Comparative Evaluation of Cigarette Smoke and a Heated Tobacco Product on Corneal Oxidative Stress in an Air/Liquid Interface Model

Sebastiano Giallongo,[1] Francesco Bellia,[2] Andrea Russo,[3] Matteo Fallico,[3] Riccardo Polosa,[4,5] Niccolò Castellino,[3] Antonio Longo,[3] Rosalia Emma,[4,5] Konstantinos Partsinevelos,[2,5] Massimo Caruso,[2,5] Arief S. Kartasasmita,[6] Giuseppe Sferrazzo,[2] Ignazio Alberto Barbagallo,[2] Rosario Caltabiano,[7] Giuseppe Broggi,[7] Amer M. Alanazi,[8] and Giovanni Li Volti[2,5]

[1]Department of Surgery and Medicine, University of Enna "Kore," Enna, Italy
[2]Department of Biomedical and Biotechnological Sciences, University of Catania, Catania, Italy
[3]Department of Ophthalmology, University of Catania, Catania, Italy
[4]Department of Clinical and Experimental Medicine, University of Catania, Catania, Italy
[5]Center of Excellence for the Acceleration of Harm Reduction (CoEHAR), University of Catania, Catania, Italy
[6]Departement of Ophthalmology Faculty of Medicine Universitas Padjadjaran (National Eye Center Cicendo Eye Hospital), West Java, Indonesia
[7]Department of Medical, Surgical Sciences and Advanced Technologies "G.F. Ingrassia," University of Catania, Catania, Italy
[8]Pharmaceutical Biotechnology Laboratory, Department of Pharmaceutical Chemistry, College of Pharmacy, King Saud University, Riyadh, Saudi Arabia

Correspondence: Giovanni Li Volti, Department of Biomedical and Biotechnological Sciences, University of Catania, Via S. Sofia 97, Catania 95125, Italy; livolti@unict.it.

Received: October 28, 2024
Accepted: March 5, 2025
Published: April 1, 2025

Citation: Giallongo S, Bellia F, Russo A, et al. Comparative evaluation of cigarette smoke and a heated tobacco product on corneal oxidative stress in an air/liquid interface model. *Invest Ophthalmol Vis Sci.* 2025;66(4):4. https://doi.org/10.1167/iovs.66.4.4

Purpose. Tobacco smoke harbors toxic combustion by-products contributing to inflammatory diseases. Cigarette smoke's impact on ocular diseases has been poorly characterized, despite conjunctival mucosa's sensitivity to these toxicants. Of note, cigarette smoke triggers redness, tearing, and discomfort, accounting as a risk factor for glaucoma, macular degeneration, cataracts, and other eye conditions. Low quit rates of cessation highlight the need for alternatives. Heated tobacco products (HTPs), may represent a less toxic alternative for those smokers. This study evaluates cigarette smoke and HTPs effects on cornea under standard and clinically relevant conditions.

Methods. Corneal tissues collected from donors and in vitro model in two different cell lines of corneal epithelium were exposed to cigarette (1R6F) smoke and HTPs vapor. Air exposure was included as a control. Tissue pathological evaluation was carried out by hematoxylin and eosin staining. Reactive oxygen species (ROS) were measured, and quantitative PCR assessed inflammatory and antioxidant genes expression. Proteome analysis was used to evaluate differentially expressed proteins related to the oxidative stress. Scratch assay measured smoke and HTPs impact on cells.

Results. Hematoxylin & eosin staining highlighted that cigarette smoke impairs corneal tissue integrity, leading to ROS accumulation and inflammation, as proved by qPCR analysis. Proteomic analysis showed that corneal tissue's proteins were differently oxidized by the different experimental conditions. HTP targeted structural intracellular proteins, whereas 1R6F affects different members of collagen family. Finally, cigarette smoke, but not HTPs, impairs epithelial cells wound closure.

Conclusions. Smoking increases oxidative stress, leading to significant corneal damage and inflammation. HTPs may offer a less toxic alternative.

Keywords: cornea, oxidative stress, cigarette smoke, tobacco heated product, proteomics

Tobacco smoke harbors several combustion-derived toxicants, including a significant number of free radicals. These account for the onset, maintenance, and progression of several diseases characterized by an altered inflammatory profile.[1] In this context, the effect of cigarette smoke on ocular diseases has been so far poorly characterized. To this regard, the conjunctival mucosa is extremely sensitive to toxicants.[2] In particular, tobacco combustion byproduct lead to mild conjunctival redness and excessive tearing, and trigger the conjunctival-free nerve endings, causing uncomfortable sensations such as stinging, burning, and prickling.[3] Smoking is also linked to various eye pathological conditions such as glaucoma, suggesting that smoking may contribute to increase intraocular pressure.[4] Further-

more, smoking is a known risk factor for age-related macular disease (AMD), a leading cause for severe vision impairment. Smoking may indeed exacerbate AMD through various mechanisms, such as promoting ocular vascular remodeling and increasing oxidative stress, two factors ultimately triggering retinal damage.[5] Other conditions such as cataracts and dry eye syndrome have been linked to cigarette smoke. Smokers have a notably higher risk of developing nuclear cataracts or alteration of the tear film, eventually triggering vision impairment.[6,7] Finally, smoking contributes to ischemic diseases, increasing the risk of thrombosis and atherosclerosis, which can adversely affect ocular health. Atherosclerotic plaques, as the ones accumulating during acute anterior ischemic optic neuropathy (AION) pathogenesis, exacerbate indeed retinal ischemic events.[8] These conditions might lead to vision loss, underscoring the detrimental effects of smoking on eye health. Quitting smoking can mitigate these risks, aligning the health outcomes closer to those of non-smokers. However, the number of smokers who are willing or able to quit is relatively low, and therefore other approaches are necessary to prevent or reduce the risk of ocular complications in heavy smokers.[9]

In recent years, products that do not require combustion to deliver nicotine, such as heated tobacco products (HTPs), are increasingly replacing conventional cigarettes worldwide. Compared to traditional cigarettes, these alternatives significantly reduce exposure to harmful chemical constituents such as phenol, formaldehyde, and acrolein.[10] For this reason, combustion-free nicotine delivery systems are being considered for smoking harm reduction.[11] However, despite the benefits provided by them, concerns have been raised involving HTPs impact on Graves' orbitopathy and dry eye diseases, shading a light on the harmful impact of these devices.[12,13] Therefore, the aim of this work is to assess the impact of cigarette combustion and HTPs on corneal tissue by using standard and clinically relevant conditions.

Material and Methods

Smoke Exposure

The 1R6F reference cigarettes (Center for Tobacco Reference Products, University of Kentucky, Lexington, KY, USA) were used for smoke exposure. These cigarettes have been reported to produce 46.8 mg TPM, 29.1 mg tar, and 1.896 mg nicotine per cigarette smoked after HCI regime (Center for Tobacco Reference Products, University of Kentucky; 2018). Cigarettes were conditioned at 22°C ± 1°C and 60% ± 3% of relative humidity for at least 48 hours according to ISO 3402:1999 guidelines. LM1 smoking machine (Borgwaldt KC GmbH, Hamburg, Germany) was used to smoke two puffs of 1R6F cigarette after HCI regime, which ensures a 55 mL, two-second duration bell-shape profile, puff every 30 seconds with filter vent blocked. The number of puffs for 1R6F or HTP derives from our previous experiments to obtain comparable amount of nicotine with a clinically relevant concentration.[14] Vapor exposure was carried out using an HTP, IQOS Lumia (Philip Morris Products, Stamford, CT, USA). Terea "Sienna Selection" was used for IQOS Lumia. Vapor exposure was performed using LM4E vaping machine (Borgwaldt KC GmbH). IQOS Lumia was vaped using modified HCI regime without blocking the filter vents, to avoid the device overheating, for two. The ENDS product batteries were fully charged before use.[14]

Cornea Isolation and Air-Liquid Interface Exposure

After ethical committee approval (Azienda Ospedaliero-Universitaria Policlinico "G.Rodolico-San Marco", n. 83/2023/PO), Human corneal tissues were obtained from cornea donors employed for Descemet stripping automated endothelial keratoplasty. For their surgical use, tissues had to be transplanted within six days upon removal from the donor. Before transplantation the tissues were stored in Iscove's modified Dulbecco's medium (no. 51471C; Sigma-Aldrich Corp., St. Louis, MO, USA) at the temperature of 20°C to 31°C. Corneal tissue, obtained from the outer corneal layer described above, was exposed to smoking/vaping aerosol at air-liquid interface (ALI). Membranes were placed on 12 mm Transwells inserts (Corning Incorporated, Corning, NY, USA), and three inserts two inserts per test product were transitioned to the exposure chamber with 20 mL of PBS in the basal compartment. This exposure method is the most physiologically relevant for epithelial cell lines exposing them to all fractions and components of smoke/vapor.[15] Moreover, 1 negative control consists of 1 seeded insert without apical media (AIR) in the incubator.

Histopathological Analysis

Corneal tissue was formalin-fixed, paraffin-embedded and treated for histological examination using a standard method.[16] Two pathologists (R.C. and G.B.) separately evaluated all histological slides, blinded to sample identity. Histological features were assessed on 5 µm-thick sections stained with hematoxylin and eosin (H&E) as previously described.[17]

Extracellular Reactive Oxygen Species (ROS) Evaluation

Upon air-liquid interface exposure, corneal tissue was left in fresh media at 37°C, 5% CO_2 for 24 hours. Upon this time, the media was collected and spun in a centrifuge to avoid any residue to influence the analysis. Therefore the media was collected and incubated, avoiding light exposure, for 30 minutes at 37 °C with 2′,7′-Dichlorofluorescein acetate 10mM (no. 35848; Sigma-Aldrich Corp.). After this time, fluorescence intensity (λ_{ex} = 505 nm; λ_{em} = 526 nm) was assessed by using Synergy HT (Bio-Tek, Winooski, VT, USA).

QPCR

Gene expression on cornea was assayed by quantitative PCR. For this purpose, Corneal sections were dissolved in TRIzol (Thermo Fisher Scientific, Waltham, MA, USA). Therefore RNA extraction was performed by chemical separation as previously described.[18] The corresponding cDNA was obtained by using High-Capacity cDNA Reverse Transcription Kit (no. 4368814; Thermo Fisher Scientific) according to manufacturer's protocol. Gene expression was analyzed using Brilliant III Ultra-Fast SYBR Green QPCR Master Mix (Agilent Technologies, Winooski, VT, USA) and Rotor-Gene Q 2plex (Qiagen, Hilden, Germany). For each sample, the relative expression level of the mRNA of interest was determined by comparison with the control housekeeping ribo-

Table. List of the Primers Used for qPCR

Gene	Forward Primer	Reverse Primer
GST	AAGTTCCAGGACGGAGACCT	GCTGGTCCTTCCCATAGAGC
HMOX-1	TGTTGGAGCCACTCTGTTCC	GCTCAAAAACCACCCCAACC
IL1β	ATGATGGCTTATTACAGTGGCAA	GTCGGAGATTCGTAGCTGGA
IL6	GAAAGCAGCAAAGAGGCACT	TTTCACCAGGCAAGTCTCCT
PTGS2	CTGGCGCTCAGCCATACAG	CGCACTTATACTGGTCAAATCCC
TGFβ	CGTCTGCTGAGGCTCAAGT	CGCCAGGAATTGTTGCTGTA
18S	CTTAGAGGGACAAGTGGCG	ACGCTGAGCCAGTCAGTGTA

somal RNA 18S using the $2^{-\Delta\Delta Ct}$ method. Primers used for this assay are reported in the Table.

Sample Preparation for Proteomic Analysis

The cornea tissue was homogenized in RIPA buffer for 60 seconds using an Ultra-Turrax homogenizer (Janke and Kunkel, Staufen, Germany) set at maximum speed (24,000 rpm). The protein content was precipitated with prechilled acetone (−20°C) for 30 minutes. After centrifugation (10,000*g*), the pellet was resuspended in 8M urea, and the protein content was quantified using the BCA assay. The protein sulfhydryl groups were reduced with 1.6 mM dithiothreitol and alkylated with 7 mM iodoacetamide. The samples were diluted with 50 mM NH_4HCO_3 to a final urea concentration of 0.8 M; the protein content was digested with trypsin at 37°C for 16 hours (enzyme ratio 1:50 w/w). The resulting samples were analyzed by liquid chromatography–mass spectrometry.

Analysis of Tryptic Digests by Liquid Chromatography–Mass Spectrometry

Hydrolytic peptides were analyzed using a UHPLC system (Dionex UltiMate 3000 RSLCnano; Thermo Fisher Scientific) coupled with an Orbitrap Fusion Tribrid mass spectrometer (Q-OT-qIT) (Thermo Fisher Scientific). The samples obtained by in-solution tryptic digestion were diluted with a 5% aqueous FA solution. Peptides were eluted on a PepMap RSLC C18 column (EASYSpray, 75 µm × 50 cm, 2 µm, 100 Å; Thermo Fisher Scientific) and separated by elution at a flow rate of 0.250 µL/min, at 40°C, with a linear gradient of solvent B (CH_3CN + 0.1% FA) in solvent A (H_2O + 0.1% FA). The eluted peptides were ionized by nanospray (Easy-spray ion source; Thermo Fisher Scientific) using a capillary temperature and voltage set to 275°C and 1.7 kV, respectively. Peptide precursor scans in the m/z range of 400–1600 were performed with a resolution of 120,000 (200 m/z) with an AGC target for Orbitrap detection of 4.0×10^5 and a maximum injection time of 50 ms. Tandem mass spectrometry (MS/MS) spectra were acquired using a normalized collision energy (HCD) of 35. The dynamic exclusion duration was set to 60 seconds with a tolerance of 10 ppm around the selected precursor and its isotopes. The AGC target values and maximum injection time (ms) for MS/MS spectra were 1.0×10^4 and 100, respectively. The mass spectrometer was calibrated using the Pierce LTQ Velos ESI positive ion calibration solution (Thermo Fisher Scientific). MS data acquisition was performed using Xcalibur software v. 3.0.63 (Thermo Fisher Scientific).

Mass spectra were analyzed using MaxQuant software (version 2.5.1.0). The initial maximum allowed mass deviation was set to 6 ppm for monoisotopic precursor ions and 0.5 Da for MS/MS peaks. The enzyme specificity was set to trypsin, defined as C-terminal to arginine and lysine, excluding proline, with a maximum of two missed cleavages allowed. Carbamidomethylcysteine was set as a fixed modification, while N-terminal acetylation and methionine oxidation were set as variable modifications. Spectra were analyzed using the Andromeda search engine against the homo sapiens Uniprot sequence database. Quantification in MaxQuant was performed using the integrated label-free quantification algorithm based on extracted ion chromatograms and using fast label-free quantification. The false discovery rate was set to 1% at the peptide level and 1% at the protein level, with a minimum peptide length of seven amino acids required. Statistical analyses were performed using Perseus software (version 2.1.3). Only proteins that were present and quantified in at least two out of three technical replicates were considered positively identified in a sample and used for statistical analyses. The protein-protein interaction network was performed using the STRING (https://string-db.org).

Cell Culture and Air Liquid Interface Exposure

Immortalized human corneal epithelial cells (HCEC) (P10863-IM; Innoprot, Bizkaia, Spain) were cultured with endothelial cell medium (P60104; Innoprot) according to manufacturer's instruction. Statens Seruminstitut Rabbit Cornea cells (SIRC) (CCL-60; ATCC, Milan, Italy) were cultured in minimum essential medium (Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific), 1% penicillin/streptomycin cocktail (ThermoFisher Scientific) and 1% nonessential amino acids (Thermo Fisher Scientific). Cells were passaged every two to three days by using trypsin 1x (Thermo Fisher Scientific) and maintained at 5% CO_2, 37°C.

Subsequently, SIRC were used as in vitro model to test smoke damage. When the 80% confluency was reached, the apical medium was removed from each insert and two inserts per test product were transitioned to the exposure chamber with 20 mL of PBS in the basal compartment to perform the ALI exposure. A negative control, consisting of one seeded insert without apical media (air) in the incubator was also included.

Scratch Assay

Scratch assay was performed as already described.[18] Briefly, cells were seeded in 24-well inserts and cultured until confluence. At this stage, cells were exposed either to

Investigative Ophthalmology & Visual Science

tobacco or heated-tobacco smoke using the ALI exposure method. Cell culture was scraped with a 10 µL micropipette tip. Wound closure was detected at 0, 24, 48, and 72 hours using Cytell cell imaging system (Imsol, Preston, UK). The uncovered wound area was measured and quantified at different intervals with ImageJ v1.37 (NIH, Bethesda, MD, USA).

Statistical Analysis

Statistical analysis was performed using Prism Software, for comparison of $n \geq 3$ groups, one-way or two-way ANOVA with Holm–Sidak post hoc test for multiple comparisons were used where appropriate (GraphPad Software Inc., San Diego, CA, USA; RRID: rid_000081). Data are expressed as mean ± SD, unless otherwise stated. For all statistical tests, P values < 0.05 were considered statistically significant.

Results

Cigarette Smoke Exposure Impairs Corneal Integrity

Smoking exposes epithelial tissues to several chemicals impairing tissue structure.[19] Therefore we sought to assess the integrity of corneal tissue 24 hours after smoke exposure. For these purpose tissues were stained with H&E to assess the integrity of the cornea epithelium after air, HTP, and 1R6F exposure. Interestingly, our data (Fig. 1) showed no evident signs of damage after air or HTP exposure. By contrast, cornea exposed to 1R6F, exhibited a significative damage on the epithelial (E) but not on stromal (S) layer of the tissue and alterations of the extracellular matrix (Fig. 1). These data show that conventional cigarette smoke might hamper epithelial corneal cells structure on corneal tissue.

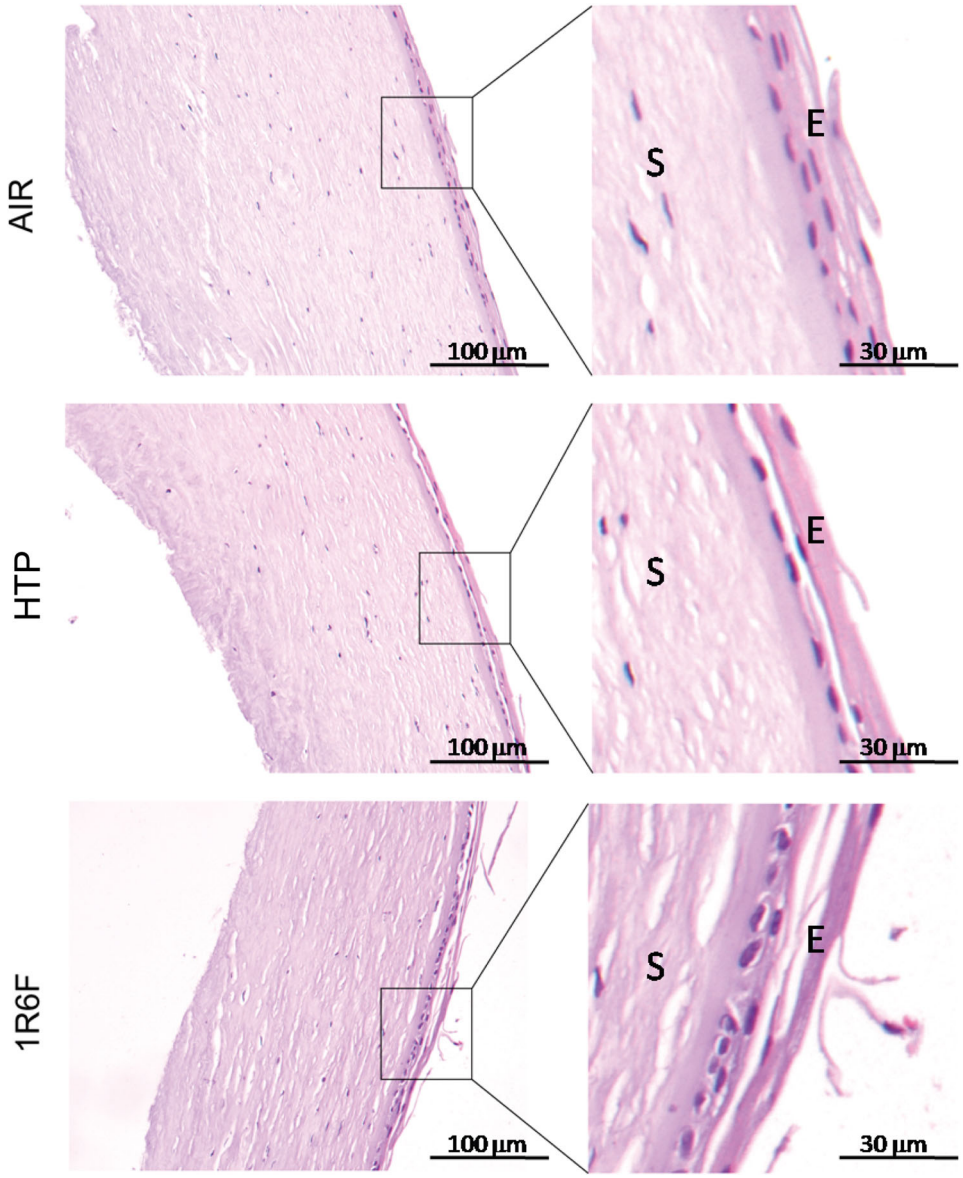

Figure 1. Cornea characterization. H&E staining for corneal epithelia. Image has been labeled marking stroma (S) and epithelium (E) Representative images showing corneal morphology after air, HTP, and 1R6F exposure. Images are representative of four different replicates.

Investigative Ophthalmology & Visual Science

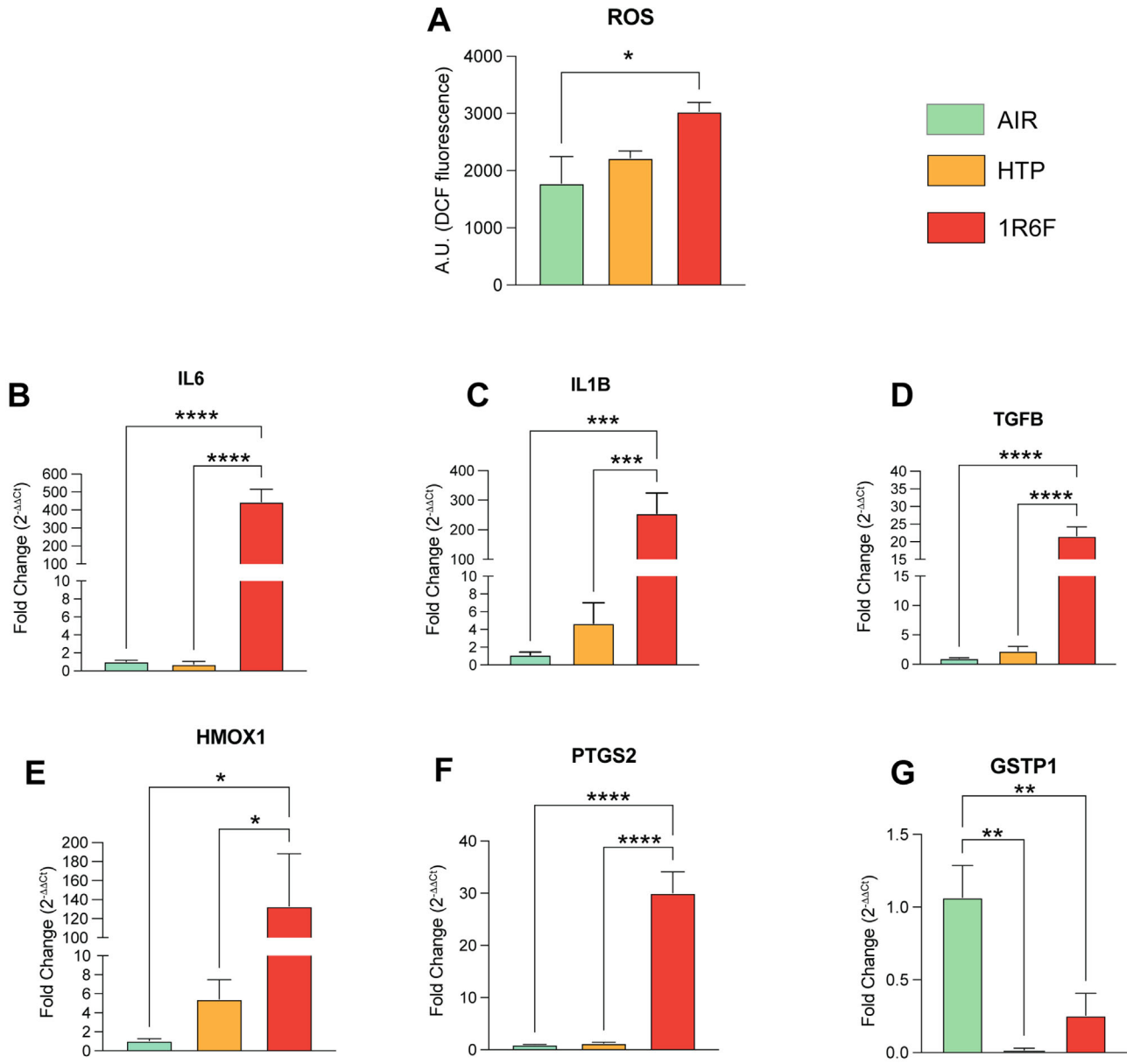

Figure 2. Evaluation of oxidative stress and inflammation on four corneal tissues. **(A)** Assessment of ROS level on cornea after three hours' air, HTP, and 1R6F exposure. **(B–G)** The qPCR evaluating IL6, IL1B, TGFB, HMOX1, PTGS2, and GSTP1 expression on 24 hours' exposure to air, HTP, and 1R6F. Images are representative of four biological replicates. (*$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$; ****$P \leq 0.0001$).

Cornea Epithelium Exhibits Increased Oxidative Stress After HTP and 1R6F Exposure

The observed damage on epithelial cells might be driven by the several chemicals contained in smoking, eventually triggering oxidative stress on epithelial cells. Therefore we assessed the level of ROS released in the extracellular media of corneal tissue 24 hours after air, HTP, and 1R6F exposure (Fig. 2). Interestingly, after 1R6F exposure we observed an increased ROS concentration in comparison to air and HTP exposure (Fig. 2A). Therefore, to further confirm the oxidative stress triggered by 1R6F exposure, we sought also to assess the expression of different genes involved in the oxidative stress signaling cascade. Interestingly, qPCR analysis showed an increase in the expression of interleukin-6 (IL6), Interleukin-1β (IL1B); Tumor growth factor-β (TGFB), heme-oxygenase 1 (HMOX1), prostaglandin-endoperoxide synthase 2 after 24 hours' 1R6F exposure compared to air and HTP counterparts (Figs. 2B–F). Of note, we also detected a marked decreased expression of glutathione S-transferase pi 1 (GSTP1) after both HTP and 1R6F exposure (Fig. 2G). Therefore these data show that 1R6F exposure significantly increase cornea oxidative stress and the inflammatory status.

Proteomic Analysis Demonstrates a Different Oxidation Pattern After HTP and 1R6F Exposure

The chemical and physical boost coming from both the HTP and 1R6F exposure may somehow affect the proteome homeostasis. To monitor differently expressed proteins (if any) upon the treatment of corneal tissue with HTP and

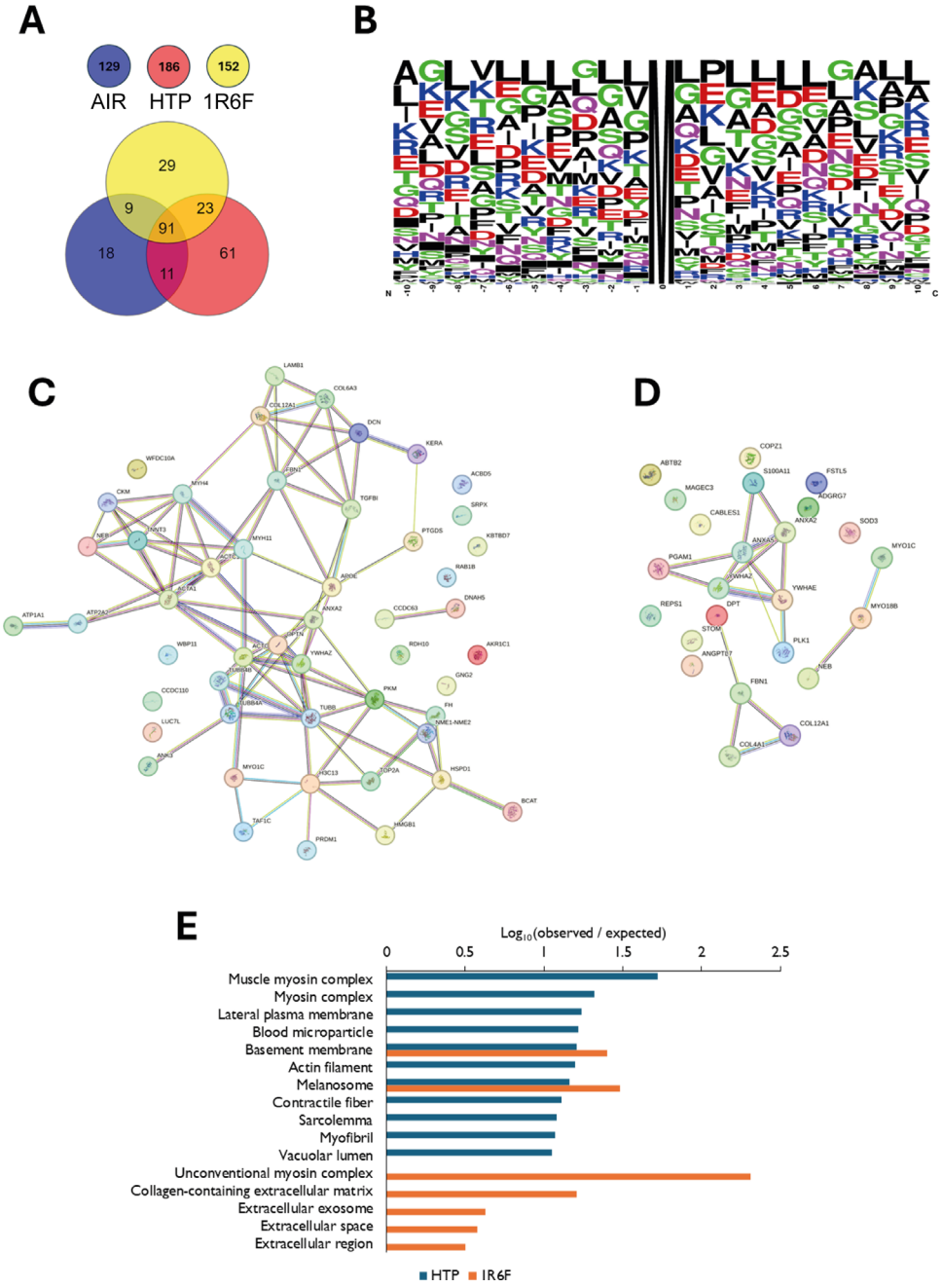

Figure 3. Mass spectrometry-based proteomic analysis on corneal tissue. **(A)** Venn diagram showing the differently oxidized proteins within the three experimental groups. **(B)** Sequence logo de analysis of the oxidized peptides, centered on the amino residues oxidized. Interacting network for HTP **(C)** and 1R6F **(D)** exclusively oxidized proteins (FC > 5; adjusted $P \leq 0.01$). **(E)** Gene ontology annotation on biological properties of the main exclusively oxidized proteins detected for treatment with HTP and 1R6F. Images are representative of four biological replicates.

1R6F, we used a proteomic approach based on MS. As many as 524 proteins were identified in all the samples analyzed. Volcano plots (Supplementary Fig. S1) show that there are no differentially expressed proteins; this means that the proteomic pattern of tissues exposed to air does not significantly change when the cornea samples were treated with the HTPs vapor or the cigarette smoke. Smoke and vapor from HTPs or conventional cigarettes also harbor a plethora of highly unstable free radicals eventually enhancing ROS levels, which might be responsible for the post-translational modifications (PTMs) of several proteins. Oxidation, nitration, citrullination, carbamylation and glycosylation are some of the PTMs mainly induced by conventional cigarettes. Oxidation is the most significant process among them, because it can affect several amino acid residues (His, Tyr, Trp, Cys, Met) and involves various other biological substrates. Therefore we sought to monitor the oxidation of all the proteins previously detected. One hundred twenty-nine out of 524 proteins are oxidized in the sample coming from control tissues (Fig. 3A). Interestingly, such a value increases to reach 186 or 152 proteins when cornea is exposed to HTP or 1R6F, respectively. A

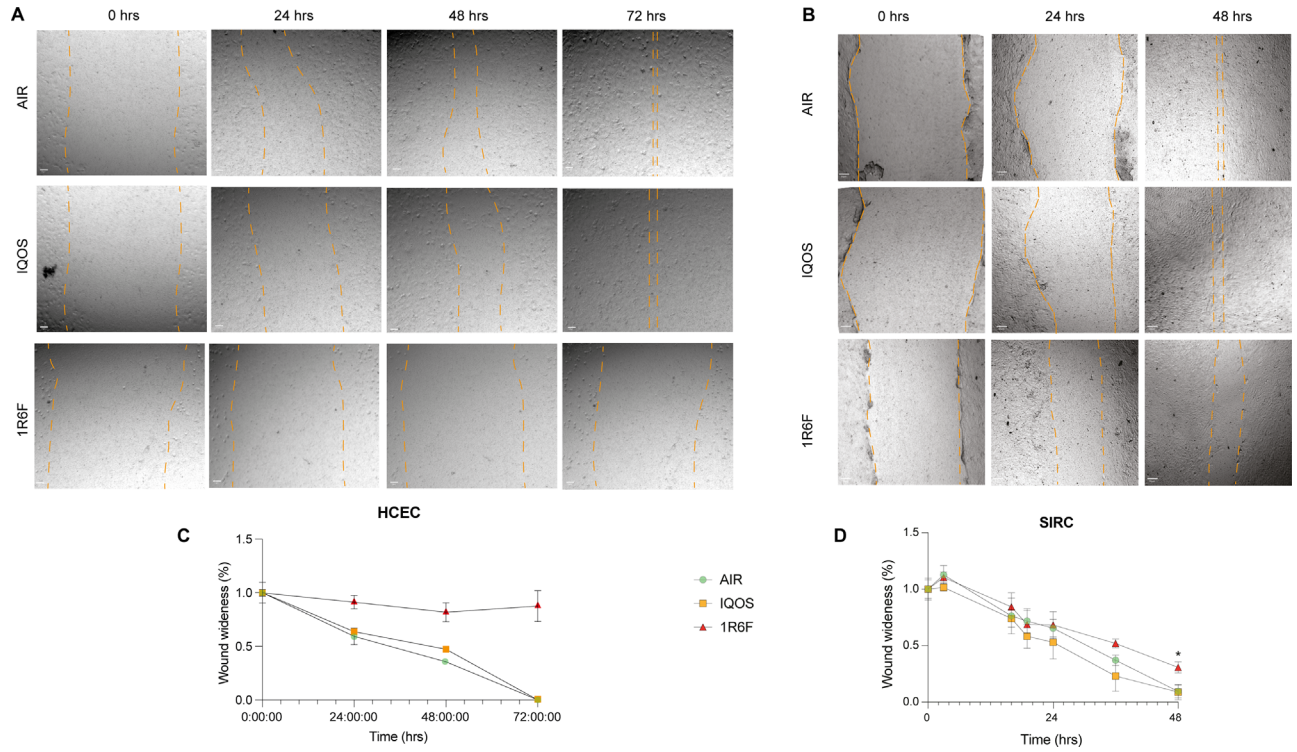

Figure 4. The 1R6F exposure impairs epithelial cell wound closure ability. **(A)** Wound healing assay on HCEC following ALI exposure to air, HTP and 1R6F. Images are representative of four time points 0, 24, 48, and 72 hours. **(B)** Wound healing assay on SIRC after ALI exposure to air, HTP and 1R6F. Images are representative of three time points 0, 24, and 48 hours. **(C)** Quantification of wound closure percentage on HCEC and **(D)** SIRC exposed to air, HTP and 1R6F. (**$P \leq 0.01$; ****$P \leq 0.0001$).

detailed analysis of the protein sequence at the oxidation site shows that methionine is the only amino acid residue affected by oxidation under our experimental conditions (Fig. 3B). Moreover, the frequency distribution of all the other residues located in the proximity of the oxidation site plainly excludes the presence of any consensus sequence. Our data also reveal 18, 61, and 29 differently oxidized proteins exclusively affected by air, HTP and 1R6F, respectively (Fig. 3A). Moreover, 11 proteins were commonly oxidized by air and HTP, whereas nine were oxidized by both air and 1R6F (Fig. 3A). Furthermore, 91 proteins were oxidized by all three experimental conditions, whereas 23 were oxidized by HTP and 1R6F (Fig. 3A).

To investigate the groups of proteins exclusively oxidized in the air, HTP and 1R6F samples, we therefore performed a network analysis. The oxidized proteins from air do not show a specific network cluster (Supplementary Fig. S2). On the other hand, the output coming from the HTP-treated sample reveals a peculiar cluster of proteins oxidized (Fig. 3C) which mostly differs from that originated by the proteins oxidized in the 1R6F sample (Figs. 3D, 3E). With our data taken together, we showed that both HTP and 1R6F profoundly change the oxidation pattern of corneal epithelia.

The 1R6F and HTP Exposure Impair Human and Rabbit Corneal Epithelial Cells Wound Healing

Given the evidence collected on corneal tissues we sought to assess the impact of HTP and 1R6F exposure on two different in vitro models of human and rabbit corneal epithelial cells. Therefore we performed a scratch assay to evaluate how air, HTP, and 1R6F affect cell proliferation (Figs. 4A, 4B). Measuring the wound closure percentage on HCEC we observed a complete closure upon 72 hours for cells exposed to air and HTP. Interestingly, 1R6F-exposed cells were not able to close the wound (Fig. 4C). Once exposed to air and HTP, SIRC completely closed the wound in 48 hours. The 1R6F-exposed cells, on the other hand, were not able to close the wound at this time point (Fig. 4 D). Overall, these data show that 1R6F exposure impairs wound healing ability of HCEC and SIRC.

Discussion

Tobacco smoke harbors a complex mixture of different chemicals, eventually being responsible for the oxidative stress characterizing smokers.[20] As a consequence, smoke has been associated to several pathologies characterized by an enhanced inflammatory response.[21] Among them, several ocular diseases have been linked to the toxic effects of smoke exposure on corneal epithelium, in turn triggering an inflammatory and oxidative stress response.[22] In the latest years, novel devices named HTPs, emerged as a potential alternative to conventional cigarettes, eventually reducing the exposure to cigarettes' harmful chemical components.[11] Therefore HTPs emerged as a combustion-free nicotine delivery system considered for smoking harm reduction, in turn also releasing an aerosol expected to be much less toxic for the eye.[11] To investigate this aspect, we explored the impact of conventional cigarettes and HTPs on the cornea by using standard and clinically relevant conditions.

Investigative Ophthalmology & Visual Science

By H&E staining we detected significant damage to human corneal epithelial cells after conventional cigarette exposure, characterized by a significant spall of the outer area of the cornea itself. This outcome might be related to the large amount of oxidative chemicals in cigarettes, which have been extensively reported to lead to severe tissue damage.[23] In particular, cigarette smoke has been described as a major risk factor for pulmonary fibrosis through a mechanism involving alterations in alveolar epithelial cells, eventually becoming more permeable, decreasing surfactant production, and misregulating inflammatory cytokines and growth factor production.[24] Further insight highlighted the mechanisms of such an issue as relying on neutrophils and monocytes, which, once activated, impair tissue architecture by degradative enzymes derived from such inflammatory cells.[25] Inflammation and oxidative stress, therefore, are two of the main issue associated to smoke, which might also be responsible for tissue damage.[24,25] Corroborating this, our data show a significative enhancement of ROS concentration following 1R6F exposure, supported by an increased expression of IL6, IL1B, TGFB, HMOX1, and PTGS2, but not of GSTP1, in turn decreased upon exposure to conventional cigarettes. These outcomes are relatable to the different works characterizing issues associated to cigarette smoke and claiming a significative number of highly unstable free radicals increasing ROS production and therefore leading to an enhancement of oxidative stress and inflammation.[1,20,26] Corroborating our analysis it has been also reported that inflammatory and oxidative markers as IL6, IL1B, TGFB, HMOX1 and PTGS2 are upregulated by conventional cigarette in different models.[27–30] Interestingly, GSTP1 downregulation has been associated to be induced following a dramatic increase in ROS concentration as the one we observed following 1R6F exposition.[31] Of note, the same trend was not evidenced for HTP, which did not alter IL6, IL1B, TGFB, HMOX1, and PTGS2 expression of those markers. Interestingly, prolonged inflammation in the cornea is associated with persistent epithelial defect, eventually linking tabagism with an increased risk of eye-related diseases.[32] The increased oxidative stress might also lead to post-translational modifications, eventually affecting several pathways which might impair corneal homeostasis. The MS-based proteomic investigation after 1R6F and HTP exposure revealed several proteins differently oxidized by HTP and conventional cigarettes. Of note, the oxidized proteins from air do not show a specific network cluster meaning that the oxidation process might have an unspecific origin, like the natural propensity of methionine to be oxidized during the sample treatment. The oxidation induced by HTP, on the other hand, mainly addresses proteins linked to the tubulin and myosin networks HTP, whereas 1R6F mainly affects the oxidation of collagen chains and extracellular protein components. Interestingly, it has been reported that, once oxidized, collagen network impairs connective tissue, an event usually associated to aging and eventually supporting the results we observed by H&E staining.[33] On the other hand, we found tubulin, myosin and actin among the proteins oxidized by HTP, an outcome related to cytoskeleton rearrangement.[34] Interestingly, further functional approach aiming to provide the outcomes given by protein oxidation on their functionality would be the gold standard to dissect the impact of cigarette smoke and HTPs impact on corneal proteome. Moreover, we also assayed the impact of conventional cigarettes and HTP on an in vitro model of rabbit epithelial corneal cells. The detection of wound closure after the exposition unveiled a similar trend for human and rabbit corneal epithelial cells exposed to air or HTP, whereas 1R6F-exposed cells were impaired in their ability of closing the wound. The impairment in wound healing might be relatable to the significant amount of ROS triggered by 1R6F exposition, as corroborated by the in vivo data obtained on a model zebrafish (*Danio Rerio*) larva.[35] Therefore the stress induced by cigarettes might also impair cell proliferation and their ability in closing the wound.

Our data, based on two different experimental models, demonstrated using standard and clinically relevant conditions that smoking has to be accounted as a risk factor for epithelial cornea related diseases. Smoke might indeed trigger chronic inflammation, delaying wound healing and exacerbating dry eye syndrome by reducing tear production and damaging the ocular surface. Furthermore, chemicals in tobacco smoke can harm the corneal epithelium and impair its function.[36] Corroborating this, we show here that conventional cigarettes enhance oxidative stress, in turn oxidizing several proteins downstream. As a result, the epithelium undergoes a significative damage, showing also a marked grade of inflammation. In this context, even if HTP might represent a valuable alternative to conventional cigarettes, both the smoking approaches are oxidative stress inducers that an in-deep proteomic investigation of PTMs clearly revealed. This is the reason why quitting smoke remains the most reliable strategy to avoid smoke-related complications.

Acknowledgments

This work has been conducted thanks to tools and high-end equipment property of and kindly provided free of charge by ECLAT Srl, a spin-off of the University of Catania that delivers solutions to global health problems with particular emphasis on harm minimization and technological innovation. This work was funded by the researchers supporting project funds (No. RSP2024R261), King Saud University, Riyadh, Saudi Arabia. The authors gratefully acknowledge the Bio-nanotech Research and Innovation Tower (BRIT) financed by Italian Ministry of Education e and Research (MIUR).

Author Statement: RP is professor of Medicine and Director of the Institute for Internal Medicine at Catania University. He has received the following EU and governmental competitive grants: U-BIOPRED, AIR-PROM, Integral Rheumatology & Immunology Specialists Network (IRIS), and Ministero dell Universita' e della Ricerca (MUR) PNRR 3277/2021, PNRR 341/2022, and PNRR 411/2021 funded by NextGenerationEU of the European Commission. He has also received investigator initiated grants from Foundation for a Smoke Free World, Pfizer, GlaxoSmithKline, CV Therapeutics, NeuroSearch A/S, Sandoz, Merk Sharp & Dohme, Boehringer Ingelheim, Novartis, Arbi Group Srl., Duska Therapeutics, and Forest Laboratories. He is founder of the Center for Tobacco Prevention and Treatment and of the Center of Excellence for the Acceleration of Harm Reduction at Catania University. He has been consulting for Pfizer, Boehringer Ingelheim, Duska Therapeutics, Forest Laboratories, CV Therapeutics, Sermo Inc., GRG Health, Clarivate Analytics, Guidepoint Expert Network, and GLG Group. He receives textbooks royalties from Elsevier and EDRA. He is also Chair of the European Technical Committee for Standardization on "Requirements and test methods for emissions of electronic cigarettes" (CEN/TC 437; WG4) and scientific advisor at RIDE2Med Foundation.

Disclosure: **S. Giallongo**, None; **F. Bellia**, None; **A. Russo**, None; **M. Fallico**, None; **R. Polosa**, None; **N. Castellino**, None; **A. Longo**, None; **R. Emma**, None; **K. Partsinevelos**, None; **M. Caruso**, None; **A.S. Kartasasmita**, None; **G. Sferrazzo**,

None; **I.A. Barbagallo**, None; **R. Caltabiano**, None; **G. Broggi**, None; **A.M. Alanazi**, None; **G. Li Volti**, None

References

1. Emma R, Caruso M, Campagna D, Pulvirenti R, Li Volti G. The impact of tobacco cigarettes, vaping products and tobacco heating products on oxidative stress. *Antioxidants*. 2022;11:1829.
2. Rashidi M, Mohammadpoorasl A, Sahebihagh MH. Environmental tobacco smoke and educational self-regulation and achievement in first grade high school students. *J Med Life*. 2020;13:229–234.
3. Cometto-Muniz JE, Cain WS. Sensory irritation. Relation to indoor air pollution. *Ann N Y Acad Sci*. 1992;641:137–151.
4. Perez-de-Arcelus M, Toledo E, Martinez-Gonzalez MA, Martin-Calvo N, Fernandez-Montero A, Moreno-Montanes J. Smoking and incidence of glaucoma: The SUN Cohort. *Medicine*. 2017;96:e5761.
5. Velilla S, Garcia-Medina JJ, Garcia-Layana A, et al. Smoking and age-related macular degeneration: review and update. *J Ophthalmol*. 2013;2013:895147.
6. Tariq MA, Amin H, Ahmed B, Ali U, Mohiuddin A. Association of dry eye disease with smoking: a systematic review and meta-analysis. *Indian J Ophthalmol*. 2022;70:1892–1904.
7. Raju P, George R, Ve Ramesh S, Arvind H, Baskaran M, Vijaya L. Influence of tobacco use on cataract development. *Br J Ophthalmol*. 2006;90:1374–1377.
8. Chung SM, Gay CA, McCrary JA, 3rd. Nonarteritic ischemic optic neuropathy. The impact of tobacco use. *Ophthalmology*. 1994;101:779–782.
9. Toll BA, Rojewski AM, Duncan LR, et al. "Quitting smoking will benefit your health": the evolution of clinician messaging to encourage tobacco cessation. *Clin Cancer Res*. 2014;20:301–309.
10. Upadhyay S, Rahman M, Johanson G, Palmberg L, Ganguly K. Heated tobacco products: insights into composition and toxicity. *Toxics*. 2023;11:667.
11. Polosa R, Morjaria JB, Prosperini U, et al. Health outcomes in COPD smokers using heated tobacco products: a 3-year follow-up. *Intern Emerg Med*. 2021;16:687–696.
12. Aranyosi JK, Galgoczi E, Erdei A, et al. Different effects of cigarette smoke, heated tobacco product and e-cigarette vapour on orbital fibroblasts in Graves' orbitopathy; a study by real time cell electronic sensing. *Molecules*. 2022;27:3001.
13. Makrynioti D, Zagoriti Z, Koutsojannis C, Morgan PB, Lagoumintzis G. Ocular conditions and dry eye due to traditional and new forms of smoking: A review. *Cont Lens Anterior Eye*. 2020;43:277–284.
14. Caruso M, Emma R, Distefano A, et al. Electronic nicotine delivery systems exhibit reduced bronchial epithelial cells toxicity compared to cigarette: the Replica Project. *Sci Rep*. 2021;11:24182.
15. Azzopardi D, Haswell LE, Foss-Smith G, et al. Evaluation of an air-liquid interface cell culture model for studies on the inflammatory and cytotoxic responses to tobacco smoke aerosols. *Toxicol In Vitro*. 2015;29:1720–1728.
16. Broggi G, Verzi AE, Caltabiano R, Micali G, Lacarrubba F. Correlation between in vivo reflectance confocal microscopy and horizontal histopathology in skin cancer: a review. *Front Oncol*. 2021;11:653140.
17. Broggi G, Salvatorelli L, Reibaldi M, et al. Solitary fibrous tumor of the orbital region: report of a case with emphasis on the diagnostic utility of STAT-6. *Pathologica*. 2020;112:195–199.
18. Longhitano L, Giallongo S, Orlando L, et al. Lactate rewrites the metabolic reprogramming of uveal melanoma cells and induces quiescence phenotype. *Int J Mol Sci*. 2022;24:24.
19. Yamaguchi NH. Smoking, immunity, and DNA damage. *Transl Lung Cancer Res*. 2019;8:S3–S6.
20. Caliri AW, Tommasi S, Besaratinia A. Relationships among smoking, oxidative stress, inflammation, macromolecular damage, and cancer. *Mutat Res Rev Mutat Res*. 2021;787:108365.
21. Lee J, Taneja V, Vassallo R. Cigarette smoking and inflammation: cellular and molecular mechanisms. *J Dent Res*. 2012;91:142–149.
22. Kulkarni A, Banait S. Through the smoke: an in-depth review on cigarette smoking and its impact on ocular health. *Cureus*. 2023;15:e47779.
23. Soleimani F, Dobaradaran S, De-la-Torre GE, Schmidt TC, Saeedi R. Content of toxic components of cigarette, cigarette smoke vs cigarette butts: a comprehensive systematic review. *Sci Total Environ*. 2022;813:152667.
24. Checa M, Hagood JS, Velazquez-Cruz R, et al. Cigarette smoke enhances the expression of profibrotic molecules in alveolar epithelial cells. *PLoS One*. 2016;11:e0150383.
25. McCusker K. Mechanisms of respiratory tissue injury from cigarette smoking. *Am J Med*. 1992;93:18S–21S.
26. Isik B, Ceylan A, Isik R. Oxidative stress in smokers and non-smokers. *Inhal Toxicol*. 2007;19:767–769.
27. Aldaham S, Foote JA, Chow HH, Hakim IA. Smoking status effect on inflammatory markers in a randomized trial of current and former heavy smokers. *Int J Inflam*. 2015;2015:439396.
28. Churg A, Zhou S, Wang X, Wang R, Wright JL. The role of interleukin-1beta in murine cigarette smoke-induced emphysema and small airway remodeling. *Am J Respir Cell Mol Biol*. 2009;40:482–490.
29. Baglole CJ, Sime PJ, Phipps RP. Cigarette smoke-induced expression of heme oxygenase-1 in human lung fibroblasts is regulated by intracellular glutathione. *Am J Physiol Lung Cell Mol Physiol*. 2008;295:L624–636.
30. Martey CA, Pollock SJ, Turner CK, et al. Cigarette smoke induces cyclooxygenase-2 and microsomal prostaglandin E2 synthase in human lung fibroblasts: implications for lung inflammation and cancer. *Am J Physiol Lung Cell Mol Physiol*. 2004;287:L981–991.
31. Singh RR, Mohammad J, Orr M, Reindl KM. Glutathione S-transferase pi-1 knockdown reduces pancreatic ductal adenocarcinoma growth by activating oxidative stress response pathways. *Cancers*. 2020;12:1501.
32. Dutta T, Sangwan J, Mondal M, et al. Prolonged inflammation and infectious changes in the corneal epithelium are associated with persistent epithelial defect (PED). *Pathogens*. 2023;12:261.
33. Henrotin Y, Deberg M, Mathy-Hartert M, Deby-Dupont G. Biochemical biomarkers of oxidative collagen damage. *Adv Clin Chem*. 2009;49:31–55.
34. Farah ME, Sirotkin V, Haarer B, Kakhniashvili D, Amberg DC. Diverse protective roles of the actin cytoskeleton during oxidative stress. *Cytoskeleton*. 2011;68:340–354.
35. Alvarez M, Chavez MN, Miranda M, Aedo G, Allende ML, Egana JT. A novel in vivo model to study impaired tissue regeneration mediated by cigarette smoke. *Sci Rep*. 2018;8:10926.
36. Xiao C, Wu M, Liu J, et al. Acute tobacco smoke exposure exacerbates the inflammatory response to corneal wounds in mice via the sympathetic nervous system. *Commun Biol*. 2019;2:33.