isoform mRNA and protein expression, suggests that AhR signaling is an integral component of O_3-induced induction of CYPs.

Human experimental studies

Extrapolating the effects of O_3 on murine skin to humans may not be appropriate in that the former has fewer viable epidermal cell layers, a somewhat different lipid composition and a lower transepidermal permeability [63]. Two controlled exposure studies in healthy volunteers have however also reported acute responses [48, 64]. For example, on exposing human forearm skin (female Caucasian volunteers aged 18 to 55 years old) for to 0.8 ppm O_3 2 hours, He et al. reported a significant reduction in vitamin E (70%), compared with the 20-60% reduction in skin antioxidants observed in the murine model [34, 36], and a 230% concomitant increase in lipid hydroperoxides (LPO) in the SC. Exposure created a measurable LPO gradient within the SC, with the highest concentration localized to the surface layer of the SC and a rapid decrease with tissue depth. Higher doses of O_3 (5 or 10 ppm for 2 h) were needed to induce MDA content in mouse skin [34], suggesting that biochemically, human SC may be more sensitive to the effects of O_3. A second study exposed subjects' forearms to 0.8 ppm for 3 hours/d for 5 days and following biopsy analysis reported significant increases in 4-HNE-PAs and 8-iso-prostaglandin-$F_{2\alpha}$ (8-iso $PGF_{2\alpha}$), another known byproduct of lipid peroxidation [48]. Again a clear gradient pattern in oxidation products was found, with high concentrations of 4-HNE in the upper epidermis and lower levels in the dermis (Figure 3). Further evidence of

oxidative stress previously observed in murine models included an activation of NF-κB and increase in MMP-9. Owing to their role in maintaining skin elasticity and resilience, collagen I and III were also assessed and both were found to decrease (by 64% and 60% respectively) and the authors postulated that this mainly reflected oxidation of the proteins rather their degradation.

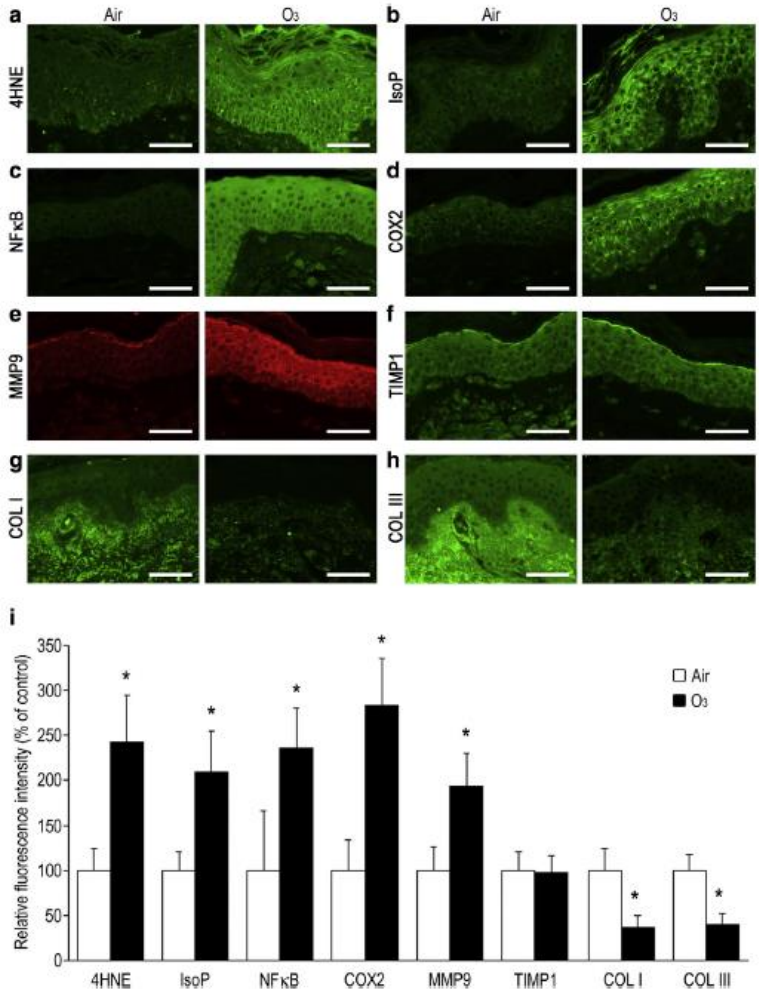

Figure 3. Ozone-induced damage to human skin. Reproduced from Valacchi. [48].

Particulate matter

Toxicology

Different hypotheses have been put forth concerning the initiation of PM-induced detrimental effects on cutaneous tissues. These could be attributable to an indirect 'inside-outside' systemic effect via the lungs and systemic circulation and/or to a more direct 'outside-inside' regional effect on the skin. The latter would of course rely on transcutaneous uptake and this will be discussed in the following section. Toxicology studies employing various in vitro systems have uncovered

interconnected cytotoxic events occurring through oxidative stress and inflammatory mechanisms, involving the activation of a series of mediators of the NADPH oxidase family, and up-regulation of redox sensitive transcription factors within cell nuclei [65-69]. For instance in human epidermal keratinocytes (HaCaT cells) urban PM (SRM 1649b; 1000 μg/ml) exposure induces ROS generation that is mediated through the AhR/p47phox/NADPH oxidase pathway, which in turn activates ERK1/2, p38/NF-κB and JNK/AP-1, and ultimately induces pro-inflammatory mediators such as IL-1α and cyclooxygenase 2 (COX-2) [69]. Magnani et al used a reconstructed human epidermis (RHE) model to evaluate the ability of concentrated ambient particles (CAPs; size range of 0.1–2.5 μm; 25 or 100 μg/ml for 24 or 48 h) to promote oxidative stress in skin tissue and assess the possible mechanisms involved. A RHE tissue consists of a differentiated epidermis (without dermis), with histological features similar to those observed in vivo and posses a basal, spinous and granular layer and a stratum corneum [70]. CAP exposure significantly affected RHE viability in a time and dose related pattern, as demonstrated by LDH release, the observation of apoptotic cells as well as DNA fragmentation. Increased concentrations of markers of oxidative damage included those of F_2-α isoprostane and 4-HNE, whilst particle surface characterisation and the use of deferoxamine (DFO) in human keratinocytes showed this to be dependent upon iron present on the surface of the CAPs. Furthermore and in line with the findings of Lee et al [71], NFκB nucleus translocation increased, as well as cytochrome P450, IL-1α and COX-2.

It is well recognized that ROS become cytotoxic only when their concentration exceeds a certain critical threshold and that the ability of the cell to curtail oxidative damage heavily relies on the efficiency of the Nrf2 /ARE pathway which plays a key role in preserving cellular homeostasis following chemical or oxidative challenges in all human cells including keratinocytes [72]. The latter modulate the expression of fundamental redox defense enzymes, such as GR and GPx, NADP(H) quinone oxidoreductase (NQO1) and SOD whose role it is to quench altered oxidative stress. On investigating the cellular mechanisms *beyond* oxidative changes, Romani et al. focused their attention on the Nrf2 pathway (Figure 4) [66]. Exposure of cultured HaCaT cells to CAPs (0.1-2.5 μm; 5, 10, 25 μg/ml) decreased viability, increased concentrations of 4-HNE PAs and stimulated IL-1α release. The dose and time dependent increased in 4-HNE PAs were significantly attenuated by DFO, again suggesting a key role of iron in the CAP induced oxidative damage. These responses were accompanied by a time and dose dependent significant increase in NF-kB and Nrf2 translocation from the cytoplasm to the nucleus but no increase in either gene expression or enzymatic activity of Nrf2 sensitive defensive enzymes (GR, GPx, NQO1). Further examination of the ineffective Nrf2-driven response of keratinocytes suggested that this might be explained by the ability of CAPs to alter binding of Nrf2 to the ARE DNA sequence. The researchers then went on to hypothesize a responsible mechanism involving for example the inhibition of transcriptional cofactors of the Keap1-Nrf2 regulatory pathway such as small MAF proteins, owing

to their involvement in keratinocyte differentiation [73], and the degradation of nuclear Nrf2 through sumolynation and ubiquitination [74].

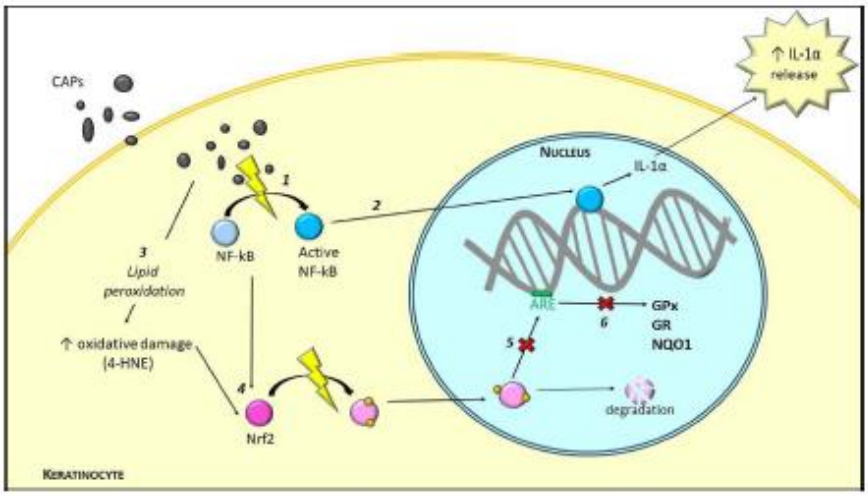

Figure 4. Proposed model of CAPs-induced oxi-inflammation in human keratinocytes. Reproduced from Romani et al. [66].

Another study that evaluated the impact of ultrafine particles rich in metallic elements ($PM_{0.3\text{-}2.5}$; 3, 15 and 30 μg/cm^2) on a RHE, reported oxidative stress and inflammatory response that could potentially contribute to a tissue slackening following the degradation of the extracellular matrix and cell-cell junctions [67]. The higher dose increased concentrations of 4-HNE, up-regulated HMOX1, MT1G and MT1E (3 genes involved in oxidative stress) and stimulated production of IL-1α and IL-8 in a dose-dependent fashion. These responses were accompanied by increased concentrations of two (MMP-1 and MMP-3) of nine MMPs tested, suggesting an acceleration of the extracellular matrix and a decrease in loricrin, a major protein component of terminally differentiated epidermal cells. Cell-cell junction, anchorage and terminal differentiation genes were mainly down regulated following PM-exposure. Analysis of apoptotic genes revealed a down regulation of BIRC5 (a gene that blocks apoptosis and regulates the cell cycle), whilst CDKN2 A expression was doubled and CASP3 was highly stimulated. CASP3 and CDKN2 A are both pro-apoptotic genes and expression of the latter has been shown to directly correlate with chronological ageing of human skin in vivo [75].

To understand the molecular alterations elicited in human epidermal keratinocytes (NHEK-Ad) following chronic exposure to diesel particulate extract (DPE; 0.05% [v/v]; 20 days) or its vapour (DPE-V), Rajagopalan et al employed a quantitative proteomics approach [68]. Increased expression of Nrf2 was observed along with widespread proteomic alterations. A total of 4490 proteins were identified, of which 374 and 201 were significantly dysregulated in NHEK-AD-DPE and NHEK-AD-DPE-V cells respectively. Proteins significantly downregulated by DPE and its

vapour included those involved in cornification (cornifin A), wound healing (antileukoproteinase) and differentiation (suprabasin). In addition, protein network analysis revealed that DPE vapor dysregulated mitochondrial oxidative phosphorylation proteins, including components of mitochondrial complex I that are the primary source of ROS in a variety of pathological conditions including ageing [76], as well as those that maintain cell-cell and cell-matrix interactions such as integrins, extracellular matrix proteins, MMP-14 and TIMP2. Treatment with vitamin E (9 IU/ml) decreased expression of Nfr2 in both DPE and DPE-V exposed cells as well as partially restoring expression of altered proteins.

Penetration

Occupational exposure studies, analysing specific populations such as coke oven workers [77], asphalt-paving workers [78, 79] asphalt roofing workers [80] and chimney sweeps [81], all indicate that dermal uptake is a direct route of pollutant contamination. The ability of ambient particulate pollutants to penetrate human skin is however debatable. It is possible that, depending upon particle size, hair follicles, by forming intrusions in the stratum corneum, may provide a route for penetration. In a study focused on the use of microparticles for drug delivery rather than the detrimental effect of PM on cutaneous tissue, it was reported that particles less than 1.5 μm in diameter do penetrate efficiently into the hair follicles up to a depth > 2 mm [82]. Such a transfollicular route may enable ambient particles and/or their surface bound constituents such as metal and PAHs, to reach viable cells, such as melanocytes, in deeper skin layers (Figure 5).

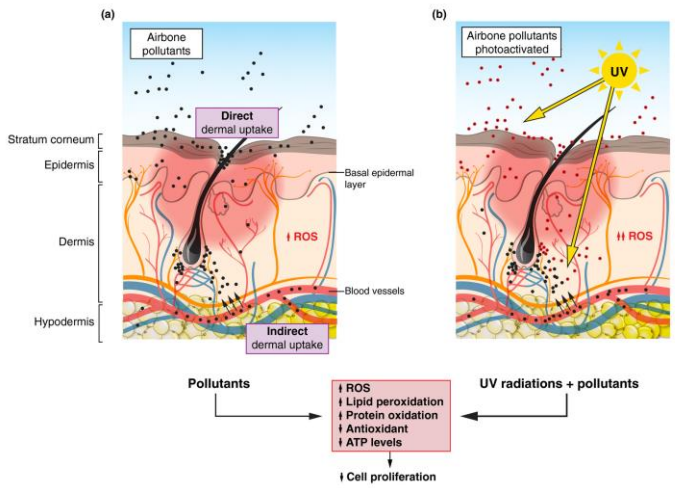

Figure 5. Direct and indirect pollutant uptake into cutaneous tissue. Reproduced from Araviiskaia et al. [83].

A previously described in vitro reconstructed human skin model also included a penetration analysis, identifying CAPs (0.1-2.5 μm; at 100 but not at 25 μg/ml) in the

upper (near SC) cell layer after a 24 h exposure and in deeper cell layers following 48 h [65]. However, in addition to the high concentration of particles required to demonstrate penetration, it is likely that such a model has an incomplete and weak barrier function. The penetrative capacity of lower concentrations of CAPs (5-50 μg/ml) has also been detected in keratinocytes after 24 h exposure, with diffuse particles visible in the cellular space, from the membrane to the cytoplasm [66]. Jin et al (2018) used in vitro (cultured keratinocytes) and in vivo models (BALB/c mice with barrier-intact or disrupted dorsal skin) to study the penetrative capacity of PM (< 1 μm) collected during a winter in Seoul [84]. Following a 24 h incubation of keratinocytes (40 μg/ml PM), particles were found to accumulate in vesicles, surmised to be secondary lysosomes, suggesting that the PM was endocytosed. Increased ROS production and IL-8 and MMP-1 mRNA expression and protein levels were also observed. To examine if PM penetrates the epidermis, 8 $\mu g/cm^2$ of PM was applied to the skin of mice using transparent film dressing for 6 h. Regardless of the barrier state of the skin, PM was observed in every follicle in the PM treated group and in the barrier-disrupted (but not intact) skin, PM was found in intercellular space of the follicular epidermis. On examining the epidermis of barrier-disrupted skin under TEM, amorphous shaped particles with heterogeneous electron-densities were identified in the spinous layer that lies above the basal layer of the epidermis.

Studies also indicate that for certain indoor pollutants, such as phthalate esters that leach from household good into the home environment, dermal absorption from air is comparable to or greater than inhalation. In a series of experiments, human subjects were exposed for 6 hours in a chamber containing elevated air concentrations diethyl phthalate (DEP) and di(*n*-butyl) phthalate (DnBP). The participants either wore a hood and breathed filtered air or did not wear a hood and inhaled chamber air [85]. By extrapolating parent phthalate intakes from urinary metabolites of DEP and DnBP, investigators found that for DEP and DnBP respectively, median dermal uptakes were 4.0 and 3.1 $\mu g/(\mu g/m^3$ in air) whilst inhalation intakes were 3.8 and 3.9 $\mu g/(\mu g/m^3$ in air). Moreover, the investigation revealed that dermal absorption of VOCs increases with age, with subjects over 60 years old having five times higher concentrations of the phthalates in urine compared to 30-year-old subjects [85]. Whether clothing influences the cutaneous uptake of compounds from polluted air has also been studied [86, 87]. Clean cotton clothes were only able to protect the skin by about 20-30% from absorption of airborne phthalates, and if not changed daily, they became a source of pollutant accumulation, increasing cutaneous uptake.

Regarding the ‘supply’ of pollutants to the dermis and deep epidermis via blood circulation, Moreau et al. observed that 8 hours post-injection in rats, BaP accumulated in lung, adipose and skin tissues, where it remained detectable 24 hours later [88]. Furthermore, Sykes et al. demonstrated dermal accumulation of gold nanoparticles or quantum dots (diameter of approximately 30 and 10 nm respectively) in rats following intravenous injection [89].

Strategies for protection

If the cutaneous impact of pollution occurs via both surface interactions and contamination of deeper layers of the skin from inside, prevention strategies would need to combine products that are able to protect at both of these levels. Surface protection of human skin against air pollution-induced skin damage may consist of unspecific as well as specific measures. Unspecific measures include the daily cleansing to remove particle load as well as topically applied emollients to improve skin barrier functions and hence reduce or prevent cutaneous load and/or penetration. In addition, since as discussed in the following section evidence exists that that UVR may potentiate the detrimental effects of air pollution on human skin, photo protective measures may represent another protection strategy.

Specific protection has been investigated by Valacchi and colleagues, who focused on antioxidant supplements as a defensive approach against O_3-generated oxidative stress [46, 47, 90]. Initial studies evaluated effect of β-carotene dietary supplementation on tumour necrosis factor alpha, macrophage inflammatory protein 2, iNOS and HO-1 on the skin of hairless mice exposed to O_3 for 7 days (0.8 ppm; 6 h/day) [90]. The results showed that supplementation provided protection against O_3-induced skin oxidative stress and inflammation. Another approach has employed a topical cocktail of antioxidants (MIX1: 15% L-ascorbic acid + 1% α-tocopherol + 0.5% ferulic acid; MIX2: 10% L-ascorbic acid + 2% phlorectin, + 0.5% ferulic acid) to investigate protective effects against O_3-induced oxidative stress damage in human keratinocytes [46]. Results showed that pretreatment with the tested compounds protected the cells from O_3-induced cytotoxicity and decreased the formation of HNE PAs, ROS, and carbonyls concentrations. Furthermore, a significant activation of Nrf2 and decreased activation of NF-κB were observed. The same investigators evaluated the ability of the antioxidant mixtures (MIX1: 15% vitamin C + 1% vitamin E + 0.5% ferulic acid; MIX2: 10% vitamin C + 2% phloretin + 0.5% ferulic acid; MIX3: 1% resveratrol + 1% vitamin E + 0.5% baicalin] to prevent the noxious effects of O_3 on a RHE (0.4 or 0.8 ppm for 4h) and in vivo on human forearm skin (0.8 ppm for 3 h/day for 5 consecutive days) [47, 48]. Again the benefits of the mixtures were demonstrated, preventing HNE-PA and 8-iso-$PGF_{2\alpha}$ formation, activating Nrf2 and attenuating the activation of NF-κB, the expression of MMP-9 and COX-2 and collagen marker loss.

Other protection strategies, selected on the basis of their potential to stimulate detoxification pathways, include various marine-based compounds to protect human keratinocytes from $PM_{2.5}$-induced apoptosis via inhibiting ROS generation [91, 92]. A diverse selection of compounds tested specifically for their protective effects against PM-induced ageing in human skin cells including (-)-epigallocatechin gallate (EGCG), the most abundant catechin in green tea [93], fermented fish oil derived from mackerel [94] and 7,3',4'-trihydroxyisoflavone (734THI), a secondary metabolite derived from daidzein in soybean [95]. Results have demonstrated intracellular scavenging of ROS, decreased expression of MMPs via regulation of

NF-κB, AP-1 and/or MAPKs signaling pathways and in the case of EGCG, a dose-dependent recovery of collagen synthesis and an inhibition of intracellular elastase and collagenase activities.

Synergy with sunlight

Traditionally, environmental factors believed to influence extrinsic skin ageing have been investigated separately, such that interactions between distinct factors and the resulting consequences are not well understood. The skin however is continuously and simultaneously exposed to several oxidative stressors that can have additive, if not synergistic, effects (Figure 5). The studies summarized below include those that have evaluated whether the photoageing process can be accelerated in various polluted environments.

UV & PAH

Particulate matter originating from fuel combustion contains PAH, which can be highly photo-reactive and induce strong oxidative stress under UV exposure. In a recent review, Burke and Wei reported that even low concentrations of BaP, in combination with UVA, contributes to increased oxidative damage in the skin, increasing extrinsic ageing and tumorigenicity [96]. The dose-dependent photocytotoxicity of BaP and six of its phase 1 metabolites in HaCaT keratinoctyes irradiated (1.0 J/cm^2 UVA light) in the presence of methyl linoeate has also been investigated [97]. The formation of LPOs was partially inhibited by SOD, whilst electron spin resonance spin trapping experiments indicated that both singlet oxygen and the superoxide radical anion were generated from UVA photoirradiation of BaP trans-7,8-diol-anti-9,10-epoxide in a light dose responding manner. Studies performed in NHEK and a reconstructed epidermis have also confirmed that photo-pollution is more dangerous than by pollution alone [98]. NHEK exposed to daily UVA1 (350–400 nm) combined with various PAHs, in the range of those reported in blood of pollution-exposed people, showed significant synergistic phototoxicity damage. The generation of ROS within cells and in the inner mitochondrial compartment, mitochondrial membrane depolarization and/or reduced ATP production were also noted.

UV and Ozone

Ultraviolet radiation penetrates into the epidermis (UV-B) or into the dermis (UV-A) and is known to stimulate the release of tissue-degrading enzymes even at suberythemal exposures. Since O_3 only oxidizes biological systems at the skin surface it is feasible that together, these environmental factors could exert an additive photo-ageing effect in cutaneous tissues. For example, a compromised skin barrier following UV irradiation may be enhanced by O_3-induced perturbation of SC lipid constituents that are critical determinants of barrier function. One study to investigate such a

putative additive/synergistic effect of environmental oxidative stressors evaluated effects in the SC of hairless mice exposed UVR and O_3 alone or in combination [99]. Whilst a significant depletion of α-tocopherol was observed after individual 2-hour exposures to either 1 ppm O_3 or a minimal erythemal dose of UV, the combination did not increase the effect of UV alone. A lower dose of O_3 (0.5 ppm; 2 h) with no effect when used alone did however significantly enhance the UV-induced depletion of vitamin E, suggesting additive oxidative stress within the SC in response to concomitant exposure to low doses of UV and O_3 at levels near those that humans can be exposed.

UV & traffic

In urban environments, human skin is routinely exposed to both UVR and traffic related air pollution, prompting Huls et al to use 834 elderly German women from the SALIA cohort to investigate an interplay of these two ubiquitous environmental factors in contributing to facial lentigines [23]. Measures of UV exposure included UVB-whole-day that is based on the ambient erythemally weighted UVB dose from the whole daylight period and the maximal UVR (UVI-peak) [100]. Whilst UVI-peak includes UVB and UVA radiation, it is primarily governed by UVB dose. UVI-peak and PM were associated with more facial lentigines. Associations were only slightly attenuated after adjustment for the second stressor and robust against additional adjustment for the time participants spent outdoors. A strong negative interaction between PM and UVB-whole-day was evident, such that a positive association between UVB-whole-day and lentigines was only visible at low PM exposure concentrations. Conversely, associations between PM and lentigines became weaker with increasing UVB-whole-day exposures. Possible explanations for this interaction included a shielding effect of photochemical smog or a photochemical reaction in which UVR can be absorbed by PAHs [98] and diesel exhaust particles [101]. In contrast no interaction between PM and UVI-peak was identified (eg the adverse effects of UVI peak were not modified by the PM concentration), possibly explained by the fact maximal UV exposure at midday does not coincide with (hence is less likely to be shielded or absorbed by) the higher concentrations of TRAP generally experienced during morning and afternoon rush hours.

Discussion

There is now epidemiological evidence that exposure to traffic-related air pollution including PM [24, 25], NO_2 [23] and ground level O_3 [22] is associated with pigment spot and wrinkle formation in Caucasians and East Asians. Evidence also indicates that indoor air pollution from cooking with solid fuels may accelerate skin aging in Chinese women [26]. For PM and NO_2, the strongest correlations with extrinsic skin ageing were for pigment spots and less pronounced for wrinkles. In contrast associations between O_3 exceedances and coarse facial wrinkles were found, but not for pigment spots. These discrepancies may, as previously discussed, relate to the

higher oxidative potential and shorter lifetime of the O_3 molecule, and reflect differences in the pathophysiologic pathways for various air pollutants [14].

In turn, these epidemiological findings help to put into context the results and suppositions that have arisen from experimental studies whilst the latter offer a plausible link between ambient pollutant exposure and the exacerbated appearance of accelerated skin ageing. For example, the recently reported connection between long-term exposure to O_3 and skin health [22] was found in earlier animal and in vitro studies that have described an interaction with unsaturated lipids in the upper layers of the skin to generate ROS, deplete antioxidant defense and in turn, induce a cascade of cellular stress reactions in deeper layers of the skin. Of particular relevance, these studies have shown that O_3 triggers the activation of MMPs [37, 48, 49], enzymes involved in collagen degradation and as a consequence wrinkling. An overexpression of MMPs and a downregulation of TIMP have also been demonstrated in keratinocytes exposed to diesel particulate [68], whilst a negative impact of PM on fundamental skin functions such as tissue anchorage has been observed [67]. One pathway that activates MMPs is via activation of the AhR [102], which in addition to its documented promotion of wrinkle development [103], also contributes to skin pigment formation [104, 105]. Whilst in vitro studies have shown that O_3 and PM activates the AhR in keratinocytes [56, 69], Vierkötter et al. reported more pronounced associations between extrinsic ageing traits and soot, a mixture of carbon particles covered by organic AhR ligands such as PAHs [24]. The activation of AhR signaling therefore has the potential to be involved in a crosstalk within cutaneous cells, which then causes the development of wrinkles and lentigines in human skin. No experimental studies have been conducted to investigate the effects of NO_2 on epidermal cells. However, it is known that NO_2 can undergo photolysis, with generation of reactive breakdown products that can affect lungs and potentially the skin. NO_2-exposed human bronchial epithelial cells exhibit an increased generation of proinflammatory mediators, such as nitric oxide/nitrite and cytokines [106] accompanied by an increased expression of HO-1 [107].

Outdoor and indoor pollutants are widespread within ambient air with which we physically interact and once inhaled, these pollutants can be distributed throughout the whole body via the systemic circulation. As a consequence, both the air-exposed, superficial and deep skin layers are potential pollutant targets. An important question raised by epidemiological studies is whether the reported manifestations of skin ageing are attributable to a systemic effect from chronic inflammation via the lungs and/or attributable to more regional effects on the skin. Huls et al. did not however detect a significant association between concentrations of NO_2 and the number of lentigines on other environmentally exposed areas such as the forehead, dorsal hand, or forearm, suggesting the operation of a regional effect [23]. However, whilst experimental evidence indicates that dermal uptake may constitute a route of entry [65, 66, 84], this may be very limited in healthy tissue since transcutaneous penetration would necessitate particles passing through the sebum and several stratum

layers before reaching living keratinocytes. A recent paper addressing the absorption of nanoparticles into skin concluded that PM measuring between 20 and 45 nm can only permeate and penetrate damaged skin and larger particles remain on the surface of the skin [108].

There is evidence that interactions between genetic factors and environment insults are an integral part of skin aging. For example, after accounting for skin type and sun exposure, wrinkle severity has been reported in elderly women to be associated with decreased lung function (specifically the ratio of forced expiratory volume to forced volume capacity), but only in individuals who carry specific MMP promoter variants [109]. This association was significant and independent of smoking or air pollution. It has also been suggested that responses to air toxicants are age related [110], and studies have shown that skin responses to pro-oxidant pollutants are modulated by age possibly owing to a higher baseline level of oxidative stress. For example, the association between concentrations of NO_2 and cheek lentigines in two separate populations, one in Germany and the other in China, were observed both individuals over 50 years old [23]. Furthermore, young and old mice exposed to O_3 exhibit differences in oxidative stress and MMP/TIMP responses [49, 50].

Ultra-violet radiation, cigarette smoking and air pollution are the three main factors that have been implicated in skin ageing. A study conducted in Australia was the first to formally follow adult volunteers and evaluate the impact of sunscreen use on photoageing [111]. The daily sunscreen group showed no detectable increase in skin aging after 4.5 years and skin aging from baseline to the end of the trial was 24% less in the daily sunscreen group than in the discretionary sunscreen group. In relation to cigarette smoking, prevention recommendations are simple - to avoid smoking and cigarette contaminated environments. With regard to air pollution, benefits of measures as diverse as wearing a mask, reducing cooking, using an air filtration unit, taking supplemental antioxidants and applying topical formulations based on targeting detoxification pathways remain unproven. Rather, sustained clean air policies remain the most effective and efficient solutions to reduce any health effect that air pollution may target.

Declarations of interest

None.

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