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Special Issue Reprint

Cigarette Smoke and Oxidative Stress

Edited by
Lorenzo Loffredo

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Guest Editor

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About the Editor

Lorenzo Loffredo

Lorenzo Loffredo is a Full Professor of Internal Medicine at Sapienza University of Rome. His research focuses on cardiovascular prevention and oxidative stress, particularly the mechanisms through which oxidative processes and inflammation contribute to vascular injury and cardiovascular disease.

His work aims to improve the understanding of cardiovascular risk and inform strategies for disease prevention. Additionally, he is interested in the systemic effects of smoking and their implications for human health.

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Editorial

Beyond Combustion: Oxidative Stress as a Shared Pathway of Harm Across Conventional Cigarettes, Heated Tobacco Products, E-Cigarettes, and Waterpipe Smoking

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1. Introduction

Tobacco smoking is an established cardiovascular risk factor and is closely associated with the development and progression of atherosclerosis, as well as complications such as myocardial infarction and stroke [1–3]. In recent years, there has been a rapid spread of alternatives to conventional cigarettes—including heated tobacco products (HTPs), e-cigarettes, and water pipes—especially among adolescents and young adults [4–7]. Such a spread has been favored, at least in part, by the perception that the absence of combustion or the filtration of smoke through the water substantially reduces the health risk compared with conventional cigarettes [8,9].

The available evidence indicates, however, that these products are not free of harmful effects [10,11]. Nicotine, reactive aldehydes, ultrafine particles, volatile organic compounds, and metals present in their aerosols can impair endothelial function, promote inflammation, and contribute to cardiovascular, breathing and systemic damage, even in the absence of combustion [9,10,12]. Consequently, several scientific societies have reiterated that, as well as traditional cigarettes, e-cigarettes, HTPs, and waterpipes should also not be considered safe products and that complete cessation of tobacco and nicotine exposure remains the priority goal, prioritizing counseling and validated drug therapies over their use as harm reduction tools [8,12–16].

Oxidative stress appears to be one of the main mechanisms shared by these different smoking products. Exogenous oxidants and the activation of cellular sources of reactive oxygen species (ROS)—including NADPH oxidases and mitochondria—can reduce antioxidant defenses, impair nitric oxide bioavailability, and trigger proinflammatory responses [10]. Persistence of this redox alteration can result in cellular dysfunction and multiorgan damage [10,17,18]. However, the mechanisms through which different types of exposure induce and maintain oxidative stress and systemic inflammation are not yet fully defined. This Special Issue and this Editorial aim to critically analyze the evidence relating to the effects of conventional cigarettes and new tobacco and nicotine products on redox balance, delving into their potential links with persistent inflammation and consequent organ damage.

2. Overview of Published Articles

2.1. Effect of Smoking and Increase in Oxidized Stress Markers

2.1.1. Conventional Cigarettes

The damage caused by conventional cigarettes well established [10,11]. As reported in several previous studies, and in the one reported in this Special Issue, the conventional

cigarette is associated with a greater increase in oxidative stress markers such as NOX2, isoprostanes, and H_2O_2 and a reduction in antioxidant status highlighted by the reduction in NO [10,19,20] [Contribution 1]. This increase occurs in subjects exposed to both second-hand smoke and active smoke. A novelty reported in this Special Issue is that conventional cigarette smoking can cause an alteration of the intestinal microbiota, favoring the passage of lipopolysaccharide (LPS)—a membrane component of Gram-negative bacteria—into the systemic circulation, with subsequent binding to Toll-like receptor 4 (TLR4) and activation of oxidative stress through NADPH oxidase type 2 (NOX2) (Figure 1) [Contribution 1]. This increased intestinal permeability could perpetuate and contribute to the increase in oxidative stress seen in smokers (Figure 1) [Contribution 1]. Of particular interest is the damage found in children exposed to conventional tobacco cigarettes [19] [Contribution 1].

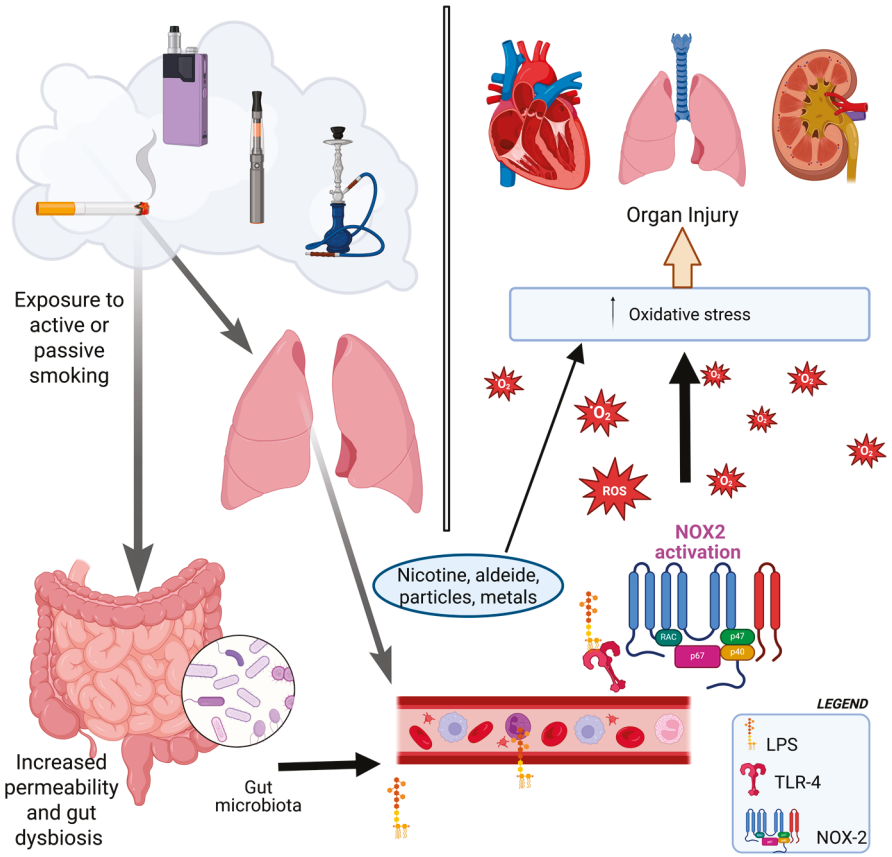

Figure 1. Proposed mechanisms linking exposure to conventional, heated tobacco and electronic smoking products to systemic oxidative stress and organ injury. Active or passive exposure to cigarette smoke, electronic cigarettes, heated tobacco products, and waterpipe tobacco smoke introduces nicotine, reactive aldehydes, particulate matter, and metals into the respiratory tract. These constituents may directly increase reactive oxygen species (ROS) formation and promote NOX activation. Smoking exposure may also increase intestinal permeability and induce gut dysbiosis, facilitating the translocation of bacterial lipopolysaccharide (LPS) into the circulation [Contribution 1]. LPS binding to Toll-like receptor 4 (TLR4), together with smoke-related toxicants, activates intracellular signaling pathways that promote the assembly of the NOX2 complex through the recruitment of Rac and the cytosolic p47phox, p67phox, and p40phox subunits to the membrane-bound catalytic complex. Activated NOX2 generates superoxide, thereby amplifying systemic oxidative stress. The resulting redox imbalance and inflammatory response contribute to cardiovascular, pulmonary, and renal injury.

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2.1.2. HTPs

Like conventional tobacco cigarettes, HTPs also cause increased oxidative stress and a reduction in antioxidant status [19,21] [Contribution 1]. Of pivotal importance is the lack of significance in blood concentrations of LPS and zonulin between subjects actively and passively exposed to traditional cigarette smoke and those exposed to HTPs, which highlights the detrimental effect of both of these products on the systemic concentration of oxidative stress and NOX2 activation [Contribution 1].

Another interesting in vitro experimental study, conducted on human bronchial epithelial cells exposed for 24 h to emissions from three different HTPs, reports that such emissions can induce mitochondrial oxidative stress [Contribution 2]. Although HNBC resulted in less extensive overall alterations in gene expression than the conventional cigarette, all examined products significantly increased the production of ROS at the mitochondrial level [Contribution 2]. This is in line with the review by Morosini [Contribution 3] and with other previous studies on the damage caused by these products in terms of increased inflammatory and oxidative status [Contribution 3] [22,23].

2.1.3. Electronic Cigarettes

Compared to other devices with more or less homogeneous characteristics, one of the problems with e-cigarettes is their variability among devices in generating aerosol; there are also numerous types (approximately 8000) of liquid flavors available [11].

Despite these variabilities, e-cigarettes produce volatile harmful products such as formaldehyde, acrolein and fine particles [11] and are also not free from pro-oxidant and inflammatory effects [24–26]. Thus, e-cigarettes can activate NOX2 and increase oxidative stress, as assessed by the higher levels of isoprostanes with reduced bioavailability of NO and blood levels of Vitamin E [26,27]. Sater's review shows that e-cigarette aerosols contain numerous potentially pro-oxidant components and that, in humans, vaping has been associated with alterations in antioxidant defenses and early indicators of vascular damage [Contribution 4].

The main alterations observed concern the following:

- The reduction in superoxide dismutase and catalase;
- The alteration of the ratio between reduced and oxidized glutathione;
- The increased activity of NOX-2, a ROS-producing enzyme;
- The increase in malondialdehyde, an indicator of lipid peroxidation;
- The increase in 8-isoprostanes;
- Increased 8-OHdG, an indicator of oxidative DNA damage.

Nicotine appears to play a significant role in the effects of the damage, but the compounds contained in e-cigarette aerosols could also contribute to it.

2.1.4. Waterpipes

In recent years, an increase in the use of waterpipes with tobacco (WTS) has been observed worldwide, especially among young people, both of Arabic origin and belonging to other populations [16,28,29]. The perception, unsupported by scientific evidence, that WTS is less harmful than conventional cigarette smoke—because the smoke is filtered through water—has contributed to the spread of this practice. However, this belief is not supported by studies, which instead highlight the danger of this practice. In fact, WTS sessions last approximately 45 min, with inhalation of several liters of waterpipe smoke, corresponding to approximately 10 times the absorption of CO and particulate matter, 27 times the formaldehyde, and 80 times the lead compared to a single conventional cigarette [28]. The growing popularity of tWTS among younger age groups led the World

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Health Organization to define it, in its 2015 advisory note, as an emerging public health problem [28].

In the study by Shaukat et al., published in this Special Issue, the concentration of 8-oxoguanine (8-oxoGua) in exhaled breath condensate (EBC) was analyzed before and after a WTS session [Contribution 5]. 8-oxoGua, a product of DNA guanine oxidation, represents a biomarker of oxidative DNA damage mediated by ROS. After exposure to waterpipe smoke, a significant increase in 8-oxoGua levels was found, indicative of an acute increase in oxidative stress and subsequent oxidative DNA damage [Contribution 5].

2.2. Effect of Smoking on Oxidative Stress and Organ Damage

2.2.1. Cardiovascular System

The harmful cardiovascular effects of conventional cigarettes have been well established; numerous meta-analyses have demonstrated a close relationship with coronary heart disease, stroke, and sudden death, making tobacco smoking a classic cardiovascular risk factor [30,31].

Although some studies report that HTPs release lower amounts of several toxic substances than conventional cigarettes, the reduction in exposure does not equate to an absence of health risk [10,11]. The available evidence indicates that their emissions continue to promote oxidative stress, inflammation, endothelial dysfunction and other cellular alterations associated with the development of cardiovascular diseases [19,21] [Contribution 1]. HTPs should therefore not be described as safe products; any harm reduction concerns only comparison with the continuation of conventional cigarette smoking and not with complete abstinence from tobacco products [Contribution 3].

A further element of concern relates to passive exposure. Higher concentrations of cotinine, circulating LPS, and zonulin were found in children passively exposed to HTP emissions or conventional cigarette smoke compared to unexposed controls, with no significant differences between the two forms of exposure. Elevated zonulin may suggest increased intestinal permeability, which may promote the translocation of bacterial LPS into the bloodstream and lead to low-grade endotoxemia [32]. LPS was also associated with markers of NOX2-mediated oxidative stress, reduced nitric oxide bioavailability, and increased platelet activation. These alterations could contribute to endothelial dysfunction and, in the long term, to an increased cardiovascular risk. The study did not, however, directly analyze the intestinal microbiota; therefore, it does not allow us to state that secondhand smoke causes dysbiosis, but identifies an impairment of the intestinal barrier compatible with this hypothesis [Contribution 1].

Another possible mechanism of cardiovascular damage is described in the review by Sabath de Oliveira Bernardes et al. [Contribution 6]. Analysis of clinical and experimental studies shows that exposure to conventional cigarettes and e-cigarettes is associated with increased oxidative stress and impaired cardiac autonomic control [Contribution 6]. The latter is highlighted by the reduction in heart rate variability (HRV), the decrease in parasympathetic modulation and the shift in autonomic balance towards a sympathetic predominance [Contribution 6]. Oxidative stress, inflammation, and sympathetic hyperactivation may promote vascular dysfunction, hypertension, arrhythmias, and other cardiovascular events [Contribution 6]. Although the mechanism is biologically plausible, large studies simultaneously measuring oxidative stress and HRV in smokers are lacking; its prognostic relevance will therefore need to be confirmed by larger longitudinal studies [Contribution 6].

E-cigarettes are also associated with cardiovascular complications [11]. Previous studies showed endothelial dysfunction and reduced coronary reserve after their use [26,33].

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Waterpipe smoking has also been associated with cardiovascular changes such as increased heart rate and blood pressure [34]. Additionally, increased coronary artery disease was reported in heavy waterpipe smokers (more than 40/50 sessions per year) compared to less frequent smokers [35].

2.2.2. Renal System

The review by Dzięgiel et al. describes a biologically plausible mechanism supported by cellular, animal, and observational studies through which smoking—and nicotine in particular—can contribute to kidney damage [Contribution 7]. Nicotine, by binding to nicotinic receptors present in podocytes and other renal cells, may promote the activation of NADPH oxidase and the consequent production of reactive oxygen species (ROS) [Contribution 7]. Increased oxidative stress may compromise the integrity of podocytes and the glomerular filtration barrier, promoting proteinuria, inflammation, cell apoptosis, and extracellular matrix deposition, leading to progression of renal fibrosis [Contribution 7]. Clinical evidence therefore suggests that, in patients with glomerulopathy or chronic kidney disease, smoking represents a modifiable risk factor potentially associated with a more rapid decline in renal function [Contribution 7]. However, because much of the mechanistic evidence comes from preclinical models, and clinical studies are predominantly observational, larger longitudinal studies are needed to more precisely define the causal relationship and quantify the renal benefits of smoking cessation [Contribution 7].

Since smoking products such as conventional, electronic and heated tobacco cigarettes and waterpipes contain nicotine, it cannot be ruled out that nicotine-mediated mechanisms could determine potential harmful effect on the kidneys [36,37]. However, differences in nicotine delivery, patterns of use, and exposure to other toxicants preclude the assumption of an equivalent degree of renal risk across different tobacco and nicotine products.

2.2.3. Respiratory System

The systematic review by Cinicola et al. evaluated the relationship between passive smoking exposure, oxidative stress, and pediatric respiratory diseases. The authors describe the harmful effects that secondhand smoke causes in children, contributing to increased oxidative stress and persistent inflammation that causes persistent allergic rhinitis, greater wheezing severity, poorer asthma control and reduced responsiveness to corticosteroids [Contribution 8].

In parallel, a higher proportion of active smokers among adolescents has been reported with e-cigarette or vaping-associated lung injury (EVALI) [38,39].

Children and adolescents are increasingly actively and passively exposed to smoke from new devices [10]. It is therefore of fundamental importance to inform parents about the potential harm resulting from secondhand smoke and to promote smoking cessation campaigns among adolescents which lead to a restoration of the early respiratory damage caused by exposure to smoke [40].

3. Conclusions

All currently available types of smoking are characterized by increased oxidative stress and systemic inflammation. E-cigarettes, HTPs, and waterpipes are often identified as less harmful products, a perception that may contribute to their increasing use. However, reduced exposure to certain toxicants should not be equated with an absence of health risks, and current evidence does not support the consideration of these products as harmless.

Therefore, it is of fundamental importance to promote preventive campaigns that highlight the possible damage mediated by oxidative stress at the respiratory, cardiovascu-

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lar and renal levels. Larger prospective studies will be needed to evaluate the long-term systemic effects of these products.

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Tobacco Smoke Exposure and Oxidative Stress: The Role of Circulating Lipopolysaccharides in Heated and Conventional Products

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Abstract: Background: Exposure to tobacco smoke, from conventional tobacco cigarettes (CTC) or heated tobacco products (HTPs), increases oxidative stress, causing endothelial dysfunction and higher cardiovascular risk. It is unclear whether smoke exposure also promotes low-grade endotoxemia, potentially activating NADPH oxidase and further impairing endothelial function. This study assessed serum lipopolysaccharide (LPS) levels in children and adults actively or passively exposed to conventional cigarette smoke or HTPs, compared with non-exposed controls. **Methods:** We conducted a cross-sectional study comprising 26 children passively exposed to HTPs, 26 children exposed to CTC, and 26 unexposed controls, as well as 20 adult chronic HTP users, 20 chronic CTC, and 20 non-smoking adults. Circulating LPS was measured alongside oxidative stress markers (NOX2, H_2O_2), endothelial function, intestinal permeability (zonulin), and nicotine exposure (serum cotinine). **Results:** Exposed children had higher cotinine, LPS, and zonulin than controls, with no differences between HTP and CTC groups. Multiple linear regression analysis identified cotinine ($\beta = 0.343$; $p = 0.005$) and zonulin ($\beta = 0.441$; $p < 0.001$) as independent LPS predictors. In adults, LPS and zonulin were higher in both smoker groups versus controls; zonulin ($\beta = 0.477$; $p < 0.001$) and nitric oxide bioavailability ($\beta = -0.307$; $p = 0.007$) independently predicted LPS. **Conclusions:** Passive and active exposure to CTC or HTPs increases low-grade endotoxemia and zonulin, potentially driving NOX2-mediated oxidative stress.

Keywords: smoking; heated tobacco products; cigarettes; LPS; lipopolysaccharides; endothelial dysfunction; flow-mediated dilation; NOX-2; NADPH-oxidase 2; atherosclerosis

1. Introduction

Tobacco consumption remains one of the leading preventable causes of morbidity and mortality worldwide, primarily through its contribution to cardiovascular complications and cancer [1,2]. Traditional combustible cigarettes release thousands of toxic compounds, including reactive aldehydes, heavy metals, and particulate matter, which contribute to systemic inflammation and the development of chronic diseases [1]. In recent years, heated tobacco products (HTPs) have been marketed as reduced-risk alternatives to conventional smoking, based on the rationale that aerosol generation at lower temperatures may decrease exposure to harmful and potentially harmful constituents [1,3]. However, accumulating evidence suggests that both cigarette smoke and HTPs aerosols may still exert detrimental effects on human health [1,4].

A key mechanism linking tobacco exposure to systemic disease is low-grade endotoxemia, characterized by chronically elevated plasma levels of lipopolysaccharide (LPS), a structural component of Gram-negative bacteria [5]. Smoking-induced gut dysbiosis and impaired intestinal barrier integrity facilitate LPS translocation into the circulation [6], which is further aggravated by the direct presence of endotoxins adsorbed onto smoke particles [7]. Even low-grade increases in circulating LPS can activate Toll-like receptor 4 (TLR4) signaling, initiating immune responses that amplify systemic inflammation [5].

Among the downstream mediators, NADPH oxidase 2 (NOX2) plays a pivotal role in promoting systemic oxidative stress [8,9]. LPS-TLR4 engagement activates NOX2 in innate immune cells, leading to enhanced production of reactive oxygen species (ROS) [5]. Excessive ROS contributes to oxidative stress, endothelial dysfunction, and vascular injury, thereby creating a mechanistic link between low-grade endotoxemia and the cardiovascular complications observed in smokers [5,10]. Increased circulating levels of NOX2-derived markers have been described not only in active smokers but also in individuals passively exposed to second- and third-hand smoke [11–13].

Children exposed to secondhand smoke represent a particularly vulnerable population. Passive exposure during critical windows of immune and metabolic development may predispose to long-term inflammatory phenotypes and NOX2-driven oxidative damage, thereby amplifying the burden of chronic disease in adulthood [12,13]. Meanwhile, chronic smokers, whether using conventional cigarettes or HTPs, may exhibit distinct profiles of endotoxemia, NOX2 activation [11], and endothelial dysfunction, although comparative human data remain scarce.

In this context, studying the impact of traditional and heated tobacco smoking on low-grade endotoxemia, with a focus on LPS-induced NOX2 activation, in both passive pediatric exposure and chronic smokers is crucial.

The aim of this study was to evaluate serum LPS levels in children and adults who were actively or passively exposed to conventional cigarette smoke or HTPs, compared with non-exposed controls, and to investigate the correlation between LPS levels, oxidative stress, and endothelial function.

2. Materials and Methods

The populations of children exposed to passive smoking by HTPs and traditional tobacco and chronic smokers of HTPs or traditional tobacco were taken from 2 studies previously published by the same authors [11,12].

2.1. Aim of the Study

This study was designed as an observational cross-sectional investigation.

The primary aim was to assess circulating LPS levels in first-hand smokers (active smokers) and second-hand smokers (individuals exposed to passive smoke) of heated tobacco products (IQOS, Philip Morris, New York, NY, USA) and traditional combustible tobacco.

A secondary objective was to investigate the relationship between circulating LPS and several biomarkers, including soluble NOX2-derived peptide (sNOX2-dp), serum isoprostanes, hydrogen peroxide (H_2O_2) concentrations, and hydrogen peroxide breakdown activity (HBA, a measure of antioxidant capacity). Associations were also explored with markers of endothelial dysfunction—namely nitric oxide (NO) bioavailability and flow-mediated dilation (FMD)—as well as with platelet activation, assessed by serum levels of soluble CD40 ligand (sCD40L) and soluble P-selectin (sP-selectin).

Two populations, including both children and adults, were selected to evaluate the effects of active and passive exposure to traditional tobacco cigarettes or HTPs on endotoxemia levels, and to correlate these with markers of oxidative stress, endothelial dysfunction, and platelet activation.

2.2. Study Population: Passive Smoke Exposure and Controls

To investigate the effects of passive smoke exposure, 78 children were recruited: 26 exposed to passive smoke from HTPs (IQOS, Philip Morris, New York, NY, USA), 26 exposed to traditional combustible tobacco smoke, and 26 non-exposed children serving as controls.

Specifically, the eligibility criteria included children and adolescents aged 2 to 18 years, in apparent good health (defined as the absence of known chronic or acute diseases), who had been exposed or not exposed to passive smoking for at least six months.

Exclusion criteria were: obesity or severe underweight, chronic inflammatory disease, diabetes mellitus, dyslipidemia, active or past smoking, cardiopulmonary disease, severe nephropathy, liver disease, neuromuscular disorders, or vitamin supplementation. Children with one parent smoking traditional tobacco and the other using HTPs were also excluded.

Written informed consent was obtained from parents in accordance with Italian regulations. The study protocol was approved by the Ethics Committee of Sapienza University of Rome (ref. no. 4929; protocol no 173/18, date of approval 22 February 2018) and conducted in accordance with the Declaration of Helsinki.

2.3. Study Population: Chronic Active Smokers and Controls

To investigate the effects of chronic active smoking (first-hand exposure), 60 healthy adult participants were recruited and blood donors who provided written informed consent.

Of these, 20 had smoked HTPs for at least one month, 20 had smoked tobacco combustible cigarettes for at least one month, and 20 were non-smokers serving as controls. All HTP users were former conventional cigarette smokers who had. switched exclusively to HTPs. Their mean duration of HTP use was 1.5 ± 0.5 years. To be classified as chronic smokers, participants had to use only HTPs for more than one month [11].

Participants had not taken vitamin E, antioxidant supplements, or antiplatelet drugs during the month preceding the study. Female participants were not menstruating at the time of blood collection. The study was approved by the Ethics Committee of Sapienza University of Rome (ref. no. 3241; protocol no. 813/14, date of approval 26 June 2014) and conducted in compliance with the Declaration of Helsinki.

2.4. Sample Collection

Fasting blood samples were collected between 8:00–9:00 a.m. from the antecubital vein into Vacutainer tubes (with or without anticoagulants). Plasma and serum aliquots were separated and stored at −80 °C until analysis.

2.4.1. Biochemical and Functional Assessments

NO_2^-/NO_3^- (Serum Nitric Oxide Metabolites)

Serum nitric oxide (NO) concentration was determined using a colorimetric assay based on the quantification of nitrite/nitrate (NO_2^-/NO_3^-) (Arbor Assays, Ann Arbor, MI, USA). Nitrate (NO_3^-) in the sample was reduced to nitrite (NO_2^-) by nitrate reductase, after which the total nitrite was detected by reaction with Griess reagents, leading to the formation of a colored azo compound with absorbance measured at 540 nm. Results were expressed in μM. Intra- and inter-assay coefficients of variation (CVs) were both <10%.

2.4.2. 8-Isoprostane (8-Iso-$PGF_2\alpha$)

Serum concentrations of 8-iso-Prostaglandin F2α (8-iso-PGF2α) were measured using commercial competitive enzyme-linked immunosorbent assay (ELISA) kits (Abcam, Cambridge, UK; DRG International, Springfield, NJ, USA). Serum samples and standards were added to microplate wells pre-coated with anti–8-iso-PGF2α antibodies and incubated with an enzyme-conjugated tracer. After unbound components were removed by washing, a colorimetric substrate was applied. The enzymatic reaction generated a color change inversely proportional to the 8-iso-PGF2α concentration. Absorbance was read at 450 nm (Abcam) or 405 nm (DRG) using a microplate reader. Results were expressed in pg/mL. Intra- and inter-assay CVs were <10%.

2.4.3. Soluble NOX2-Derived Peptide (sNOX2-dp)

The soluble NOX2-derived peptide (sNOX2-dp) was measured by ELISA. This peptide, released upon platelet activation, is recognized by a monoclonal antibody directed against amino acids 224–268 of the extracellular portion of NOX2, the catalytic subunit of NADPH oxidase. Enzymatic activity was quantified spectrophotometrically by the increase in absorbance at 450 nm. Results were expressed in pg/mL, with intra- and inter-assay CVs of 8.95% and 9.01%, respectively.

2.4.4. Hydrogen Peroxide (H_2O_2)

Serum H_2O_2 concentration was determined using a colorimetric assay (Abcam, Cambridge, UK), according to the manufacturer's instructions [14]. The final product was read at 450 nm, and results were expressed in μM. Intra- and inter-assay CVs were <10%.

2.4.5. Hydrogen Peroxide Breakdown Activity (HBA)

Serum antioxidant activity was assessed using the HBA kit (Hydrogen Peroxide Breakdown Activity, Aurogene, code HPSA-50), which quantifies the ability of samples to degrade H_2O_2. Results were expressed as percentage HBA, calculated according to the kit formula.

2.4.6. Soluble P-Selectin

Plasma soluble P-selectin concentration was measured using a commercial ELISA kit (DRG International, Springfield, NJ, USA). Results were expressed in ng/mL. Intra- and inter-assay CVs were 4.3% and 6.1%, respectively.

2.4.7. Soluble CD40 Ligand (sCD40L)

Serum sCD40L levels were determined by solid-phase ELISA (DRG International, Springfield, NJ, USA). Results were expressed in ng/mL. Intra- and inter-assay CVs were 3.2% and 4.3%, respectively.

2.4.8. Cotinine

Serum cotinine was measured using a human cotinine ELISA kit (Sigma Aldrich, St. Louis, MO, USA), based on a solid-phase competitive immunoassay. Samples and cotinine–enzyme conjugate were incubated in wells coated with anti-cotinine antibodies. After substrate addition, color intensity was inversely proportional to sample cotinine concentration. Absorbance was read at 550 nm, and results were expressed in ng/mL. Intra- and inter-assay CVs were <10%.

2.4.9. Lipopolysaccharide (LPS)

Serum LPS concentrations were quantified using a commercial ELISA kit (Cusabio, Wuhan, China). Standards and samples were added to a microplate pre-coated with an anti-LPS antibody and incubated for 2 h at room temperature. Following incubation, absorbance was measured at 450 nm. Results were expressed as pg/mL, with both intra- and inter-assay coefficients of variation below 10%.

2.4.10. Zonulin

Serum zonulin levels were measured using a commercial ELISA kit (Elabscience, Houston, TX, USA). A zonulin-specific antibody was pre-coated onto a microplate; 100 µL of standards and patient serum samples were added and incubated for 90 min at 37 °C. A biotinylated zonulin-detection antibody and horseradish peroxidase (HRP)–avidin conjugate were then added. Results were expressed in ng/mL. Intra- and inter-assay CVs were <10%.

2.5. Flow-Mediated Dilation (FMD)

Ultrasound evaluation of baseline brachial artery diameter and endothelium-dependent dilation (FMD) was performed according to published guidelines [15–17] and previous reports [9]. In brief, assessments were performed between 8:00 a.m. and 10:00 a.m. with participants resting in the supine position in a temperature-controlled room (22.8 °C). A 7.5-MHz linear array transducer ultrasound system (HS30, Samsung, Seoul, Republic of Korea), equipped with electronic calipers, was used to measure the brachial artery 3–7 cm above the antecubital crease. Dedicated vascular software provided two-dimensional imaging as well as color and spectral Doppler. A sphygmomanometer cuff was placed on the forearm and inflated to 50 mmHg above systolic pressure for 5 min to occlude brachial artery inflow and induce a flow stimulus. Vasodilation was evaluated at end-diastole. A high-resolution ultrasound system equipped with a 7.5-MHz linear transducer (Samsung HS30, Samsung, Seoul, Republic of Korea) and electronic caliper was used for FMD measurements.

2.6. Statistical Analysis

- Passive smoke exposure group: Continuous variables were expressed as mean ± standard deviation; categorical variables as percentages. Normality was assessed with the Kolmogorov–Smirnov test. Between-group differences were analyzed using chi-square for categorical variables and ANOVA with Bonferroni correction for normally distributed variables. Bivariate correlations were evaluated by Spearman's test. Variables with $p < 0.10$ were included in multivariate linear regression (stepwise procedure). Variables with $p < 0.10$ were included in multivariate linear regression (stepwise procedure). Multivariate linear regression models, adjusted for age, sex, BMI, mean

arterial pressure and cotinine were used to compare non-smokers vs. conventional smokers, and non-smokers vs. HTP users. Statistical significance was set at $p < 0.05$. Analyses were performed using SPSS v25.0 (IBM, Armonk, NY, USA) and GraphPad Prism 7 (GraphPad Software, San Diego, CA, USA). Sample size was calculated based on a two-sided Student's t-test: a difference (δ) of 2.5% in FMD between exposed and unexposed children, standard deviation of 2.5%, $\alpha = 0.05$, and power $(1 - \beta) = 0.95$, yielding 26 patients per group.

- Active smokers group: Continuous variables were reported as median (IQR), categorical as counts (%). Group comparisons were performed using Kruskal–Wallis tests and Wilcoxon–Mann–Whitney tests. Correlations were analyzed by Spearman's test and visualized with clustered heatmaps. Multivariate linear regression models, adjusted for age, sex, BMI, mean arterial pressure, total cholesterol, and cotinine, were used to compare non-smokers vs. conventional smokers, and non-smokers vs. HTP users. Comparisons between conventional smokers and HTP users were further adjusted for smoking duration and cigarettes/day. A two-tailed $p < 0.05$ was considered statistically significant. Analyses were conducted with SPSS v18.0 (SPSS, Chicago, IL, USA), Stata 13 (StataCorp, College Station, TX, USA), and R 3.5.3 (R Foundation for Statistical Computing, Vienna, Austria).

3. Results

3.1. Passive Smoking

In the first part of the study, we analyzed children exposed to passive smoke from traditional tobacco cigarettes, heated tobacco products, and a control group. The clinical characteristics of the three groups are summarized in Table 1. As previously reported, there were no significant differences among the groups in terms of age, fasting blood glucose, systolic and diastolic blood pressure, or body mass index (BMI) (Table 1). As expected, children exposed to passive smoking had significantly higher serum cotinine levels compared to the control group (Table 1). No significant difference in serum cotinine levels was observed between the traditional tobacco (TT) and HTPs groups [12].

Table 1. Characteristics of the children and adult cohorts.

	Children		
Variable	**Controls (N = 26)**	**TT (N = 26)**	**HTPs (N = 26)**
Age	9 ± 3	9 ± 3	10 ± 3
Female Gender	19 (73%)	16 (62%)	17 (65%)
FMD (%)	7.93 ± 2.30	5.78 ± 2.92	5.51 ± 3.00
NO (μM)	60.69 ± 11.44	48.12 ± 11.15	49.92 ± 9.01
sNox2-dp (pg/mL)	17.65 ± 7.92	25.96 ± 5.26	24.87 ± 7.64
H_2O_2 (μM)	23.19 ± 5.41	32.35 ± 7.61	29.04 ± 6.13
sCD40L (ng/mL)	1.83 ± 0.48	2.78 ± 0.81	2.44 ± 0.84
sP-selectin (ng/mL)	5.10 ± 1.74	6.77 ± 1.92	6.33 ± 1.20
Platelet aggregation (%)	25.60 ± 3.78	39.60 ± 5.90	37.00 ± 7.18
Cotinine (ng/mL)	1.45 ± 1.25	36.59 ± 9.42	33.46 ± 6.58
LPS (pg/mL)	21.95 ± 3.01	25.89 ± 3.95	26.88 ± 4.31
Zonulin (ng/mL)	1.94 ± 0.77	2.55 ± 0.86	2.71 ± 0.90
8-iso-PGF2α (pg/mL)	142.50 ± 20.89	176.43 ± 43.75	178.5 ± 36.26
HBA (%)	53.37 ± 7.68	40.95 ± 6.37	45.11 ± 5.73

Table 1. *Cont.*

Adults			
Variable	**Controls (N = 20)**	**TT (N = 20)**	**HTPs (N = 20)**
Age	28 (23–33)	27 (24– 30)	33 (28–44)
Female Gender	11 (55%)	10 (50%)	12 (60%)
FMD (%)	7.1 (2.8–11.5)	1.6 (0–3.9)	3.3 (2.4–6.0)
NO (μM)	41 (38–49)	10 (9–13)	10 (8–13)
sNox2-dp (pg/mL)	19 (15–23)	46 (41–50)	40 (34–41)
H_2O_2 (μM)	8.8 (7.2–11.9)	33.5 (19.5–52.7)	26.7 (21.9–33.8)
sCD40L (ng/mL)	1.6 (1.1–2.1)	3.2 (2.5–4.4)	3.0 (2.5–3.3)
sP-selectin (ng/mL)	3.0 (2.0–3.9)	9.2 (6.7–12.0)	8.1 (5.5–9.2)
Platelet aggregation (%)	62 (58–70)	80 (77–80)	76 (70–80)
Cotinine (ng/mL)	2 (2–3)	139 (130–148)	137 (103–163)
LPS (pg/mL)	21.68 ± 4.82	26.62 ± 4.58	27.43 ± 4.31
Zonulin (ng/mL)	1.68 ± 0.38	2.28 ± 0.53	2.55 ± 0.92

Serum levels of LPS and zonulin were significantly elevated in children exposed to passive smoke from both traditional and heated tobacco sources (Figure 1). No statistically significant differences were observed between children exposed to heated tobacco product (HTP) emissions and those exposed to conventional cigarette smoke.

Simple linear regression analysis showed that serum LPS levels were positively correlated with zonulin (Rs = 0.580, $p < 0.001$), sCD40L (Rs = 0.224, $p = 0.04$), p-selectin (Rs = 0.286, $p = 0.01$) and cotinine (Rs = 0.550, $p < 0.001$), and negatively correlated with HBA (Rs = −0.325, $p < 0.001$).

Multivariable linear regression analysis identified cotinine (standardized coefficient β = 0.343; SE = 0.029; $p = 0.005$) and zonulin (standardized coefficient β = 0.441; SE = 0.452; $p < 0.001$) as the only independent predictors significantly associated with LPS levels ($R^2 = 0.20$) (Table 2).

Table 2. Multiple linear regression for the dependent variable LPS in children. (β = regression coefficient; SE = standard error).

	β	**SE**	***p***
Zonulin (ng/mL)	0.441	0.452	<0.001
sCD40L (ng/mL)	0.042	0.504	0.665
sP-Selectin (ng/mL)	0.017	0.262	0.861
HBA (%)	−0.027	0.054	0.798
Cotinin (ng/mL)	0.343	0.029	0.005

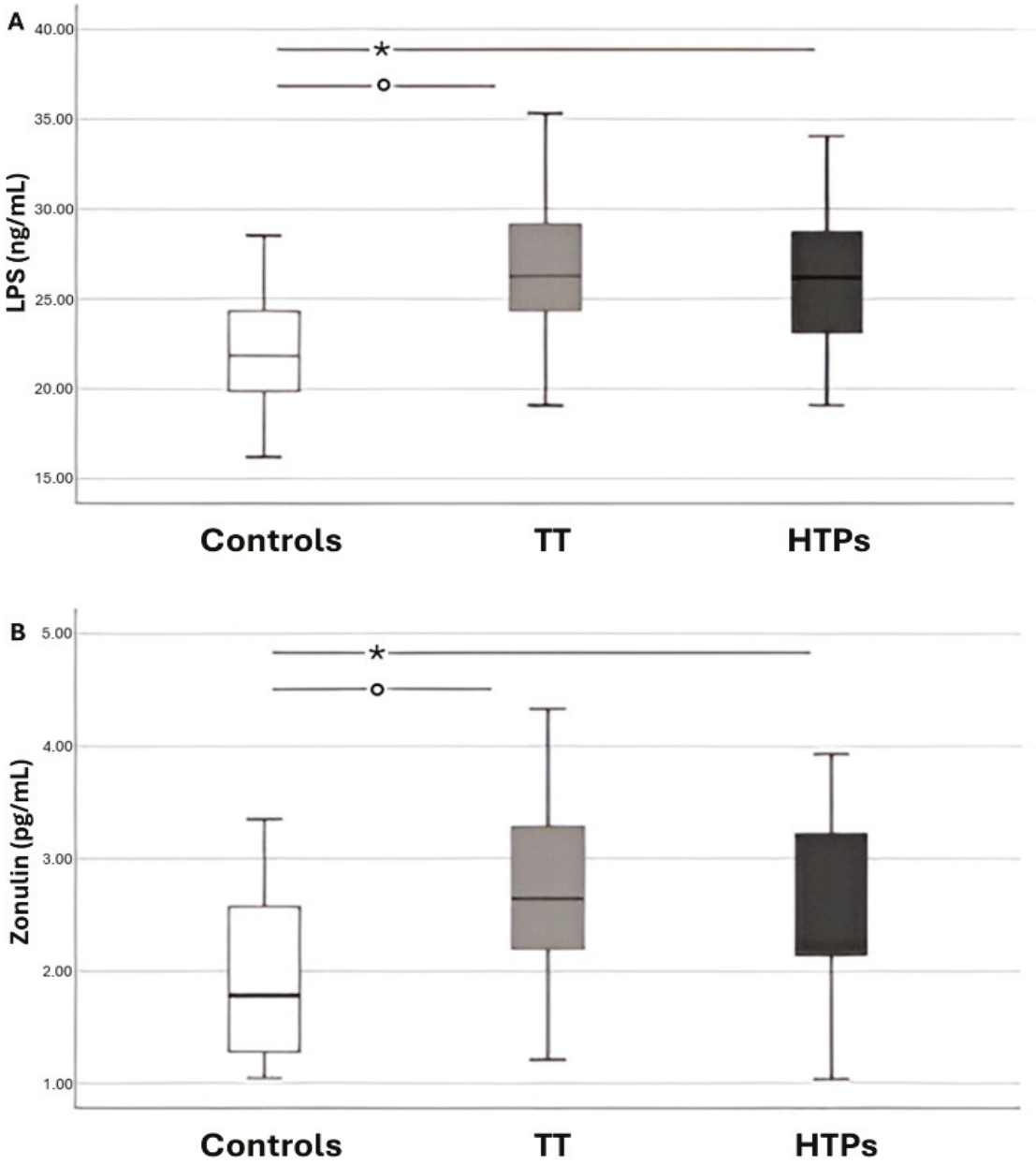

Figure 1. Box–and–whisker plots showing data from children divided into three groups: controls, those exposed to traditional tobacco cigarette smoke (TT), and those exposed to heated tobacco product emissions (HTPs). Panel **A** displays circulating lipopolysaccharide (LPS) levels, while Panel **B** shows circulating zonulin levels. The boxes represent the interquartile range (IQR; 25th–75th percentile), with the horizontal line indicating the median. Whiskers extend to values from the box to the minimum and maximum values. Panel (**A**): * $p < 0.001$; ° $p < 0.001$. Panel (**B**): * $p = 0.006$; ° $p = 0.003$.

3.2. Active Smokers

In the second part of the study, we investigated the effects of smoking on low-grade endotoxemia in a population of cigarette smokers, including users of heated tobacco products, conventional tobacco, and healthy controls [11].

The clinical characteristics of this population are reported in Table 1.

No statistically significant differences were observed between the two types of active smokers (traditional tobacco vs. heated tobacco) in terms of LPS and zonulin levels. However, significant differences were found when comparing both smoking groups to the control subjects (Figure 2).

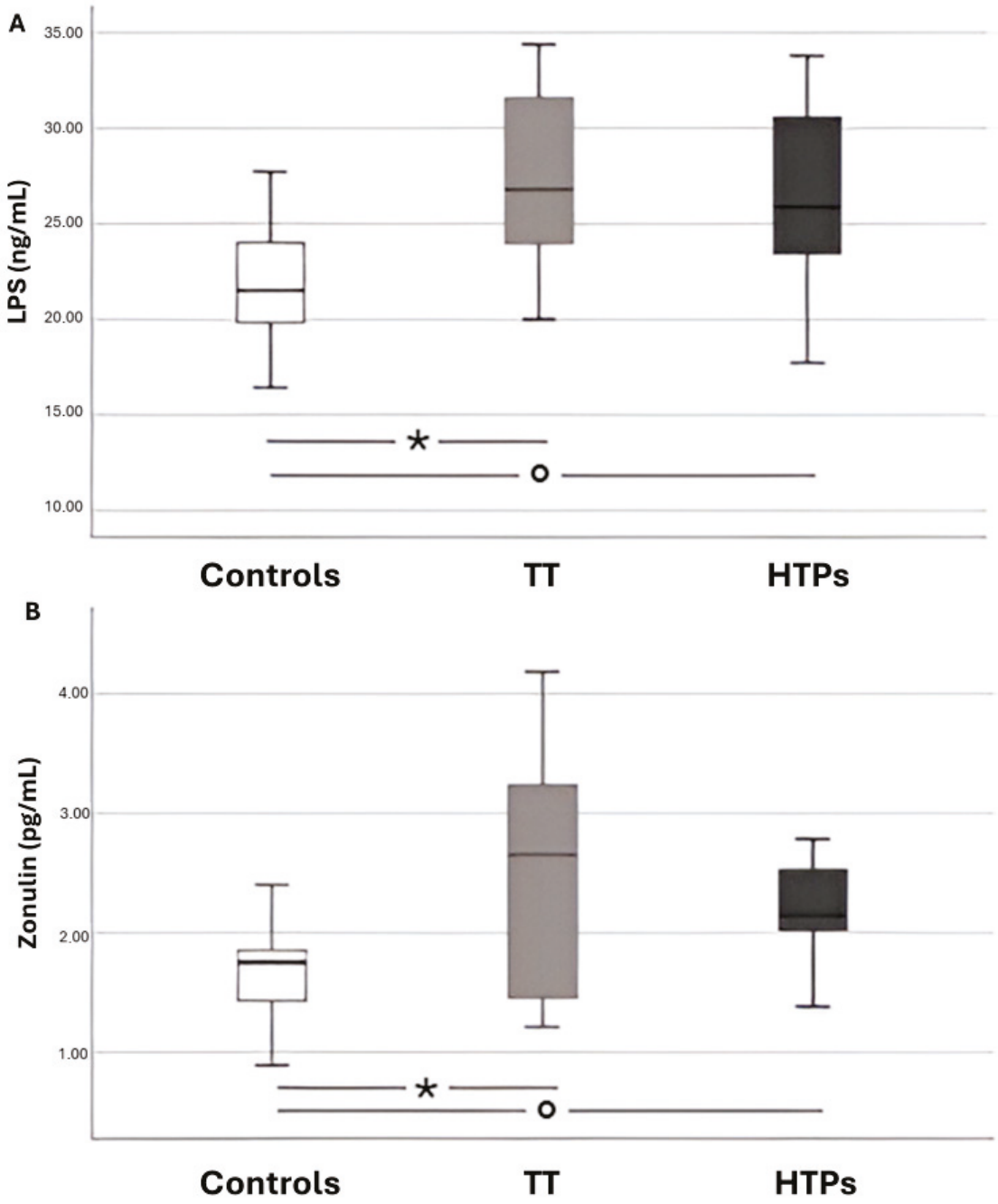

Figure 2. Box-and-whisker plots illustrating data from adults across three groups: healthy controls, chronic smokers of conventional tobacco cigarettes (TT), and users of heated tobacco products (HTPs). Panel **A** shows circulating levels of LPS, while Panel **B** reports circulating levels of zonulin. Panel (**A**): * $p < 0.001$; ° $p = 0.001$. Panel (**B**): * $p = 0.002$; ° $p < 0.001$.

A bivariate analysis revealed a linear correlation between LPS and the following variables: zonulin (Rs = 0.581, $p < 0.001$), H_2O_2 (Rs = 0.398, $p = 0.002$), NOX2 (Rs = 0.494, $p < 0.001$), NO (Rs = −0.520, $p < 0.001$), sCD40L (Rs = 0.286, $p = 0.02$), P-selectin (Rs = 0.437, $p < 0.001$), and cotinine (Rs = 0.485, $p < 0.001$).

A multiple linear regression analysis identified zonulin (standardized coefficient β = 0.477; SE = 0.751; $p < 0.001$) and nitric oxide (standardized coefficient β = −0.307; SE = 0.033; $p = 0.007$) as independently associated with serum LPS levels (Table 3).

Table 3. Multiple linear regression for the dependent variable LPS in adults. (β = regression coefficient; SE = standard error).

	β	SE	*p*
Zonulin (ng/mL)	0.477	0.751	<0.001
NO (μM)	−0.307	0.033	0.007

4. Discussion

This study demonstrates that exposure to both passive and chronic smoking, from either HTPs or conventional cigarettes, increases low-grade endotoxemia. To our knowledge, circulating LPS levels have not previously been investigated in either conventional cigarette smokers or HTP users, making our results particularly novel.

The effects of conventional cigarette smoking on the gastrointestinal tract are well established. Cigarette smoke contains thousands of chemicals, including reactive aldehydes, hydrocarbons, and free radicals, which exert toxic effects not only on the lungs but also on the gut. Previous studies have shown that smoking alters the composition of the gut microbiota, reduces microbial diversity, and increases the relative abundance of potentially pathogenic species [18]. At the same time, smoking promotes systemic and local inflammation and oxidative stress. These changes may explain the elevated levels of circulating LPS observed in smokers in our study, since LPS originates from the outer membrane of Gram-negative bacteria and enters the circulation when barrier integrity is compromised [19].

Animal models provide strong mechanistic support. Chronic smoke exposure has been shown to damage intestinal villi, reduce tight junction proteins such as claudins and occludin, and promote bacterial translocation [20,21]. These structural and functional changes collectively lead to "leaky gut," which facilitates the entry of LPS into the bloodstream [20,21]. Other studies demonstrated increased gut permeability and serum LPS in mice exposed to cigarette smoke, confirming a causal link between smoking and endotoxemia. In addition to direct mucosal damage, smoke-induced hypoxia may represent another mechanism. Cigarette smoke reduces oxygen availability, and hypoxic conditions activate hypoxia-inducible factors (HIF-1α), which impair epithelial renewal, promote vascular dysfunction, and exacerbate barrier leakiness [22–25]. Furthermore, both mainstream and sidestream smoke contain measurable amounts of LPS, which may provide a direct exogenous source of endotoxin [26].

A novel aspect of our study is the evaluation of HTPs. These devices are marketed as reduced-risk alternatives to conventional cigarettes because they heat rather than burn tobacco. However, our findings challenge this assumption, showing that HTP exposure is also associated with increased circulating LPS and zonulin levels. The observation that passive exposure to HTP emissions results in similar changes emphasizes the potential health risks for non-users.

The mechanism underlying these findings may involve gut microbiota dysbiosis. Tian et al. demonstrated that 90-day HTP exposure in mice significantly reduced Lactobacillus abundance, a genus known to support epithelial integrity, produce anti-inflammatory metabolites, and compete against pathogens [27]. Reduced Lactobacilli could predispose to increased LPS release and absorption [27–30]. Our data, showing elevated LPS and zonulin in HTP users, are consistent with this interpretation. Interestingly, industry-sponsored studies have reported opposite results, with increased Lactobacilli following the switch from cigarettes to HTPs [31]. Discrepancies may reflect differences in experimental design, exposure duration, or conflicts of interest. Independent replication in human cohorts is urgently needed.

Zonulin plays a central role in the regulation of intestinal permeability by reversibly disassembling tight junctions [32]. Elevated serum zonulin is considered an indirect biomarker of enhanced permeability [32]. In our study, zonulin levels were increased in both conventional and HTP smokers, as well as in passively exposed individuals, strongly supporting the notion of barrier dysfunction. Importantly, the correlation between zonulin and LPS suggests that smoke-induced permeability changes may directly mediate systemic endotoxemia. However, as zonulin is not a perfect marker, and LPS could potentially derive from extra-intestinal sites such as the oral cavity or the lungs, further mechanistic investigations are required.

In our previous studies [11,12], we demonstrated that exposure to both conventional cigarette smoke and heated tobacco products induces oxidative stress, endothelial dysfunction, and platelet activation in exposed subjects. In the current study, the pathophysiological implications of elevated LPS are significant. LPS activates TLR4 on immune and endothelial cells, initiating an inflammatory cascade mediated by NF-κB [33]. In our study, circulating LPS correlated with NOX2 activation, hydrogen peroxide levels, reduced nitric oxide bioavailability, sCD40L and p-selectin. This suggests a mechanistic link in which smoke-induced endotoxemia activates NOX2, thereby amplifying oxidative stress, impairing endothelial function, and promoting platelet activation, as evidenced by the simple and multiple linear correlations observed in this study. Such processes are central in the pathogenesis of atherosclerosis and thrombosis, providing a plausible explanation for the increased cardiovascular risk observed in smokers (Figure 3). An additional potential influence on this pathological process may have been the exposure of study participants to nicotine. Previous research has shown that nicotine induces dysbiosis, which could, in turn, contribute to elevated circulating levels of LPS [34,35]. Although HTPs do not involve combustion as TTs do, they may still exert comparable deleterious effects on intestinal integrity, potentially leading to similar levels of LPS release into the bloodstream [36]. This appears plausible considering that both TTs and HTPs contain comparable nicotine concentrations, and that individuals who switch from TTs to HTPs often increase their overall consumption [37]. Furthermore, it should be noted that HTPs reach temperatures high enough to enable nicotine absorption through pyrolysis processes.

Our study has limitations. The sample size was relatively small, and larger studies are needed to validate these results. The mechanistic pathways of LPS translocation were not directly examined, and we cannot exclude alternative sources. We also did not assess gut microbiota composition, which would have provided direct evidence for dysbiosis. Nonetheless, the consistent increase in both LPS and zonulin, and their correlation, supports the hypothesis of impaired gut barrier function; however, Zonulin serves as an indirect marker, and we cannot rule out other potential sources of serum LPS. Another limitation of the study is the lack of data on the duration of conventional cigarette use prior to switching to HTPs. The participants' long-term history of traditional cigarette use before transitioning to heated tobacco products may have confounded or influenced the results observed in the HTPs user group. Further studies involving smokers who have exclusively used HTPs are needed to assess this potential influence.

The clinical implications are considerable. Low-grade endotoxemia has been linked to metabolic syndrome, insulin resistance and cardiovascular disease. The fact that both conventional and HTPs exposure, including passive exposure, increase endotoxemia underscores the importance of public health interventions. While dietary strategies such as probiotics [38], prebiotics or antioxidants may help reduce endotoxemia [39], smoking cessation and avoidance of passive exposure remain the most effective strategies to prevent the downstream consequences of LPS elevation.

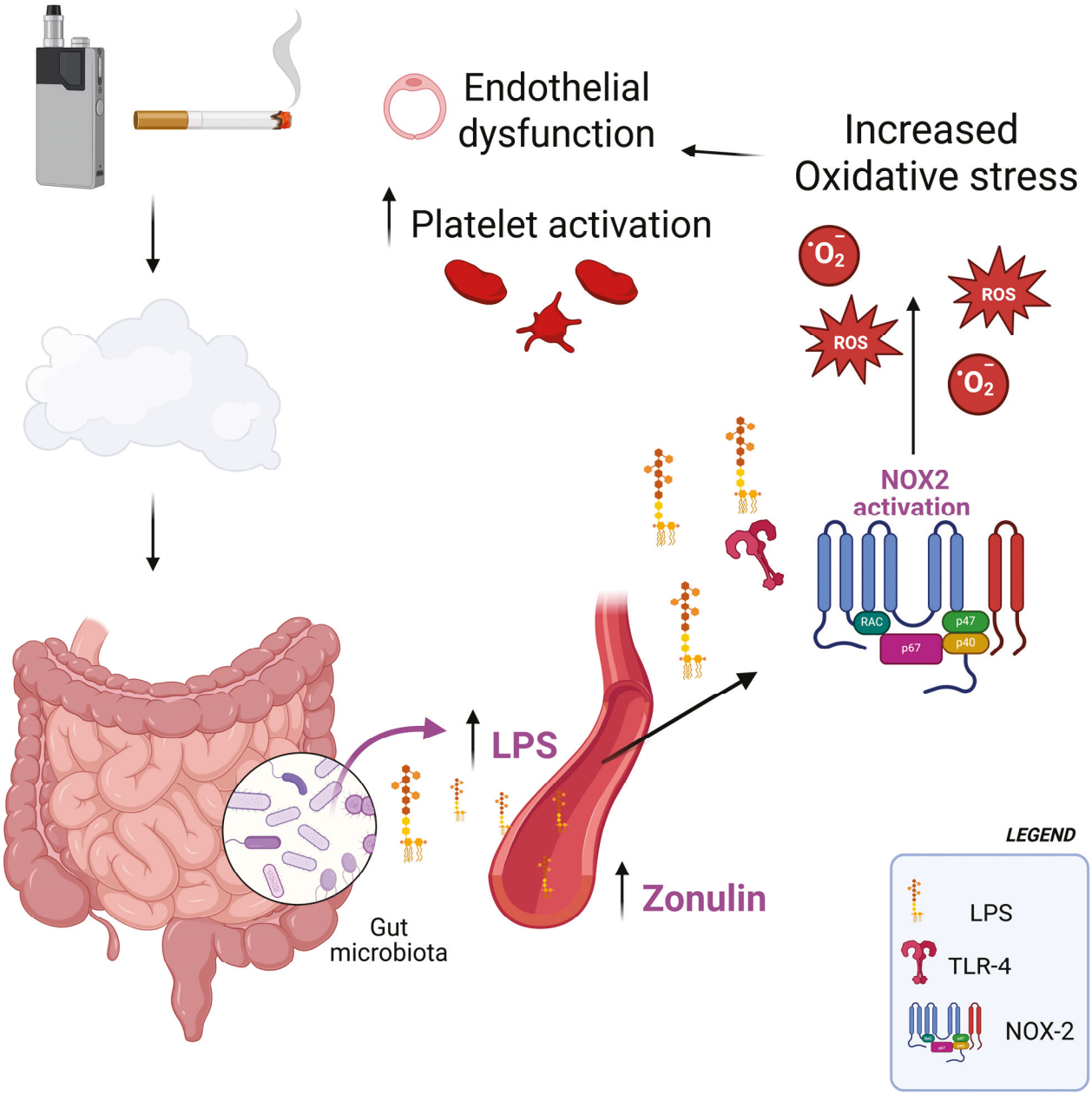

Figure 3. Following exposure to first-, second-, or third-hand smoke—whether derived from heated tobacco products or from conventional combustible cigarettes—there is an increase in circulating levels of lipopolysaccharides (LPS), most likely as a consequence of enhanced intestinal epithelial permeability. Elevated concentrations of even low-grade LPS promote the activation of the enzyme NADPH oxidase 2 (NOX2), which in turn drives excessive production of reactive oxygen species (ROS). This oxidative imbalance contributes to endothelial dysfunction and enhances platelet activation, two interrelated processes that are widely recognized as pivotal mechanisms in the development and progression of cardiovascular risk.

Future research should address several points: (i) longitudinal studies to determine whether LPS and zonulin levels normalize after smoking cessation or switching from HTPs to abstinence; (ii) microbiome sequencing to identify specific bacterial taxonomy involved; (iii) mechanistic studies linking LPS-TLR4 signaling to NOX2 activation and cardiovascular outcomes; and (iv) interventional trials testing whether microbiota-targeted therapies can mitigate smoke-induced endotoxemia.

In conclusion, this study demonstrates that both conventional cigarettes and HTPs, including passive exposure, are associated with increased low-grade endotoxemia and elevated zonulin levels. These alterations likely indicate impaired gut barrier integrity and may contribute to NOX2-mediated oxidative stress, endothelial dysfunction, and heightened cardiovascular risk. Therefore, heated tobacco products, much like traditional cigarettes, may exert a detrimental effect on low-grade endotoxemia and related pathophysiological processes.

Author Contributions: Conceptualization, L.L.; Methodology, E.G., G.F. and G.B.-Z.; Validation, A.S., P.P. and F.V.; Formal Analysis, E.M. and C.B.; Data Curation, S.B., A.M. and C.M.T.; Writing—Original Draft Preparation, L.L., E.M. and R.C.; Writing—Review & Editing, L.L., E.M., R.C. and A.A.; Visualization, B.L.C., F.A. and A.L.; Supervision, A.D., A.A. and A.M.Z.; Project Administration, L.L. The Smoking Prevention Study Group contributed to the recruitment of the patients. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of the Azienda Ospedaliera Universitaria Policlinico Umberto I (ref. no. 4929; protocol no 173/18, date of approval 22 February 2018; ref. no. 3241, protocol no. 813/14, date of approval 26 June 2014).

Informed Consent Statement: Informed consent was obtained from all individual participants included in the study.

Data Availability Statement: The original contributions presented in this study are included in the article Further inquiries can be directed to the corresponding author (Request to: Prof. Lorenzo Loffredo; e-mail: lorenzo.loffredo@uniroma1.it).

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Article

Effects of Emissions from Heated Tobacco Products and Reference Cigarettes on Gene Expression and Mitochondrial Function in Human Lung Epithelial BEAS-2B Cells †

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‡ These authors contributed equally to this work.

Abstract: Heated tobacco products (HTPs) are marketed as lower-risk alternatives to conventional cigarettes; however, their toxicological impacts remain insufficiently characterized. This study evaluated the effects of HTP emissions on gene expression and mitochondrial function in comparison with conventional cigarettes. Whole cigarette smoke condensates (WCSCs), comprising both gas and particulate phases, were prepared from three commercially available HTPs and from 3R4F reference cigarettes. Human lung epithelial BEAS-2B cells were exposed to WCSCs at 3 μg nicotine/mL for 24 h, followed by transcriptome profiling using RNA sequencing. Principal component analysis demonstrated that HTP-WCSCs induced weaker gene expression changes than 3R4F-WCSC, with only modest variation among HTPs. Gene set enrichment analysis revealed that both HTP- and 3R4F-WCSCs significantly downregulated oxidative phosphorylation (OXPHOS)–related pathways, indicating potential mitochondrial impairment. Functional assays confirmed that both exposures elevated mitochondrial reactive oxygen species (ROS), while mitochondrial morphology, ATP production, membrane potential, and cytosolic ROS were largely unaffected. Collectively, these results show that although HTP emissions elicit weaker transcriptomic perturbations than conventional cigarette emissions, both converge on mitochondrial targets by suppressing OXPHOS gene expression and increasing mitochondrial ROS. Mitochondrial dysfunction may therefore represent a common mechanism underlying tobacco product toxicity.

Keywords: heated tobacco products; cigarettes; cytotoxicity; mitochondria; whole cigarette smoke condensates

1. Introduction

Heated tobacco products (HTPs), also referred to as heat-not-burn cigarettes, are devices that heat tobacco at lower temperatures than conventional cigarettes [1]. Typically, HTPs heat tobacco to below 350 °C to generate an aerosol rather than smoke, whereas

conventional cigarettes combust tobacco at temperatures approaching 900 °C [2]. This technology is intended to reduce the harmful effects of cigarette smoking, primarily through reduced tar yields. Since their commercial introduction in the 2010s, HTPs have rapidly gained popularity and market share, often promoted as potentially less harmful alternatives to conventional cigarettes [3,4]. However, their widespread adoption has also raised public health and regulatory concerns regarding their true health impact [5].

Several studies suggest that HTPs emit lower levels of toxicants than conventional cigarettes [6–8], but the evidence remains incomplete and sometimes contradictory. Lower heating temperatures can decrease toxicant formation [9], and in 2020, the U.S. Food and Drug Administration authorized certain HTPs as modified-risk tobacco products based on reduced exposure to selected harmful chemicals [10]. Despite these advantages, HTPs still release a range of potentially harmful constituents, including nicotine and other toxicants, some of which are absent from conventional cigarette smoke [2]. Their toxicities and underlying mechanisms remain insufficiently defined, and a comprehensive toxicological profile is required to accurately assess their potential health risks.

Transcriptomic profiling provides a powerful approach to investigate the toxicological characteristics of HTPs. Alterations in gene expression can reveal cellular and molecular responses to toxicant exposure and identify disrupted biological pathways [11–14]. Advances in next-generation sequencing technologies now permit comprehensive transcriptomic analyses, also enabling comparisons of gene expression changes induced by HTPs versus conventional cigarettes. Previous RNA sequencing studies have shown that HTPs generally cause fewer transcriptomic alterations than conventional cigarettes [15,16]. Nevertheless, important limitations persist. For example, exposure levels have not always been clearly defined, and functional validation of transcriptomic changes has often been insufficient [17–19]. Moreover, comparative studies using conventional cigarettes as controls are essential to strengthen the toxicological interpretation of transcriptomic findings.

In this study, we investigated the effects of HTP emissions on gene expression in a cultured cell system. Whole cigarette smoke condensates (WCSCs) were prepared from three commercially available HTPs as well as the 3R4F reference cigarette, and exposures were carried out in human bronchial epithelial BEAS-2B cells, a relevant model since the respiratory epithelium is a primary site of inhalation exposure [20]. Transcriptomic alterations were assessed by means of RNA sequencing and bioinformatic analysis. Our goal was to characterize transcriptomic responses to HTP exposure at lower, clinically relevant levels compared with those used in earlier studies, thereby more closely reflecting realistic smoker exposure [18,21,22]. In addition, based on the transcriptomic findings, we evaluated mitochondrial functions, focusing on oxidative phosphorylation (OXPHOS) and reactive oxygen species (ROS) formation, to determine whether transcriptomic alterations were accompanied by functional changes in mitochondria. This combined approach provides novel insights into mitochondrial involvement in HTP-induced toxicity.

2. Materials and Methods

2.1. Reagents

Lil [Korea Tomorrow & Global (KT&G), Daejeon, Republic of Korea], IQOS (Philip Morris International, Neuchâtel, Switzerland), and Glo (British American Tobacco, London, UK) were purchased from a local cigarette retailer. 3R4F reference cigarettes were obtained from the Center for Tobacco Reference Products, University of Kentucky (Lexington, KY, USA). MitoTracker Red CMXRos, MitoSOX Red, dihydrorhodamine 123 (DHR123), tetramethylrhodamine methyl ester (TMRM), 10-acetyl-3,7-dihydroxyphenoxazine (Amplex Red), 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid acetoxymethyl ester (BAPTA-AM), bicinchoninic acid (BCA) protein assay kit, and cell culture reagents, in-

cluding Roswell Park Memorial Institute (RPMI) 1640 medium, fetal bovine serum (FBS), penicillin–streptomycin, and Hank's balanced salt solution (HBSS), were obtained from Thermo Fisher Scientific (Waltham, MA, USA). Primary antibodies against dynamin-related protein 1 (Drp1), phospho-Drp1 (Ser616), mitofusin-1 (Mfn1), and mitofusin-2 (Mfn2), as well as horseradish peroxidase (HRP)-conjugated anti-rabbit and anti-mouse IgG secondary antibodies, were acquired from Cell Signaling Technology (Danvers, MA, USA). Carbonyl cyanide m-chlorophenyl hydrazone (CCCP), HRP, and crystal violet were obtained from Sigma-Aldrich (St. Louis, MO, USA). Additional reagents were as follows: Complete protease inhibitor cocktail, PhosSTOP phosphatase inhibitor cocktail, and X-tremeGENE HP DNA transfection reagent (Roche Diagnostics, Mannheim, Germany); Seahorse Mitostress test kit and XF RPMI medium (Agilent, Santa Clara, CA, USA); pCS2+MLS-HyPer7 plasmid and pLJM1 lentiviral vector (Addgene, Cambridge, MA, USA); Premix WST-1 (Takara Bio, Kusatsu, Japan); RNeasy kit (Qiagen, Hilden, Germany); NEBNext Ultra II Directional RNA Library Prep kit (New England Biolabs, Ipswich, MA, USA); Poly(A) RNA selection kit (Lexogen, Vienna, Austria); Immobilon Western chemiluminescent HRP substrate (Donginbiotech, Seoul, Republic of Korea); CellTiter-Glo luminescent cell viability assay kit (Promega, Madison, WI, USA); and radioimmunoprecipitation assay (RIPA) buffer (Biosolution, Suwon, Republic of Korea). All other chemicals were of the highest available purity and obtained from standard commercial suppliers.

2.2. Preparation of WCSCs

WCSCs were prepared as previously described [23], with minor modifications. Cigarettes were conditioned at 22 ± 1 °C and 60 ± 2% relative humidity for 48 h in accordance with ISO 3402 standards [24]. Emissions were generated from 80 sticks of HTPs (Lil, IQOS, and Glo) using their respective heating devices or from 15 sticks of 3R4F reference cigarettes using an SG-300 cigarette smoke generator (Sibata, Saitama, Japan), following the Health Canada Intense smoking protocol: 55 mL puff volume, 2-s puff duration, 30-s puff interval, and 100% filter vent blocking [25].

Particulate matter was trapped on a 44 mm Cambridge filter pad (GE Healthcare, Little Chalfont, UK), while the remaining gas phase was absorbed into 50 mL of Dulbecco's phosphate-buffered saline at a flow rate of 1.65 L/min for 5 min using an impinger and sampling pump, yielding the cigarette smoke extract (CSE). Particulate matter retained on the filter pad was extracted with 0.5 mL methanol, evaporated under nitrogen, and resuspended in dimethyl sulfoxide to generate the total particulate matter (TPM). WCSCs were prepared by combining TPM and CSE and stored at −80 °C until use.

The measured TPM concentrations were 24.4, 27.5, 29.0, and 20.6 mg/mL for Lil-, IQOS-, Glo-, and 3R4F-WCSCs, respectively, with corresponding nicotine concentrations of 1.81, 2.45, 2.26, and 1.10 mg/mL (Table 1). Nicotine concentrations were determined using a GC-2010 gas chromatography system (Shimadzu, Kyoto, Japan) according to established protocols [23].

Table 1. TPM and nicotine concentrations in WCSCs.

	Lil	**IQOS**	**Glo**	**3R4F**
TPM	24.4	27.5	29.0	20.6
Nicotine	1.81	2.45	2.26	1.10

Values are shown as mg/mL.

2.3. Cell Culture

The human bronchial epithelial cell line BEAS-2B was obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured in RPMI 1640 medium

supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. Cells were maintained at 37 °C in a humidified incubator with 5% CO_2 and subcultured at 80–90% confluence.

2.4. Assessment of Cell Viability and Growth

Cell viability and growth were assessed using the WST-1 and crystal violet staining assays [26,27]. For the WST-1 assay, cells were treated with WCSCs for 24 or 48 h, followed by incubation in 200 μL phenol red-free medium containing 20 μL Premix WST-1 for 1 h at 37 °C. For the crystal violet staining assay, WCSC-treated cells were fixed with 4% paraformaldehyde, stained with 0.1% crystal violet, washed, and dissolved in 1% sodium dodecyl sulfate (SDS). Absorbance was measured at 450 nm for the WST-1 assay and at 590 nm for the crystal violet assay using a SpectraMax M3 microplate reader (Molecular Devices, San Jose, CA, USA).

2.5. RNA Isolation and Library Preparation

Total RNA was extracted from WCSC-treated cells using the RNeasy kit. RNA integrity was evaluated using a 2100 Bioanalyzer (Agilent Technologies, Amstelveen, The Netherlands), and RNA concentration was determined with an ND-2000 spectrophotometer (Thermo Fisher Scientific).

Poly(A)+ RNA was enriched using the Poly(A) RNA Selection kit, and libraries were prepared using the NEBNext Ultra II Directional RNA Library Prep kit following the manufacturer's instructions. RNA was fragmented, reverse-transcribed into cDNA, indexed with Illumina indexes 1–12, and amplified by polymerase chain reaction (PCR). Library quality was verified with TapeStation HS D1000 ScreenTape (Agilent), and quantification was performed using a library quantification kit and the StepOne real-time PCR system (Life Technologies, Carlsbad, CA, USA). Sequencing was carried out on a NovaSeq 6000 platform (Illumina, San Diego, CA, USA) with paired-end 100 bp reads.

2.6. RNA Sequencing Analysis

2.6.1. Preprocessing

Raw read count matrices were imported into R (version 4.2.3; R Foundation, Vienna, Austria). Genes with low expression were filtered using the filter_genes function of the RNAseqQC package (version 0.2.1), retaining only those with a minimum of five counts in at least four replicates.

2.6.2. Differential Expression Analysis

Differential expression analysis was performed using DESeq2 (version 1.38.3), with the control group as the reference. Count normalization was conducted using the median-of-ratios method, and log2 fold changes were stabilized with the apeglm shrinkage estimator. Genes were considered differentially expressed if they met the criteria of adjusted p-value < 0.05 and absolute log2 fold change ≥ 0.58 (~1.5-fold). Volcano plots were generated using ggplot2 (version 3.5.2) to visualize significantly up- and downregulated genes, with the most prominent candidates annotated.

2.6.3. Principal Component Analysis (PCA) and Sample Correlation

Variance-stabilizing transformation (VST) and regularized log (rlog)-transformation of normalized counts were performed using DESeq2 (version 1.38.3) to reduce heteroscedasticity across samples. PCA was conducted on the VST-transformed expression matrix to visualize global transcriptomic variation among groups. Sample-to-sample correlations were computed from rlog-transformed counts using the Pearson correlation coefficient,

and the resulting correlation matrix was visualized as a clustered heatmap generated with the ComplexHeatmap package (version 2.24.1).

2.6.4. Gene Set Enrichment Analysis (GSEA)

Hallmark gene sets were retrieved from the Molecular Signatures Database (MSigDB) using the msigdbr package. Gene-level statistics were derived from DESeq2 results by ranking genes according to log2 fold change. For genes with duplicate symbols, the entry with the largest absolute log2 fold change was retained. GSEA was performed with the fgsea package (version 1.24.0), using 10,000 permutations and restricting gene set sizes to 10–500 members. Pathways with an adjusted *p*-value (false discovery rate, FDR) < 0.05 were considered significantly enriched. Normalized enrichment scores (NES) indicated the direction of regulation, with positive values representing upregulated pathways and negative values representing downregulated pathways. Enrichment results were visualized using ggplot2 by plotting all pathways ordered by |NES|, with significant pathways (FDR < 0.05) highlighted in blue.

2.7. Analysis of Mitochondrial Morphology

Cells were stained with 100 nM MitoTracker Red CMXRos for 30 min following 24 h of WCSC treatment. Images were acquired using an Eclipse Ti-U inverted microscope equipped with an S Fluor 100× oil-immersion objective lens (Nikon, Tokyo, Japan), a Lambda DG-4 filter changer with a xenon arc lamp (Sutter Instruments, Novato, CA, USA), and an Evolve 512 EMCCD camera (Photometrics, Tucson, AZ, USA). Fluorescence was detected at excitation and emission wavelengths of 475 and 560 nm, respectively. Images were analyzed using Meta Imaging System (version 7.7.6; Molecular Devices) and the Mitochondria Analyzer plugin in ImageJ (version 1.54f; National Institutes of Health, Bethesda, MD, USA) [28,29].

2.8. Immunoblot Analysis

Protein expression and phosphorylation of mitochondrial dynamics regulators were examined by Western blotting [30]. Cells were lysed in RIPA buffer supplemented with a Complete protease inhibitor cocktail and a PhosSTOP phosphatase inhibitor cocktail, and centrifuged at 16,200× *g* for 30 min. Supernatants were collected, and protein concentrations were determined using the BCA protein assay kit. Equal amounts of protein were separated by means of SDS–polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membranes, probed with primary and HRP-conjugated secondary antibodies, and visualized using Immobilon Western chemiluminescent HRP substrate. Chemiluminescent signals were detected with the ChemiDoc XRS+ system and quantified using Image Laboratory software (version 3.0; Bio-Rad Laboratories, Hercules, CA, USA).

2.9. Measurement of Mitochondrial ROS (mtROS), Intracellular ROS, and Mitochondrial Membrane Potential

mtROS, intracellular ROS, and mitochondrial membrane potential were quantified using fluorescent probes coupled with flow cytometric analysis. Cells exposed to WCSCs for 24 h were stained with 5 μM MitoSOX Red for 20 min, 10 μM DHR123 for 30 min, or 100 nM TMRM for 30 min to detect mitochondrial ROS, intracellular ROS, and mitochondrial membrane potential, respectively. Stained cells were analyzed using a BD FACSAria III cell sorter (BD Biosciences, San Jose, CA, USA) and FlowJo software (version 7.6; BD Biosciences). Excitation/emission settings were 488/575 nm for MitoSOX and TMRM and 488/519 nm for DHR123.

mtROS was further assessed using the genetically encoded, mitochondria-targeted H_2O_2 sensor MLS-HyPer7. The construct was cloned into a pLJM1 vector and transfected

into BEAS-2B cells using X-tremeGENE HP DNA transfection reagent. After 48 h, cells were seeded onto coverslips, treated with WCSCs for 24 h, and imaged with an Eclipse Ti-U inverted microscope (Nikon). Fluorescence images were acquired at excitation wavelengths of 405 and 480 nm with emission at 528 nm, and were analyzed using the Meta Imaging System (version 7.7.6; Molecular Devices).

Intracellular ROS production was further evaluated with the Amplex Red assay. Cells were incubated with 10 μM Amplex Red and 0.1 U/mL HRP in phenol red-free RPMI 1640 medium. Fluorescence was measured at excitation/emission wavelengths of 560/580 nm under kinetic mode for 60 min using a SpectraMax M3 microplate reader (Molecular Devices).

2.10. Measurement of ATP Levels

Cellular ATP levels were quantified using the CellTiter-Glo luminescent cell viability assay kit (Promega). Cells were exposed to WCSCs for 24 h, treated with CellTiter-Glo reagent, and stabilized for 10 min. Luminescence was measured using a Glomax Multi+ detection system (Promega).

2.11. Bioenergetic Profiling

Mitochondrial bioenergetic function was evaluated using the Seahorse XF HS Mini Analyzer with the Mitostress test kit. Cells were seeded into XFp miniplates (Agilent) at a density of 4.0×10^4 cell/well, exposed to WCSCs for 24 h, and equilibrated in Seahorse XF RPMI medium. Oxygen consumption rate (OCR) and extracellular acidification rate were measured following sequential injection of electron transport chain modulators: oligomycin (1.0 μM), carbonyl cyanide-4-(trifluoro-methoxy) phenylhydrazone (FCCP, 1.0 μM), and rotenone/antimycin A (1.5 μM). Parameters, including ATP production-linked respiration, maximal respiration, proton leak, and non-mitochondrial respiration, were calculated. OCR values were normalized to protein content per well, as determined by the BCA protein assay kit.

2.12. Statistical Analysis

Data are expressed as mean ± standard error. Statistical analyses were performed using one-way analysis of variance followed by Dunnett's post hoc test. All analyses were conducted with SigmaPlot (version 13; Systat Software, San Jose, CA, USA). A p-value < 0.05 was considered statistically significant.

3. Results

3.1. Overall Effects of HTP-WCSCs on Gene Expression in BEAS-2B Cells

To evaluate the impact of HTP aerosols on the respiratory epithelium, transcriptomic profiling was performed in BEAS-2B cells exposed to WCSCs. WCSCs were prepared from three commercially available HTPs (Glo, IQOS, and Lil) as well as from 3R4F reference cigarettes, and applied to cells at 3 μg nicotine/mL for 24 h. Transcriptome-wide analysis was conducted by RNA sequencing. This exposure concentration corresponds to the approximate nicotine level in a single puff of 3R4F cigarette smoke [31–33]. Cell viability and growth were unaffected for up to 48 h under these conditions (Figure 1).

PCA revealed substantial changes in gene expression following WCSC exposure (Figure 2A). Alterations induced by HTP-WCSCs were less pronounced than those caused by 3R4F-WCSC, and transcriptomic profiles clustered more closely among HTP groups than between HTP- and 3R4F-exposed groups. Pairwise correlation analysis further supported this observation, demonstrating strong correlations within HTP-treated cells but weaker correlations between HTP- and 3R4F-exposed groups (Figure 2B).

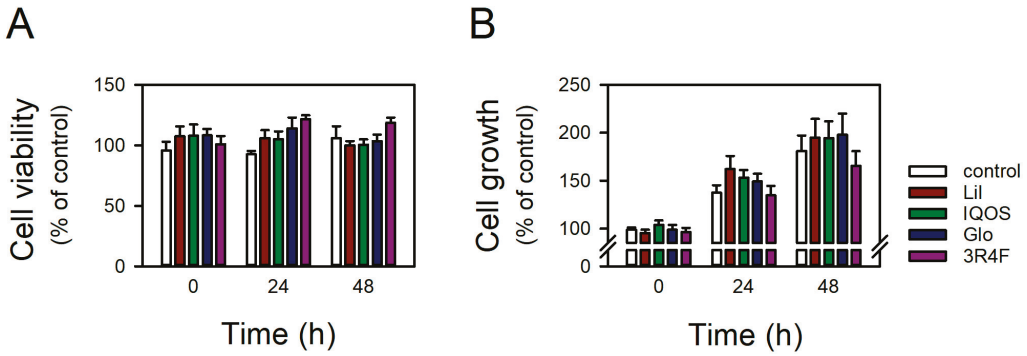

Figure 1. Effects of WCSCs on the viability and growth of BEAS-2B cells. (**A**) Cell viability and (**B**) growth were assessed using WST-1 and crystal violet assays. Cells were treated with 3 μg nicotine/mL WCSCs for up to 48 h. Values from the control group at 0 h were regarded as 100%. Data are presented as mean ± standard error (n = 6 for WST-1 assay; n = 5 for crystal violet assay).

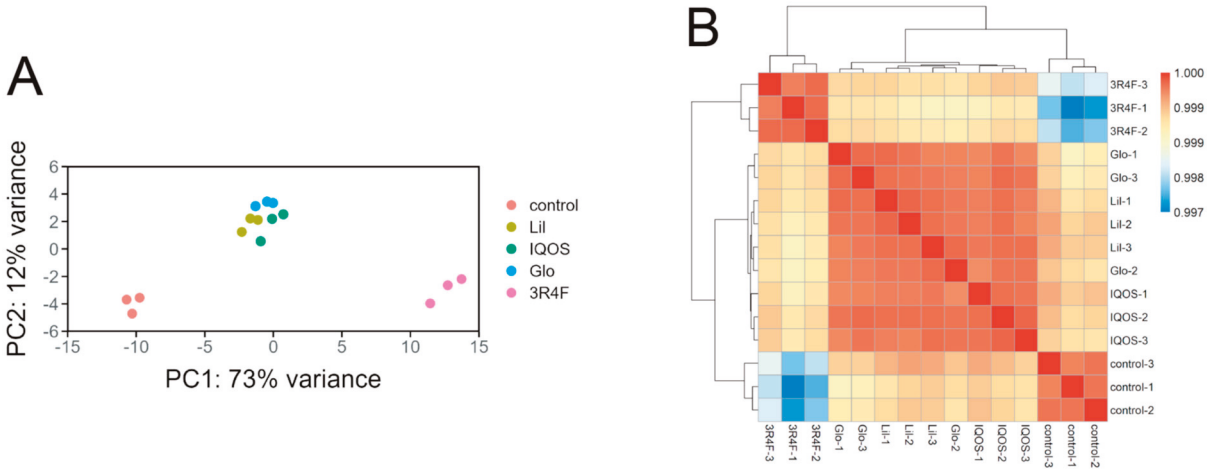

Figure 2. Transcriptomic profiles of cells exposed to HTP- and 3R4F-WCSCs. RNA sequencing was conducted on cells treated with WCSCs for 24 h. (**A**) PCA of normalized transcriptomes showing distinct clustering among experimental groups. (**B**) Heatmap of pairwise sample correlations based on variance-stabilized counts (n = 3 per group).

Differentially expressed gene (DEG) analysis highlighted these differences in greater detail. Volcano plots revealed substantially fewer significantly altered genes in HTP-treated cells compared with 3R4F-treated cells (Figure 3). The numbers of altered genes at ≥1.5- and ≥2.0-fold changes were as follows: Lil, 127 and 24; IQOS, 171 and 33; and Glo, 225 and 40. In contrast, 3R4F-WCSC altered 1019 genes at ≥1.5-fold and 249 genes at ≥2.0-fold (Table 2). Overall, the greatest transcriptomic impact was observed with 3R4F-WCSC, followed by Glo, IQOS, and Lil, while differences among the HTP groups were relatively modest.

Table 2. Quantitative analysis of DEGs in BEAS-2B cells exposed to HTP- and 3R4F-WCSCs.

	FC > 1.5		**FC > 2**	
	Up	**Down**	**Up**	**Down**
Lil	90	37	24	0
IQOS	104	67	32	1
Glo	113	112	36	4
3R4F	307	712	91	158

Values indicate the number of DEGs with $p < 0.05$ compared with the control.

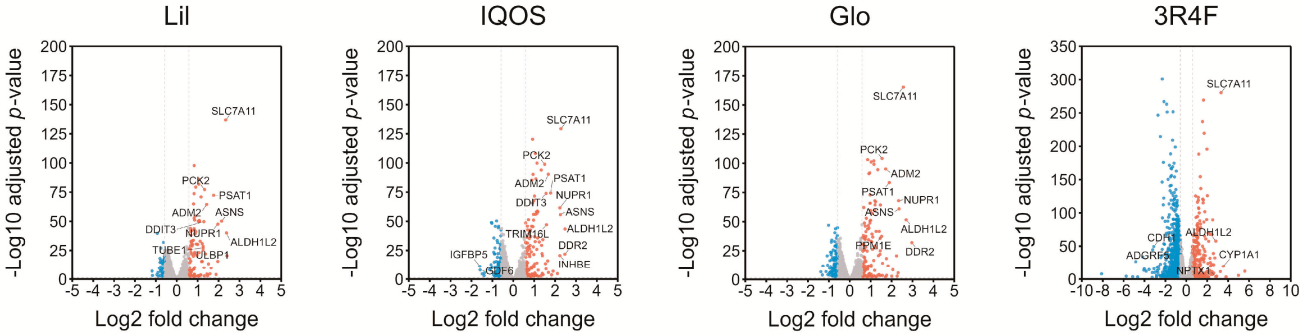

Figure 3. Differential gene expression induced by HTP- and 3R4F-WCSCs. Volcano plots depict DEGs in cells exposed to HTP- or 3R4F-WCSCs. The x-axis represents log2 fold change, and the y-axis denotes −log10 adjusted *p*-value. Genes meeting the criteria of log2 fold change ≥0.58 or ≤−0.58 and adjusted $p < 0.05$ are highlighted in red or blue, respectively. Genes that do not meet the fold-change cutoff (|log2 fold change| < 0.58) are displayed in gray, indicating non-significant expression changes. Please note that the y-axis scale of the 3R4F plot differs from those of the Lil, IQOS, and Glo plots.

3.2. Enriched Biological Pathways Identified by GSEA

GSEA was performed using Hallmark gene sets from MsigDB to identify pathways perturbed by WCSC exposure (Figure 4). The top ten enriched pathways were ranked by NES for each exposure group. Across all conditions, epithelial–mesenchymal transition, TGF-β signaling, and apical junction pathways were consistently enriched. Notably, OXPHOS ranked among the top ten pathways in all HTP-treated groups but not in the 3R4F-WCSC group, suggesting a relatively greater emphasis on OXPHOS-related alterations in HTP exposures. In contrast, angiogenesis and KRAS signaling were more prominent in 3R4F-exposed cells, underscoring pathway-level distinctions between HTPs and conventional cigarettes (Table 3).

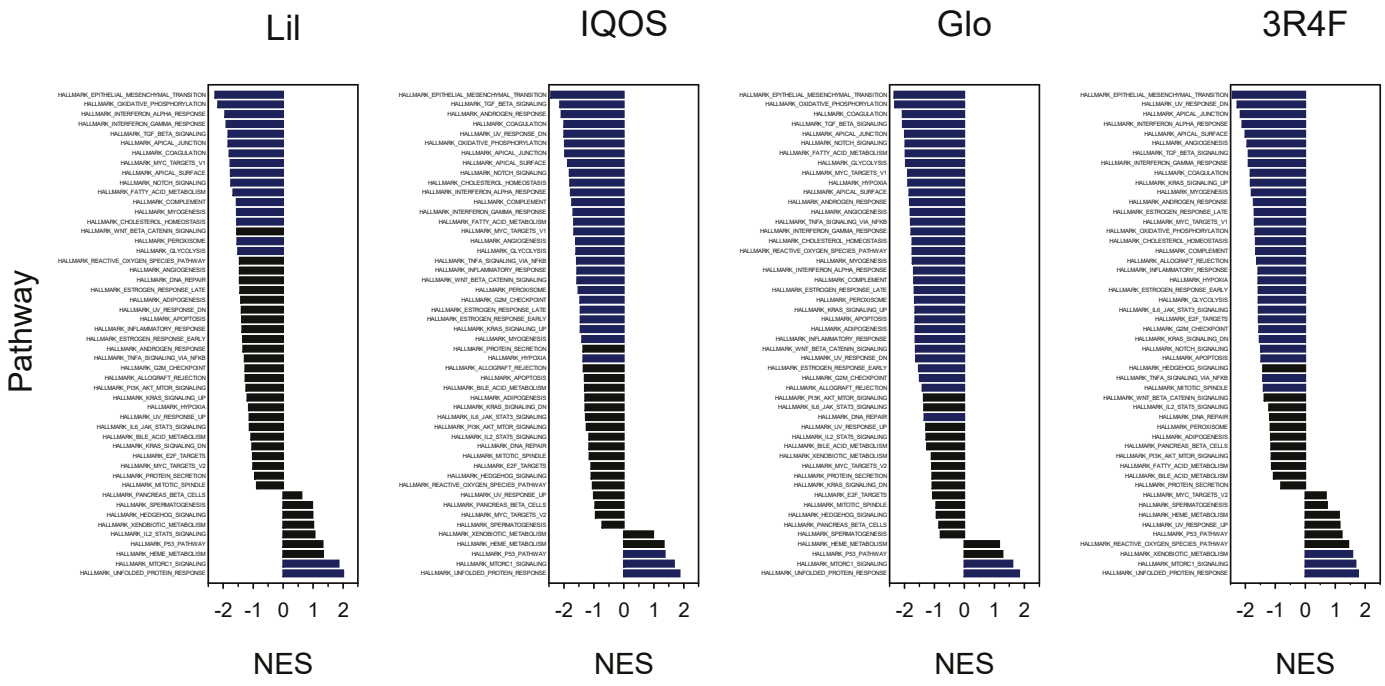

Figure 4. GSEA of Hallmark pathways affected by WCSCs. NES are shown for pathways in WCSC-exposed cells. Positive NES values indicate upregulation, whereas negative NES values represent downregulation. Pathways with adjusted $p < 0.05$ are highlighted in blue.

Table 3. Significantly enriched Hallmark pathways in WCSC-exposed groups.

Pathway	Rank (NES)			
	Lil	**IQOS**	**Glo**	**3R4F**
Epithelial–mesenchymal transition	1 (−2.27)	1 (−2.43)	1 (−2.34)	1 (−2.43)
Oxidative phosphorylation	2 (−2.18)	6 (−1.98)	2 (−2.32)	17 (−1.67)
Unfolded protein response	3 (2.01)	9 (1.86)	12 (1.84)	12 (1.76)
Interferon-α response	4 (−1.94)	12 (−1.78)	20 (−1.70)	4 (−2.11)
Interferon-γ response	5 (−1.90)	14 (−1.69)	16 (−1.77)	8 (−1.89)
MTORC1 signaling	6 (1.85)	15 (1.68)	30 (1.61)	16 (1.68)
TGF-β signaling	7 (−1.84)	2 (−2.14)	4 (−2.06)	7 (−1.90)
Apical junction	8 (−1.83)	7 (−1.97)	5 (−1.99)	3 (−2.16)
Coagulation	9 (−1.80)	4 (−2.00)	3 (−2.06)	9 (−1.83)
MYC targets_v1	10 (−1.77)	17 (−1.66)	9 (−1.89)	15 (−1.69)
Apical surface	11 (−1.76)	8 (−1.88)	11 (−1.84)	5 (−2.01)
Notch signaling	12 (−1.74)	10 (−1.82)	6 (−1.97)	false
Fatty acid metabolism	13 (−1.67)	16 (−1.67)	7 (−1.97)	false
Complement	14 (−1.56)	13 (−1.74)	21 (−1.69)	19 (−1.65)
Myogenesis	15 (−1.55)	28 (−1.40)	19 (−1.73)	11 (−1.80)
Cholesterol homeostasis	16 (−1.54)	11 (−1.80)	17 (−1.74)	18 (−1.65)
Peroxisome	17 (−1.53)	23 (−1.52)	23 (−1.66)	false
Glycolysis	18 (−1.51)	19 (−1.59)	8 (−1.96)	25 (−1.57)
Androgen response	false	3 (−2.09)	13 (−1.82)	13 (−1.73)
UV response_down	false	5 (−1.99)	29 (−1.62)	2 (−2.27)
Angiogenesis	false	18 (−1.61)	−14 (1.81)	6 (−1.94)
TNFα signaling via NF-κB	false	20 (−1.57)	15 (−1.79)	31 (−1.42)
Inflammatory response	false	21 (−1.57)	27 (−1.63)	21 (−1.58)
WNTβ catenin signaling	false	22 (−1.57)	28 (−1.63)	false
G2M checkpoint	false	24 (−1.46)	32 (−1.50)	28 (−1.55)
Estrogen response_late	false	25 (−1.45)	22 (−1.67)	14 (−1.70)
Estrogen response_early	false	26 (−1.45)	31 (−1.53)	24 (−1.57)
KRAS signaling_up	false	27 (−1.45)	24 (−1.65)	10 (−1.83)
p53 pathway	false	29 (1.37)	false	false
Hypoxia	false	30 (−1.36)	10 (−1.88)	23 (−1.57)
Reactive oxygen species pathway	false	false	18 (−1.74)	false
Apoptosis	false	false	25 (−1.64)	30 (−1.47)
Adipogenesis	false	false	26 (−1.64)	false
Allograft rejection	false	false	33 (−1.40)	20 (−1.62)
DNA repair	false	false	34 (−1.34)	false
Xenobiotic metabolism	false	false	false	22 (1.57)
IL6-JAK-STAT3 signaling	false	false	false	26 (−1.56)
E2F targets	false	false	false	27 (−1.56)
KRAS signaling_down	false	false	false	29 (−1.53)
Mitotic spindle	false	false	false	32 (−1.40)

Significantly enriched pathways (adjusted $p < 0.05$) identified by GSEA are listed in descending order of |NES|, beginning with those significant in Lil, and subsequently including additional significant pathways from IQOS, Glo, and 3R4F groups. false: adjusted $p > 0.05$.

Comparative GSEA between HTP- and 3R4F-treated cells revealed distinct patterns of pathway enrichment (Figure 5). Across all three HTPs, the OXPHOS pathway consistently exhibited the highest comparative NES, indicating a significantly stronger downregulation of mitochondrial respiratory gene sets relative to the 3R4F group. This trend was repro-

ducible across HTPs, suggesting that suppression of mitochondrial oxidative metabolism represents a common transcriptomic feature specific to HTP exposure.

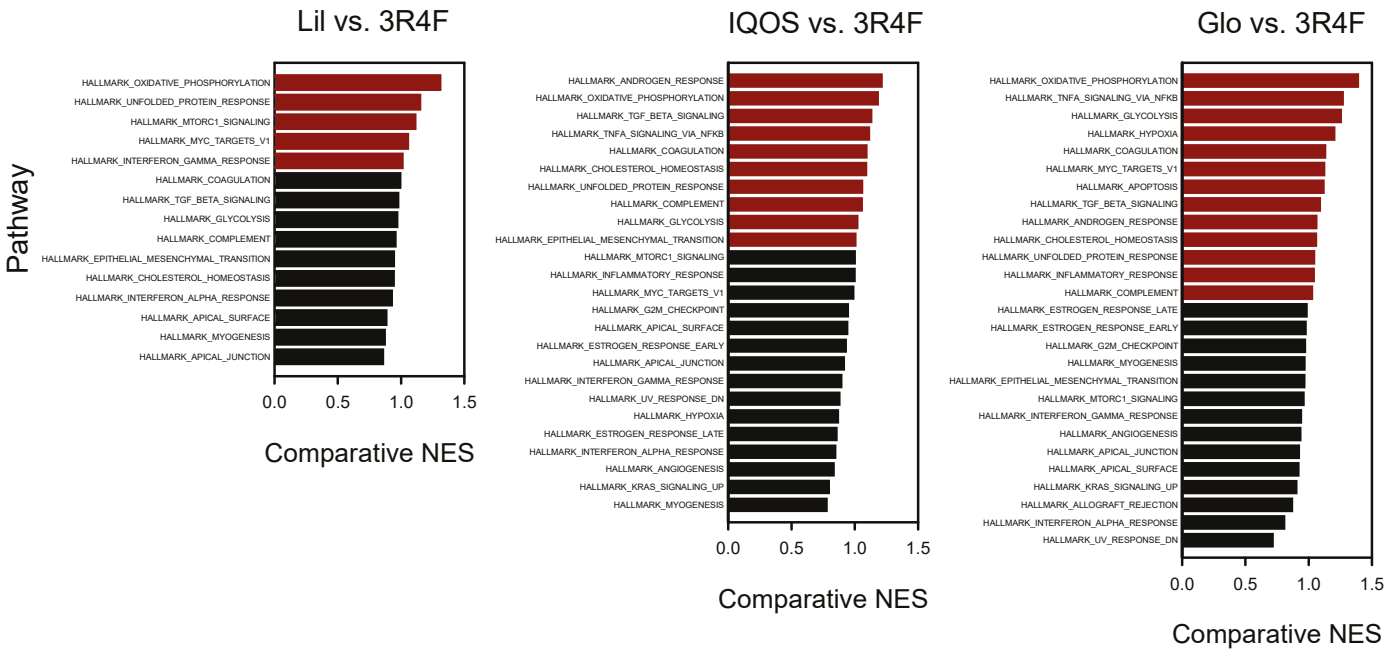

Figure 5. Comparative enrichment of Hallmark pathways by HTP- versus 3R4F-WCSCs. Comparative NES values illustrate relative pathway enrichment between HTP-WCSC- and 3R4F-WCSC-treated cells. Pathways more enriched in HTP-WCSC-exposed cells (comparative NES > 1) are shown in red.

3.3. Changes in OXPHOS Pathway-Related Gene Expression

The OXPHOS gene set comprises 197 genes encoding subunits of the mitochondrial respiratory complexes. Expression changes were visualized in a heatmap organized by complex, with additional genes categorized as "others" (Figure 6). WCSC exposure broadly downregulated genes across multiple complexes, indicating widespread suppression of mitochondrial respiratory capacity. Most of these changes represented statistically significant downregulation, while only a few genes were significantly upregulated. These transcriptomic signatures prompted subsequent experiments to determine whether WCSCs also induced functional alterations in mitochondrial physiology.

3.4. Effect of WCSCs on Mitochondrial Morphology and Dynamics

The impact of WCSCs on mitochondrial morphology and dynamics was assessed in treated cells. Mitochondrial morphology was quantified by evaluating five parameters: area, perimeter, form factor, aspect ratio, and solidity [28,34]. Under control conditions, mitochondria typically exhibited an elongated morphology and formed interconnected networks throughout the cytoplasm (Figure 7A, control images). None of the WCSC exposures significantly altered these parameters (Figure 7A,B), indicating negligible morphological effects. In contrast, the positive control BAPTA-AM induced mitochondrial fragmentation and fission, characterized by rounded structures and disrupted networks, consistent with previous findings [35,36].

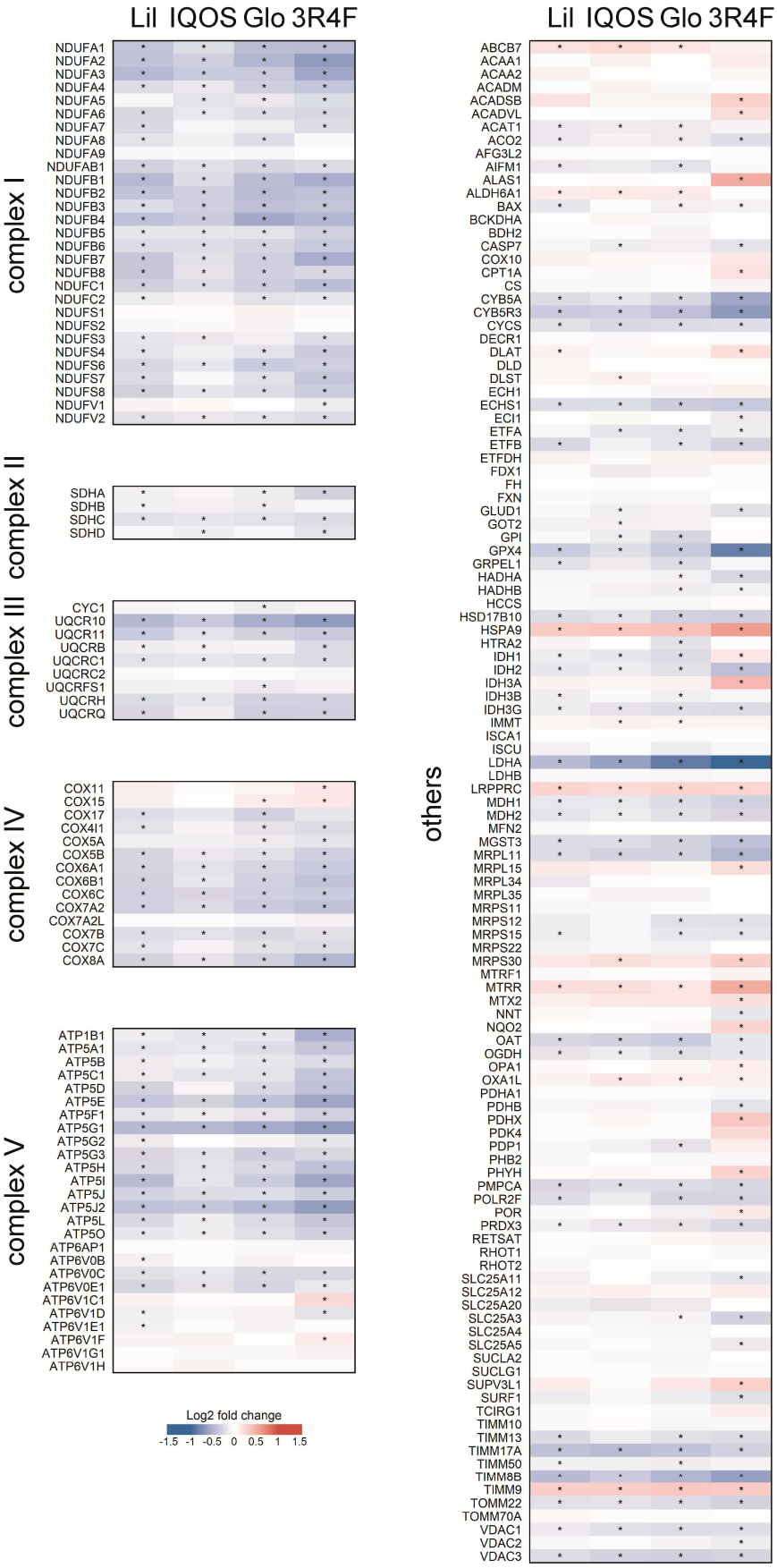

Figure 6. Downregulation of OXPHOS pathway genes by WCSCs. Heatmap of expression changes in genes associated with OXPHOS, categorized by mitochondrial respiratory complexes I–V and "others". The color scale represents log2 fold change values. * adjusted $p < 0.05$.

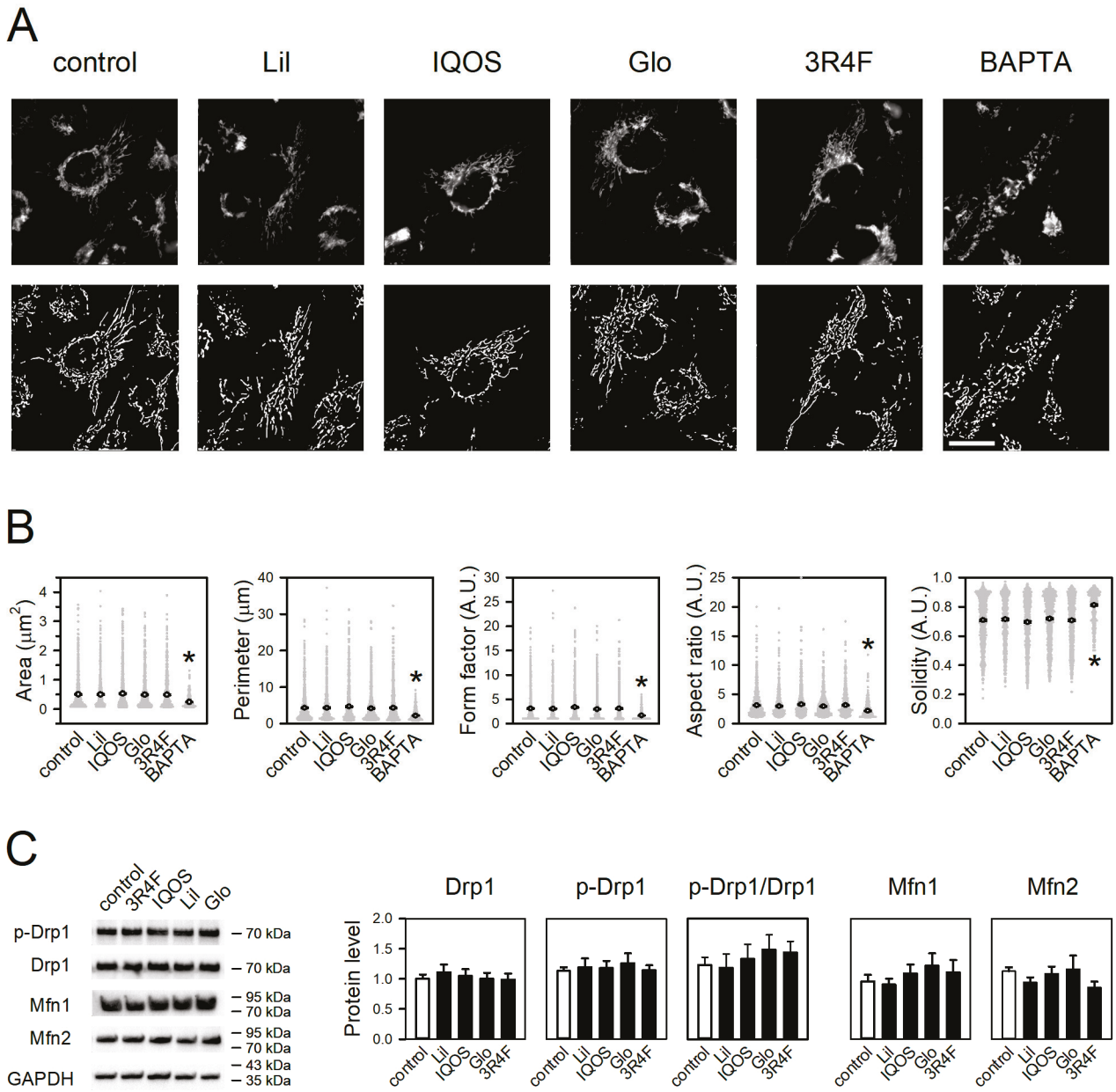

Figure 7. Effects of WCSCs on mitochondrial morphology and dynamics. Cells were treated with WCSCs for 24 h. (**A**) Representative images of mitochondria. Upper panels show raw images of mitochondria stained with MitoTracker Red CMXRos, and lower panels show processed images generated by ImageJ for quantitative morphology analysis. Scale bar = 5 μm. (**B**) Quantitative analysis of mitochondrial morphology across five parameters. (**C**) Effects of WCSCs on mitochondrial fission and fusion proteins. Phosphorylation of Drp1 at Ser616 and expression levels of Drp1, Mfn1, and Mfn2 were examined by immunoblotting. Representative blots and quantified band intensities normalized to untreated groups are shown. Values are mean ± standard error [n = 1028, 671, 739, 993, 740, and 489 for control and Lil-, IQOS-, Glo-, 3R4F-WCSC-, and BAPTA-AM-treated groups, respectively (**B**); n = 10, 8, 8, 8, and 9 for control and Lil-, IQOS-, Glo-, and 3R4F-WCSC-treated groups, respectively (**C**)]. * $p < 0.05$ vs. control.

In line with these observations, WCSCs exerted minimal effects on the expression of Drp1, a central mediator of mitochondrial fission, or on its phosphorylation at Ser616 (Figure 7C) [37]. Similarly, the expression of Mfn1 and Mfn2, key regulators of mitochondrial fusion [38], remained unchanged following WCSC treatment.

3.5. Effect of WCSCs on mtROS Formation

The effects of WCSCs on mtROS generation were evaluated using the mitochondrial superoxide indicator MitoSOX Red. All WCSC exposures significantly increased mtROS levels (Figure 8A). Comparable results were obtained with MLS-HyPer7, a genetically encoded biosensor specific for mtROS [39] (Figure 8B). In contrast, WCSCs did not elevate cytosolic ROS, as determined by DHR123 (Figure 8C) or Amplex Red (Figure 8D). The positive controls, antimycin A and hydrogen peroxide, significantly increased mtROS and cytosolic ROS, respectively, confirming assay validity [40]. These findings indicate that WCSC exposure selectively stimulates ROS generation within mitochondria without concomitant elevation of cytosolic ROS.

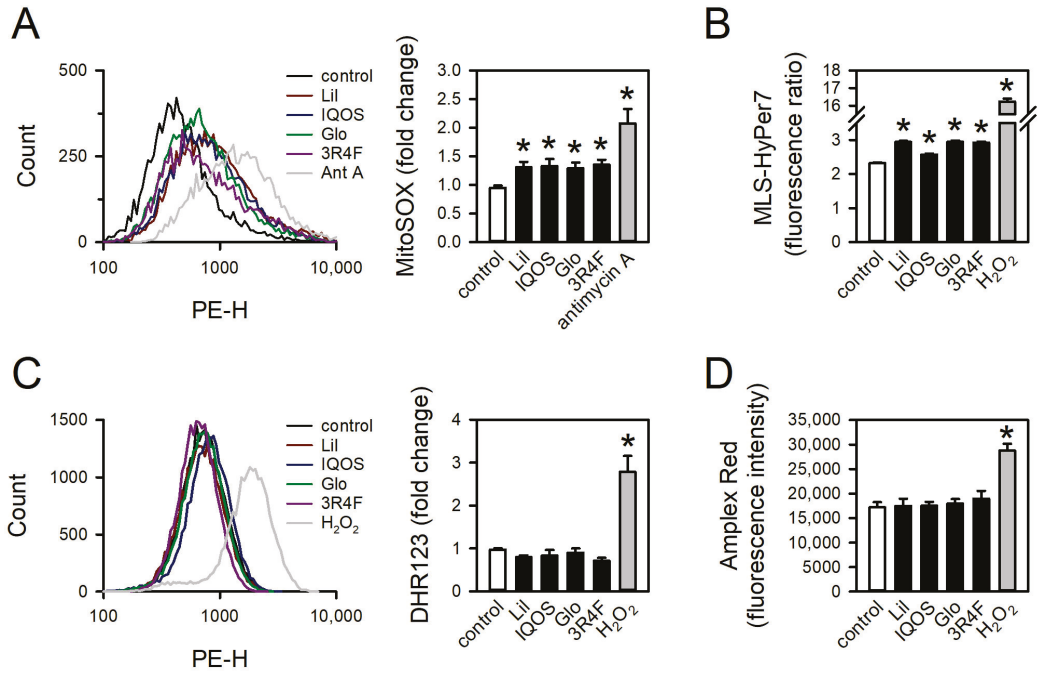

Figure 8. Increase in mtROS but not cytosolic ROS by WCSCs. Cells were treated with HTP- or 3R4F-WCSCs for 24 h. mtROS were detected with (**A**) MitoSOX Red or (**B**) MLS-HyPer7, and cytosolic ROS were measured with (**C**) DHR123 or (**D**) Amplex Red. (**A**,**C**) Flow cytometry histograms and quantification of mean fluorescence intensity normalized to untreated groups. Ant A: antimycin A. (**B**,**D**) Quantification of (**B**) MLS-HyPer7 ratio and (**D**) Amplex Red fluorescence intensity. Values are mean ± standard error [n = 7 for (**A**); n = 755, 876, 815, 712, 1088, and 166 for (**B**); n = 4 for (**C**); and n = 5 for (**D**)]. * $p < 0.05$ vs. control.

3.6. Effect of WCSCs on ATP Levels, Mitochondrial Respiration, and Membrane Potential

Mitochondrial respiratory function was examined by measuring the OCR [41]. No significant differences in OCR traces were observed across WCSC-treated groups (Figure 9A). WCSCs did not induce significant changes in any respiratory parameters, including basal respiration, ATP-linked respiration, proton leak, maximal respiration, reserve capacity, or non-mitochondrial respiration (Figure 9B). Consistent with the minimal effects on bioenergetic function, intracellular ATP levels also remained unchanged, whereas the positive control antimycin A markedly reduced ATP content (Figure 9C).

The effects of WCSCs on mitochondrial membrane potential were evaluated using the fluorescent indicator TMRM, given that impairment of OXPHOS often results in membrane depolarization [42]. None of the WCSCs significantly altered mitochondrial membrane potential (Figure 9D). As expected, the positive control CCCP, a mitochondrial uncoupler, markedly reduced membrane potential [43]. Collectively, these results indicate that

WCSCs increase mtROS without substantially disrupting mitochondrial respiration, ATP production, or membrane integrity.

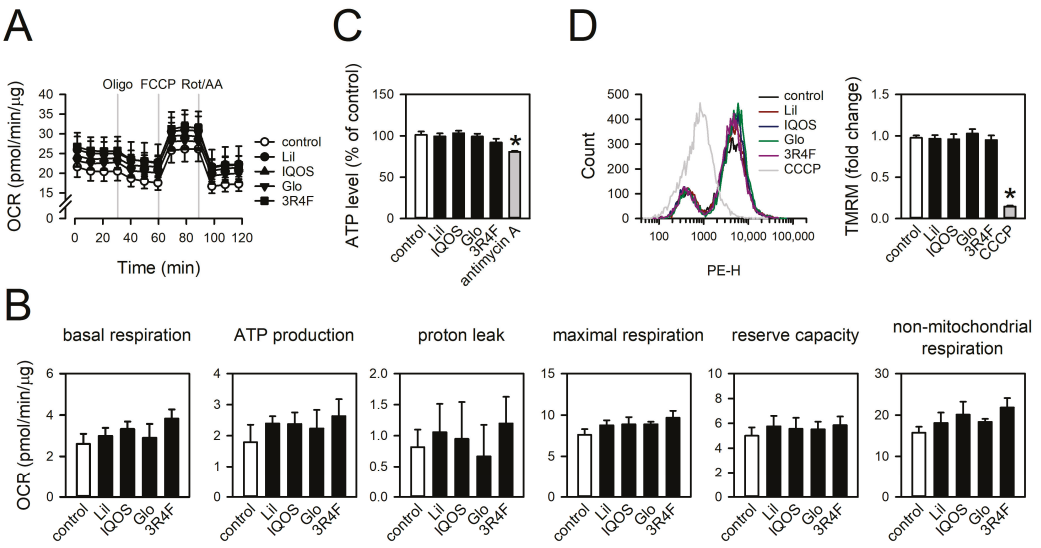

Figure 9. Effects of WCSCs on bioenergetic function, intracellular ATP levels, and mitochondrial membrane potential. Cells were treated with WCSCs for 24 h. (**A**) OCR traces representing bioenergetic functions. Oligo: oligomycin. (**B**) Quantification of mitochondrial respiration parameters, including basal respiration, ATP production, proton leak, maximal respiration, reserve capacity, and non-mitochondrial respiration. (**C**) Intracellular ATP levels normalized to untreated groups. (**D**) Flow cytometry histogram and normalized fluorescence intensity of TMRM-stained cells to assess mitochondrial membrane potential. Values are mean ± standard error [n = 8 for (**A**,**B**); n = 4 for (**C**), except CCCP-treated group (n = 3); and n = 9 for (**D**)]. * $p < 0.05$ vs. control.

4. Discussion

This study investigated the responses of bronchial epithelial cells to emissions from three HTPs (IQOS, Glo, and Lil) as well as the 3R4F reference cigarette, with particular emphasis on transcriptomic alterations. All WCSCs tested induced significant changes in gene expression. Overall, however, these alterations were less pronounced for HTP-WCSCs than for 3R4F-WCSC, and transcriptomic profiles were more similar among HTP-exposed groups than between HTP- and 3R4F-exposed groups (Figures 2 and 3, Table 2). Although HTP-WCSCs affected diverse gene sets associated with distinct cellular functions, several pathways were commonly enriched across the different HTPs (Figures 4 and 5). Among these, the mitochondrial OXPHOS pathway was one of the most consistently and significantly affected (Figure 5, Table 3). Both HTP-WCSCs and 3R4F-WCSC downregulated genes encoding OXPHOS complexes (Figure 6), leading to elevated mtROS with minimal effects on mitochondrial morphology or biogenesis (Figures 7–9). These findings suggest that HTP use, irrespective of product type, can alter transcriptomic profiles in the respiratory epithelium and that mitochondria may represent a common toxicological target.

The present study examined three major HTPs. IQOS and Glo are the most widely marketed and studied globally, whereas Lil, produced by KT&G, is the leading HTP in Korea and is expanding internationally [44–48]. Although nicotine and TPM yields varied slightly among products (Table 1), their cellular effects converged on mitochondrial pathways, specifically OXPHOS gene downregulation and mtROS elevation. This convergence suggests that compositional or quantitative differences in emissions may not directly translate into differential cellular stress, underscoring the importance of overall chemical composition rather than individual constituents.

Determining appropriate exposure concentrations remains a challenge, particularly for complex mixtures such as HTP emissions or cigarette smoke. Nicotine is frequently used as a reference compound to standardize exposure across products, as it is the principal driver of dependence and a consistent marker of smoking intensity [49,50]. In this study, nicotine was used to normalize WCSC exposures across products and to facilitate direct comparison with 3R4F reference cigarettes. Because nicotine is a critical determinant of addiction and dependency, equivalent nicotine consumption was assumed to reflect comparable smoking intensity across product types. According to Health Canada Intense machine-smoking data, the nicotine concentration per puff of a 3R4F reference cigarette is approximately 0.199 mg, corresponding to ~3.62 μg/mL [31–33,51]. On this basis, a concentration of 3 μg nicotine/mL was employed in the present study. Preliminary experiments using lower concentrations (100 ng nicotine/mL WCSCs, reflecting a 1:30 dilution in the lung airways [15,52]) elicited only minimal transcriptomic changes. Thus, 3 μg nicotine/mL likely represents a threshold concentration sufficient to elicit measurable transcriptomic alterations in vitro. This nicotine-based approach offers advantages over TPM-based normalization, as TPM levels vary substantially among products. Notably, the concentration used here is considerably lower than that employed in many prior transcriptomic studies, which often relied on cytotoxic levels without appropriate justification [18,53,54]. While the clinical relevance of this chosen concentration remains uncertain, it is more directly linked to per-puff nicotine levels and therefore provides a more realistic representation of smoker exposure than most previous investigations.

The comparative enrichment analysis further highlights the distinct mitochondrial signature associated with HTP exposure (Figure 5). The OXPHOS pathway was consistently ranked among the top affected pathways in HTP-WCSC–exposed cells but showed a comparatively lower ranking in 3R4F-WCSC–treated cells (Figure 4, Table 3). Suppression of the OXPHOS pathway was stronger and more statistically significant in the HTP-treated groups (NES = −2.18 for Lil, −1.98 for IQOS, and −2.32 for Glo) than in 3R4F-treated cells (NES = −1.67) (Table 3). Indeed, the relative NES difference compared with 3R4F was greater for OXPHOS than for any other pathway (Figure 5). Despite these differences, both HTP- and 3R4F-WCSCs consistently downregulated OXPHOS-related genes and elevated mtROS levels. This lack of clear distinction suggests that even subtle effects of WCSC exposure may be sufficient to disrupt mitochondrial function. Because 3R4F-WCSC caused a broader and more extensive transcriptomic response—altering more than 1000 genes by ≥1.5-fold compared with fewer than 250 genes in each HTP-WCSC group (Table 2)—its impact on OXPHOS may be modulated by other transcriptomic changes. These findings are consistent with earlier transcriptomic studies reporting that HTPs generally induce fewer changes than conventional cigarettes [15,18,53], although direct comparisons remain limited by variability in experimental exposure conditions. Nonetheless, certain cellular processes may be more selectively or extensively affected by HTP exposure.

Cigarette emissions are capable of generating ROS both directly, through chemical interactions with aqueous media [55], and indirectly, by stimulating endogenous cellular sources such as NADPH oxidases [56,57]. Notably, in this study, WCSCs were completely removed after 24 h of exposure, prior to analysis. Thus, the observed elevation of mtROS reflects WCSC-induced genetic alterations rather than the direct chemical activity of WCSCs. Both HTP- and 3R4F-WCSCs suppressed expression of OXPHOS-related genes across multiple complexes, indicating widespread mitochondrial impairment. Interestingly, only mtROS levels were elevated, while mitochondrial morphology, OCR, ATP production, and membrane potential remained largely unaffected. These findings suggest that the exposure conditions employed were insufficient to induce collapse of mitochondrial integrity or overt mitochondrial dysfunction. Instead, mtROS elevation may represent an early biomarker of

mitochondrial perturbation. Further studies are needed to determine whether prolonged or repeated exposures exacerbate these effects and ultimately lead to more pronounced mitochondrial dysfunction.

This study has several limitations. As with most in vitro experiments, reliance on an immortalized cell line limits direct extrapolation to in vivo airway physiology. Nevertheless, such models provide a controlled and practical system for elucidating novel mechanisms, including the mitochondrial effects of HTPs demonstrated herein. Moreover, exposures were conducted over a short-term, 24-h period, whereas chronic or repeated exposures more closely reflect habitual smoking. It remains uncertain, however, whether extended in vitro treatment across multiple passages can adequately model chronic human exposure, given the complex physiological interactions among diverse airway cell types. Finally, although we identified mtROS elevation as a consequence of OXPHOS suppression, downstream toxicological pathways—including redox signaling, antioxidant defenses, and inflammatory responses—were not examined. Future studies should address these aspects to clarify the clinical significance of HTP-induced transcriptomic and mitochondrial alterations.

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Abbreviations

The following abbreviations are used in this manuscript:

HTPs	Heated tobacco products
WCSCs	Whole cigarette smoke condensates
OXPHOS	Oxidative phosphorylation
ROS	Reactive oxygen species
KT&G	Korea tomorrow & global
DHR123	Dihydrorhodamine 123
TMRM	Tetramethylrhodamine methyl ester
Amplex Red	10-Acetyl-3,7-dihydroxyphenoxazine
BCA	Bicinchoninic acid
RPMI	Roswell Park Memorial Institute
FBS	Fetal bovine serum
HBSS	Hank's balanced salt solution
Drp1	Dynamin-related protein 1
Mfn1	Mitofusin-1
Mfn2	Mitofusin-2

HRP	Horseradish peroxidase
CCCP	Carbonyl cyanide m-chlorophenyl hydrazone
RIPA	Radioimmuno-precipitation assay
CSE	Cigarette smoke extract
TPM	Total particulate matter
SDS	Sodium dodecyl sulfate
PCR	Polymerase chain reaction
GSEA	Gene set enrichment analysis
FDR	False discovery rate
NES	Normalized enrichment score
mtROS	Mitochondrial ROS
OCR	Oxygen consumption rate
FCCP	Carbonyl cyanide-4-(trifluoro-methoxy) phenylhydrazone
PCA	Principal component analysis
DEG	Differentially expressed gene
MsigDB	Molecular Signatures Database

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Review

Heated Tobacco Products: Emerging Health Outcomes and Insights into Oxidative Stress and Pro-Inflammatory-Driving Mechanisms

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Abstract

With over 8 million deaths annually, smoking remains one of the most impactful global public health burdens. Heated tobacco products (HTPs) offer smokers the possibility of inhaling nicotine by heating tobacco, resulting in a significant reduction in the levels of toxic and potentially toxic compounds released. However, a growing number of studies indicate that exposure to HTPs increases oxidative stress and triggers pathophysiological changes, mirroring cellular mechanisms well-documented in conventional cigarette smoke exposure. In particular, evidence from animal models suggests that the use of heated tobacco products (HTPs) may represent a risk factor for respiratory and cardiovascular diseases, as well as a potential contributor to reduced male and female fertility. The following review summarizes the current state of knowledge on HTP toxicity, focusing on both preclinical and emerging clinical data on the health impact of HTP use and the putative harm reduction aspects.

Keywords: heated tobacco products; electronic cigarettes; oxidative stress; respiratory system; cardiovascular risk; neurotoxicity; fertility

1. Introduction

Although there has been a significant decrease in the overall tobacco consumption in several countries, smoking still remains one of the main health issues worldwide, making smoking cessation a critical world health priority [1]. While pharmacological and behavioral approaches to smoking cessation show that less than 10% of patients successfully quit smoking for at least 12 months, the launch in the market of Electronic Nicotine Delivery Systems (ENDS) initially diverted many smokers wishing to quit to consider switching to the use of such devices, maintaining the typical gestures associated with traditional cigarettes. Initially, they were advertised as almost risk-free even in the absence of proper scientific evidence-based data [2] and it should not surprising that their popularity has grown dramatically especially among young people [3]. To date, the most widely distributed devices can be divided into two main categories: "liquid e-cigs" (electronic cigarettes; e-cigs) that include an atomizer that vaporizes a liquid composed of vegetable glycerin (VG), polypropylene glycol (PG), with the possible addiction of nicotine and flavorings, and the "Heat-not-Burn systems" (HnB) or "Heated Tobacco

Products" (HTPUSERS), which heat tobacco sticks through contact with an electronic heating metal blade [4]. The latter are of particular interest considering the Food and Drug Administration (FDA) authorized their marketing as Modified-Risk Tobacco Products based on the considerably lower levels of potential carcinogens and toxic chemicals that can harm the respiratory or reproductive systems [5]. It is important to mention here that a considerable number of independent studies confirms that these devices release lower concentrations of toxic compounds; however, here the main question is if that reduction can be sufficient for considering them a valid aid for people who wish to stop smoking, or who are trying to cut down as the UK National Health System (NHS) guidance recommends [5]. Numerous basic studies highlighted how exposure to ENDS mainstream aerosol could be associated with a higher risk for chronic non-communicable diseases, including impacts on the cardiovascular system, airways inflammation, lung damage, impairment of gonadal function, and an increased risk of cancer [6]. At the same time, there is insufficient evidence to support the efficacy of ENDS for smoking cessation [7].

Over the years, nicotine use has declined in U.S. teens as a result of intense tobacco control efforts targeting this age group. However, the recent popularity of e-cigarettes has been complicating efforts to further reduce nicotine use, and vaping use rose dramatically among teens [8]. Data from some observational studies showed how ENDS consumers, especially young vapers, are more likely to become HTP users [9].

Further, it was recently discussed that regardless of youths' intentions, those who used e-cigarettes were 4.6 times more likely to use cigarettes and become tobacco smokers one year later, showing how current e-cigarette users were five times more likely than non-current users to become cigarette users [10,11].

The premise that the introduction of electronic cigarettes would constitute an important tool to promote smoking cessation is not currently supported by concrete evidence. On the other hand, emerging studies are exploring and characterizing the toxicological risks associated with these devices; this aspect becomes even more significant if not counterbalanced by a hypothetical long-term benefit, such as abstaining from tobacco products.

The present review will specifically focus on HTPs, which are among the most rapidly spreading nicotine delivery systems in Western countries, particularly among younger individuals. It will present the most relevant findings concerning their impact on the respiratory, cardiovascular, and reproductive systems, discussing the underlying pathogenic mechanisms reported in the literature, paying particular attention to the pro-oxidant and pro-inflammatory effects associated with HTP use.

2. ENDS Devices as a Harm-Reduction Strategy for Smokers: Their Evolution and Key Regulation Acts in the USA and Europe

This chapter aims to provide an overview of the introduction and development of electronic nicotine delivery systems (ENDS), describing their main functional characteristics and highlighting the key milestones that have shaped their current regulatory status and commercial use. Although the present manuscript focuses specifically on heated tobacco products (HTPs), it is important to offer the reader a broader perspective on the evolution of these devices in order to facilitate a clearer understanding of the current state of the art.

The term "electronic cigarette" and its related nicknames (e-cigarettes, e-cigs, vape pens) are beginning to spread and attract the attention of smokers or former smokers who see these new devices as a way to consume nicotine, maintaining the typical gestures associated with traditional cigarettes, but without the well-known health risks associated with tobacco product consumption. In this regard, the advertising campaigns that accompanied the launch of these products have certainly played a key role in the success of these devices. E-cigarettes were initially promoted as a risk-free aid to quit smoking. In the following

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years, some studies reported no clear evidence that e-cigarettes are an effective smoking cessation aid, while a growing and convincing evidence suggests that they can act as a gateway to cigarette smoking [12]. Alongside the increasingly widespread distribution of these products, they are beginning to undergo a true evolution in order to offer the consumer a more varied vaping experience, capable of attracting an ever-growing number of people, including younger individuals.

E-cig devices are mainly composed of a battery, an e-liquid mixture cartridge, and an electronic heating atomizer equipped with a coil to vaporize the e-liquid mixture to create an aerosol. The e-liquid typically contains propylene glycol (PG), vegetable glycerin (VG), nicotine, flavors, and other additives [4]. Although the main technical features have remained largely unchained over the time, the market witnessed a marked change, moving from the first generation devices, designed to mimic the appearance of combustible tobacco cigarettes, often called "cig-a-like", to the second generation characterized by a much-larger-capacity battery and cartridge than the first generation, until reaching a third generation of devices that shares no commonality with conventional cigarettes in appearance and, most importantly, allowing the users to adjust voltage, atomizer power, and the resistance of the coil in terms of Ohm. Voltage and the resistance adjustment applied to the atomizer, as well as customization of the liquid composition by modifying the ratio of VG and PG, enable a high degree of personalization of the vaping experience; however, it also results in considerable variability in the concentrations of emitted toxicants, and consequently in the overall health impact [4].

The newest generation of e-cigarettes is a compact capsule-like device, also known as "Pod Mods", which typically utilizes nicotine salts rather than free-base nicotine as in the previous generations. Next to these products, electronic devices have been developed to heat tobacco via conduction, allowing the user to inhale vapors produced by heating rather than by combustion. These brand-new devices were successfully distributed under the name of heated tobacco products (HTPs) or "heat-not-burn" e-cigarettes. With the possibility to fully release the active substances in tobacco by heating it instead, HTPs not only meet people's demand for tobacco products, but they are also strongly advertised as lower-risk products [13]. Although HTPs are certainly influenced by factors such as the specific processing of the tobacco used and the presence or absence of flavorings, the number of variables involved in HTP systems is significantly lower compared to e-cigs, enhancing the reproducibility of specific datasets.

Figure 1 summarizes the main differences between HTPs and liquid e-cigs.

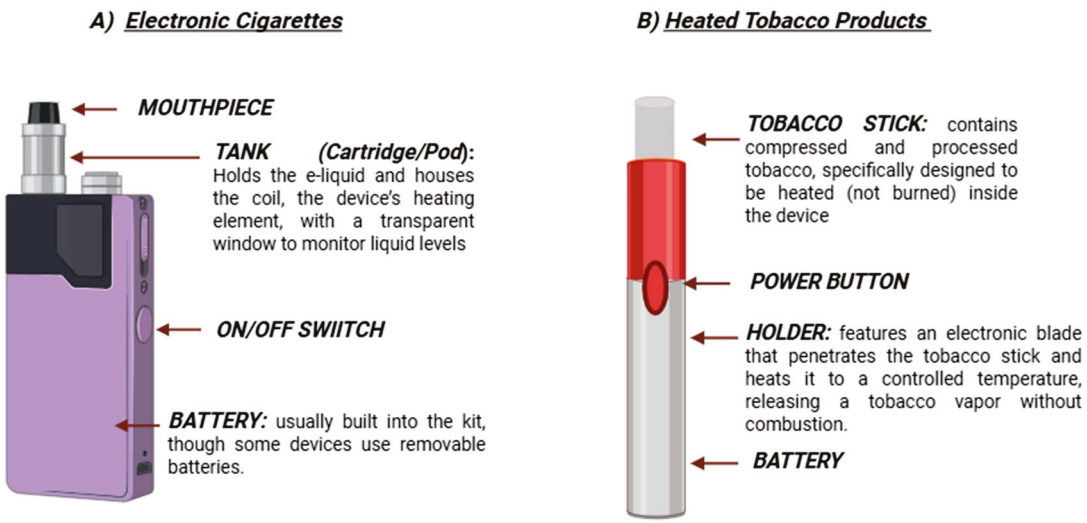

Figure 1. Schematic representation of e-cigs and HTPs.

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Comparison between electronic cigarettes (A) and heated tobacco products (B). Electronic cigarettes consist of a tank (cartridge/pod) that contains e-liquid and a heating coil, powered by an integrated or removable battery, to generate an aerosol inhaled through the mouthpiece. Heated tobacco products, instead, employ specially designed tobacco sticks containing processed tobacco, which are heated by an electronic blade within the holder to a controlled temperature, releasing a nicotine-containing aerosol without combustion. These differences underline their distinct modes of operation and contribute to the toxicity associated with their use. Created in BioRender. Morosini, C. (2025) https://BioRender.com/cfts9hp (Academic License).

From the point of view of the key regulations on the US market, it is possible to outline the main regulatory stages as follows:

- In 2008, the FDA claimed these products fell under its jurisdiction and required their approval before they could be sold.
- In 2011, the FDA drafted a guidance document asking any company planning to, or already having, brought e-cigarettes to market to submit documentation of substantial equivalence if the product was similar enough to a tobacco product already on the market, or a premarket tobacco product application (PMTA) if the product was substantially different.
- With the rapid growth of the electronic cigarette market and under the pressure from the scientific community, in 2016 the FDA published a new guidance known as Deeming Rule imposing additional restrictions on the sale of tobacco products by mandating age verification (including online), while also requiring manufacturers to register new products, submit PMTAs, disclose ingredients, and include health warnings on the packaging. In the same year, Philip Morris International (PMI) submitted an application to the FDA to approve the marketing of its new HTPs as a "Modified Risk Tobacco Product" (MRTP).
- In 2017, the FDA delayed the PMTA submission deadline until 2022. Already dealing with the rising youth vaping crisis and record numbers of adolescent vapers, the American Academy of Pediatrics put pressure on the FDA and suggested a ban on flavored products (except for tobacco, menthol, and mint).
- In 2018, the number of hospitalizations due to e-cigarette- or vaping-associated lung injury (EVALI) increased [14], and a new FDA guidance on e-cigarette flavors was introduced: the guidance emphasized prioritizing the ban on flavored cartridges or pod-based e-cigarettes to reduce their appeal to minors, though several exceptions were made (e.g., tobacco- and menthol-flavored pod-based e-cigarettes, flavored disposable e-cigarettes, and flavored e-liquids for refillable e-cigarettes). As a result, many flavored e-cigarette products remain available, contributing to a surge in disposable e-cigarette use among middle and high school students from 2019 to 2020 [15].
- On 7 July 2020, the FDA partially authorized PMI's MRTP application. While it concluded that the data submitted showed that the device may reduce the release of harmful substances, it did not agree that it reduces the risk of disease and death, compared to smoking cigarettes, and so had failed to meet the higher standard of "risk modification". European countries lay down rules for electronic cigarettes sold as consumer products by Article 20 of the Tobacco Products Directive (2014/40/EU). The Directive sets a maximum nicotine concentration and volume for cartridges, tanks, and nicotine liquid containers. E-cigarettes should be child-resistant and tamper-evident and have a mechanism that allows refilling without spillage to protect consumers. E-cigarette ingredients must be of high purity, and e-cigarettes should deliver the same amount of nicotine when puffed at the same strength and duration. Health warnings for e-cigarettes advising consumers that they contain nicotine and should not be used

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by non-smokers are mandatory. Packaging must also include a list of ingredients contained in the product, information on the product's nicotine content, and a leaflet with instructions for use and information on adverse effects, risk groups, addictiveness, and toxicity. Promotional elements are not allowed on e-cigarette packaging, and cross-border advertising and promotion of e-cigarettes is prohibited.

The phenomenon of novel ENDS devices has evolved very rapidly, leaving regulatory stakeholders with limited time to define and manage their use. This has often led to confusion, particularly regarding the terminology used to refer to the different types of electronic devices. In the present review, the term *ENDS* will be used as a general descriptor for any electronic device available for purchase in retail outlets specializing in tobacco products, thus excluding all medical-grade electronic devices [9].

This work will specifically focus on modified-risk tobacco products (MRTPs) equipped with heat-not-burn (HnB) technology, which will hereafter be referred to as heated tobacco products (HTPs). It is important to emphasize, for the sake of clarity, that the data presented herein refer exclusively to HTPs and should not be extrapolated to other devices, even those with seemingly similar mechanisms of action [15].

The specific characteristics of each product category (e.g., liquid-based electronic cigarettes vs. HTPs) influence not only the user's vaping experience but also the observed outcomes in both experimental models and clinical settings. For this reason, any comparative extrapolation must be approached with the utmost caution.

Overview of a Heated Tobacco Product (HTPs) and "Liquid" Electronic Cigarette (E-Cigs)

HTPs are the last electronic cigarette model launched on the market; they use an external heat source to heat the tobacco at a temperature typically below 350 °C to release a flavor without tobacco combustion. HTPs are operated by a battery-powered heating device with a heating blade that is inserted into a stick containing reconstituted tobacco (HTP sticks). Alternatively, the tobacco stick can be heated from the outside through a cylindrical heated chamber [16]. HTP sticks come in different flavors (e.g., menthol, tobacco, chocolate, berry, citrus), as do e-cig liquids. Heating versus combustion is the basic operational difference between HTPs and traditional tobacco products. In 2022, global "liquid" e-cigarette sales reached 18.85 billion US dollars, while HTPs reached 32.38 billion US dollars, showing a more significant market share increase for HTPs compared to the older models [16,17].

The tobacco industry claims that due to this operational difference, HTP aerosol contains fewer toxic chemicals compared to traditional cigarette smoke and, hence, it is a safer alternative to conventional smoking. To date, several independent studies have confirmed the significant reduction in numerous harmful and potentially harmful substances. Table 1 reports a comparative range of harmful and potentially harmful constituents recorded in mainstream heated tobacco products and the smoke of traditional combustible cigarettes.

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Table 1. Comparative review of the main HPHCs reported in the literature for HTP mainstream and traditional cigarette (TC) smoke.

HPHC	Mainstream HTP	TC Smoke
Nicotine (mg)	0.3–1.5	0.7–2.1
Formaldehyde (μg)	0.9–22.6	3.2–74.4
Acetaldehyde (μg)	128.5–301. 5	567.0–1534
Acetone (μg)	18.8–48.37	95.5–775.6
Acrolein (μg)	0.9–13.1	1.1–160.9
Propionaldehyde (μg)	7.8–22.3	29.6–124.0
Crotonaldehyde (μg)	0.7–6.4	10.10–65.7
Methacrolein (μg)	6.5	85.5
Butyraldehyde (μg)	14.9–30.7	22.2–65.0
Valeraldehyde (μg)	20.1	-
Glyoxal (μg)	3.1	-
Methyl glyoxal (μg)	33.5	-
2-Butanone (μg)	4.2–6.5	11.0–220.5
Polycyclic aromatic hydrocarbon (PAH)		
Benzo(a)pyrene (ng)	−0.8	6.7–20
Naphthalene	1.6	1105
Acenaphthylene (ng)	1.9	235
Acenaphthene (ng)	145	49
Fluorene (ng)	1.5	371
Anthracene (ng)	0.3	130
Phenanthrene (ng)	2.0	292
Fluoranthene (ng)	7.3	123
Pyrene (ng)	6.4	89
Benz[a]anthracene (ng)	1.8	33
Chrysene (ng)	1.5	48
Benzo[b]fluoranthene(ng)	0.5	24
Benzo[k]fluoranthene(ng)	0.4	4.3
Total particulate matter	12.9–55.8	9.8–37.7
Tar	7.5–16.6	8.0–25.50
Carbon monoxide	0.3–0.5	2–33.0

Range of harmful and potentially harmful constituents (HPHC) per stick or tobacco cigarette are reported for heated tobacco product mainstream (HTP) and traditional tobacco cigarette (TC smoke). The value ranges derived from the following studies: [16,18–25]. The puffing regimen used in the different studies is summarized below: International Organization for Standardization—ISO 3308:2000 (ISO): puff volume: 35 mL; duration: 2 s; interval: 60 s; six puffs per HTP stick; six puffs per TC. Health Canada Intense puffing regimens—T-115 (HCI): puff volume: 55 mL; duration: 2 s; interval: 30 s; 12 puffs per HTP stick; 8–12 puffs per TC. Cooperation Center for Scientific Research Relative to Tobacco—N°64 (CORESTA): puff volume: 75 mL; duration: 2.5 s; interval: 30 s; 7–12 puffs per HTP stick; 11 puffs per TC. The study by Auer et al. followed International Organization for Standardization standards for puff volume (35 mL) at 2 puffs per minute, based on observation of HTP users who took a mean of 14 puffs during 5 to 6 min. (-). The symbol indicates that the marker was not reported or below the detection level. TC smoke data were obtained from reference-grade (3R4F, 1R6F) cigarettes or Marlboro Red cigarettes.

The above-mentioned studies highlight several crucial aspects, although a significant reduction in the hazardousness of HPHC, but at the same time, some harmful constituents of tobacco products derived from elements from pyrolysis and incomplete combustion of the tobacco, such as acetaldehyde, benzo[a]pyrene (BaP), and carbon monoxide, are still present in the HTP mainstream. Some authors consider these elements to be sufficient to characterize the HTP mainstream aerosol as "smoke" [24]. Data confirm the presence of known carcinogens such as BaP, even though in concentrations significantly lower compared to those typically observed in traditional cigarette smoke, and it represents a key point since HTPs do not fall under the "smoking products" category, and in some countries, they are not subject to the current regulations regarding the use of cigarettes in public places. However, principle 1 for implementing article 8 of the World Health

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Organization convention on tobacco control highlights that we should reject ideas that there is a threshold value for toxic effects from second-hand smoke [26].

Again, as effectively summarized by the studies of St Helen et al., often chemical characterization studies on HTP mainstream do not cover the entire list of HPHCs of the US Food and Drug Administration. More in-depth studies on the topic show that among the not reported compounds, some of them are at least two-fold higher compared to TC smoke. Table 2 reports reviewed data from the literature, and they indicate that beyond doubt, HTPs release lower concentrations of toxicants, but at the same time they expose users to comparable or even higher levels of certain products [27].

Table 2. Approximate fold increase in HPHCs in HTP compared to 3R4F reference cigarette smoke (CS).

HPHC	Fold Increment Compared to 3R4F Smoke
1,4-Dioxane, 2-ethyl-5-methyl-	137
Hexadecanoic acid, ethyl ester	60
Trans-4-hydroxymethyl-2-methyl-1,3- dioxolane	47
Stearate, ethyl-	24
12,14-Labdadiene-7,8-diol, (8a,12E)	21
Butylated hydroxytoluene	18
Ethyl linoleate	16
Labdane-8,15-diol, (13S)	9
Propylene glycol	6
2-Furanmethanol	4
Butyrolactone	5
Methyl furoate	4
2-Cyclopentene-1,4-dione	4
2-Furanmethanol, 5-methyl-	3
2-Cyclopentene-1,4-dione	3
2-Methylcyclobutane-1,3-dione	3
Lanost-8-en-3-ol, 24-methylene-, (3beta)	3
2-Furancarboxaldehyde, 5-methyl-	3
Eicosane, 2-methyl-	3
1,2,3-Propanetriol, diacetate (diacetin)	2
Glycidol	2
Heneicosane, 2-methyl-	2

Data adapted from St Helen et al., 2018, and Upadhyay et al., 2023 [25,28]. The present list represents an extract of HPHCs recording to be more than 2-fold higher in HTP streaming compared to TC smoke.

Alongside the carcinogen compounds such as polycyclic aromatic hydrocarbons (PAHs), aromatic amines, tobacco-specific nitrosamines, and phenolic products, the presence of tar also contributes to several oxidative agents through redox-cycling reactions, when its constituents encounter molecular oxygen in the human lungs. The by-products of redox cycling in tar, such as free radicals and other oxidative agents, are linked to cancer development and increased inflammatory response. In fact, for decades, the scientific community has spread the point that "patients smoke for nicotine and die for tar" [28]. The tar phase contains several relatively stable free radicals, such as a quinone/hydroquinone (Q/QH_2) complex held in the tarry matrix. This Q/QH_2 polymer may function as an active redox system by reducing molecular oxygen in the smokers' lungs to produce $O_2^{\bullet -}$, which can eventually form other free radicals, such as H_2O_2 and $^{\bullet}OH$. The generated reactive oxygen species (ROS) fuel a vicious cycle in which smoking-related oxidative stress causes inflammation, which in turn, results in further generation of ROS, and potentially increases oxidative damage to macromolecules that may trigger carcinogenesis. The aim of this work is to summarize the current knowledge on the use of HTPs and their role as risk factors

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for oxidative stress and inflammation, as well as the impact of this phenomenon at the systemic level.

3. The Impact of HTP Consumption on the Respiratory Cardiovascular System Its Role in Airway Disease

Since the consumption of traditional tobacco products predominantly impacts the respiratory and vascular systems, this area of research has been the most extensively investigated in the international literature. Below, we summarize the most significant findings obtained through both in vitro and in vivo models, and a dedicated section will address the available clinical data. The aim of this chapter is to provide the reader with a comprehensive overview of preclinical findings, highlighting those that have been corroborated by clinical evidence.

3.1. Role of HTP Use on Airway Diseases: Results from Preclinical Studies

Increased oxidative stress induced by tobacco smoking is a well-recognized key driving mechanism involved in several respiratory diseases, including chronic obstructive pulmonary disease (COPD) and its comorbidities as asthma, cystic fibrosis, bronchiectasis, idiopathic pulmonary fibrosis (IPF), and pulmonary hypertension [29]. One of the first in vitro pilot studies showed how bronchial epithelial cells directly exposed to HTP mainstream were found to exhibit higher cytotoxicity and a lower metabolic activation compared to controls, although changes were milder compared to TC smoke exposure. Markers of inflammatory response were also measured, and interestingly, cytokine IL-1β and IL-6 were overexpressed in cells exposed to HTP mainstream, as is usually observed in smoking-induced diseases where oxidants trigger inflammation through the activation of damage-associated molecular patterns (DAMPs). Both HTP and TC exposure increased the binding activity of Nrf2 to antioxidant response elements and the expression of its downstream targets. Concordantly, HTP use induced oxidative DNA damage and significantly increased DNA strand breaks and chromosomal aberrations, eliciting a biological response very similar to that of TC in human epithelial pulmonary cells [30].

Further studies have explored in vivo the effects of HTP mainstream, confirming the increase in oxidative stress and the induction of the secretion of inflammatory cytokines, first in short-term exposure models [31] and then in sub-chronic exposure. Studies reported higher ROS concentration in lung biopsies, the rise of the main DNA, protein, and lipid oxidative damage markers, and again the activation of the downstream cytokines through MAPK and NF-κB pathways [32]. Interestingly, images from electronic microscopy showed that the lung parenchyma was markedly disrupted with granulocyte pulmonary infiltration and higher circulating blood neutrophils, typically observed in patients with stable COPD.

Focusing on glutathione, a well-known indicator of oxidative stress on lung tissue and alveolar macrophages, some studies reported that many macrophages had intracellular foaming, likely due to their activation following the HTP exposure. This observation was very similar to the lipid accumulation in alveolar macrophages observed in mice exposed to tobacco smoke, as well as e-cigarette mainstream aerosol [33]. A disruption of the homeostasis of lipid metabolism, such as lung surfactants, was observed, resulting in lung inflammation and immune system compromise, and similar effects are triggered by HTP exposure [31]. The effects of increased oxidative stress stimulate the production of inflammatory cytokines. Similar to what is observed in human alveolar and tracheal epithelial cells exposed to combustible cigarette extracts, data from cellular and in vivo models confirm the increased expression of the GM-CSF gene and macrophage inflammatory protein CCL3. This, in turn, can stimulate the alveolar macrophages to secrete pro-inflammatory cytokines and may be associated with COPD progression. Additionally,

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the up-regulation of CCL3 facilitates the recruitment of macrophages into the airways [32]. These data suggest that HTP use may trigger a mechanism whereby increased oxidative stress and the release of pro-inflammatory factors result in tissue damage, which can significantly contribute to the exacerbation of respiratory diseases such as COPD [34].

Comparing the effect of short-term exposure to HTPs versus TC smoke in mice, some results indicate that the total number of lung-infiltrating leukocytes was equivalent after exposure to both HTP aerosol or CS smoke, but it was significantly increased compared to air-exposed controls. Moreover, a significant increase in $CD4^+ROR\gamma t^+$ T cells was observed in both groups. The RORγt receptor plays a critical role in the differentiation of Th17 lymphocytes and is implicated in the pathogenesis of autoimmune disorders and inflammatory responses. These findings are consistent with those previously described, leading to the conclusion that both HTPs and conventional cigarettes induce comparable lung damage and pro-inflammatory alterations [35]. Supporting these findings, the increase in CXCL8 chemokine from human bronchial epithelial cells and primary human airway smooth muscle cells following HTPs, liquid e-cigarette or CSs, and the increased release of collagen 1 and fibronectin strengthen the hypothesis that e-cigarettes and HTPs, similar to classic cigarettes, can increase oxidative stress, inflammation, and airway reconstruction [36].

In contrast, the study by Wong et al. reported an accumulation of macrophages, neutrophils, and $CD4^+$ and $CD8^+$ lymphocytes in the lungs, along with significantly elevated levels of inflammatory mediators in the bronchoalveolar lavage fluid (BALF) of mice exposed to conventional cigarette smoke, but not in those exposed to HTP aerosols. Similarly, impaired lung function and emphysematous alterations were observed exclusively in the CS-exposed group [37].

3.2. Influence of Heated Tobacco Product (HTP) Exposure on Cardiovascular Function: Results from Preclinical Studies

The link between smoking and cardiovascular disease is well established; endothelial dysfunction is a critical indicator of cardiovascular damage and is considered an early predictor of cardiovascular events and prognosis in smokers. The pathophysiological processes driving smoking-induced endothelial dysfunction (SIED) are complex, as summarized in Figure 2. A pivotal role is played by oxidative stress, mainly generated by the accumulation of ROS, which diminishes the bioavailability of nitric oxide (NO). Interestingly, in smoking conditions, ROS comes from both the inhaled fraction and from endogenous cellular mechanisms. One of the main ROS-generating cellular pathways involves nicotinamide adenine dinucleotide phosphate oxidase (NOX), which transfers electrons from cytoplasmic NADPH to molecular oxygen, increasing the formation of O_2^- and H_2O_2, and reducing the nitric oxide (NO) reservoir, creating a state of oxidative stress. Studies on tobacco products indicate that smoke enhances the recruitment and activation of leukocytes by increasing the expression of adhesion factors such as ICAM-1, VCAM-1, and E-selectin in endothelial cells [38]. Furthermore, exposure to foreign toxicants such as those present in CS or HTP mainstream can trigger the secretion of various immune mediators, including cytokines from the pulmonary compartment, inducing a condition of systemic inflammation, thereby affecting the circulatory system and contributing to an increased risk of atherosclerosis [39]. Some evidence seems to confirm the pro-oxidative and pro-inflammatory effects of HTP exposure, in particular, an in vitro 3D cardiovascular co-culture model.

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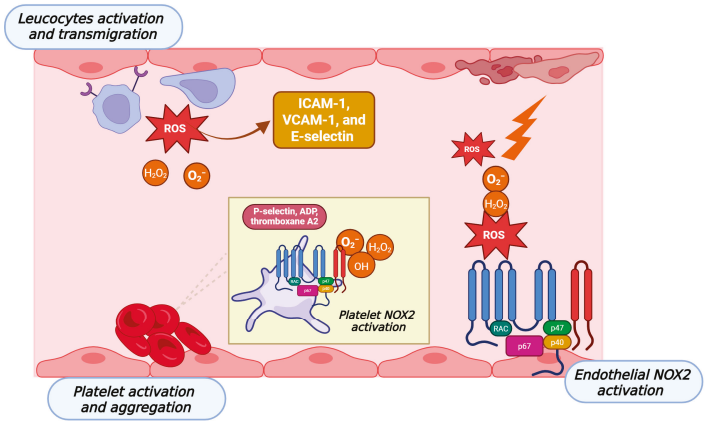

Figure 2. Shared redox-dependent mechanisms of smoking-induced endothelial dysfunction. The figure depicts the main pathophysiological processes driving smoking-induced endothelial dysfunction that are shared by conventional tobacco cigarettes, electronic cigarettes, and heated tobacco products. Exposure to smoke or aerosol increases reactive oxygen species (ROS) levels through both inhaled oxidants and enhanced endogenous production, primarily mediated by NADPH oxidases (NOX), including NOX2 in platelets and endothelial cells. The resulting redox imbalance, characterized by increased superoxide and hydrogen peroxide, promotes oxidative stress and reduces nitric oxide bioavailability. ROS-dependent endothelial activation induces the expression of adhesion molecules (ICAM-1, VCAM-1, and E-selectin), facilitating leukocyte activation and transmigration. In parallel, platelet NOX2 activation enhances platelet activation and aggregation through the release of pro-thrombotic mediators such as P-selectin, ADP, and thromboxane A_2. Together, these oxidative and inflammatory pathways contribute to endothelial dysfunction, vascular inflammation, and thrombus formation, representing a common cardiovascular mechanism across different smoking and vaping products. Created in BioRender. Morosini, C. (2026) https://BioRender.com/9f8dafr (Academic Lincense).

It was shown that HTPs, although to a significantly lesser extent compared to CS, lead to a glutathione depletion and an increase in inflammatory markers, along with higher monocyte adhesion following exposure. These results suggest a putative increase in cardiovascular risk through a pathway of oxidative stress, inflammation, and endothelial dysfunction [40]. More recently, in an in vitro model of monocyte adhesion using a vasculature-on-a-chip platform mimicking the tissue-resident macrophage-mediated endothelial activation, the authors showed how HTP exposure increased monocyte adhesion and the release of TNF-α and IL-1β due to the pro-inflammatory cytokine secretion from macrophages, indicating a risk of atherosclerosis, which, nonetheless, was found to be markedly reduced in comparison to exposure to CS [41].

Since phosphorylation of eNOS at Ser1177 positively regulates endothelial nitric oxide synthase (eNOS) activity and NO generation, the impact of HTPs on eNOS phosphorylation was explored. Some HTP tests disrupt eNOS and NO content, indicating that HTP consumption, mirroring the effect of CS, can increase the risk for endothelial dysfunction [42].

Some animal models on short-term or chronic exposure to HTPs showed alterations in endothelial function similar to those induced by traditional cigarette smoke. A single exposure to HTPs can rapidly impair endothelial function in rats comparably to smoke from a cigarette [43].

The preclinical data and the results from clinical trials available to date, despite the discrepancies among studies, seem to indicate that a complete switch from CS to HTPs confers benefits in terms of reducing markers of inflammation and oxidative stress, as well as improving key clinical parameters associated with cardiovascular risk. A key challenge lies in accurately assessing the risk associated with new or emerging HTP consumers. While studies clearly indicate a reduction in harmful and potentially harmful constituents

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(HPHCs) in the aerosol emitted by HTPs, emerging factors with potential toxicological relevance further complicate the picture—such as the presence of toxic metals released due to device wear and degradation. A recent review reveals that nickel and cadmium may contribute to oxidative stress, promoting DNA damage and contributing to the impairment of the endothelial cells' function, along with interference with the ion balance affecting vascular tone and cardiac contraction [4]. Currently, for these reasons, and in the absence of long-term clinical data, all major heart international societies do not recommend the use of electronic or heated tobacco products as a means to quit smoking.

3.3. Data from Clinical Studies on HTP Exposure and Biological Effects: Biomarkers Associated with Cardiovascular and Respiratory Function

In this paragraph, the major clinical studies that have evaluated established exposure biomarkers will be analyzed in order to assess the role of HTPs in potential risk reduction compared to the use of combustible tobacco products.

To date, clinical trials aimed at investigating the impact of HTPs on human health are still scant, and for ethical reasons, mainly only cohorts of patients switching from the use of traditional tobacco products to HTP or HTP native users can be considered.

Table 3 provides the main clinical trials focusing on exposure and biological effects biomarkers related to oxygen delivery, genotoxicity, inflammation, oxidative stress, lipid metabolism, endothelial function, platelet activation, and cardiovascular and respiratory function.

Table 3. Key evidence from clinical studies on HTP use.

Authors	Nicotine Delivery System	Study Design	Intervention Period	Key Findings	Main Redox Biomarkers
Polosa et al., 2021 [44]	Switch from CS to HTPs	COS	3 years	↓ COPD exacerbations ↑ CAT score ↑ 6 MWD	carbon monoxide carboxyhemoglobin
Roethig et al., 2005 [45]	Switch from CS to HTPs	RCT	8 days	↓ Urine mutagenicity	↓ carbon monoxide ↓ carboxyhemoglobin
Roethig et al., 2007 [46]	Switch from CS to HTPs	RCT	8 days	↓ Urine mutagenicity	↓ carbon monoxide ↓ carboxyhemoglobin
Tricker et al., 2012 [47]	Switch from CS to HTPs vs. CS	RCT	6 days	↓ Excretion of mutagenic material in urine	↓ carbon monoxide ↓ carboxyhemoglobin
Tricker et al., 2012 [48]	Switch from CS to HTPs vs. CS	RCT	8 days		
Martin Leroy et al., 2012 [49]	Switch from CS to HTPs vs. CS	RCT	35 days	↓ WBC count ↓ 11-DTXB_2	↓ carboxyhemoglobin ↓ *hs*-CRP
Sakaguchi et al., 2014 [50]	Switch from CS to HTPs	RCT	28 days	↓ Urine mutagenicity	- carboxyhemoglobin
Shepperd et al., 2015 [51]	Switch from CS to reduced-toxicant-prototype cigarette (RTP)	RCT	6 months	↓ Urine mutagenicity ↓ 2-cyanoethylvaline hemoglobin adducts - 4-ABP hemoglobin adducts ↓ 11-DTXB_2 ↑ s-ICAM-1	- 8-iso-PGF2α type III
Ogden et al., 2015 [52]	Switch from CS to HTPs	RCT	24 weeks	↓ 4-ABP hemoglobin adducts	- carboxyhemoglobin ↑ 3-HPMA
Haziza et al., 2016 [53]	Switch from CS to HTPs vs. CS	RCT	5 days	↓ S-PMA ↓ MHBMA ↓ 3-OH-B[a]P ↓ CYP1A2 activity	↓ carboxyhemoglobin ↓ 3-HPMA

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Table 3. *Cont.*

Authors	Nicotine Delivery System	Study Design	Intervention Period	Key Findings	Main Redox Biomarkers
Haziza et al., 2017 [54]	Switch from CS to HTPs vs. CS	RCT	5 days	↓ S-PMA ↓ MHBMA ↓ 3-OH-B[a]P ↓ 4-ABP hemoglobin adducts	↓ carboxyhemoglobin ↓ 3-HPMA
Ludicke et al., 2017 [55]	Switch from CS to HTPs vs. CS	RCT	5 days	↓ S-PMA ↓ MHBMA ↓ 3-OH-B[a]P ↓ 4-ABP hemoglobin adducts	↓ carboxyhemoglobin ↓ 3-HPMA
Ludicke et al., 2018 [56]	Switch from CS to HTPs vs. CS	RCT	85 days	↓ sICAM-1 ↓ 11-DTX-B2 ↑ FEV_1 ↓ hs-CRP ↓ WBC count	↓ 8-epi-PGF2α
Gale et al., 2019 [57]	Switch from CS to HTPs vs. CS	RCT	6–7 days	↓ S-PMA ↓ MHBMA ↓ 4-ABP hemoglobin adducts	↓ 3-HPMA
Ludicke et al., 2019 [58]	Switch from CS to HTPs vs. CS	RCT	6 months	↓ WBC count ↑ FEV_1 ↑ HDL-c	↓ carboxyhemoglobin -8-epi-PGF2α
Haziza et al., 2020 [59]	Switch from CS to HTPs vs. CS	RCT	90 days	-Mutagenicity (Ames test) ↓ CYP1A2 activity ↓ S-PMA ↓ MHBMA ↓ 4-ABP hemoglobin adducts	↓ carboxyhemoglobin ↓ 3-HPMA
Loffredo et al., 2021 [60]	Switch from CS to HTPs vs. CS	RCT	3 years	-PA -sCD40L -sP-selectin -FMD -NO	↑ -hydrogen peroxide ↓ sNox2-dp
Sakaguci et al., 2021 [61]	Switch from CS to HTPs vs. CS	COS	28 days	↑ HDL-c ↓ sICAM-1 ↓ WBC count ↓ 11-DTX-B2 - FEV_1	↓ 8-epi-PGF2α
Ikonomidis et al., 2021 [62]	Switch from CS to HTPs vs. CS	COS	1 month	↓ PWV ↓ brachial systolic ↓ blood pressure ↓ heart rate ↑ FMD ↑ CFR ↑ GWI ↑ GWW ↓ GWE	↓ MDA ↓ TxB2 ↓ PC ↓ exhaled carbon monoxide
Gale et al., 2021 [63]	Switch from CS to HTPs vs. CS	RCT	6 months	↓ WBC count ↑ FeNO ↑ HDL ↓ 11-DTX-B2 ↓ sICAM-1 ↑ FEV_1	↓ 8-epi-PGF2α
Ohmomo et al., 2021 [64]	Switch from CS to HTPs vs. CS	COS	<2 years	↓ hypomethyleation of *AHRR*, *F2RL3*, and *RARA* genes ↓ *AHRR* gene expression	
Gale et al., 2022 [65]	Switch from CS to HTPs vs. CS	RCT	1 year	↓ MHBMA ↓ S-PMA ↓ 4-ABP hemoglobin adducts ↑ FeNO ↓ WBC count ↓ 11-DTX-B2 ↑ HDL-c	↓ 3-HPMA -8-epi-PGF2α ↓ exhaled carbon monoxide

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Table 3. *Cont.*

Authors	Nicotine Delivery System	Study Design	Intervention Period	Key Findings	Main Redox Biomarkers
Nishihara et al., 2023 [66]	Switch from CS to HTPs vs. CS	RCT	5 days	↓ C_{max} ↓ $AUC_{0-tlast}$ ↓ mCEQ subscale of "Smoking Satisfaction"	
Li et al., 2023 [67]	Switch from CS to HTPs vs. CS	RCT	28 days	↓ VOCs urine concentration	↓ 3-HPMA
Harada et al., 2024 [68]	Switch from CS to HTPs vs. CS	COS	3 years	-glutamate metabolism	
Spicuzza et al., 2024 [69]	Switch from CS to HTPs vs. CS	RCT	12 weeks	↑ V̇O2max maximal oxygen consumption	↓ carboxyhemoglobin ↓ exhaled carbon monoxide
Ansari et al., 2024 [70]	Switch from CS to HTPs vs. CS	RCT	12 months	↑ HDL-c ↓ 11-DTX-B2 ↓ sICAM-1 ↑ FEV_1 ↓ WBC count	↓ 8-epi-PGF2α ↓ myeloperoxidase ↓ *hs*-CRP
Lyytinen et al., 2024 [71]	HTP users vs. non-exposed subjects	COS	1 day	↑ PWV ↑ AIx75 ↑ platelet-dependent thrombus formation	
Chu et al., 2025 [72]	Switch from CS to HTPs vs. CS	RCT	21 days	↓ MHBMA ↓ CEMA	↓ 3-HPMA ↓ exhaled carbon monoxide

The studies summarized in the table represent the principal clinical trials retrieved using the following search string within the PubMed database: ["heated tobacco" OR "heat-not-burn" OR "tobacco heating" OR "unburned cigarette" OR "IQOS" OR "Ploom" OR "glo") AND "biomarker" OR "trial"]. For the purposes of the present review, we included studies that investigated biomarkers of exposure and biological effects associated with the use of heated tobacco products (HTPs). Studies specifically addressing smoking cessation were not considered in this discovery. Upward and downward arrows indicate an increase or a decrease in the biomarker, respectively, whereas a dash indicates that no significant change was observed. The following acronyms are used in order of appearance COS: Cohort study; RCS: Randomized controlled study; CAT score: changes in daily cigarette smoking, annualized disease exacerbations, lung function indices, patient-reported outcomes; 6 MWD: 6 min walk distance; hs-CRP: high sensitivity C-reactive protein; 11-$DTXB_2$: 11-dehydro-thromboxane B2; 8-iso-PGF2α: 8-iso Prostaglandin F2α; s-ICAM-1: soluble intercellular adhesion molecule 1; N-acetyl-S-3-hydroxypropylcysteine; S-PMA: S-phenylmercapturic acid; MHBMA: monohydroxybutyl mercapturic acid; FEV_1: forced expiratory volume in 1 s; sNox2-dp:soluble Nox2-derived peptide; PA: plaquet-activated; sCD40L:soluble CD40 ligand; FMD: flow-mediated dilation; VOCs: volatile organic compounds; PWV: Carotid-femoral pulse wave velocity; CFR: coronary flow reserve; PC: protein carbonyls; GWI: myocardial work index; GWW: global wasted myocardial work; GWE: global myocardial work efficiency; FeNO: fractional exhaled nitric oxide; VO2max: maximum oxygen level consumed; AIx75: augmentation index corrected for heart rate; CEMA: 2-cyanoethylmercapturic acid.

The majority of currently available clinical studies suggest that HTPs may play a role in harm reduction if smokers completely switch to HTPs from combustible cigarettes. It has been shown that there is less exposure to toxic substances like carbonyls or ROS using HTPs, and these products also show better results in clinical trials with fewer exacerbations in COPD patients switching to HTPs compared to continuing smoking [73]. An important 3-year study compared patients with COPD who quit traditional tobacco smoking or significantly limited their use in favor of HTPs with patients of the same age and sex who continued smoking. A significant reduction in the number of annual exacerbations was found in patients using HTPs, and no significant changes were recorded in COPD patients who continued to smoke [44]. Given the improvements in exacerbation rates, respiratory symptoms, and overall health status, it was not surprising that patients in the HTP user group consistently experienced downstaging (i.e., improvement) of their COPD stages throughout the whole duration of the study. In this light, for COPD patients unwilling to quit smoking, switching to HTPs should be seen as a valuable solution to the challenging problem of smoking. However, many authors are still firmly convinced that the increase in knowledge base about the respiratory health risks and benefits associated with HTP use

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is mandatory before considering HTP use as a part of clinical guidelines, a key for better policymaking, and for better quality of information for end-users.

Acute eosinophilic pneumonia (AEP) is a rare but serious condition that leads to severe respiratory failure. Typically triggered by the inhalation of allergic antigens, such as chemicals and fungi, it counts among its primary causes the smoking habit. Also, in this case, the presumed "harmless nature" of HTPs is being questioned. Although clinical studies on the putative role of HTP consumption on new AEP occurrence are still scant, some case reports showed elevated inflammatory markers and eosinophilic lung disease, suggesting HTP use may trigger respiratory issues due to its particulate emissions [74,75]. The mechanism of how smoking causes AEP has not been clarified; however, some chemical substances contained in cigarettes could contribute to activating an allergic reaction and eosinophils. In pediatric populations, exposure to cigarette smoke, whether active or passive, poses significant health risks, particularly regarding long-term cardiovascular outcomes. The deleterious effects of secondhand exposure are extensively documented in the literature pertaining to the respiratory health of children. Tobacco smoke functions as a pro-inflammatory stimulus, contributing substantially to the pathogenesis of asthma, allergic rhinitis, atopic conditions, and obstructive sleep apnea syndrome. These respiratory disorders have been associated with elevated cardiovascular risk in adulthood, likely mediated by the persistence of systemic low-grade inflammation initiated during early life exposure. Lee et al. found that HTP use in adolescents correlated with asthma, allergic rhinitis, and atopic dermatitis, implying a possible role in immune sensitization [76]. Particular attention should be paid to adolescents since, using these products less heavily than adults, they could be exposed to concentrations of allergens that are sufficient to sensitize, and not sufficient to suppress the sensitization [75].

The levels of tobacco exposure-related biomarkers were improved following HTP use, compared to smoking TS. The favorable change in biomarkers related to smoke exposure, especially those linked to inflammation and oxidative stress, could potentially contribute to improved health outcomes. Changes in adhesion molecule-1 (sICAM-1) in serum, white blood cell (WBC), 11 dehydrothromboxane B2 (11-DTX-B2), and 8-epi-prostaglandin F2 alpha (8-epi-PGF2α) have been reported as associated with smoking-related diseases such as cardiovascular diseases. In the study by Ludicke et al., healthy smokers who switched to HTPs for 26 weeks reported how, among the eight biomarkers assessed—HDL-C, white blood cell (WBC), forced expiratory volume in one second (FEV1) post-bronchodilator expressed as % predicted (FEV1%pred), carboxyhemoglobin (COHb) in blood, total 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (total NNAL), soluble intercellular adhesion molecule-1 (sICAM-1) in serum, 11 dehydrothromboxane B2 (11-DTX-B2), and 8-epi-prostaglandin F2 alpha (8-epi-PGF2α)—statistically significant improvements were observed in five of the eight co-primary endpoints after 6 months for patients who shifted to HTP consumption. This can indicate that HTP could be considered as an acceptable alternative when the smoking cessation clinical path fails [58]. However, some studies show how HTPs significantly increased inflammation, as indicated by elevated white blood cell counts and pro-inflammatory cytokines such as IL-6, IL-8, TNF-α, and CRP. This inflammatory response correlated with endothelial dysfunction, leading to increased arterial stiffness, as measured by pulse wave velocity and augmentation index [75]. The switching from traditional tobacco cigarettes to HTP use significantly reduces the carboxyhemoglobin concentration, but the white blood cell count, and data from plethysmography, such as airways resistance, specific airway resistance, and conductance, confirm a significant impact of HTPs on consumers' health compared with the non-smoking cohort [77]. In an observational cross-sectional study, chronic use of HTPs was associated with impaired endothelial function, elevated oxidative stress, and enhanced platelet activation. In particular, the

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study highlights an increased level of soluble Nox2-derived peptide (sNox2-dp), indicating sustained NOX activation supported by the higher serum hydrogen peroxide (H_2O_2) levels, underscoring oxidative stress that may, in turn, lead to reduced NO bioavailability and, consequently, contribute to the progression of atherosclerosis through endothelial dysfunction. Interestingly, at comparable cotinine levels, a recent trial found a significant reduction in flow-mediated dilatation (FMD) with no significant difference between the HTP and CS smoker groups [60]. According to another recent trial, HTP smoking has shown an acute impact on systolic and diastolic myocardial function in healthy HTP consumers, comparable to that induced by traditional cigarette smoking [78].

In line, the results from a large metabolomic study indicate that HTP users present alterations in plasma glutamate and other biomarkers included in glutamate metabolism (ie, glutamate, arginine, ornithine, and citrulline) strongly associated with smoking. Multiple studies have indicated that glutamate levels are associated with the prospective development of cardiovascular diseases and may contribute to atherosclerotic plaque formation. Alterations in the glutamatergic pathway could partially elucidate the mechanisms underlying smoking-induced cardiovascular pathology [68].

Again, investigating the miRNA profile of chronic exclusive HTP users, the authors identified potential pathogenetic signatures, suggesting a significant impact of HTPs on circulating miRNAs [79] and the trial by Belkin et al., reported clinically elevated parameters of arterial vascular stiffness as a correlate for endothelial dysfunction associated with the HTP use [80] and this evidence is supported by data from a recent trial on healthy young adults showing how HTPs have immediate adverse effects on vascular function, resulting in increased arterial stiffness and platelet thrombus formation, known risk factors for the development of atherosclerosis [71].

Among children exposed to passive smoke from HTPs or CS versus unexposed subjects, a recent trial reported significant changes in sCD40L, soluble P-selectin, and the Total Thrombus Formation Analysis System (T-TAS) as markers of platelet activation and thrombus formation. The authors conclude that the exposure to HTPs promotes oxidative stress, endothelial dysfunction, platelet activation, and thrombus formation in children [60]. Notably, increasing attention has been directed toward the effects of "thirdhand" smoke exposure involving the persistent residual pollutants that settle on surfaces and can be re-emitted into the air over time.

Nevertheless, it should be emphasized that the biomarkers considered in the studies mentioned above reflect processes on the pathway to smoking-related diseases: their predictive and discriminative power has yet to be established, so larger and longer-term population-based studies are needed to further clarify these findings [73].

In conclusion, although the current evidence supports the use of non-combustible smoking alternatives such as HTPs, which show evidence of improved levels of both exposure and biological effects, some results are conflicting, and confirmatory data are needed, so this remains a fertile research area in the coming years.

4. Raising Evidence of the Effects of HTP Exposure on the Central Nervous System (CNS)

Since their introduction into the market, the majority of the studies have mainly focused on the respiratory and circulatory systems. Recently, the World Health Organization (WHO) stressed the urgency of filling critical knowledge gaps, particularly with regard to the potential effects of ENDS on the central nervous system (CNS) and the association between ENDS use and nicotine dependence, as well as its impact on brain development in fetal life [81].

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Nicotine is a major contributor to the immediate increase in regional cerebral blood flow (rCBF) caused by smoking, and recent data from animal models confirm that both smoking cigarettes and HTP aerosol extracts increase the rCBF, disrupting cerebrovascular health, suggesting that the effects on cortical vessels, i.e., vasodilation, are mediated by nicotine-induced α4β2-nAChR activation, setting aside toxic non-nicotine constituents of these two extracts as potential mediators of this effect [82]. Although the magnitude of the effects triggered by the HTPs is smaller than that induced by the nicotine intake from cigarette smoking. These data are in line with those presented in the previous chapter and confirm the impact of HTPs on vascular systems, including the cerebrovascular one. To further explore the potential impacts on cerebrovascular health, a recent study focused on the effects of CS versus HTPs on microglial function under conditions of hypoxia/reoxygenation (H/R). Ischemic stroke, caused by the obstruction of cerebral blood vessels leading to reduced perfusion of brain tissue, remains a leading cause of mortality and long-term disability across Western countries. Neuroinflammation and oxidative stress are key mediators of secondary brain injury following ischemia, further highlighting the substantial health and socioeconomic burden associated with stroke-related morbidity and mortality [83]. Results from an in vitro model of H/R confirm that CS exposure under H/R conditions reduces microglia activation, an essential step in the neuroinflammatory response to brain injury. HTP exposure maintained cellular function, suggesting potentially lower cytotoxicity of HTPs. Both CS and HTP aerosols show an upregulation of RNA-binding proteins, indicating a potential adaptive response to oxidative damage modulating oxidative stress-related proteins, albeit through distinct mechanisms: HTP aerosol and nicotine preserved nuclear translocation of Nrf2, a key regulator of the antioxidant response, whereas cigarette smoke impaired Nrf2 activation—suggesting elevated oxidative stress and increased potential for cellular injury [84]. The differential activation of the Nrf2-mediated antioxidant pathway implies that HTPs may exert a lower oxidative burden compared to conventional cigarettes. However, the health impact and associated risk in individuals who are primary or only HTP users need further investigation.

HTP exposure significantly increases ROS production in the prefrontal cortex of adult rats, along with an increase in pro-inflammatory cytokine and the down-regulation of PPARα and PPARγ nuclear receptors as negative regulators of oxidative stress-induced inflammation [84]. Again, HTP exposure increased the level of 8-hydroxyguanosine, a reliable marker of DNA oxidation that is positively associated with smoking habit, xeroderma pigmentosum group C protein complex (XPC), and 8-oxoguanine DNA glycosylase-1 (OGG-1), two crucial proteins involved in nucleotide excision repair (NER) and DNA base excision repair (BER). The pro-oxidant and neuroinflammatory effects of HTP exposure were also observed in human microglial cells; however, the comparison with CS treatment confirms again that HTP use can be associated with a lesser degree of microglial toxicity, with reduced ROS production, lipid peroxidation, oxidative DNA damage, and mitochondrial dysfunction [85].

Several studies suggest the relationship between lung stressors such as air toxicants and CNS, and the inflammation and oxidative stress play a partial mediating role in the lung–brain axis [86]. A growing body of evidence indicates that chronic inflammation, encompassing both systemic and neuroinflammation, is critically involved in the neuropathological progression of Alzheimer's disease (AD) and in order to better explore this filed, in a recent study employing a mouse model mimicking prodromal AD, the authors observed a minimum impact on amyloid pathology; on the other hand, HTPs were found to affect gene modulations associated with the activity of neuropeptide hormones like arginine vasopressin, oxytocin, and galanin, a hormone known to modulate mesolimbic dopaminergic neurotransmission and associated with nicotine addiction [78] similarly

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to what previously observed in studies on CS [87]. Interestingly, early and progressive dysfunctions of the dopaminergic system from the Ventral Tegmental Area (VTA) have been described in AD [88] and recently, the exposure to HTP mainstream was found to increase KDM6B levels, along with downregulation of both PPARα and PPARγ gene expression in the VTA of exposed rats suggesting a putative mechanism associate with the pro-inflammatory effects [89]. Furthermore, the reduced expression of PTEN in exposed animals has already been documented in AD brains [90]. The increased AKT activity observed suggests a positive contribution of HTPs to neuroinflammation and virtually to AD progression, considering that a hyper-activation of GSK-3β due to AKT leads to tau hyper-phosphorylation, an important event in AD pathogenesis [91] (Figure 3).

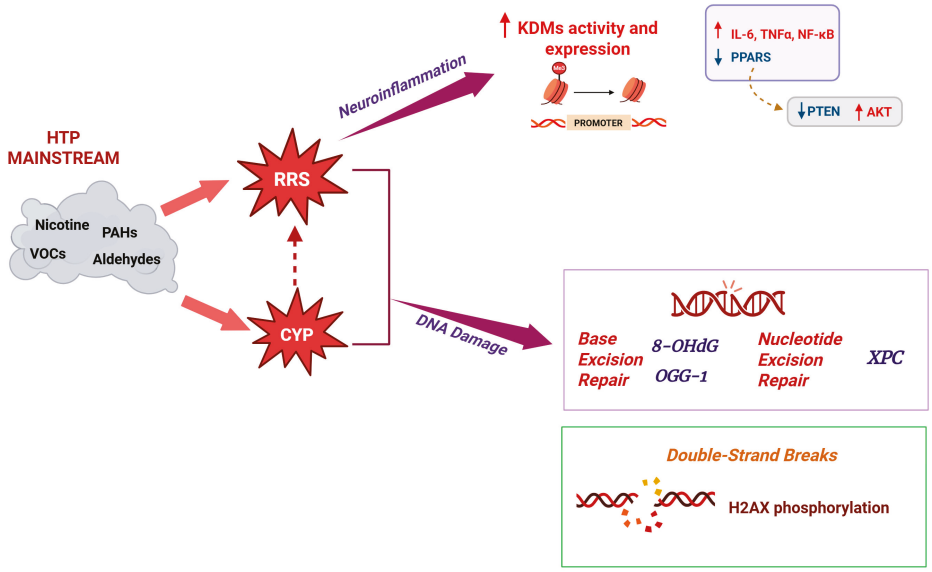

Figure 3. Effects of mainstream smoke from HTPs. Compounds present in HTP mainstream include numerous chemicals that can undergo CYP-mediated bioactivation to mutagenic or carcinogenic intermediates. Both this process and direct exposure to HTP aerosols contribute to the generation of reactive radical species (RRS), including superoxide and hydrogen peroxide. The resulting redox imbalance promotes neuroinflammation through epigenetic modulation of gene promoters by histone demethylases such as KDMs, which enhances the transcription of NF-κB–dependent pro-inflammatory mediators (IL-6, IL-1β, TNFα) and concomitantly reduces the expression of PPARs, key negative regulators of oxidative stress–induced inflammation. Although the precise mechanisms are not yet fully understood, an increase in both the activity and gene expression of KDMs has been reported after HTP exposure. In parallel, RRS-induced genotoxicity leads to multiple forms of DNA damage—such as oxidative lesions (e.g., 8-oxo-dG) and strand breaks—that activate DNA repair pathways. This is reflected by the increased expression of BER- and NER-associated proteins, including OGG1 and XPC, which are essential for counteracting the mutagenic consequences of oxidative and structurally distorting DNA lesions. Created in BioRender. Morosini, C. (2026) https://BioRender.com/z652ydp (Academic License).

Clinical data on the putative effects of HTP consumption on CNS pathologies are extremely scant. Noteworthy is an independent trial that surprisingly found that HTP-only users showed the highest proportion of anhedonia and depressed mood. CS-only users had the highest proportion of individuals who had trouble sleeping, while dual users had a higher proportion of those with fatigue and appetite problems [92]. These findings gain greater significance when interpreted in light of recent evidence showing that among adolescents, almost a third of those who had experimented with HTPs continued to use them [93], confirming that while HTPs may serve as a potential strategy for smoking

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cessation, they also pose a risk of acting as a gateway for non-smokers to initiate the use of these devices.

5. Emerging Data on HTP Use and Reproductive Health

Tobacco smoking still remains one of the leading causes of male infertility, due to the presence of nicotine, tar, carbonic monoxide, PAHs, and heavy metals, which are also partially present in HTP mainstream products. Spermatozoa are particularly susceptible to oxidative stress due to the high content of polyunsaturated fatty acids in membranes, the limited antioxidant capacity, as well as the ability of spermatozoa to generate ROS contributes to testicular inflammatory response affecting spermatogenesis and testosterone synthesis [94]. The deleterious effects of smoking on male gonadal function and fertility were extensively investigated [95]; however, the introduction of e-cigarettes has substantially heightened the scientific community's interest in this area of investigation.

Tobacco consumption leads to exposure to over 100 carcinogens and mutagens, including PAHs, aldehydes, and heavy metals, which ultimately lead to DNA damage and testicular cytotoxicity, and some of them, as previously discussed, are still present in HTP mainstream despite concentrations still being significantly lower compared to CS. Although the studies on this topic are still scant, some animal models showed that parentally HTP-exposed male offspring mice exhibited changes in testicular morphology and decreased spermatogenesis, suggesting a significant impairment of male testicular function or delayed sexual maturation. In particular, the exposure during the organogenetic period leads to delayed spermatogenesis in juveniles and an increased incidence of abnormal seminiferous tubule morphology. These effects may be attributable to delayed sexual maturation or may result from a broader developmental delay. Surprisingly, the study reported no adverse effects of prenatal CS (3R4F) exposure on spermatogenesis or increases in the incidence of seminiferous tubule damage in male offspring [96]. These data are of particular interest because they represent one of the rare investigations showing a worsening effect associated with HTPs compared to CS. Although the concentrations of tobacco-specific toxicants in HTPs mainstream are significantly lower than those in CS, some constituents, such as 4-aminobiphenyl (4-ABP), for which sufficient evidence is available about its carcinogenicity, have been shown to cross the placenta, and it has also been shown that 4-ABP binds to fetal DNA [97]. This may offer a plausible explanation for the observed data, although the hypothesis has yet to be confirmed. Supporting data on toxic effects as a significant increase in oxidative stress markers—particularly those indicative of DNA damage—was observed in exposed animals. This was accompanied by the upregulation of NF-κB-dependent pro-inflammatory mediators, including TNF-α, IL-1β, IL-6, and COX-2. Additionally, the inactivation of key androgenic enzymes, such as 3β-hydroxysteroid dehydrogenase and 17β-hydroxysteroid dehydrogenase, along with a reduction in testosterone synthesis, suggests a potential disruption of male gonadal function [98]. In contrast, histological analysis of testes from rats exposed to treatments for 28 days showed normal Sertoli and Leydig cells along with regular arrangement of spermatogenic cells [99], indicating a neutral impact of HTP exposure on the male reproductive system. However, it should be pointed out that the HTPs used in the study were test samples from the New Tobacco Products Engineering and Technology Research Center of Sichuan Province, Chengdu, China, and not devices commercially available.

Further insights on oxidative stress-mediated testis impairment come from OECD 90-day sub-chronic exposure, which showed how both HTP aerosol and CS increased oxidative stress in the testes, as evidenced by an increase in ROS generation and MDA levels along with a reduction in antioxidant capability. Pro-inflammatory factors such as IL-1, IL-6, and TNF were found to be increased in both HTP and CS exposed animals. TNF

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and interleukins play a key role in coordinating immune cell function and supporting spermatogenesis. However, under certain pathological conditions, elevated levels of pro-inflammatory cytokines can shift the homeostatic balance toward enhanced immune and inflammatory responses. This may lead to the recruitment of immune cells to the testes or their activation within germ cells, potentially resulting in germ cell apoptosis and impaired spermatogenesis [94]. Moreover, the authors reported an increased activation of NLRP3 inflammasome through the ROS/NLRP3/Caspase-1 pathway, leading to pyroptosis, a programmed lytic cell death characterized by rapid plasma membrane rupture [100].

5.1. Clinical Data on HTP Impact on the Male Reproductive System

Given that atherosclerotic lesions are often irreversible, the observed improvement in erectile function following smoking cessation is likely attributable primarily to the reduction in oxidative stress. The current literature lacks comparative data on the prevalence of erectile dysfunction among users of e-cigarettes or HTPs versus conventional cigarette smokers or non-smokers, thereby precluding definitive conclusions regarding the impact of these alternative nicotine delivery systems on erectile function. However, a recent systematic review and other original investigations indicate that both e-cigarette and HTP consumption may impair erectile function [101]. This speculation is supported by the fact that nicotine itself is a known vasoconstrictor reported to cause endothelial impairment through oxidative stress and impair sexual and erectile function in animal models and humans [102]. However, to date, there are no relevant clinical data available on the effects of HTP use on erectile dysfunction nor on the potential benefits of switching from conventional cigarettes to HTPs. Similarly, clinical studies investigating the potential effects of HTP use on semen quality are currently lacking.

5.2. Clinical Data on HTP Impact on the Female Reproductive System

A recent clinical trial reports that the use of HTPs negatively affected fertility, leading to reduced ovarian quality and ovarian reserve. It had a detrimental impact on both the quality and quantity of oocytes retrieved during intracytoplasmic sperm injection cycles (ICSI). Additionally, users of HTPs exhibited decreased levels of anti-Müllerian hormone (AMH) [103].

In line, pregnant mothers who smoked HTPs were more at risk of preterm birth, similar to cigarette smokers (CS) [104], and those who smoked HTPs exclusively during pregnancy were at higher risk of small for gestational age (SGA) than those who had never smoked [105].

Overall, studies tackling the effect of HTP consumption on reproductive health are not yet sufficient, and no clear conclusion can be drawn on the potential detrimental effects of HTP use on reproduction and offspring health.

6. Conclusions and Future Outlook

Given the growing public emphasis on detrimental health effects associated with CS, the shift from conventional cigarette use is rapidly turning into an expanding phenomenon, particularly in industrialized countries, and is especially appealing to younger individuals. While the perception of a less harmful product may play a significant role, the adoption of these devices may also be driven by social imitation and peer influence.

Based on the relevant literature, reviewed here, it is clear that HTPs allow for nicotine inhalation at concentrations very similar to those observed with conventional cigarettes, while exposing users to significantly lower levels of HPHC. On this point, there is substantial agreement in the literature. However, it is equally clear that, based on the reduced HPHC content, it was initially assumed—at least in the early phases—that these devices

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would be inherently safer. Evidence from basic research studies and clinical trials strongly suggests that exposure to HTPs may contribute to the onset or exacerbation of certain diseases typically associated with cigarette smoking. Notably, the cellular and molecular mechanisms underlying the effects of HTPs often mirror those observed with conventional cigarette smoke exposure, as summarized in Table 4. Current evidence indicates that exposure to HTPs is associated with an increase in oxidative stress, and it represents a key mechanism through which tobacco products impair organ function. In addition, a further common feature of HTP exposure is its pro-inflammatory effect. These two non-negligible aspects are central to the etiology of various smoking-related diseases and indeed form the basis of many of the studies discussed above.

It is important to clearly reiterate that, with few exceptions, comparative studies—including clinical trials—consistently report that the use of HTPs has a lower impact compared to conventional cigarette smoking. This inevitably leads to a discussion on the potential benefit that a smoking patient might derive from switching to HTP use. However, long-term data to support this hypothesis are currently lacking. The mere observation of less pronounced pathological effects in HTP users over the short term does not provide definitive predictive value. Furthermore, HTP users are often considered former smokers, overlooking the possibility that these devices may also serve as a gateway to tobacco use for new users. In this regard, the role of HTPs in smoking cessation is under debate, and recent clinical studies indicate that HTPs fail to help smokers quit or prevent former smokers from relapsing [106–108]. This should not come as a surprise, since HTPs mimic traditional cigarettes in terms of nicotine and gestural behavior. Furthermore, the dual-use pattern is often overlooked. While extrapolating current data on HTP-only users remains uncertain, the situation becomes even more complex when considering dual-users.

As a whole, to date, the risk and safety evaluation of HTPs needs further research, in particular:

- While there is a rich and detailed body of literature on the reproductive effects of smoking and fetal development, data regarding the use of HTPs remain very limited.
- Many studies compare the effects of HTPs with conventional smoking (CS) and focus their conclusions on this comparison, often neglecting the investigation of the intrinsic toxicity of the devices themselves.
- An increase in cohort studies, exploring in greater depth, the early warning toxicity markers of exposure as well as epigenetic changes in cancer-regulating genes.

Since we are far from definitively understanding the health impact of HTPs, even compared with cigarettes, there is a need for longer-term, unbiased clinical trials and epidemiological studies to assess: the long-term effects of HTP use under real-world conditions and the health consequences in the increasing population of non-smokers who are adopting these products.

Table 4. Key effects associated with the use of heated tobacco products (HTPs) and their corresponding biomarkers.

Health Concern	Molecular Pathways/Biomarkers Involved	Studies
↑ Inflammation, cellular damage, and oxidative stress	↑ Cytokine IL-1β, IL-6 expression ↑ activation of DAMPs ↑ Nrf2 antioxidant response ↑ DNA oxidative damage	In vitro [29]

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Table 4. *Cont.*

Health Concern	Molecular Pathways/Biomarkers Involved	Studies
↑ Lung and tracheal remodeling, inflammation, and oxidative response	↑ NF-κB pathways ↑ Granulocytes pulmonary infiltration ↑ Circulating blood neutrophils ↑ CCL expression ↑ GM-CSF	In vivo [30–32]
↑ Lung parenchyma alteration	↑ Lipid accumulation in alveolar macrophages	In vivo [33]
↑ Lung infiltrating leukocytes	↑ $CD4^+$ ↑ $ROR\gamma t^+$ receptor ↑ CXCL8	In vivo [33]
↑ Increased risk of atherosclerosis, oxidative stress, and endothelial dysfunction	↓ Glutathione ↑ Inflammatory markers ↑ Monocyte adhesion ↑ TNF-α ↑ IL-1β ↑ Monocyte adhesion	In vitro [40,41]
↑ Endothelial dysfunction	↓ Phosphorylation of eNOS at Ser1177 ↓ eNOS ↓ NO generation	In vitro [42]
↓ COPD flare-up	↓ Exacerbation rates ↓ Respiratory symptoms	Clinical data [44]
↑ Risk of acute eosinophilic pneumonia (AEP)	↑ Eosinophils infiltration ↑ Allergic reaction	Clinical data [74,75]
↑ Risk of asthma ↑ Allergic rhinitis ↑ Atopic dermatitis		Clinical data [76]
↓ Cardiovascular risk	↑ HDL-C ↓ White blood cell (WBC) ↑ Forced expiratory volume ↓in one second (FEV1) ↓ Carboxyhemoglobin (COHb) ↓ Total 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (Total NNAL)	Clinical data [58]
↓ COPD-associated symptomatology	↓ COPD exacerbation rate	Clinical data [73]
↓ Oxidative stress	↓ Carboxyhemoglobin (COHb)	Clinical data [77]
↓ Endothelial function ↑ Elevated oxidative stress ↑ Enhanced platelet activation	↑ Nox2-derived peptide (sNox2-dp) ↑ Serum hydrogen peroxide (H_2O_2) levels ↓ NO bioavailability ↓ Flow-mediated dilatation (FMD)	Clinical data [60]
↑ Cardiovascular risk	↓ Systolic and diastolic myocardial function	Clinical data [78]
↑ Cardiovascular risk	↑ Plasma biomarkers of glutamate metabolism (i.e., glutamate, arginine, ornithine, and citrulline).	Clinical data [68]
↑ Cardiovascular risk and endothelial dysfunction	↑ Arterial vascular stiffness ↑ Platelet thrombus formation	Clinical data [79,80]
↑ Cortical vessel dilatation	↑ Nicotine-induced α4β2-nAChR activation	In vivo [82]
↑ Oxidative stress ↑ Neuroinflammation	↑ Nrf2 antioxidant response ↑ Microglia activation	In vitro [84,109]

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Table 4. *Cont.*

Health Concern	Molecular Pathways/Biomarkers Involved	Studies
↑ Oxidative stress ↑ Neuroinflammation	↑ ROS production ↑ PPARα and PPARγ nuclear receptors ↑ 8-hydroxyguanosine ↑ Xeroderma pigmentosum group C protein complex (XPC) ↑ 8-oxoguanine DNA glycosylase-1 (OGG-1)	In vivo [85]
↑ Alzheimer's disease risk	↑ Mesolimbic dopaminergic neurotransmission	Clinical data [78]
↑ Neuroinflammation	↑ KDM6B ↓ PPARα and PPARγ PTEN ↑ AKT	In vivo [89]
↑ Psychiatric disorders risk	↑ Anhedonia ↑ Depressed mood	Clinical data [92]
↓ Male gonadal function	↓ Spermatogenesis ↑ Sexual maturation time	In vivo [96]
↓ Male gonadal function ↓ Testosterone levels ↑ Oxidative stress	↑ NF-κB ↑ TNF-α ↑ IL-1β ↑ IL-6, ↑ COX-2 ↓ 3β-hydroxysteroid dehydrogenase ↓ 17β-hydroxysteroid dehydrogenase	In vivo [98]
↓ Male gonadal function ↑ Inflammation ↑ Oxidative stress	↑ ROS ↑ MDA ↑ IL-1 ↑ IL-6 ↑ TNF	In vivo [94]
↓ Female fertility	↓ Anti-Müllerian hormone (AMH) ↓ Quality and quantity of oocytes retrieved during Intracytoplasmic sperm injection cycles (ICSI)	Clinical data [103]
↓ Female fertility	↑ Preterm birth ↑ Risk of small for gestational age (SGA)	Clinical data [104,105]

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Review

Oxidative Stress as a Potential Mechanistic Bridge Between Electronic Cigarette Components and Chronic Disease: A Combined Narrative and Bibliometric Analysis

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Abstract

Despite their introduction two decades ago as cessation tools, vaping devices and electronic cigarettes (ECs) have surged dramatically among adolescents and adults worldwide. The widespread misconception that ECs are harmless has raised substantial concern about their potential contribution to chronic diseases associated with oxidative stress (OS) pathways. To map this rapidly growing field, we combined a bibliometric analysis of human research on vaping-associated OS over the past decade with a narrative review of EC component toxicology and regulatory updates. The bibliometric analysis of 189 human studies identified four keyword clusters reflecting clinico-epidemiological, biological/mechanistic, methodological, and acute-event research themes. The results of this bibliometric analysis structure the narrative synthesis, which evaluates the chemical constituents of ECs, including e-liquid constituents, device-derived contaminants, and their aerosol by-products, and links them to pathways associated with OS development. Oxidative stress was the most frequent bridging keyword, reflecting predominant academic trends as research shifted from acute-focused investigations toward mechanistic chronic disease research. No prior review has integrated EC component toxicology with bibliometric evidence to map this landscape. This combined approach provides researchers, clinicians, and policymakers with an integrated evidence base and identifies priority areas for future investigation, including long-term exposure thresholds, age- and sex-specific outcomes, and the urgent need for harmonized global regulation.

Keywords: electronic cigarette; vaping; aerosol; oxidative stress; e-cigarette regulations; nicotine; chronic diseases; bibliometric analysis; public health

1. Introduction

The credit for the first electronic smoking device is attributed to Herbert A. Gilbert, who was acutely aware of the health consequences of tobacco smoking in the early 1960s. Ahead of his time, Gilbert described a "smokeless non-tobacco cigarette" that "heated moist, flavored air" [1] that no manufacturer was prepared to commercialize, a concept at that time. Forty years later, the Chinese pharmacist Hon Lik brought the electronic cigarette (EC) to market as an "electronic atomization cigarette," explicitly designed to reduce nicotine consumption and assist with smoking cessation [2,3]. ECs are built on

four core components: (1) a mouthpiece, (2) a cartridge, pod, or tank containing the e-liquid, (3) a heating element or atomizer, and (4) a rechargeable battery. Each of these constituents, particularly the metallic heating elements, can release compounds under certain conditions of use; therefore, any associated health risk is a function of exposure rather than the mere presence of the material. The operating principle of ECs involves heating the e-liquid at temperatures ranging from 100 °C to 315 °C through a metal coil, generating a vapor or aerosol that is inhaled by the user [4]. Unlike conventional cigarettes, ECs do not generate sidestream smoke but instead produce an exhaled aerosol, whose composition and concentration are influenced by respiratory retention and dilution in ambient air [5,6].

The chemical transformation of e-liquid components into aerosol-containing solid particles has raised substantial global health concerns. E-liquids consist principally of propylene glycol, glycerol, flavorings, and, in many but not all products, nicotine. During heating, these components can undergo thermal degradation to byproducts such as formaldehyde, acrolein, and acetaldehyde [7,8]. Although ECs do not generate the tar and carbon monoxide associated with conventional tobacco cigarettes, the chronic health consequences of aerosol inhalation remain an area of intensive investigation aimed at establishing global safety, toxicological, and regulatory standards.

The prevalence of vaping is now high among young people who have never smoked [9] and societal trends have distorted the original cessation rationale behind EC design, shifting its predominant use away from the original harm-reduction rationale toward a broader public health concern among never-smokers [10]. Discrepancies between declared and actual nicotine levels in e-liquids compound this problem. Indeed, products advertised as nicotine-free frequently contain measurable and even high levels of tobacco-derived or synthetic nicotine [11,12]. Motivations for EC use are multifactorial and include social acceptability, cost-effectiveness, the appeal of novel flavors, and the misperception of reduced harm relative to traditional cigarettes [13]. In interpreting these trends, it is important to distinguish the absolute risk of EC use among non-smokers and adolescents from the relative risk among adult smokers who switch completely from traditional cigarettes to ECs. Only a limited number of organizations have comprehensively documented countries that have implemented EC regulations [14,15], and elaborating a global policy consensus on preventing youth use remains an ongoing challenge [16]. Indeed, this concern is especially acute for adolescents and young non-smokers. As recently reviewed, ECs, which represent complex chemical emission sources, have been characterized as "wolves in sheep's clothing" [17], reflecting that the diversity of chemical components and flavors they can deliver makes these devices far more than simple nicotine vehicles [18]. This framing applies to absolute risk in never-smokers and adolescents and is distinct from the question of relative benefits among adult smokers who switch completely from traditional cigarettes [19].

Understanding the toxicological consequences of chronic EC use is therefore essential, as aerosol-contained substances—metals, metalloids, carbonyl compounds, and reactive oxygen species (ROS)—adversely affect multiple physiological systems, particularly the respiratory, cardiovascular, and digestive tracts [20].

Despite this extensive background on electronic cigarettes and chronic disease, no prior review has simultaneously synthesized the chemical toxicology of EC components from a mechanistic perspective while systematically mapping the structure, scope, and thematic evolution of that literature. This review addresses this gap through two complementary approaches: (1) a bibliometric analysis that quantitatively characterizes the global landscape of vaping-associated OS in human populations over the past decade, revealing the global research landscape, thematic clusters, and knowledge gaps; and (2) a narrative synthesis structured around these bibliometric findings evaluates the latest evidence on EC chemical

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components, aerosol toxicology, and pathways associated with OS development underlying the clinical clusters observed in the bibliometric analysis. Together, these approaches provide researchers, clinicians, and policymakers with an integrated, evidence-based roadmap of the field.

2. Bibliometric Landscape of Vaping and Chronic Disease Research (2016–2026)

2.1. Bibliometric Methods

2.1.1. Data Selection and Search Strategy

The comprehensive bibliometric search was conducted in the Scopus database on 20 March 2026, using a targeted query combining terms for electronic cigarettes, chronic diseases, and human studies, while explicitly excluding in vitro and in vivo model studies (Figure 1). Scopus was selected for its broad coverage of biomedical journals, modern data volume, and standardized affiliation and keyword indexing. The search was restricted to a 10-year publication window (2016–2026), peer-reviewed journal articles at the final publication stage, and English-language documents, yielding 495 initial records.

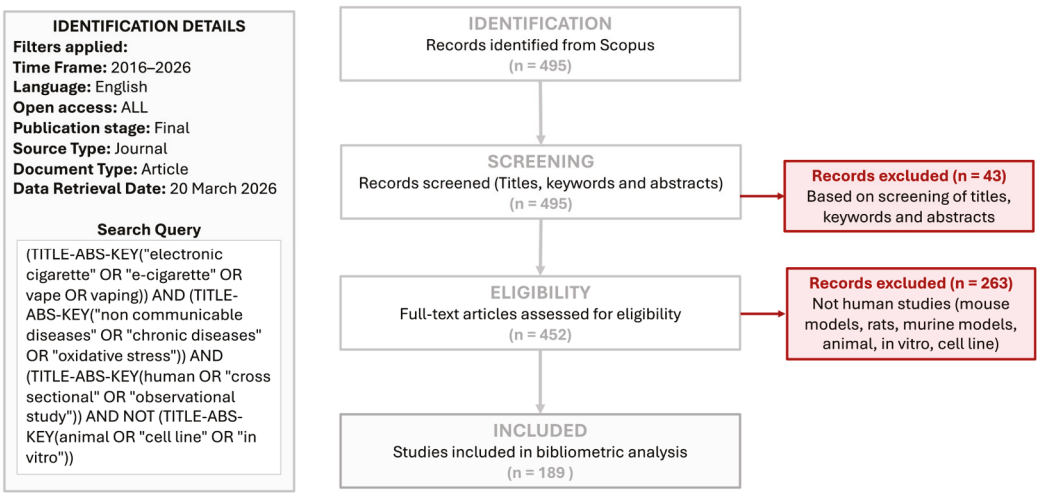

Figure 1. Flow diagram of the literature search and selection process for bibliometric analysis.

2.1.2. Exclusion and Inclusion Criteria

Title, abstract, and keyword screening led to the immediate exclusion of 43 irrelevant documents. Full-text evaluation during the eligibility phase eliminated an additional 263 studies due to duplication, a lack of thematic relevance, or non-human study methodology. Ultimately, 189 publications met all predefined criteria and were included in the final bibliometric synthesis strictly limited to human clinical and epidemiological studies.

2.1.3. Data Visualization

Bibliometric data were visualized using the Biblioshiny interface within the R package 'bibliometrix' (R Project 4.5.3), VOSviewer (version 1.6.19) and Mermaid Live Editor. Visualizations include annual production curves, geographic distribution maps, journal source analyses, cited document analyses, keyword word clouds, trend topic charts, and co-occurrence networks. Keyword analyses were performed on Scopus-indexed keywords, which combine author keywords with controlled descriptors assigned by the database. No thesaurus file or stop-list was applied; synonyms were not harmonized, and all generic indexing descriptors are reported.

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2.2. General Research Growth and Geographic Landscape

The annual scientific production in the field of OS-associated disease from vaping shows two distinct phases, as illustrated in Figure 2. Phase 1 is marked by steady and rapid growth, with a productivity of 3 articles in 2016 to a peak of 27 articles in 2020. Phase 2 is marked by stabilization, with annual publications fluctuating between 24 and 28 articles between 2020 and 2025, and a sharp decline in growth rates. The transition from Phase 1 to Phase 2 coincides with the EVALI outbreak that triggered global interest in this research, which remained active but not exponential. A 3-year moving average indicates that the apparent decline in 2021 was not a true deceleration but instead a normal inter-annual variation. This pattern demonstrates a classic lifecycle observed in bibliometric analyses, where the focus progresses from the identification of a global health burden to achieving scientific recognition.

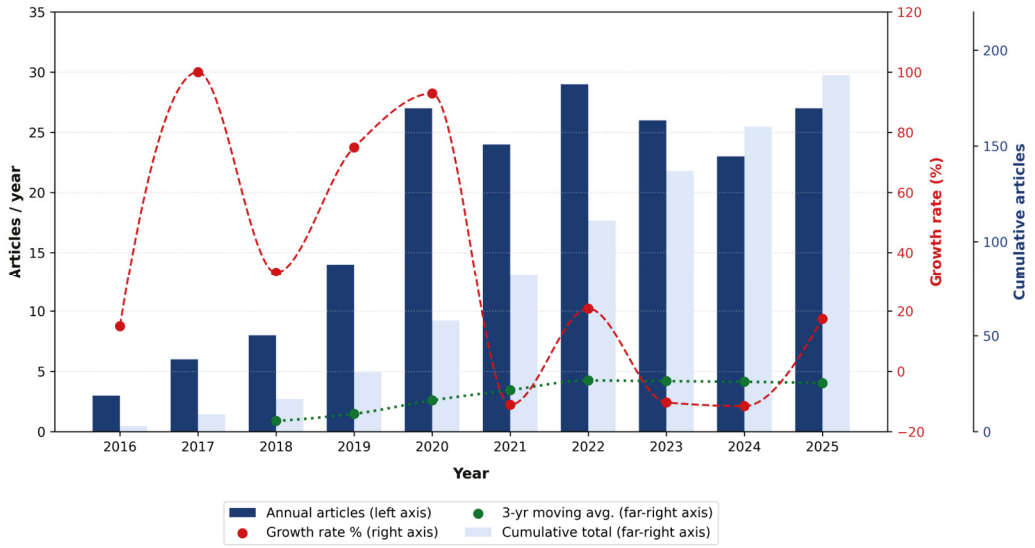

Figure 2. Annual scientific production on OS-associated chronic disease from vaping (Scopus, 2016–2026).

2.3. Geographic Landscape, Contributing Countries and Institutions

As shown in Figures 3 and 4A, the United States (US) largely dominates the global output of research on vaping-associated OS with 461 country-affiliation occurrences. This metric represents the number of times the country appears among author affiliations, which can exceed the number of articles because a single article may list authors affiliated with several countries. The US is followed by Italy with 67, the United Kingdom (UK) with 45, and South Korea with 23 country-affiliation occurrences. Although the US also leads with a total of 2650 citations and an average of 30.1 citations per article, Italy has the highest average of 46.2 citations per article. Belgium achieves nearly the same total citation average per article while having far fewer articles than the US (Figure 4B). Surprisingly, Japan and China, which are usually among the world's top research producers, do not show the expected ratios based on their recognized research capacity in this field. This may be due to an Anglo-Saxon language bias, indexing bias, or an artifact in the database used. Notably, Lebanon's fifth position suggests that the country has a small output (4 articles) but a notable research impact with an average of 11.2 citations per article from a total of 45 citations. This suggests that research impact is not necessarily a function of a country's resources or size. The US also ranks first for publication output over time for the selected period, with a notable surge after 2019 (Figure 4C), but has the lowest level of international collaboration compared to other countries (Figure 4D). However, the overall SCP/MCP analysis shows that despite the global health burden associated with vaping, research

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remains predominantly intra-country and siloed, with a very low level of international collaboration reflected by the multi-country publications (MCP). This observation explains why the ten top contributing institutions in the field of OS-associated vaping disease are American. These are led by the University of California, followed almost equally by the CDC's National Center for Environmental Health and the FDA's Center for Tobacco Products (FDA) (Table 1). Their leading role is expected as they are the main regulatory bodies for tobacco marketing controls, toxic exposure, and non-infectious environmental hazard surveillance. In contrast, Italy and the UK show a high MCP fraction consistent with their extensive European collaborative network.

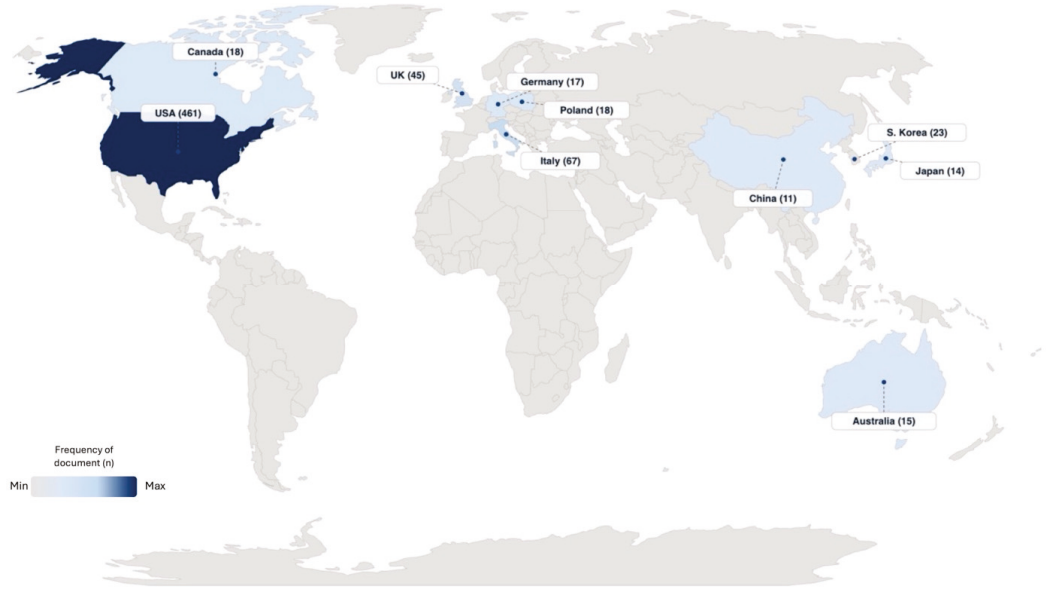

Figure 3. World choropleth map of scientific production by country (2016–2025).

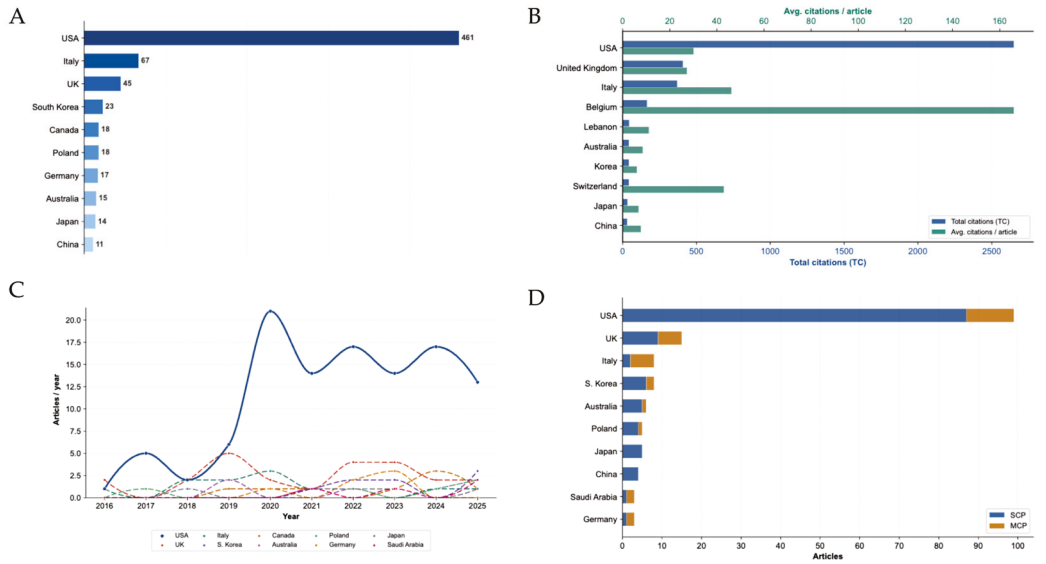

Figure 4. Scientific production on OS-associated disease from vaping. (**A**) Top 10 countries by country-affiliation occurrences; (**B**) Total citations (TC) and average citations per article for the 10 most cited countries; (**C**) Country Scientific production over time; (**D**) Single-country publication (SCP) and multiple-country publication for the 10 leading corresponding-authors countries (MCP).

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Table 1. Top 10 Most Relevant Affiliations.

Rank	Affiliation	Articles
1	University of California	8
2	Roswell Park Comprehensive Cancer Center	6
3	New York University	6
4	Florida International University	6
5	David Geffen School of Medicine (UCLA)	5
6	Geisel School of Medicine at Dartmouth	5
7	National Center for Environmental Health (CDC)	5
8	Center for Tobacco Products (FDA)	5
9	University of Catania	5
10	Johns Hopkins University School of Medicine	4

2.4. Most Relevant Sources and Authors

Nicotine and Tobacco Research and Tobacco Induced Disease rank first among the 10 top journals publishing on vaping-associated OS and chronic diseases. They are followed by interdisciplinary journals such as the American Journal of Preventive Medicine and PLOS ONE (Figure 5A). These sources demonstrate consistent cumulative publication growth, especially from 2019 onward for Nicotine and Tobacco Research and notably the International Journal of Environmental Research in line with the EVALI outbreak (Figure 5B). The multi-scope pattern of these sources reflects the multidimensional nature and broad spectrum of the vaping-related health outcomes.

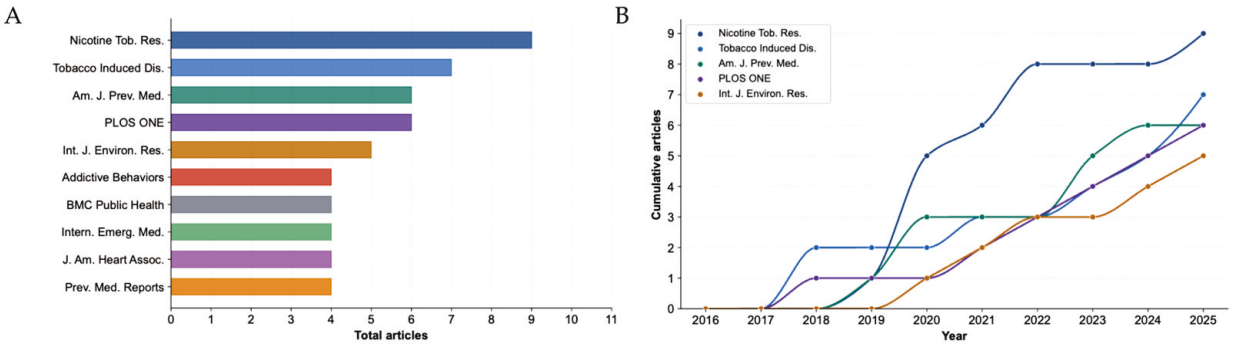

Figure 5. Publication sources. (**A**) Top 10 journals by article count; (**B**) Cumulative journal publication over time (2016–2025).

Figure 6 shows a tie among Gornbein J, Polosa R and Sargent JD, the most productive authors in the field with 6 articles each, followed closely by seven authors with 5 articles each. This distribution suggests that vaping-associated cardiovascular and respiratory disease research is a highly competitive domain. Fractionalized scores below 1 for all these authors highlight the abundant co-authorship, a common trait in clinical and epidemiological research fields. Interestingly, these authors were not among the contributors of the most cited articles (Figure 7). Indeed, the most cited article by far, with 1437 citations, is by Arnett DK et al., published in 2019 in the Journal of the American College of Cardiology (JACC) [21]. This work is the 2019 ACC/AHA Guideline on the Primary Prevention of Cardiovascular Disease. It provides a general cardiovascular prevention guideline rather than a guideline specific to vaping, which may explain its high overall citation count. The next two most cited articles are by McConnell R et al. (2017, Am J Respir Crit Care

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Med, TC = 322) and Bhatta DN et al. (2020, Am J Prev Med, TC = 231) [22,23], addressing vaping-associated pulmonary disease in adolescents and cardiovascular disease at the population level, respectively. The normalized total citations (NTC) were the highest for the article by Arnett DK (NTC = 9.37), followed by Bhatta (NTC = 4.54) [22], Wang JB (NTC = 3.36) [24], and Werner AK (NTC = 3.34) [25], showing that these papers have been rapidly gaining citations (Figure 7).

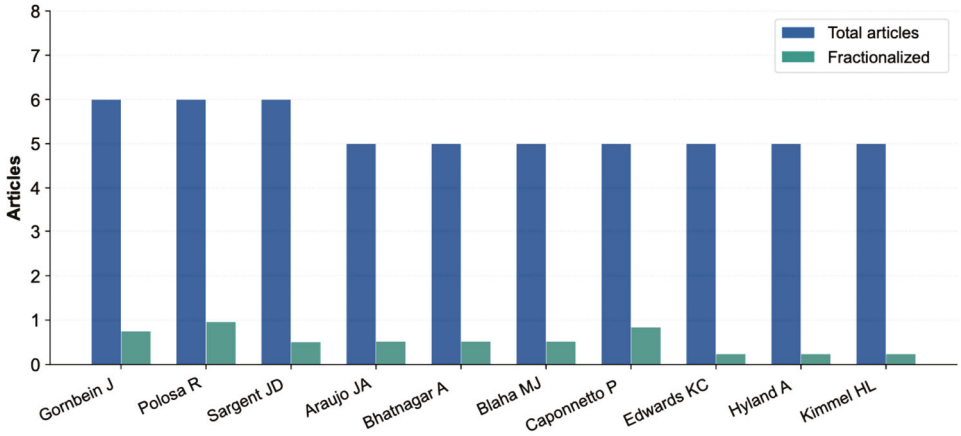

Figure 6. Ten most productive authors by article count (blue) and fractionalized count (teal) side by side.

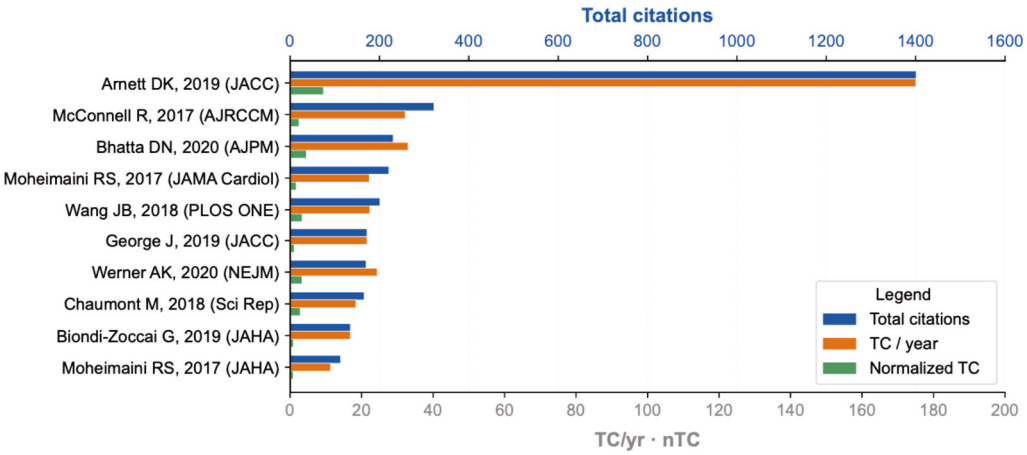

Figure 7. Ten most globally cited documents ranked by total citation count [21–30].

2.5. Keyword Structure, Thematic Landscape and Temporal Evolution

Combined Figures 8 and 9 present an overall well-structured and dynamic research landscape over time on vaping and electronic cigarettes in human populations organized into four groups. First, the keyword group surrounding EC products themselves is dominated by interchangeable terms such as electronic nicotine delivery systems, electronic cigarette, vaping, cigarette smoking, and tobacco product, which appear frequently throughout the entire study period (2016–2025). The second large group relates to the populations studied, including terms such as human, female, male, adult, adolescent, young adult, and middle-aged, with human (321 occurrences), adult, male, and female (145 each) being the most frequent in the word cloud (Figure 8A,B). This reflects the significant focus on age groups within populations, balanced with sex representation, in this research field. A third group of keywords encompasses those related to the types of studies employed in this research field, noted in prior articles (154 occurrences), including terms such as controlled study, cross-sectional study, major clinical study, questionnaire, and cohort analysis. This highlights a chronological evolution of observational studies in the earlier years of the

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study period toward more focused clinical and epidemiological studies as the field matured (Figure 9). The last group of keywords includes terms related to health outcomes, including chronic obstructive lung disease, pulmonary disease, chronic obstructive, adverse event, asthma, COPD, and oxidative stress. In the timeline/Gantt-style chart (Figure 9), the term oxidative stress retained the highest frequency with 34 occurrences in the three-year period 2019–2022, reflecting its position as the most prominent bridging theme within this corpus. Because oxidative stress was the primary search term used to build this study's collection, its high frequency is expected. This result reflects our search strategy rather than providing an independent confirmation of the underlying biological mechanism. Figure 9 also shows a chronological "maturation" of the field with earlier research, from 2017 to 2020, centered on methodological aspects from exposure to drugs and nicotine, and acute effects on disease exacerbation, toward a broader spectrum of epidemiological studies oriented around the OS pathways. This shift concurred with the change in study types mentioned above. In other words, topics such as EVALI, nicotine dependence, and acute lung inflammation dominated in the early period up to 2019. After 2020, diseases related to the cardiovascular and renal systems and long-term exposure outcomes appeared in the timeline landscape. This new predominance of chronic disease in the research field indicates the emerging awareness among researchers that vaping injury may be more than solely limited to acute events such as EVALI and highlights the importance of developing diagnostic tools based on etiology.

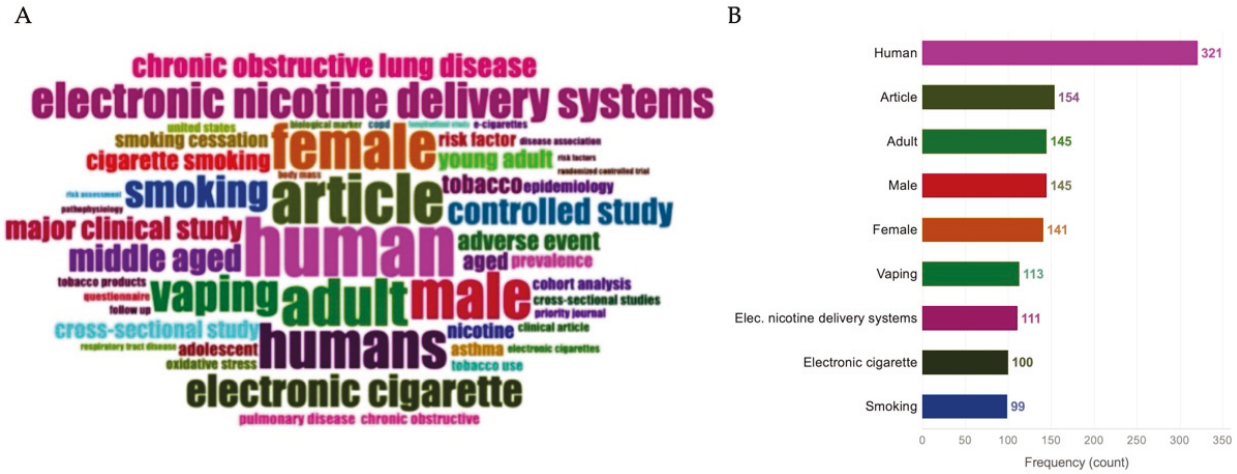

Figure 8. Keyword analysis of the OS-associated disease from vaping. (**A**) Word cloud; (**B**) frequency chart.

The keyword co-occurrence network (Figure 10) confirms the landscape patterns suggested by Figures 7 and 8 to visualize OS-associated chronic disease from vaping in humans. In Figure 10A, the size of each circle represents the frequency at which a keyword appears, and the lines between circles show how often two keywords appear together in the same study. Based on these parameters, four main clusters have been identified and simplified from this complex network in Figure 10B. The blue cluster groups disease severity terms within the demographic cluster, with terms such as severity, outcome assessment, COPD, pulmonary disease, age, and middle-aged. It reflects the consistent trend of associating age groups within populations with the severity of vaping-associated lung disease. The red cluster encompasses epidemiological and disease-related terms such as epidemiology, risk factor, asthma, COPD, bronchitis, cohort study, and chronic disease, reflecting the systematic association between vaping exposure and pulmonary and systemic diseases at the population level. The largest and densest cluster is the green one, which is the most complex to interpret. It captures a combination of clinical terms

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such as pathophysiology, adverse event, biological markers, inflammation, oxidative stress, and includes central terms such as human, male, and smoking. Notably, oxidative stress appears as the most connected node within the green cluster, and the most connected of the four clusters identified. This position indicates that OS functions as a shared organizing framework across the clinical, epidemiological, and mechanistic literature. It should be interpreted as a description of the research landscape rather than independent evidence of a biological mechanism. Indeed, its peripheral location within the green cluster suggests that it is not restricted to a single specific pathway. Finally, the yellow cluster, the smallest and located at the bottom of the map, groups terms related to acute conditions of lung injury such as lung injury, etiology, cannabis, dyspnea, and EVALI, reflecting the early period of research focused on acute disease before the shift toward chronic disease outcomes, discussed in Figure 9.

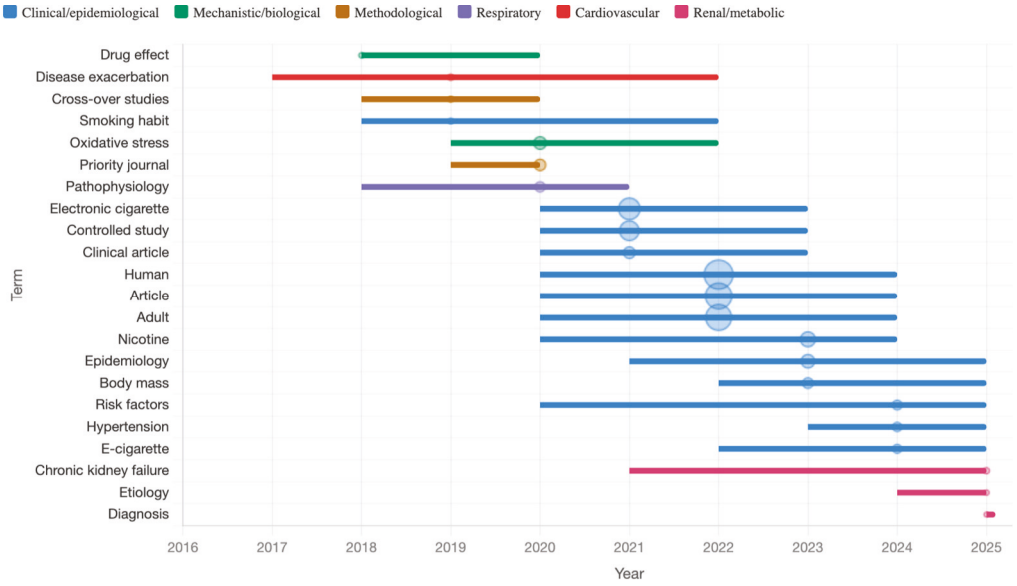

Figure 9. Trend topics over time (2016–2025). Bars represent Q1–Q3 IQR bars. Bubble sizes represent term frequency (smallest size = 5, intermediate size = 100, and largest size = 150).

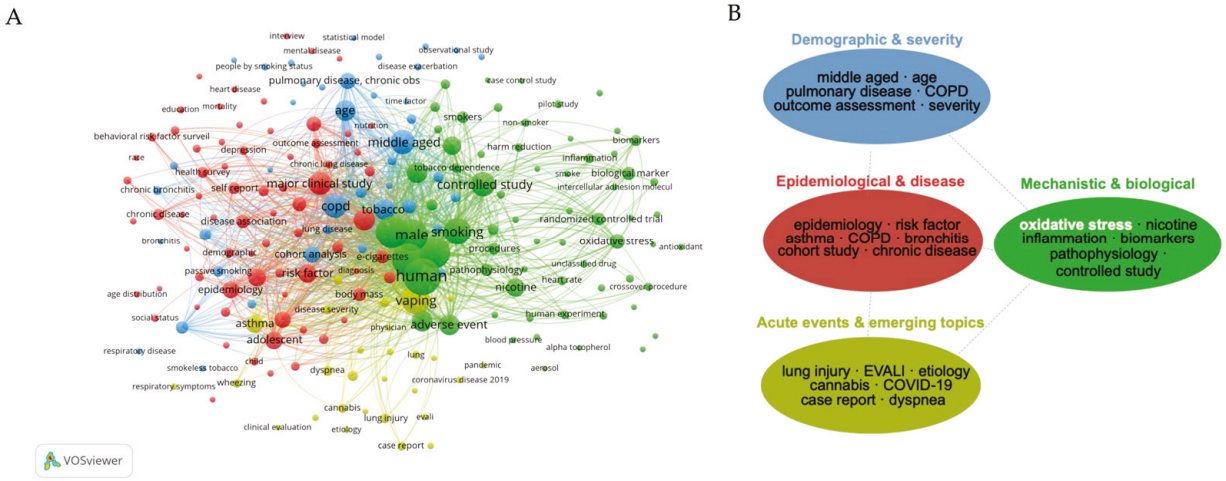

Figure 10. Keyword co-occurrence network. (**A**) Network map generated using VOSviewer; (**B**) cluster summary.

Together, this analysis gives a comprehensive picture of the research field of OS-related disease from vaping that started with a narrow focus on acute lung injury and demographic description, with a progressive transition toward a broader, mechanistic approach based

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on the association of vaping exposure and chronic disease. Oxidative stress has emerged as the central core unifying these two dimensions. These findings reflect the growing global awareness among researchers, clinicians, and regulatory bodies that vaping-related harm is no longer restricted to acute events, and that OS represents a relevant, measurable target for future diagnostic and therapeutic strategies in this field.

For the next narrative sections, the literature published within the past five to ten years was identified through searches of PubMed and Scopus. Search strategies utilized combinations of the keywords "electronic cigarette", "vaping", "e-liquid", "aerosol", "oxidative stress", "antioxidant", and "chronic disease". These strategies were supplemented by hand-searching the reference lists of key retrieved reviews for each component analyzed in Sections 3 and 4. Studies were selected based on EC research addressing OS in human cohorts and in vitro human cell models. Both significant results and experimental regimens not reported in the original studies are presented in the subsequent sections.

3. Components of Electronic Cigarettes

For clarity, the constituents discussed below are grouped by origin, as illustrated in Figure 11: those added to the e-liquid, distinguishing standard components from non-nicotine products and illicit additives; and those found in the aerosol, either generated during heating and thermal degradation or introduced as device-derived metallic contaminants that leach from the coil and the wick.

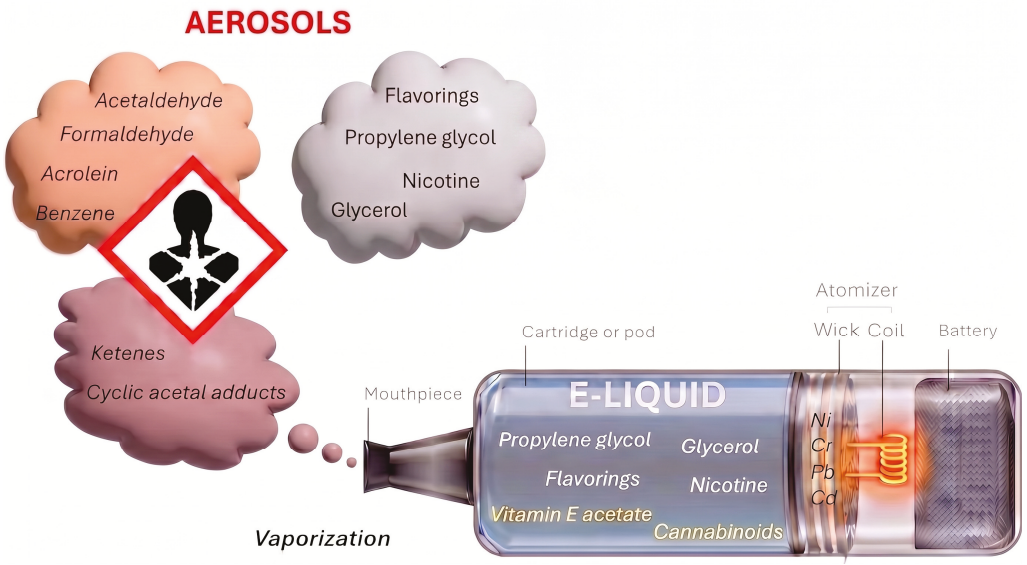

Figure 11. A simplified drawing that depicts aerosol compounds, contaminants, and potentially hazardous chemicals generated during the vaporization of e-liquid components, which occurs due to heating and thermal degradation initiated by the puffing process. The schematic distinguishes between unaltered evaporated carrier ingredients (gray cloud) and hazardous thermal degradation byproducts generated by heat (orange cloud), flagged with the GHS health-hazard pictogram to indicate that several of these byproducts carry individual regulatory hazard classifications. Non-nicotine and illicit additives (yellow text) present in the e-liquid generate pyrolytic emissions (brown cloud). Device-derived metallic contaminants that leach from the heating elements are nickel (Ni), chromium (Cr), cadmium (Cd), and lead (Pb).

3.1. E-Liquid Constituents, Non-Nicotine Products, and Illicit Additives

E-liquids are complex mixtures of glycerol (GL), propylene glycol (PG), nicotine, and flavorings, whose potential toxicity is driven by their thermal degradation products rather than their native chemical forms. These standard components are evaluated alongside non-nicotine vaping products containing cannabinoids and illicit additives like vitamin E

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acetate (VEA). This section reviews the characteristics and properties of these compounds and summarizes the most recent evidence regarding human health risks associated with chronic exposure.

3.1.1. Alcohol-Derived Chemicals

The food industry uses PG and GL widely, as both are manufactured alcohol-derived chemicals used as thickeners, humectants, preservatives, and sweeteners owing to their hygroscopic properties. GL (glycerin; E422) is derived from vegetable oils and fats [31]. PG (1,2-propanediol; E1520), which has an acceptable dietary intake of 25 mg/kg body weight in adults [32], is manufactured from propylene oxide, a recognized carcinogen. Although the U.S. Food and Drug Administration (FDA) has classified both substances as "Generally Recognized as Safe" (GRAS) for food and pharmaceutical uses [20,33,34], their safety profile upon inhalation has never been formally established. Concentrations of GL and PG in commercially available e-liquids range from 0.3% to 98% of total composition, underscoring the potential inhalation exposure during vaping [34].

3.1.2. Flavorings

Approximately 15,000 unique flavoring agents are currently used to enhance the appeal of e-liquids [8,35,36]. While these substances are deemed safe when ingested as food additives, compounds such as diacetyl (2,3-butanedione) and pentanedione raise concerns upon chronic inhalation, particularly in adolescents [37]. An additional layer of complexity arises from the thermal decomposition of flavorings during vaporization, which generates byproducts of unknown toxicity at undefined safe limit concentrations [35]. Aldehydic flavorings, including vanillin, berry, and fruit compounds, undergo chemical reactions with PG and GL to form cytotoxic cyclic acetal adducts detected in EC aerosols [38–40]. Several countries, including Australia, have enacted restrictive regulations limiting tobacco flavorings in e-liquids to discourage vaping among non-smokers, though such bans risk driving users back to conventional tobacco products [41,42].

3.1.3. Nicotine

Nicotine is a highly addictive natural alkaloid present in many, though not all, e-liquids. Reported concentrations ranging from 0 to 100 mg/mL vary widely, and permitted maxima differ by jurisdiction, such as the 20 mg/mL limit in the European Union, with less than 1% of products being genuinely nicotine-free [43]. During vaping, the transformation of nicotine into salts by its protonation with benzoate produces byproducts that are more efficiently absorbed into the bloodstream, increasing the risk of potential addiction [44]. A growing body of evidence challenges the cessation rationale for ECs, suggesting that these devices may drive rather than attenuate nicotine dependence [45–47]. Nicotine acts on the dopaminergic reward circuit and may facilitate addiction to other substances via epigenetic mechanisms [48] or maladaptive neuroplasticity. Paradoxically, reducing nicotine concentrations in e-liquid prompts longer and more frequent puffing, increasing user exposure to carbonyl compounds [49]. In response to the public health concerns posed by flavored nicotine products, the World Health Organization has called for an urgent ban, and fifty countries have already implemented such measures [50].

3.1.4. Non-Nicotine Vaping Products: Cannabinoids

Cannabis-derived substances used in e-liquids include the non-psychoactive cannabidiol (CBD)—approved for selected neurological indications—and the psychoactive Δ9-tetrahydrocannabinol (THC), which remains illegal in many jurisdictions. In e-liquids, CBD is dissolved in PG:GL mixtures; however, thermal conversion of CBD to THC during vaping has been demonstrated and shown to depend on coil temperature, solvent ratio,

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and vaping frequency [51–53]. The absence of transparent and accurate CBD labeling on EC products reinforces concerns that these devices can function as vehicles for illicit drug delivery [54], particularly as recreational cannabis markets expand [55].

3.1.5. Illicit Additives and Adulterants: Vitamin E Acetate

Vitamin E acetate (VEA; alpha-tocopheryl acetate), a thickening agent used in dietary supplements, food additives, and skin care products, is illicitly added to cannabis vape products [56,57]. Despite its antioxidant reputation in nutritional contexts, VEA has been directly implicated in the outbreak of EC-associated acute lung injury (EVALI) [58,59]. The National Institute on Drug Abuse (NIDA) and the FDA have issued national alerts recommending that individuals avoid any product containing VEA or THC obtained from informal sources [60]. As with cannabinoids, VEA contamination is largely confined to informal or illicit supply chains, but because there is no comprehensive market control, these separate product categories often mix on the unstandardized vaping market [56].

3.2. Aerosol By-Products and Device-Derived Contaminants

Although EC aerosols are not chemically equivalent to cigarette smoke, they are nonetheless potentially harmful. Produced through the heating and vaporization of e-liquid, aerosols contain all constituents of the e-liquid—PG, GL, flavorings, and nicotine—alongside byproducts generated during heating and thermal degradation, including aldehydes, psychoactive substances, and benzene (Figure 11). The wicking material (cotton, ceramic, or silica) further contributes to aerosol heterogeneity by emitting different compounds depending on heat resistance and chemical stability [61]. The coil and wick additionally release metallic contaminants into the aerosol.

3.2.1. Aldehydes

Acrolein is a highly toxic aldehyde found in traditional cigarette smoke [62] and is also detected in EC aerosols at concentrations exceeding those in tobacco smoke under high-power or dry-puff conditions, whereas under normal-use conditions, reported yields are generally lower than in traditional cigarettes [53]. As few as fifteen puffs can emit 3–61.5 μg of acrolein—a potent stimulator of respiratory distress and cardiovascular disease [63]. Formed during the oxidation of PG and GL, acrolein is an important contributor to EC-related lung toxicity, asthma exacerbation, and lung cancer risk [64]. Formaldehyde and acetaldehyde are produced in quantities that exceed the lowest-observed-adverse-effect level for respiratory harm during chronic vaping [49,53]. Regarding its carcinogenic status, acetaldehyde is classified by IARC (International Agency for Research on Cancer) as a Group 1 human carcinogen in the context of alcoholic-beverage consumption, while as an isolated chemical agent, it has historically been evaluated separately as Group 2B [65]. These yields depend strongly on coil temperature, device power, and puff topography. Comparative human data indicate that toxicant exposure may be lower in EC users than in combustible-cigarette smokers, although some inflammatory effects may be comparable [66,67].

3.2.2. Benzene

Benzene, a potent volatile aromatic carcinogen [68], is formed during the pyrolysis of PG and GL. A recent analysis of 43 e-liquids and aerosol samples confirmed the consistent presence of benzene and ethylbenzene at quantifiable concentrations [57]. Reported benzene levels are markedly lower than in traditional-cigarette smoke and increase with device power. This indicates that benzene formation depends on exposure conditions rather than being intrinsic to EC use [57].

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3.2.3. Ketenes

Ketenes, highly reactive carbon–carbon compounds, arise from the pyrolysis of acetate forms, principally VEA, during vaping. Metal components of the wick catalyze this thermal transformation, and the resulting ketenes are inhaled by both EC users and bystanders [69]. While classified among the most toxic emissions identified to date in EC aerosols, VEA-derived ketenes represent a significant risk factor for acute lung injury [70]. However, ketene formation has been demonstrated principally with acetate-containing additives such as VEA and has not been reported at comparable levels in standard nicotine vaping.

3.2.4. Cyclic Acetal Adducts

E-liquids contain diverse chemicals capable of interacting to form novel cyclic acetal adducts. These arise primarily through condensation reactions between the carrier solvents (PG and GL) and aldehyde flavorings, yielding cytotoxic compounds exhaled as aerosols and potentially introducing localized environmental exposure pathways for users and bystanders [71–73]. Cinnamaldehyde and benzaldehyde—despite not being listed as EC constituents—have demonstrated cytotoxic effects on the respiratory epithelium [74,75]. Additionally, acrolein-derived DNA adducts (α- and γ-OH-Acr-dG) are potentially carcinogenic and have recently been implicated in the early carcinogenic transformation of buccal cells [76,77].

3.2.5. Metal Elements

A wide array of metals—including arsenic, chromium, aluminum, selenium, iron, lead, titanium, cobalt, molybdenum, nickel, and manganese—has been identified in EC cartridges, tanks, and wicks [78]. Chromium and lead are classified as probable carcinogens by both the US Environmental Protection Agency and the IARC. Systematic review data indicate that most EC-derived metals are absorbed and detectable in saliva, urine, and serum at higher concentrations than those associated with conventional cigarette use [78]. Corroborating these findings, a Korean cohort confirmed elevated serum lead, cadmium, and mercury in EC users compared to traditional smokers, which was attributable to coil leaching during heating [79]. Certain metals, particularly chromium and aluminum, further catalyze the formation of cytotoxic acetals and aldehydes from PG [72]. The presence and release of these device-derived contaminants depend heavily on device type, wattage settings, and coil aging; therefore, the inhalation risk is a function of specific exposure conditions rather than the absolute presence of these elements in the hardware [53,78].

4. From Aerosols to Disease: Oxidative Stress Mechanisms, Endogenous Antioxidant Defense Responses, and Translational Implications of EC Toxic Components

4.1. Oxidative Stress Mechanism of EC Toxic Components

Inadequate quality control, inaccurate labeling, the enormous diversity of flavoring agents, and variability in coil and wick composition collectively impede the establishment of global safety standards [80]. Despite mounting evidence of health risks linked to vaping, the toxic components of e-liquids and e-liquid-derived aerosols remain subject to evolving regulation in several jurisdictions, such as the US FDA's Premarket Tobacco Product Application (PMTA) and the EU tobacco products directive. However, rather than a single missing standard, three distinct issues coexist: first, the absence of dedicated legislation in some jurisdictions; second, the heterogeneity of existing regulatory frameworks; and third, the lack of harmonization at the global level, which together leave vapers and bystanders exposed to incompletely characterized risks.

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Although the preclinical studies underlying the mechanisms presented in Table 2 and Figure 12 vary considerably depending on the device, power, coil, wick composition, puff topography, e-liquid composition, exposure system, and dose normalization, the overall pattern remains consistent. Indeed, virtually every class of e-liquid-derived aerosol byproducts, including aldehydes, aromatic hydrocarbons, heavy metals, flavorings, and cannabis additives, converges on oxidative stress through diverse mechanisms. Specifically, cellular membrane lipid peroxidation is driven by acrolein, mitochondrial dysfunction is induced by diacetyl, ROS-mediated DNA strand breaks are driven by benzene, and glutathione depletion is caused by coil-leached heavy metals. However, while standardized protocols (such as ISO 20768) exist to govern mechanical aerosol generation and puffing regimens, these standards do not extend to biological in vitro exposure design. As shown in Table 2, the available preclinical studies largely do not specify a standardized puffing regimen, nor do they report dry-puff verification, air-liquid interface configuration, or dose normalization for in vitro exposure models. This heterogeneity limits direct quantitative comparison across studies, though not qualitative comparison. Notably, several of these attributions concerning compounds such as formaldehyde, acetaldehyde, benzene, ketenes, and chromium or nickel rely on general emission data or inference from non-EC research rather than on EC-specific focused research models. Therefore, these results should be interpreted as converging lines of evidence of differing strengths instead of directly comparable quantitative findings.

Table 2. Vaping-associated oxidative stress: Toxic components, mechanisms and disease outcomes.

Toxic By-Product	Chemical Reaction on E-Liquid Substrate	Oxidative Stress Mechanism	Associated Disease	Evidence Base and Reporting Gaps	Ref.
ALDEHYDES and CARBONYLS					
Acrolein	Oxidation of PG and GL	Lipid peroxidation; respiratory epithelial ROS; mitochondrial stress	Lung toxicity, asthma exacerbation, lung cancer	In vitro, not ISO-specified; dry-puff NR	[62–64]
Formaldehyde	Thermal degradation of PG and GL	DNA adduct formation; mucosal oxidative inflammation	Respiratory harm; Probable carcinogenesis	Emissions only; no cellular model	[49,53]
Acetaldehyde	Thermal degradation of PG and GL	Protein carbonylation; DNA adduct formation; mitochondrial dysfunction	Probable carcinogenesis; chronic airway injury	Emissions only; no cellular model	[49,53,65]
AROMATIC HYDROCARBONS					
Benzene	Pyrolysis of PG and GL	ROS via epoxide metabolites; DNA strand breaks; bone marrow toxicity	Hematological cancers; lung cancer risk	Emissions only; no cellular model	[57,68]
CYCLIC ACETAL ADDUCTS					
Cyclic acetals (PG/GL; aldehydes)	Condensation of PG/GL with aldehyde flavorings	Cytotoxicity to respiratory epithelium; DNA adduct formation	Airway inflammation; oral/buccal carcinogenesis	In vitro, dose normalization NR	[38,71–73]
Acrolein-DNA adducts	Acrolein reaction with guanine bases in DNA (Michael reaction)	DNA adduct formation	Early carcinogenic transformation of buccal cells	Human biomonitoring	[76,77]
Cinnamaldehyde/ Benzaldehyde	Thermal decomposition of flavoring agents	Electrophilic attack on respiratory epithelium; thiol depletion	Cytotoxicity; airway epithelial injury	In vitro, dry-puff NR	[74,75]
CANNABIS ADDITIVES					
Vitamin E Acetate (VEA)	Ketene formation	Cellular lipid membrane disruption; surfactant layer damage	EVALI (e-cigarette/vaping-associated lung injury)	In vitro/simulation; clinical case series	[56,58–60]
Ketenes (VEA-derived)	Metal-catalyzed pyrolysis of VEA during vaping	Highly reactive oxidants; rapid protein and lipid alkylation	Acute lung injury; secondhand bystander exposure	Emissions only; no cellular model	[69,70]

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Table 2. *Cont.*

Toxic By-Product	Chemical Reaction on E-Liquid Substrate	Oxidative Stress Mechanism	Associated Disease	Evidence Base and Reporting Gaps	Ref.
HEAVY METALS					
Lead (Pb), Cadmium (Cd)	Coil/wick leaching during heating	Redox cycling; mitochondrial ROS; Glutathione depletion; catalysis of aldehyde formation	Systemic toxicity; probable carcinogenesis; cardiovascular harm	Human biomonitoring; mechanism inferred	[78,79]
Chromium (Cr), Nickel (Ni)	Coil/wick leaching during heating	Cr (VI) oxidative DNA damage; Ni epigenetic ROS dysregulation; catalysis of acetals	Lung carcinogenesis (IARC probable); endothelial damage	Emissions only; no cellular model	[72,78,79]
NICOTINE and NEUROACTIVE					
Nicotine benzoate	Protonation (Nicotine Salt Formation)	Dopaminergic ROS; epigenetic dysregulation; maladaptive neuroplasticity	Nicotine dependence; cross-addiction; cardiovascular oxidative harm	Human studies; OS mechanism inferred	[44–46,48]
Nicotine	Principal e-liquid alkaloid (0–100 mg/mL)	Dopaminergic ROS; epigenetic dysregulation; maladaptive neuroplasticity	Nicotine dependence; cross-addiction; cardiovascular oxidative harm	Human controlled exposure; OS mechanism inferred	[45,46,48]
FLAVORING AGENTS					
Diacetyl/ Pentanedione	Butter Cream flavoring agents	Bronchiolar oxidative inflammation; mitochondrial dysfunction in club cells	Obliterative bronchiolitis ('popcorn lung'); irreversible airway fibrosis	Occupational epidemiology; EC evidence limited	[37]

Abbreviations: ROS, reactive oxygen species; PG, propylene glycol; GL, glycerol; VEA, vitamin E acetate; EVALI, e-cigarette/vaping-associated lung injury; DNA, deoxyribonucleic acid; EC, electronic cigarette; ISO, International Organization for Standardization; NR, not reported.

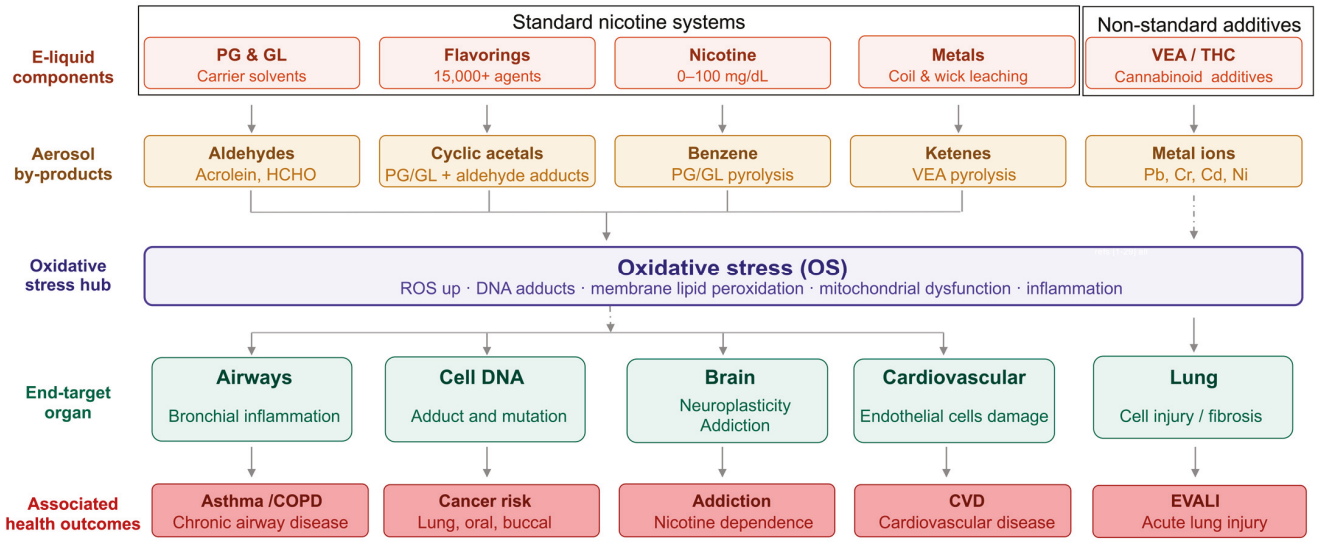

Figure 12. The schematic suggests how thermal degradation of e-liquid chemicals generates distinct aerosol byproducts and contaminants converging on oxidative stress (OS). The category of non-standard additives (VEA/THC), which encompasses non-nicotine products and illicit adulterants, is structurally distinguished from standard nicotine systems. Downstream cellular responses propagated through the OS hub affect specific end-target organs, driving associated health outcomes across respiratory, nervous, and cardiovascular systems, as well as cellular DNA injuries. Abbreviations: PG, propylene glycol; GL, glycerol; HCHO, formaldehyde; EVALI, e-cigarette or vaping product use-associated lung injury; VEA, vitamin E acetate; THC, tetrahydrocannabinol; Pb, lead; Cr, chromium; Cd, cadmium; Ni, nickel; COPD, chronic obstructive pulmonary disease; ROS, reactive oxygen species; DNA, deoxyribonucleic acid; CVD, cardiovascular disease.

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4.2. Alteration of the Antioxidant Defense Mechanisms by Exposure to EC Components

The key mechanism by which the human body responds to oxidative stress is mostly driven by the transcription factor NRF2 (nuclear factor erythroid 2-related factor 2), which is normally kept inactive in the cytoplasm by the regulatory protein KEAP1 (Kelch-like ECH-associated protein 1) [81,82]. Upon exposure to acrolein or heavy metals that modify KEAP1, NRF2 is released and translocated to the nucleus, where it promotes the transcription of antioxidant enzymes such as HO-1 (heme oxygenase-1) and NQO1 (NADPH quinone oxidoreductase 1).

Human studies of EC users report reduced superoxide dismutase (SOD) and catalase below the levels observed in non-smokers, a reversal of the glutathione redox ratio (GSH:GSSG), and an upregulated NOX-2-derived ROS appearance, consequently accelerating ROS accumulation [67,83]. Read together with the NRF2/KEAP1 mechanism characterized in other chronic-exposure settings [81,82], these observations are compatible with a biphasic NRF2 mechanism, featuring an adaptive response to short-term exposure to acrolein, aldehydes, and heavy metals that leads to NRF2 activation, triggering an initial protective antioxidant response. The second phase would be characterized by progressive NRF2 exhaustion due to sustained and chronic exposure overwhelming the antioxidant defense mechanisms. This NRF2 exhaustion is suggested to prompt a transcriptional switch that downregulates antioxidant enzymes involved in the detoxification and elimination of reactive oxygen species (ROS).

In humans, controlled and observational studies have linked EC use to endothelial cell dysfunction and vascular oxidative stress, including reduced flow-mediated vasodilation and elevated markers of oxidative injury and inflammation. A three-period randomized crossover trial conducted in tobacco smokers reported that vaping with nicotine impaired microvascular vasodilation and increased arterial stiffness and OS, whereas neither sham vaping nor vaping of the PG-GL vehicle alone modified these parameters [26]. Furthermore, an open-label, randomized, crossover study showed that smokers acutely exposed to ECs with nicotine exhibited disruption of the autonomic nervous system by a shift toward sympathetic dominance and a compromised antioxidant defense compared to vaping without nicotine [27]. The SUR-VAPES 2 randomized trial found that acute EC exposure raised soluble NOX-2-derived peptide and reduced flow-mediated dilation, although to a lesser extent than that observed in traditional tobacco smokers [28]. A recent systematic review and narrative synthesis integrated this clinical evidence, linking exposure to ECs and heated-tobacco products to the activation of OS and to the early onset of atherosclerotic and cardiovascular alterations in humans [84]. Specifically, the overproduction of ROS can be clinically monitored through distinct specimens containing excreted biomarkers, allowing the tracking of endothelial and macrovascular alterations (Figure 13). Human clinical evidence outlines that the upregulation of NOX-2-derived peptides directly mirrors the systemic oxidative burden that triggers early vascular damage. This pro-oxidant state induces lipid peroxidation and DNA damage, which are captured by elevated concentrations of plasma malondialdehyde (MDA), urinary 8-isoprostanes, and serum or urinary 8-hydroxydeoxyguanosine (8-OHdG). Nevertheless, it is important to consider that these controlled human exposures indicate nicotine as the main driver of the acute vascular and oxidative response. In contrast, the carrier solvents, flavorings, and device-derived metals rely largely on in vitro human cell models, as presented in Table 2, and have not yet been confirmed in controlled human protocols. Beyond these acute effects, the transition from acute adaptation to chronic antioxidant insufficiency may explain the progressive organ damage observed in chronic disease onset, as shown in Figure 12. This underscores that vaping injury is not solely restricted to acute events but instead increases over time through the "NRF2 exhaustion model". Because these studies documenting OS and toxicant exposure in EC users do not provide direct human evidence for

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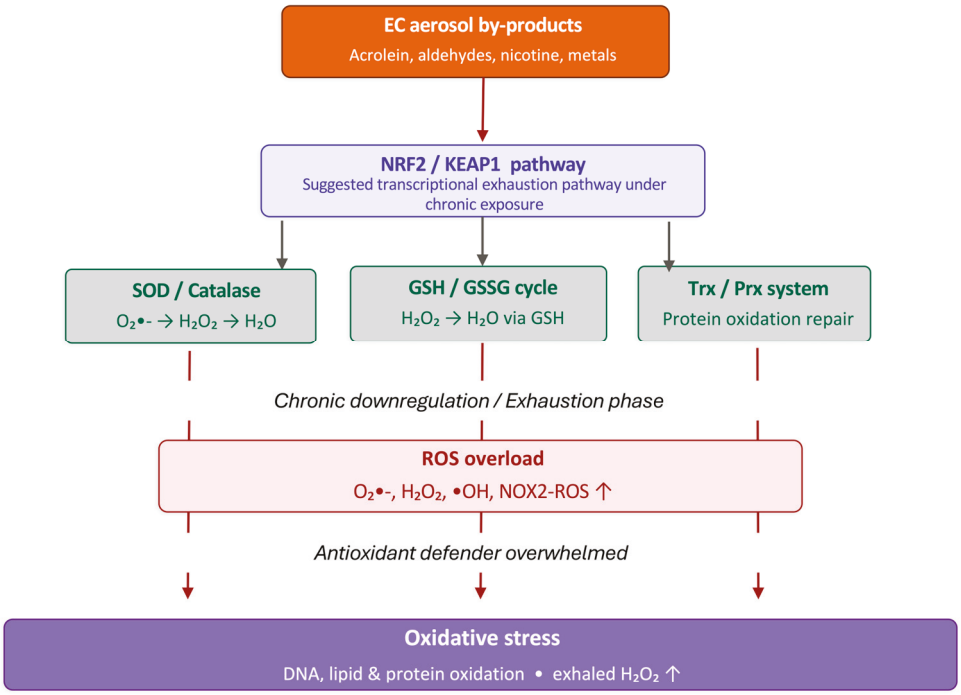
the complete NRF2/KEAP1 exhaustion sequence, the framework illustrated in Figure 13 is therefore presented as a suggestive synthetic model of converging mechanisms rather than a strict established evidence strength.

Although these chronic human cohort studies converge toward the same conclusion of OS-associated chronic disease, their interpretation requires caution. Most participants, classified as EC users, were former smokers, and several biomarkers in EC users fell between those of current smokers and non-users instead of being exclusively linked to EC use [85]. Conversely, longitudinal data from Wave 1 of the PATH (Population Assessment of Tobacco and Health) study cohort showed that adult former smokers who switched exclusively to ECs had levels of biomarkers of inflammation and OS, including urinary F_2-isoprostane, similar to those of former smokers who had stopped all tobacco use, and to those of the never-users [86]. Smoking history and time since cessation are important covariates in this literature, discriminating between acute and chronic exposure.



Measurable biomarkers in EC users

Biomarkers	Observed alterations in EC users vs non-smokers	Specimen	Study design	Ref
Catalase activity	Decrease	Erythrocytes	Crossover, single-blind	a
SOD activity	Decrease	Erythrocytes	Crossover, single-blind	a
Exhaled H_2O_2	Increase	Breath	Clinical	b
MDA	Increase	Plasma	Descriptive cross-sectional	c
Nox-2-derived peptide	Increase	Serum	Cross-sectional	d
8-isoprostanes	Increase	Urine	Cross-sectional	e
8-OHdG	Increase	Urine, serum	Cross-sectional	f

Figure 13. Proposed framework for NRF2 pathway exhaustion under e-cigarette (EC) aerosol exposure. Chronic exposure to aerosol byproducts is hypothesized to parallel a biphasic shift from initial endogenous antioxidant defense activation to possible transcriptional downregulation of the NRF2/KEAP1 master regulatory pathway and its downstream enzymatic defenses—superoxide dismutase (SOD)/catalase, the glutathione peroxidase (GPx)/glutathione (GSH) cycle, and the thioredoxin (Trx)/peroxiredoxin (Prx) system. This exhaustion is associated with reactive oxygen species (ROS) overload and measurable oxidative stress across specific human cohorts. Biomarker changes observed in human EC users are indicated vs. non-smoking controls. Letters in the righ-most column correspond to studies in the literature: a, [83]; b, [87]; c, [84]; d, [28]; e, [86]; f, [20,86,88].

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4.3. Biomarkers to Assess Disruption of Antioxidant Defense Associated with EC Exposure

Beyond the NRF2/KEAP1 pathway, several other antioxidant defense molecules are targeted by the toxic EC components during oxidative damage. These include 8-hydroxydeoxyguanosine (8-OHdG), which reflects the oxidative damage of nuclear DNA; malondialdehyde (MDA) and 8-isoprostanes, which indicate lipid peroxidation; and hydrogen peroxide (H_2O_2), which reveals systemic oxidation. All these biomarkers, measurable from accessible specimens such as blood [83], plasma [85], serum [87], urine [21,67,88], and exhaled breath [66], can provide only an estimate of the OS burden in EC users (Figure 13) as no thresholds have been designated.

Together, these data from this narrative review underscore that the transformation of EC chemical components into byproduct aerosols raises concerns that are not yet fully addressed by current safety regulations. Furthermore, chronic exposure to these toxic aerosols for both EC users and bystanders carries a long-term disease risk that remains to be fully quantified.

4.4. Convergence of Data from Combined Bibliometric Analysis and Narrative Review and Future Perspectives

The bibliometric analysis and narrative review align conceptually in highlighting OS as the central and bridging theme linking EC exposure to chronic disease. This relationship is evaluated through different forms of evidence. However, because OS was utilized as a baseline search term, this structural alignment maps a shared academic focus rather than demonstrating an independent finding.

As described in Section 3, EC aerosol components trigger OS through direct ROS generation and progressive NRF2/KEAP1 exhaustion, shifting the antioxidant defense from an initial protective response to a chronic antioxidant defense deficit that worsens with sustained exposure duration. This timing directly mirrors the chronological pattern observed in Figure 9, in which earlier studies (2017–2020) focused on acute events such as EVALI and short-term exposure, while after the outbreak, the research landscape shifted toward chronic disease targeting the cardiovascular and renal systems. This reveals a striking thematic convergence between global literature trends and molecular toxicity mechanisms, in which the chronological maturation observed in the bibliometric timeline parallels the expanding academic focus on the hypothesized NRF2 biphasic framework. Indeed, early cross-sectional studies captured mostly short-term vapers whose initial adaptive response was asymptomatic, while the chronic antioxidant system deficiency and progressive cellular insufficiency become detectable as organs become the end-targets of the long-duration EC exposure. As researchers and clinicians have progressively investigated NRF2, the chronology of these scientific advances has aligned with the conceptual stages of the "NRF2 biphasic response". To synthesize this cross-disciplinary mapping effectively, it is necessary to distinguish between the bibliometric body of literature that is strictly limited to human observational studies, and the molecular signaling pathways heavily relying on in vitro human cell models presented in the narrative review. These distinct levels of evidence, although fundamentally complementary, are not interchangeable. Thus, any extrapolation from preclinical protocols to human clinical applications must be made with caution. Our data track a clear structural shift in publication trends over the last decade, developing across three observable intervals. An initial exploratory phase occurred between 2016 and 2018, characterized by limited publication activity. This baseline shifted into a rapid expansion phase from 2019 to 2022, marked by substantial increases in annual output and growth rates. Most recently, the 2023–2025 window indicates a period of academic consolidation during which publication productivity stabilized at a relatively high level while cumulative scientific knowledge continued to grow. These structural

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trends confirm that vaping-associated OS has become an established area of research with sustained international scientific interest.

Convergence is further confirmed by the keyword co-occurrence analysis presented in Figure 10, where OS is revealed as the most connected node across all four clusters, bridging epidemiological, clinical and mechanistic research. Strikingly, the key finding of this combined analysis is that OS is the main biological mechanism explored in the narrative review and the most frequent bridging keyword in the bibliometric research landscape. These two different perspectives, one mechanistic and one bibliometric, align to reflect a common research focus from which scientific communities across disciplines and study designs have independently and progressively identified OS as an important factor in EC-associated chronic disease.

The biomarkers described in Figure 13 translate this mechanistic evidence into measurable parameters across noninvasive specimens. Their integration into future longitudinal studies could address the critical gap identified in this combined analysis: the absence of dose–response data and exposure thresholds across sexes, age groups and device types for e-liquid components and aerosols. Moving forward, future research agendas must prioritize three areas: (1) standardizing air-liquid interface preclinical protocols incorporating device power, coil and wick materials, puff topography, and dose normalization, to enhance study reliability and comparability; (2) executing long-term human cohort studies to monitor and track exposure thresholds across age, sex, and device types using non-invasive biomarkers like urine, exhaled breath, and saliva; and (3) separately studying regular nicotine ECs and illicit black-market cannabinoids to distinguish their health impact. These data are urgently needed to provide a regulatory framework and justify the control of EC component composition and emission standards.

Together, this bibliometric analysis and narrative review indicate that the field of OS-related chronic diseases from vaping has matured during the last decade from a narrow focus on acute lung injury toward a mechanistic, multidisciplinary understanding to guide future diagnostic, therapeutic, and regulatory strategies.

4.5. Limitations

Several limitations of this integrated review merit acknowledgment. The narrative component is necessarily selective; the EC literature is vast and growing, and no review can claim to capture every relevant study. The bibliometric analysis was based exclusively on the Scopus database, which does not index all published literature, and a single search date (20 March 2026), introducing indexing-dependent selection effects. Studies published in languages other than English were systematically excluded. A significant limitation involved the bibliometric search itself because "oxidative stress" was included as a baseline search term to meet the objective of the study. This has led to a high frequency and high network centrality, which should be cautiously interpreted solely within the context of the bibliometric dataset of this review; thus, OS should not be interpreted as an independent validation of the biological mechanism linking ECs to chronic disease. As noted earlier, the deliberate choice to rely solely on the Scopus database was motivated by its broader coverage, multidisciplinary scope, and built-in citation tracking over Web of Science and PubMed. While merging these three databases would have made our study more rigorous and prevented coverage-dependent effects, Scopus was deemed sufficient for the scope of this analysis. Another limitation lies in the divergence between the bibliometric analysis mapping human clinical and epidemiological data, and the narrative review, which intentionally selected the most recent preclinical in vitro human models, necessary for explaining signaling pathways. The exclusion of animal studies was a deliberate choice to maximize clinical and epidemiological data. This implies that the present study omits mech-

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anistic evidence that has not yet been validated in human populations. Finally, bibliometric co-occurrence networks visualize topical proximity but do not establish the directionality, quality, or causal weight of associations between the two research themes.

4.6. Research Gap and Future Directions

Our bibliometric analysis identifies four gaps that the narrative review cannot close on its own. First, the very low multi-country publication fraction, together with a concentration of the ten leading institutions in the US, highlights the need for coordinated international consortia and harmonized reporting to foster a global perspective on universal standards. Second, since 2020, research has frequently linked heart and kidney health risks together, yet the corresponding mechanistic evidence is missing. Future research must therefore prioritize how OS damages the kidneys and the vasculature upon EC exposure. Third, the acute events of the EVALI outbreak stand out as a distinct pattern driven by the illicit use of VEA as an adulterant in unregulated THC vaping products, separate from daily EC use. This distinction prevents these separate products categories from being wrongly conflated with the toxicological profiles of regulated nicotine products. Fourth, current human research lacks critical evidence on how vape dose, device type, and frequency of use determine long-term exposure thresholds and clinical outcomes. To resolve this data gap, long-term monitoring studies, through international collaborations, should track real-world users over time, stratified by age, sex, and the specific devices and products they use, using non-invasive OS biomarkers measured in exhaled breath or urine. Together, these approaches would improve the comparability on which such thresholds depend.

5. Conclusions

The significance of this review lies in its combined approach, as it is the first to combine a bibliometric analysis and a narrative synthesis to provide an integrated picture of how EC exposure has been associated with chronic disease through OS-related pathways. Although electronic cigarettes were initially introduced as a promising cessation tool for nicotine-dependent smokers, our findings indicate that this original promise has been complicated by the rapid increase in EC use among never-smokers and adolescents and by unresolved toxicological concerns. The bibliometric analysis describes a research field that has grown rapidly over the last decade, progressively shifting from early, acute-centered research toward a broader focus on chronic disease outcomes; within this corpus, oxidative stress was the most frequent bridging keyword and the most connected theme in the global research landscape; however, this prominence does not independently confirm the mechanism. In turn, the narrative synthesis indicates that ECs deliver a diverse and incompletely characterized mixture of components that have been associated with markers of OS across multiple body systems, particularly the cardiovascular, pulmonary, digestive, and immune systems. These conclusions rest on a selective synthesis and do not establish causation. Furthermore, our findings highlight geographical inequities and a disciplinary gap between epidemiology and mechanistic research around oxidative stress. By addressing the “what”, “who”, “where”, “how”, and “how much” of OS-associated chronic disease from vaping, this review provides researchers with a clearer and more comprehensive picture of the current state of the science on this emerging public health concern. Ultimately, it offers clinicians a synthesis of the most relevant disease mechanisms proposed to link vaping-associated oxidative stress to chronic disease and equips policymakers with an evidence-based framework to identify the regulatory gaps necessary for an effective response to the rapid global increase in EC use.

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Abbreviations

CBD	Cannabidiol
COPD	Chronic obstructive pulmonary disease
EC	Electronic cigarette
EVALI	E-cigarette or vaping product use–associated lung injury
FDA	Food and Drug Administration (U.S.)
GL	Glycerol
OS	Oxidative stress
PG	Propylene glycol
ROS	Reactive oxygen species
THC	delta-9-tetrahydrocannabinol
VEA	Vitamin E acetate

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Analyzing 8-Oxoguanine in Exhaled Breath Condensate: A Novel Within-Subject Laboratory Experimental Study on Waterpipe Smokers

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Abstract: Introduction: This study aimed to analyze exhaled breath condensate (EBC) for 8-oxoguanine (8-oxoGua), an oxidative stress biomarker among waterpipe (WP) smokers. Methods: In a within-subject pre-post exposure design, thirty waterpipe smokers completed two 45 min laboratory sessions. EBC was analyzed for 8-oxoGua before and after WP smoking. Median differences between time points (pre vs. post) were assessed using the Wilcoxon sign rank test, with significance defined as $p < 0.05$. Results: The analysis included 59 WP smoking sessions. Participants had a median age of 24 years (IQR: 21–25), with 62.1% being female. Most had a bachelor's degree or less (62.1%), and over half were students (55.2%), while 34.5% were employed. The average age for first WP use was 18.6 years, with participants reporting a median of three WP smoking sessions per month. Results indicate a median increase in 8-oxoGua among participants from 5.4 ng/mL (IQR: 8.8) before the smoking session to 7.6 ng/mL after (IQR: 15.7; $p < 0.001$). Conclusions: This study is the first to examine 8-oxoGua in EBC. Findings provide strong evidence of WP smoking's contribution to oxidative stress in the airways. It justifies the use of EBC to study the exposure to markers of oxidative stress with emerging tobacco use methods such as the waterpipe.

Keywords: exhaled breath condensate; 8-oxoguanine; oxidative damage; waterpipe

1. Introduction

Waterpipe (WP; Hookah) is one of the emerging global tobacco products and in the United States (US), especially among young adults [1,2]. The Centers for Disease Control and Prevention (CDC) reported that in 2018, approximately 7.8% of high school students and 12.3% of young adults aged 19–30 years had used a WP in the previous year [3]. Recent data from the Population Assessment of Tobacco and Health (PATH) study (2013–2021) showed increasing trends in WP smoking among young adults (aged 18–24) compared to other age groups [4]. This trend is alarming, especially given that WP smoking is substantially higher among women and youth compared to older adults [5,6]. Several factors drive the rise in waterpipe smoking: (1) misleading marketing that falsely promotes

it as a safer alternative to cigarettes; (2) social acceptance in homes, cafés, and restaurants; (3) its usage normalization through social media; and (4) the lack of waterpipe-specific regulations [1,2,7].

WP smoke contains many toxicants derived mainly from the burning of charcoal and the heating of tobacco [8,9]. These toxicants include carbon monoxide (CO), nicotine, particulate matter, volatile organic chemicals such as formaldehyde and acrolein, arsenic, and heavy metals like lead [8–11]. When inhaled, these toxicants generate excessive reactive oxygen species (ROS), which are known to cause DNA damage [12,13]. When the level of ROS exceeds the body's antioxidant defense response, it disrupts the normal redox balance, leading to the generation of oxidative stress [14]. Oxidative stress is one of the potential mechanisms by which smoking predisposes to a range of health problems, such as respiratory conditions (asthma, chronic bronchitis, and cystic fibrosis), cardiovascular diseases, neurological disorders, and cancer [1,8,9,15–17].

Previous research has focused on identifying oxidative stress markers in various tobacco-related diseases [8,13,14,17,18]. Among those, 8-oxoguanine (8-oxoGua), an oxidation product of DNA's guanine base, is a biomarker of ROS-mediated DNA damage [14]. Only a few studies have investigated 8-oxoGua levels in the blood and urine of WP smokers [19,20]. For instance, Jebai et al. found higher urinary 8-oxoGua concentrations after WP smoking [19]. However, due to delayed effects and influences from other sources like environmental factors and nutrition, and complex interactions affecting systemic levels of markers of oxidative stress, it is difficult to determine the precise impact of WP smoking on 8-oxoGua levels. Furthermore, direct airway sampling could clarify this, but traditional methods such as bronchoscopy are often invasive and costly [21]. Therefore, developing non-invasive, site-specific tools to assess early lung changes after WP exposure, especially in young users, presents a critical opportunity.

The main objective of this study is to measure 8-oxoguanine (8-oxoGua), an oxidative stress marker in the exhaled breath condensate (EBC) of young waterpipe smokers using a within-subject pre-post WP smoking session design. EBC is a collection of aerosolized droplets from exhaled air that contains thousands of identified metabolites [21]. It is an easy-to-collect, non-invasive, and affordable biological matrix for sampling airways [22]. Measuring 8-oxoGua in EBC may therefore serve as a non-invasive method to understand the molecular pathological changes occurring in the respiratory tract following WP smoking [21,22]. The analysis of EBC allows us to directly link WP smoking to changes in oxidative stress within the respiratory tract, a method that has not been explored in prior studies.

2. Methods

2.1. Participants

Thirty current WP smokers (defined as smoking WP at least once a week in the past six months) aged 21–35 years were recruited from Miami, Florida, via flyers, word of mouth, and online advertisements. We excluded participants with self-reported history of chronic health concerns or psychiatric conditions, regular use of prescription medications (other than vitamins or birth control), and current use of ≥ 5 cigarettes or other tobacco/nicotine products in the past month. Women were excluded if they were breastfeeding or tested positive for pregnancy (by urine pregnancy testing) at the screening. Participants were preliminarily screened on the phone for potential eligibility, and the information was later confirmed during an in-person screening session. The Institutional Review Board of Florida International University (FIU) approved this study, and written informed consent was obtained from participants prior to their participation. Participants were compensated for their time, effort, and expenses associated with attending the lab sessions with a $100.

2.2. Study Design

All participants completed two WP smoking sessions in the clinical lab at Florida International University (FIU), preceded by ≥12 h tobacco abstinence confirmed by expired Carbon Monoxide (eCO) < 6 ppm. The sessions were separated by at least a 48 h washout period. All participants selected their preferred tobacco flavor from a variety of options offered and smoked the same flavor in both sessions. Before smoking, the WP was prepared by filling the head with 15 g of tobacco product. The head was then covered with perforated foil. Tobacco was heated by two pieces of quick-lighting charcoal lit by study staff and placed on top of the foil. Participants smoked ad libitum for up to 45 min. Participants were seated in a private room with a comfortable reclining chair, given a choice to use any electronic device, and instructed to use WP at their natural pace. The same standardized instructions were given to all participants, and there was limited interaction with participants during the WP smoking session.

2.3. EBC Collection

EBC samples were collected 10 to 15 min before and after each WP smoking session. The participants were asked to breathe for 15 min (tidal breathing) through a single-use disposable RTubeTM collector (Respiratory Research, Inc., Charlottesville, VA, USA). After sample collection, a plunger was used to pool the condensed material within a cryogenic vial (about 1–2 mL) and stored at −80 °C within approximately 5 min to minimize degradation and ensure strict sample integrity.

3. Measures

3.1. Participant Characteristics

Demographic information, including age (years), gender (male/female), race (white/non-white), ethnicity (Hispanic/non-Hispanic), and education status (high school or less/some college or associate degree, bachelor's degree/more than bachelor degree), employment status (employed, not employed) were collected at baseline.

3.2. Waterpipe Pattern Use

WP-related Measures included: age at first WP smoking experience (years); the average number of hookahs (heads/bowls) smoked per month; usual type of WP tobacco smoked (flavored or unflavored); perceived differences in smoke production between flavored and unflavored tobacco (flavored, unflavored, or no difference); average duration of a typical WP smoking session (less than 30 min, 30–60 min, or more than 60 min); shared WP with others (yes/no); and usual smoking place (home, friend's home, public places such as cafés/restaurants, others).

3.3. 8-Oxoguanine Measurements

EBC samples were analyzed by the Forensic and Analytical Toxicology Facility at FIU using Liquid Chromatography-Triple Quadrupole Mass Spectrometry (LC-QqQ-MS) [22]. Before analysis, samples were thawed and vortexed. A 500 µL sample aliquot was then transferred to a plastic Eppendorf tube. A 4 h vacufuge was performed to evaporate all the EBC matrix. The samples were then reconstituted in 75 µL of acetonitrile and spiked with 8-Oxo-2′-deoxyguanosine-[13C,15N2] as an internal standard to facilitate the quantification of 8-oxo-7,8-dihydroguanine.

The limit of detection (LOD) and limit of quantitation (LOQ) for 8-oxo-7,8-dihydroguanine were 5.2 and 15.7 ng/mL, respectively. The LOD and LOQ were calculated using the signal-to-noise ratio of 3:1 for LOD and 10:1 for LOQ. The calculations were based on three replicates per calibration point. The 8-oxoguanine measurement was validated using a

fit-for-purpose approach. Validation experiments were conducted in both acetonitrile and EBC to assess method performance. Carryover, matrix effects, and interference studies were performed to ensure specificity and reliability in batch analysis. Calibration curves were generated in triplicate to determine the LOD and LOQ, and to assess linearity. All calibration curves demonstrated strong linearity, with R^2 values consistently exceeding 0.99. No matrix effects, carryover, or interference between the analyte and other compounds were observed [23].

3.4. Data Analysis

Baseline characteristics of the study sample were summarized using means and standard deviations (SDs) for continuous variables, along with proportions for categorical variables. Pre- and post-concentrations of 8-oxoGua for each session were calculated and presented with medians and interquartile ranges (IQRs). Planned comparisons were conducted with the Wilcoxon signed-rank test to examine median differences between time points (pre vs. post). Data were analyzed using SPSS version 25, and a *p*-value greater than 0.05 was considered statistically significant.

4. Results

The analysis included 59 WP smoking sessions. Table 1 shows the sociodemographic and WP use characteristics of the study participants (n = 30). The median age was 24 years (IQR: 21–25), and the majority were female (62.1%). Most participants had a bachelor's degree or less (62.1%), and over half were students (55.2%), with 34.5% employed.

Table 1. Sociodemographic and waterpipe use-related characteristics of the participants (n = 29) *.

Variables	N (%)
Sex	
Females	18 (62.1)
Males	11(37.9)
Age in years, Median (IQR)	24 (21.25)
Education	
Bachelor's or less	18 (62.1)
Master's and above	11(37.9)
Employment Level	
Employed	10 (34.5)
Not employed	3 (10.3)
Student	16 (55.2)
Age at WP first use, Mean (SD)	18.6 (2.4)
WP month average, Median (IQR)	3 (1.5)
Average time spent on WP smoking session	
Less than 30 min	6 (20.1)
30–60 min	18 (62.1)
More than 60 min	5 (17.2)

* One participant had missing data.

The mean age at first WP use was 18.6 years (SD = 2.4). Participants reported a median of 3 WP smoking sessions per month (IQR: 1–5). Regarding typical session duration, 62.1% reported smoking for 30–60 min, 20.1% for less than 30 min, and 17.2% for more than 60 min.

Results show an increase in median 8-oxoGua among the participants, from 5.4 ng/mL (IQR: 8.8) before the WP smoking session to 7.6 ng/mL (IQR: 15.7; $p < 0.001$) after the WP smoking session (Figure 1).

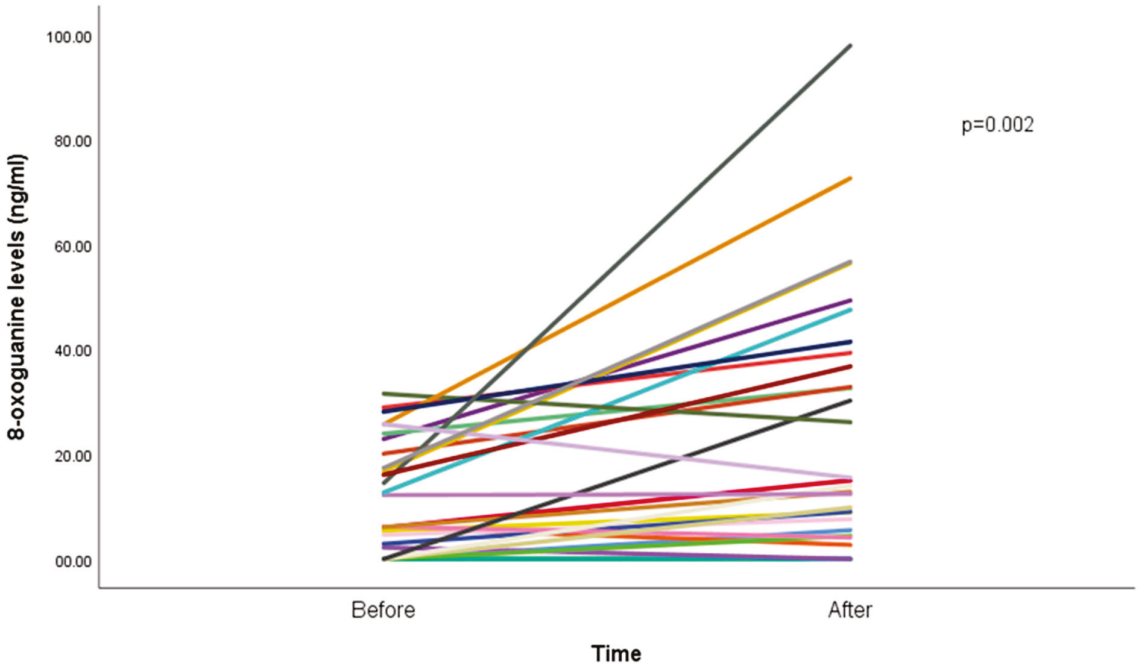

Figure 1. 8-oxoguanine concentration (ng/mL) before and after waterpipe smoking (n = 29). Each line represents a participant; overlapping lines reflect similar values. One participant was excluded due to missing post-session data.

5. Discussion

This study is the first to assess 8-oxoGua levels in EBC before and after a waterpipe (WP) smoking session in young adults. We observed a statistically significant increase in EBC 8-oxoGua concentrations post-smoking, indicating acute oxidative DNA damage in the lower respiratory tract [21]. These within-subject findings provide strong evidence of WP's immediate impact on the respiratory airways, using a non-invasive biomarker that reflects local airway-level oxidative stress. This biomarker-driven approach significantly enhances early detection of oxidative stress in the lungs and is essential for advancing clinical trials and enforcing tobacco regulation. The concerning evidence of DNA damage in the airways underscores the urgent need for targeted WP-specific policies and comprehensive educational campaigns.

Direct assessment of airway inflammation is performed using invasive techniques such as induced sputum, bronchoalveolar lavage fluid collection, or bronchial biopsy. In contrast, exhaled breath condensate (EBC), obtained by cooling exhaled air during normal breathing, is a simple, noninvasive, and safe [24]. While saliva is a convenient and fully non-invasive matrix for measuring oxidative stress markers, it predominantly reflects oral cavity and systemic sources and can be influenced by diet, oral hygiene, and local inflammatory conditions [21,25]. In contrast, EBC samples the airway lining fluid and provides a more specific assessment of lower respiratory tract oxidative stress [24], making it particularly suitable for evaluating inhalation-related exposures such as waterpipe smoking. Several studies have shown an increase in 8-oxoGua in lung tissue, plasma, and urine of cigarette and waterpipe smokers in comparison to nonsmokers [19]. For example, a previous study demonstrated that the urinary concentration of 8-oxoGua increased significantly after smoking flavored waterpipe tobacco (from 2.96 $\pm$ 0.84 to 3.45 $\pm$ 0.76 ng/mg

creatinine, $p = 0.003$) [19]. Our results are consistent with these findings, indicating a direct oxidative damage potential of WP smoking on the airway of smokers. Our new method of measuring 8-oxoGua using exhaled breath condensate (EBC) offers significant advantages over traditional urinary or plasma samples. Notably, it has the potential to eliminate systemic exposures and interactions that can interfere with the measurement of 8-oxoGua in those samples. This includes influences from chemical agents, ionizing and UV radiation, as well as environmental pollutants.

The study has several limitations, including a relatively small sample size of young adults. Despite the small sample size, we had sufficient statistical power to identify a significant difference in 8-oxoGua levels, a biomarker of oxidative stress, before and after waterpipe (WP) smoking. Additionally, the laboratory setting differs from the typical environment in which people smoke waterpipes, which may influence smoking patterns and exposure levels. To mitigate this issue, we made efforts to create a comfortable atmosphere by allowing participants to sit in reclining chairs while smoking a WP and watching movies of their choice. Another limitation is that we did not include repeated measurements of 8-oxoGua levels to assess intra-individual reproducibility, as this was not an objective of the present study. Furthermore, our study focused solely on the acute effects of waterpipe smoking; future research should explore the long-term impact of repeated WP smoking on oxidative stress using longitudinal designs. Finally, since we did not include nonsmokers in our study, we are unable to compare our results with those of nonsmokers or never-smokers. Incorporating non-smoker controls would provide a more comprehensive comparison across different demographic groups. Additionally, evaluating a wider range of oxidative stress biomarkers in EBC, such as 8-isoprostane or malondialdehyde, could provide deeper insights into the mechanisms of harm related to waterpipe use. Such evidence would help inform targeted risk reduction strategies and support the development of standardized protocols for EBC biomarker analysis in tobacco research.

6. Conclusions

In summary, while evidence of the harms of WP smoking is growing, previous studies have mainly focused on investigating systemic biomarkers, which may overlook localized airway damage. This study demonstrates that WP smoking acutely elevates 8-oxoGua levels in airway lining fluid, indicating localized oxidative DNA damage and highlighting EBC as a valuable, non-invasive method for monitoring respiratory oxidative stress. The results deliver robust and compelling evidence of WP smoking's contribution to oxidative stress in the airways of smokers. Given the alarming rise in WP use among youth and widespread misconceptions regarding its safety, immediate action is imperative to protect public health.

Author Contributions: N.S., W.M. and T.A. conceptualized and designed the study and drafted the initial manuscript. N.S. and L.M. conducted data analysis. T.F., S.R., S.C., S.F., L.M., W.M. and A.P.D. contributed to the interpretation of data and critically revised the manuscript for important intellectual content. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Review Board (or Ethics Committee) of Florida International University (Protocol #: IRB-20-0530, 11/13/20) for studies involving humans.

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Data Availability Statement: The data presented in this study are available upon reasonable request from the corresponding author. The data are not publicly available due to privacy/ethical restrictions.

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Review

Cardiac Autonomic Dysfunction and Increased Oxidative Stress in Conventional Cigarettes and E-Cigarettes: Heart Rate Variability as a Cardiovascular Predictor

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Abstract: Conventional and electronic cigarette (e-cig) users face an increased risk of cardiorespiratory diseases, driven by well-characterized pathways involving inflammation and oxidative stress (OS). Conventional cigarettes contain numerous harmful chemicals, such as nicotine and non-nicotine compounds, which produce reactive oxygen species. Although initially considered a safer alternative, the e-cig still generates toxic aldehydes that are capable of triggering oxidative responses. Heart rate variability (HRV) is an important tool for assessing autonomic function and predicting prognosis. Cardiac autonomic dysfunction, indicated by reduced HRV, has emerged as a critical cardiovascular risk factor associated with several diseases. Clinical and experimental studies show that increased OS is directly associated with heightened sympathetic activity and inversely with parasympathetic modulation. This review demonstrates that exposure to conventional cigarettes smoking and e-cigs adversely affects cardiac autonomic function, detectable by a global reduction in HRV that reflects a shift toward sympathetic dominance and a consequent increase in cardiovascular risk. These changes are associated with increased OS due to nicotine and non-nicotine compounds maintaining sympathovagal imbalance in smokers. Thus, we suggest that autonomic dysfunction, detected by HRV, correlates with oxidative responses and may be used as a modifiable risk factor in longitudinal studies involving both smoking modalities.

Keywords: tobacco smoking; vaping; oxidative stress; autonomic nervous system; heart rate variability; cardiovascular risk

1. Introduction

Over the last few decades, the nicotine-based product industry, such as conventional cigarettes, and electronic nicotine delivery systems (ENDSs), including electronic cigarettes (e-cigs), "vapes" or "pods", have undergone a significant transformation to increase their appeal and to meet new consumer demands [1]. This phenomenon has boosted the popularity of these products, particularly among teenagers and young adults, due to targeted advertising, marketing, various flavours, nicotine compositions, and a perception of reduced harm [2–8].

Regardless of the consumption method, smoking is associated with various physiological disorders and represents one of the leading causes of mortality and morbidity worldwide [9], becoming a significant public health issue [2]. ENDSs appeared in the 1970s initially as a less health-damaging option [9]. Nevertheless, in the last two decades with the effective ENDS introduction in the market, research has raised concerns about the real impacts of this new consumer demand and its possible cardiorespiratory, autonomic, and metabolic repercussions [6–10]. Although the ENDSs do not need tobacco combustion like conventional cigarettes, these devices can also deliver toxic chemical substances such as nicotine as well as reactive oxygen species (ROS), which are involved in autonomic dysfunction.

In this context, heart rate variability (HRV) has been demonstrated as a non-invasive tool for assessing alterations in autonomic balance in animals and humans [11–16]. HRV has been widely employed in different clinical conditions, including hypertension [17], chronic obstructive pulmonary disease (COPD) [18], and increases in oxidative stress (OS) [17–23]. Accordingly, the present study aims to review the literature about the impacts of conventional cigarettes and ENDS consumption on cardiac autonomic function, emphasizing the role of HRV as a sensitive tool to detect autonomic alterations associated with high levels of ROS and OS.

2. Impact of Conventional and Electronic Cigarettes on Cardiorespiratory Systems

Tobacco smoking remains a leading modifiable risk factor for cardiovascular and respiratory diseases worldwide. Particularly via tobacco combustion, conventional cigarette smoking (CCS) delivers thousands of toxic chemical substances, such as tar and nicotine, and continues to account for millions of deaths each year. Findings confirm the robust associations between tobacco exposure and cardiovascular and respiratory morbidity due to increased risk of coronary artery disease (CAD), myocardial infarction (MI), COPD, lung cancer, and other outcomes [24].

A causal relationship between genetic liability to smoking and the onset of multiple cardiovascular conditions was demonstrated by Larsson and Burgess et al. [25] in a comprehensive Mendelian randomization meta-analysis. While the odds ratio (OR) for CAD in their findings was variable across models, some analyses suggested ORs around 1.33 per unit increase in the lifetime smoking index. Similarly, recent analyses on atrial fibrillation indicate increased risk among smokers, with an OR of 1.23 (95% CI: 1.10–1.36) in specific cohorts such as Finnish populations [24]. Additionally, Al-Halabi et al. [26] demonstrated that smoking 16 to 25 cigarettes per day nearly triples the risk of acute MI. These data collectively reinforce a dose-dependent increase in cardiovascular risk due to cigarette use.

In the respiratory system, tobacco smoking is the leading contributor to COPD, typified by persistent airway inflammation, remodelling, and alveolar destruction [27]. Bircan et al. [28] and Minervini et al. [29] presented associations between smoking and COPD and other several pulmonary conditions such as asthma and COPD–asthma overlap and lung cancer. These findings align with prior meta-analytic studies reporting a lung cancer relative risk of 10.92 (95% CI: 8.28–14.40), a 4.01 risk ratio for COPD, and an OR of 1.61 (95% CI: 1.07–2.42) for adult-onset asthma among smokers [30,31].

A growing body of mechanistic evidence implicates several inflammatory biomarkers in tobacco-related damage. Caspase-1 activation, increased expression of toll-like receptor 4 (TLR4), and elevation of interleukin-6 receptor alpha (IL-6R-α) have been documented in monocytes of smokers [32,33]. In addition, ROS generated by smoking induce epithelial cell apoptosis and activate alveolar macrophages and neutrophils, sustaining a pro-inflammatory pulmonary milieu [27]. These pathways are associated with inflamma-

some activation, epithelial injury, and systemic immune dysregulation. Such molecular disruptions underpin both respiratory and vascular damage, contributing to chronic conditions such as emphysema, atherosclerosis, and heart failure.

Although initially promoted as a less health-damaging option, ENDSs are increasingly recognized as harmful, particularly to the respiratory system. Exposure to e-cig aerosols similarly results in epithelial damage, airway inflammation, increased ROS production, and endothelial dysfunction, mostly associated with high-powered devices and flavoured liquids [3]. A recent meta-analysis by Shabil et al. [34] quantified the association of e-cig use and risk of COPD: current e-cig users had an OR of 1.48 (95% CI: 1.36–1.61) for COPD compared with nonusers, and ever users had an OR of 1.79 (95% CI: 1.42–2.25). Park et al. [35] and Auschwitz et al. [36] showed that e-cig exposure disrupts epithelial integrity and immune responses in pulmonary tissue, although long-term clinical outcome data remain limited.

Recent studies indicate that e-cig use exerts measurable and deleterious effects on both the cardiovascular and respiratory systems, warranting careful scientific scrutiny and public health attention. E-cig use increases arterial pressure (AP), heart rate (HR), and arterial stiffness while reducing airflow in the lungs [37]. A large body of epidemiological evidence has also indicated that e-cig smokers have a higher risk of developing cardiovascular disease, such as cardiac arrhythmia, hypertension, acute coronary syndromes, and heart failure. In fact, ENDSs are considered an independent risk factor for increased rates of cardiovascular disease occurrence and death [38]. By analyzing the National Health Interview Survey data, Vindhyal et al. [39] found e-cig users had a 50% higher risk of developing MI (OR, 1.56; 95% CI, 1.45–1.68), 30% higher risk of developing stroke (OR, 1.30; 95% CI, 1.20–1.40), and 40% higher risk of circulatory problems (OR—1.43, 95% CI, 1.25–1.65) when compared with nonusers.

Young e-cig users show decreased lung functional capacity and cardiovascular reserve compared with non-smokers, with spirometry indicating an increased risk of respiratory obstruction [40]. Systematic reviews reveal respiratory effects including cough, phlegm, asthma symptoms, and cases of chronic bronchitis and COPD, alongside cardiovascular effects [41]. Additionally, studies indicate that e-cig aerosols also impair endothelial function by activating Nicotinamide Adenine Dinucleotide Phosphate (NADPH) oxidase and reducing flow-mediated dilation (FMD) [42]. Elevated biomarkers such as soluble intercellular adhesion molecule-1 (sICAM-1) and high-sensitivity C-reactive protein (hs-CRP) have been detected in dual users of tobacco and e-cigs, signalling vascular inflammation and endothelial compromise [43].

The mechanisms underlying cardiovascular damage include hyperlipidaemia, sympathetic dominance, endothelial dysfunction, DNA damage, and macrophage activation, with OS and inflammation serving as unifying pathways [44]. Animal studies demonstrate that long-term exposure leads to lung architectural changes, immune gene dysregulation, and low-grade inflammation [37]. These findings indicate substantial cardiorespiratory health risks associated with e-cig use. The widespread use of e-cigs has the potential to perpetuate a new epidemic of chronic cardiorespiratory disease.

Indeed, a conventional cigarette or ENDS significantly increases the risk of cardiopulmonary disease by means of well-characterized inflammation and OS [45]. In a murine model, Mancuso et al. [46] reported that e-cig exposure exacerbated cerebral ischemic injury and elicited OS comparable to that of combustible cigarettes. Additionally, both CCS and e-cig aerosols compromise mitochondrial bioenergetics and disrupt autophagic flux, thereby promoting structural and functional pulmonary decline [47].

The oxidative process also plays an important role in the development of endothelial dysfunction, a precursor to atherosclerosis. Combustible CCS impairs nitric oxide

(NO) bioavailability via endothelial Nitric Oxide Synthase (eNOS) uncoupling and heightened peroxynitrite production, contributing to vascular stiffness, hypertension, CAD, and stroke [48]. Endothelial dysfunction, frequently assessed via FMD, is consistently impaired in smokers, though partial reversibility is observed following cessation [2,48,49].

3. Effect of Conventional and Electronic Cigarettes on Oxidative Stress

Oxidative stress refers to an imbalance between the production of ROS and the body's intrinsic antioxidant defences, resulting in molecular damage to cellular components such as DNA, proteins, and lipids [27]. Tobacco use is a well-established exogenous inducer of OS and it has long been implicated in the pathogenesis of chronic diseases due to its pro-oxidant properties. Even though ENDSs were initially promoted as a less harmful alternative, growing scientific evidence demonstrates that e-cigs can equally disrupt redox homeostasis and elicit oxidative damage [27,49]. Thus, both combustible tobacco products and ENDSs contribute significantly to OS.

CCS generated by tobacco combustion contains over 7000 chemical constituents, many of which possess the capacity to generate ROS. A single puff of a cigarette emits approximately 10^{15} free radicals in both gaseous and tar phases, including hydroxyl radicals ($^{\bullet}OH$), superoxide anions ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and peroxynitrite ($ONOO^-$). These species induce direct molecular damage and activate redox-sensitive signalling cascades such as Nuclear Factor kappa-light-chain-enhancer of activated B cells (NF-κB) and Mitogen-Activated Protein Kinase (MAPK), which facilitate inflammation and apoptotic cell death (Figure 1) [27].

Tobacco smoking has been directly associated with elevated levels of OS biomarkers, including soluble NOX2-derived peptide (sNOX2-dp), F2-isoprostanes (F2-IsoP), malondialdehyde (MDA), and 8-hydroxy-2′-deoxyguanosine (8-OHdG), hallmarks of lipid peroxidation and oxidative DNA injury [21,49]. These ROS-mediated effects impair mitochondrial dynamics, decrease glutathione availability, and compromise cellular energy metabolism. Furthermore, prolonged exposure to CCS enhances the systemic expression of pro-inflammatory cytokines such as Interleukin-6 (IL-6), Tumour Necrosis Factor alpha (TNF-α), and Interleukin-1beta (IL-1β), further amplifying ROS generation and tissue injury (Figure 1) [43]. Endogenous antioxidant pathways such as the Nrf2/ARE response can also be activated by CCS, but the protective activation is often insufficient to counterbalance ROS accumulation [50].

ENDSs, by contrast, generate aerosols by means of the heating of e-liquids composed primarily of nicotine, propylene glycol, vegetable glycerine, and various flavouring agents. Although these devices do not involve combustion, thermal decomposition of their constituents produces toxic aldehydes, including formaldehyde, acrolein, and acetaldehyde, capable of triggering significant oxidative responses [42]. Some studies demonstrated that although e-cigs led to a significant degree of alterations in OS, they seemed to have less pronounced adverse effects as compared with CCS [51]. Moheimani et al. [52], Shahab et al. [53], and Christensen et al. [43] showed that e-cigs are responsible for elevated levels of F2-IsoP and low-density lipoprotein oxidizability (oxLDL), biomarkers of OS and inflammation, in comparison to non-smokers (Figure 1) [42]. Conversely, Taylor et al. [50] observed that exposure to e-cig aerosol extracts did not result in a significant rise in intracellular ROS or depletion of glutathione in bronchial epithelial cells, unlike CCS, which induced an increase of 83% in ROS production and a reduction of 98.6% in the GSH:GSSG ratio. Nonetheless, subsequent studies confirm that chronic exposure to e-cig vapour can still induce oxidative DNA damage, mitochondrial dysfunction, and overexpression of pro-inflammatory genes [47].

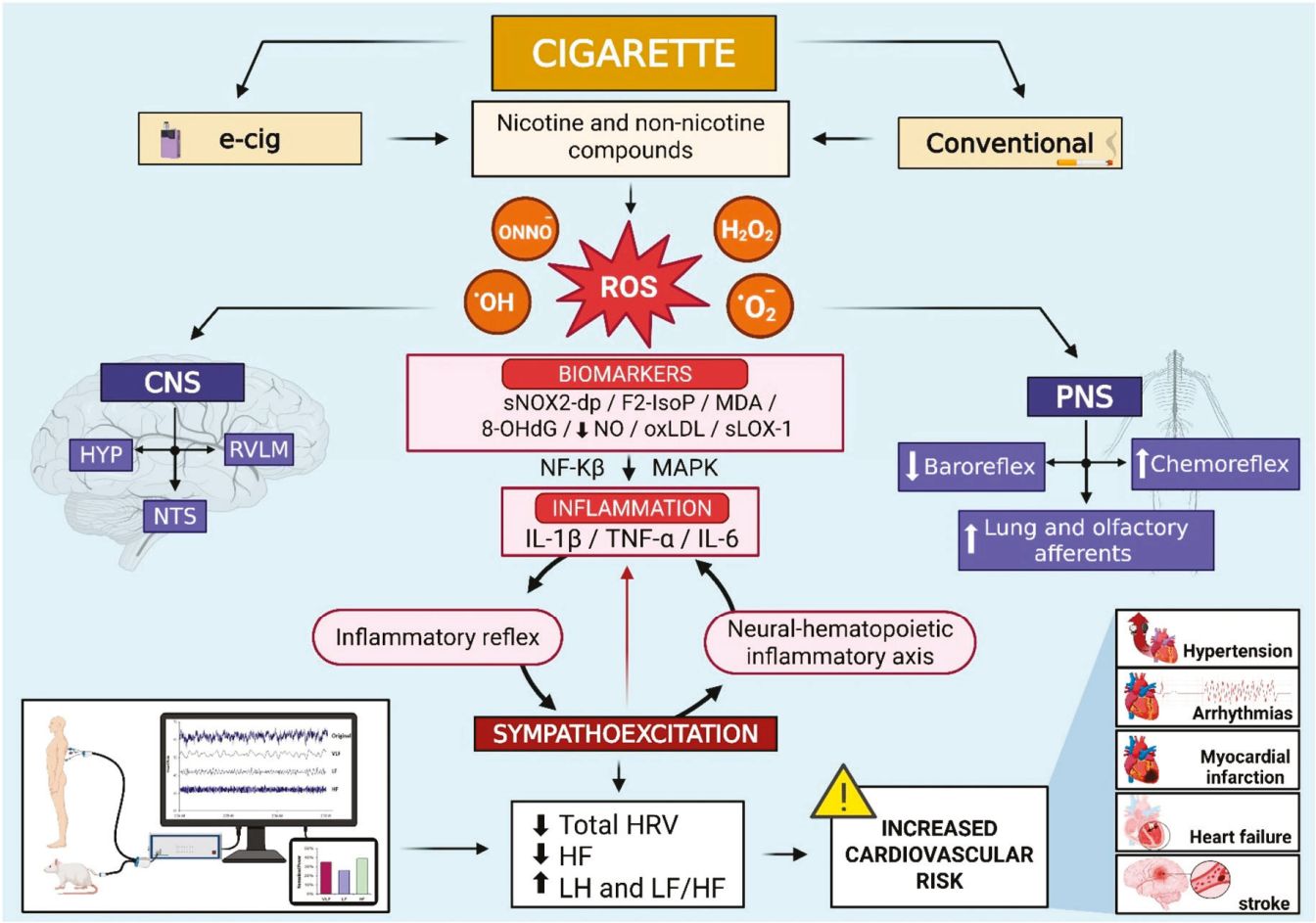

Figure 1. Schematic illustration from both human studies and experimental models suggesting that electronic and conventional cigarettes, by means of nicotine and non-nicotine compounds, increase the production of reactive oxidative species (ROS) in key central and peripheral areas of the nervous system associated with cardiovascular function and basal sympathetic activity. The activation of redox-sensitive signalling (NF-κB and MAPK) facilitates inflammation and stimulates the neural–hematopoietic inflammatory axis and the inflammatory reflex, inducing sympathoexcitation. These processes further increase oxidative stress, establishing a self-sustaining cycle between sympathetic nerve activity and increased ROS production. As a result, HRV decreases, particularly in time domain indices and HF component, while LF values and the LF/HF ratio rise. This pattern is linked to an elevated risk for cardiovascular diseases such as hypertension, arrhythmias, myocardial infarction, heart failure, and stroke. Abbreviations: $^{\bullet}$OH, hydroxyl radicals; 8-OHdG, 8-hydroxy-2′-deoxyguanosine; CNS, central nervous system; e-cig, electronic cigarette; F2-IsoP, F2-isoprostanes; H_2O_2, hydrogen peroxide; HF, high frequency; HRV, heart rate variability; HYP, hypothalamus; IL-1β, interleukin-1beta; IL-6, interleukin-6; LF, low frequency; LF/HF, low frequency/high frequency ratio; MAPK, mitogen-activated protein kinase; MDA, malondialdehyde; NF-Kβ, nuclear factor kappa-light-chain-enhancer of activated B cells; NO, nitric oxide; NTS, nucleus tractus solitarius; $O_2^{\bullet-}$, superoxide anions; $ONNO^-$, peroxynitrite; oxLDL, low-density lipoprotein oxidizability; PNS, peripheral nervous system; ROS, reactive oxygen species; RVLM, rostral ventrolateral medulla; sLOX-1, soluble lectin-like oxidized low-density lipoprotein receptor-1; sNOX2-dp, soluble NOX2-derived peptide; TNF-α, tumour necrosis factor alpha. Symbols: decrease (↓); increase (↑).

The impact of conventional cigarettes or ENDSs on the human body extends beyond the oxidative and inflammatory effects already outlined, also interfering with cardiac autonomic regulation. Continuous exposure to CCS or ENDS aerosols induces physiological changes that disturb the balance between the sympathetic and parasympathetic systems.

This imbalance may be reflected in changes in HRV, a sensitive marker of autonomic influence on cardiac function.

4. Cardiac Autonomic Regulation and Heart Rate Variability

The heart–brain axis works by means of a mechanism in which the autonomic nervous system (ANS) establishes an equilibrium between them and their function [54]. The ANS controls cardiac activity, increasing or decreasing it by means of sympathetic and parasympathetic stimulation, respectively. What modulates these signals depends on the trigger, which can be a fight-flight response, diseases, lifestyle, and many others. Since the heart is influenced by the brain, and vice versa, the activity of the ANS over the heart has become one of the sources of study to understand the influences from these stimuli and their consequences [11,55,56].

HRV is defined as method for studying instantaneous variations in HR or, more precisely, for evaluating the time-related oscillations in the interval of two consecutive heartbeats, using the R-R intervals (iRRs) [11]. Accordingly, HRV analysis is a reliable and non-invasive procedure for assessing the coupling between the autonomic nervous system (ANS) and the sinoatrial node [11,54]. Using a 24 h electrocardiogram (ECG) record, it is possible to extract successive iRRs and analyse HR over time, providing correlation with different situations in response to the ANS. This analysis has emerged as an indicator of autonomic balance and offers insights into healthy lifestyle or pathological states (Figure 2) [11,54–58].

Cardiac contraction activity occurs in a precise way, as it is a watch, primarily because of a "natural" pacemaker, the sinus node, which is responsible for controlling the heart rate [57]. In the absence of autonomic innervation, cardiac contractile activity proceeds with remarkable precision, akin to the regularity of a metronome, exhibiting consistent intervals between successive heartbeats. This phenomenon is primarily attributable to the sinoatrial node, the intrinsic pacemaker. Nevertheless, both the sympathetic and parasympathetic divisions influence the sinoatrial node function, each playing a significant role in modulating the heartbeat intervals [57].

Sympathetic innervation is responsible for increasing the activity of the sinoatrial node by means of epinephrine and norepinephrine release and β1-adrenergic receptor activation. This leads to an increase in intracellular cyclic AMP (cAMP) levels which accelerates the slow diastolic depolarization (phase 4) of the slow action potential and increase its amplitude. The underlying intracellular mechanism is an increase in inward sodium (If) and calcium currents (ICa^{2+}). Conversely, the parasympathetic system, via the vagus nerve, releases acetylcholine and activates the M2-muscarinic receptor, reducing cAMP and opening acetylcholine-dependent potassium channels, increasing outward potassium current (IKACh). This leads to greater hyperpolarization, slowing phase 4 of the action potential and reducing the depolarization rate of the sinoatrial node [11,57].

It should be pointed out that these intracellular mechanisms have significant implications for HRV. While the parasympathetic modulation of the HR occurs at higher frequencies due to rapid intracellular process that rely on potassium channels, the sympathetic activity operates within lower frequency intervals as a result of mechanisms dependent on cAMP concentration [11,57]. On that account, different approaches for measuring the oscillations of the HR are described in the literature.

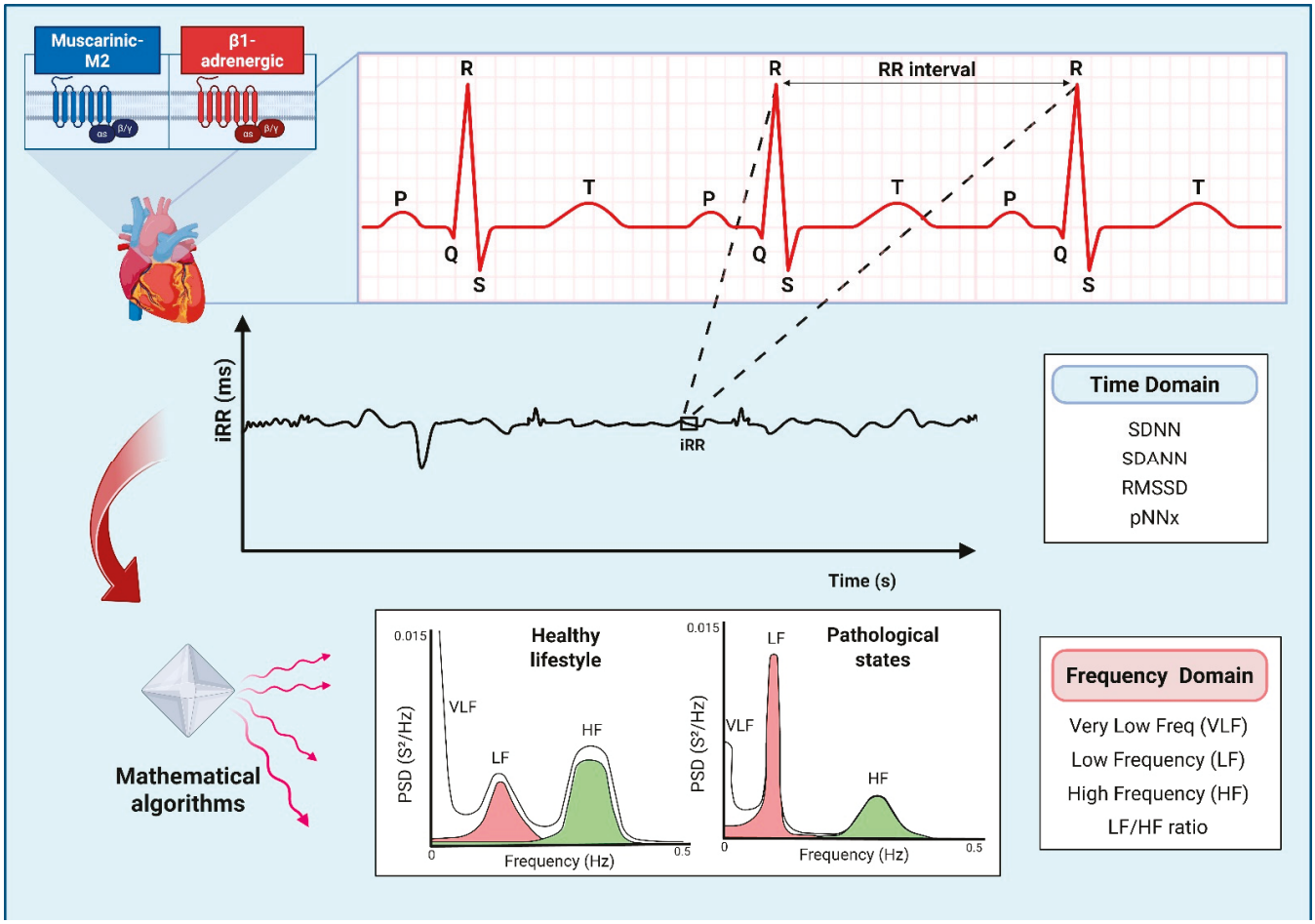

Figure 2. Diagram illustrating the Heart Rate Variability (HRV) analysis used to assess sympathetic and parasympathetic influences on the sinoatrial node by means of muscarinic M2 and β1-adrenergic receptor activation. A 24 h electrocardiogram (ECG) recording allows the extraction of successive R-R intervals (iRRs) and enables comprehensive analysis of the heart rate (HR) variations. The linear method of the HRV analysis includes the evaluation of the time and frequency domain parameters. Time domain indexes measure the HR oscillations over time, while frequency domain indexes analyze the HR frequency distribution using power spectral density (PSD). Mathematical algorithms, e.g., the Fast Fourier Transformation or autoregressive modelling, can assist in visualizing the HRV density distribution. Engaging in a healthy lifestyle is associated with elevated HRV, whereas pathological states may negatively impact it. Abbreviations: pNNx, the percentage of successive NN intervals greater than x ms; RMSSD, root mean square of the successive differences between normal iRRs; SDANNs, standard deviations of the means; SDNN, standard deviation of NN intervals.

Researchers use two primary methods to analyze HRV: the linear method, which includes the evaluation of the time and frequency domains, and the nonlinear method, based on the Poincaré plot and acceleration or deceleration capacity [11,55–58]. The linear method is the simplest and most used in the literature, offering a general assessment of heart autonomic modulation, as described below.

- Time Domain—this is the most common and the simplest way to evaluate HRV. This method seeks to understand how the HR varies in a period of time. By analyzing the iRR on the ECG, it is possible to determine what is called the Normal-to-Normal (NN) interval or the instantaneous HR, calculate the mean HR or the mean NN interval, and even compare NN intervals on the same ECG. Additionally, some parameters can be investigated using the NN intervals, such as the standard deviation of these intervals (SDNN), the standard deviations of the means (SDANNs), the root mean square of

the successive differences between normal iRRs (RMSSD), and the percentage of successive NN intervals greater than x ms (pNNx), of which the most commonly used threshold in humans is 50 ms (pNN50). The last two reflect the influence of parasympathetic autonomic modulation on the heart [11,55–58].

- Frequency Domain—unlike the time domain, data are analyzed based on the HR frequency variation and its distribution, represented by power spectral density. This method applies mathematical algorithms, e.g., the Fast Fourier Transformation or autoregressive modelling, which can assist in visualizing the HRV density distribution (Figure 2). It can correlate more closely with the sympathetic and parasympathetic influence on the heart. There are three basic spectral components of this domain and a ratio between them that can be calculated: Very Low Frequency (VLF, 0.0033–0.0400 Hz), Low Frequency (LF, 0.04–0.15 Hz), High Frequency (HF, 0.16–0.40 Hz), and LF/HF ratio. The first two correlate more with sympathetic activity, the third with parasympathetic modulation, and the last one is used as a parameter to assess the balance between sympathetic and parasympathetic activities [11,55–58].

5. Heart Rate Variability as a Health Predictor

Over the years, HRV has been widely used as a tool in research and clinical practice as it is an easily accessible and non-invasive method. Some factors such as age, physiological and pathological states, and lifestyle, genetic, and environmental factors can affect HRV and serve as a starting point to study how they can influence cardiac autonomic function, as well as to assess cardiovascular risk [56,58–60].

Each situation can manifest different HRV. After an acute MI, a reduction in HRV is expected, indicating diminished vagal activity and sympathetic predominance to the heart. These marks are associated with increased mortality, arrhythmic events, and a poor prognosis for those patients [11,55]. Likewise, hypertensive patients present an important reduction in HRV a high LF component, indicating cardiac sympathetic overactivity and reduced parasympathetic modulation. These autonomic changes induce significant structural and functional alterations in the heart, which are also associated with higher morbidity and mortality outcomes [11,55].

Diabetes can also induce severe autonomic imbalance due to diabetic neuropathy, which affects both sympathetic and parasympathetic fibres. As a result, HRV reduces due to vagal derangement, reflecting a dysautonomia, which can be associated with a higher risk for cardiovascular events, such as sudden cardiac death [55,56]. Additionally, the consumption of substances such as drugs, alcohol, and tobacco leads to an autonomic imbalance, characterized by increased sympathetic and reduced parasympathetic activity, ultimately lowering HRV [55,56]. Conversely, maintaining a healthy lifestyle increases HRV, which is associated with better overall health status (Figure 2) [16,23,55,56,58].

Physical activity has been used as a key component of treatment for several diseases due to its role in cardiac autonomic regulation [16,61]. Exercise programmes stimulate an increase in HRV, suggesting higher cardiac parasympathetic modulation and/or reduced sympathetic activity, associated with better metabolic, inflammatory, and OS parameters [61,62]. Despite the different workout programmes, moderate to highly intensive exercises demonstrated higher parasympathetic and HRV indices, indicating improved cardiac autonomic function [61–63].

HRV can also be used in other scenarios. Some studies use this method to evaluate HRV in small-for-gestational-age newborns [59], demonstrating a higher sympathetic activity in these infants compared with those appropriately sized for gestational age. In other cases, the HRV is also used to assess how a person's emotional state affects the ANS. Some psychological conditions, including anxiety and mood disorders, show a global reduction

in HRV, indicating a shift toward increased sympathetic relative to parasympathetic activity [56,60].

HRV can be used not only in humans, but also in animals; the study of HRV serves as a tool to diagnose arrhythmia in horses [64], to evaluate dysautonomia due to chagas diseases in domestic canines [65], or to observe the effects of substances on the HRV in rats [13] and in mice models [14,15,66]. Recently, Kucera et al. [67] demonstrated that mice exposed to e-cig aerosols from 5% nicotine salt showed a decrease in SDNN and RMSSD during the washout period and an increase in cardiac arrhythmias, suggesting a shift to sympathetic dominance activity and higher cardiovascular risk.

Overall, HRV stands out as an important non-invasive biomarker to assess autonomic function, being used as risk stratification and prognostic tool [68,69]. In a prospective cohort study, patients with cardiac heart failure were followed up for major adverse cardiovascular events. Those individuals who experienced these events presented reduced time domain values and lower HRV during analyses [69]. In another retrospective cohort study, analysis of the HRV parameters were also used as a predictor of mortality and admission to intensive care unit due COVID-19. In these cases, patients with higher HRV presented significantly less mortality risk and fewer admissions [70]. In patients with COPD and/or Obstructive Sleep Apnea, HRV analysis was also considered as an important biomarker to evaluate the prognosis and response to treatment, as these diseases have a significant impact on the cardiovascular system [71].

6. The Role of Oxidative Stress in Cardiac Autonomic Dysfunction

Cardiac autonomic dysfunction, detected by HRV, has been associated with OS in a variety of clinical conditions. Experimental studies have shown that ROS can stimulate sympathetic nervous system activity in the brain and in the peripheral ANS. Campese et al. [72] postulated that such an action could be the consequence of decreased NO and IL-1β cytokine activity.

Indeed, there is evidence demonstrating that ROS are produced in key central regions associated with cardiovascular function, such as the hypothalamus, nucleus of the solitary tract, and rostral ventrolateral region of the medulla. These regions play a crucial role in determining basal sympathetic activity [73–79]. Additionally, locally produced ROS in the baroreceptor region or central nervous system directly affect the baroreceptor reflex and contribute to dysautonomia [80,81]. Li et al. [80] found that ROS generated in the carotid bulb of atherosclerotic rabbits reduced the carotid sinus nerve response to elevated AP. This reduction was reproduced by exogenous ROS but prevented by their inhibition (Figure 1).

In clinical scenarios, OS can also be associated with cardiac autonomic dysfunction in patients with different chronic diseases (Table 1). Fadaee et al. [19] observed that 16 of 78 (20%) patients with chronic kidney disease (CKD) had elevated levels of F2-IsoP, which was associated with cardiac autonomic dysfunction disorder. They correlated plasma F2-IsoP with the HRV indices in both time and frequency domain and found an inverse correlation with SDNN, VLF, LF, and total power parameters. Moreover, they also detected that plasma F2-IsoP independently predicted SDNN, a global measure of HRV, indicating that CKD patients with elevated OS display evidence of autonomic dysfunction.

The association between plasma indices of OS and cardiac autonomic dysfunction has been studied in patients with prehypertension. Thyagarajan et al. [20] found that the HRV parameters in both the time and frequency domain concerning the parasympathetic system were decreased in prehypertensive young adults in comparison with control subjects. In addition, the authors showed that plasma OS parameters (TBARS and total antioxidant capacity) were deranged in prehypertensive patients. Finally, they discovered

that the RMSSD index, a marker of parasympathetic derangement, was correlated with plasma indices of OS. The study, therefore, suggested that prehypertensive patients have an association between increased OS and abnormal cardiovagal modulation.

A similar condition has been observed in patients with systemic arterial hypertension in comparison with normotensive ones. Pavithran et al. [17] examined patients newly diagnosed with systemic arterial hypertension and normotensive controls. These patients underwent HRV studies in both time and frequency domain, which were correlated with plasma measurement of OS and total antioxidant status. The authors observed that several indices of parasympathetic cardiac modulation (SDNN, RMSSD, HFabs, and HFnu) inversely correlated with MDA in hypertensive patients but not in normotensive. Furthermore, the authors also noted that MDA correlated positively with an index of sympathetic activity (LFnu) in hypertensive patients but not in those with normal systemic AP. Collectively, such data shows an inverse correlation between plasma parameters of OS and parasympathetic activity and a positive correlation between OS and sympathetic activity in patients with systemic arterial hypertension.

Recent findings by Promsrisuk et al. [18] also suggest that OS plays a potential role in impairing cardiac autonomic control in COPD patients. These authors investigated a link between MDA levels, an OS marker, pulmonary function, and parameters of HRV in clinically stable COPD patients and healthy male controls. COPD patients exhibited reduced time domain indices (SDNN and RMSSD) and HF component and increased LF component and LF/HF ratio in supine and head-up tilt positions compared with controls. In addition, MDA levels were 2.3 times greater in COPD patients and they were positively correlated with LF and LF/HF ratio but inversely correlated with SDNN and pulmonary function parameters in both positions. These findings indicated that OS may induce sympathetic overactivity in patients with COPD.

The situation seems to be somewhat different in patients with diabetes mellitus with peripheral autonomic disorder and superimposed cardiac autonomic dysfunction detected by HRV. Ziegler et al. [21] studied 22 diabetes mellitus patients with both conditions in comparison with patients without one or both and healthy subjects. They observed that the plasma parameters of OS (8-iso-PGF2α, $O_2^{\bullet -}$, and $ONOO^-$) in such patients were higher compared with control subjects but were similar to those in patients with peripheral neuropathy without cardiac autonomic dysfunction. This suggests that OS is enhanced in diabetic patients with polyneuropathy, without further significant increase in relation to superimposed autonomic neuropathy.

In fact, the rise in plasma glucose likely drives increased OS parameters, which may induce cardiac autonomic dysfunction. Thiyagarajan et al. [82] found that in prediabetic subjects, autonomic imbalance, hyperinsulinemia, insulin resistance, OS, inflammatory response, and impaired fasting glucose interact continuously—each factor reinforcing the others and contributing to disease progression. Additionally, Ziegler et al. [83] found that about one year after type 2 diabetes was diagnosed, subepidermal SOD2 expression increased by approximately 60%, likely as a compensatory response to ROS formation. The findings also reveal a correlation between this increase and longer diabetes duration, a shift towards cardiac sympathetic dominance, and reduced vagal tone, as evidenced by an elevated LF/HF ratio and decreased RMSSD. Recent findings by Al-Saoudi et al. [84] indicate that, in young adults with type 1 diabetes, lipid peroxidation correlates with an increased LF/HF ratio, while glucotoxicity is linked to reduced SDNN values.

Despite the dearth of papers on the association between the HRV indices and plasma parameters of OS, it seems that plasma parameters of OS are associated with indices of sympathetic activity and inversely correlated with those of parasympathetic activity. However, this link between oxidative metabolism and the activity of ANS is still limited and

deserves further elucidation. A recent review by Yelisyeyeva et al. [23] also discusses this relationship between autonomic regulation, HRV, and redox homeostasis during ageing.

Table 1. Key parameters of clinical studies that associated oxidative stress (OS) with autonomic dysfunction assessed by means of heart rate variability.

Reference	Clinical Condition	Size Sample (*n*)	Study Design	OS Parameters	HRV Parameters
Fadaee et al., 2016 [19]	Chronic kidney disease	Normal F2-IsoP $n = 62$ Elevated F2-IsoP $n = 16$	Cross-sectional	↑ F2-IsoP ($n = 16$) ↔ GPx ↔ TAC	↓ SDNN ↓ Total power ↓ VLF ↓ LF ↓ LF/HF ratio
Thyagarajen et al., 2013 [20]	Prehypertension	Prehypertensive $n = 97$ Normotensive $n = 81$	Cross-sectional	↑ TBARS ↓ TAC	↓ SDNN ↓ RMSSD ↓ pNN50 ↓ HFnu ↑ LFnu ↑ LF/HF ratio
Pavithran et al., 2008 [17]	Hypertension	Hypertensive $n = 36$ Normotensive $n = 14$	Cross-sectional	↑ MDA ↓ TAC	↓SDNN ↓ RMSSD ↓ RR triangular index ↓ LF ↓ HF ↓ Total power
Promiusk et al., 2023 [18]	COPD	COPD $n = 50$ Controls $n = 50$	Cross-sectional	↑ MDA	↓ SDNN ↓ RMSSD ↓ HFnu ↑ LFnu ↑ LF/HF ratio
Ziegler et al., 2004 [21]	Type 1 and 2 diabetes with PN and/or CAN	PN^-/CAN^- $n = 62$ PN^+/CAN^- $n = 105$ PN^+/CAN^+ $n = 22$ Control $n = 85$	Cross-sectional	↑ 8-iso-$PGF_{2\alpha}$, ↑ $O_2^{\bullet -}$, ↑$ONOO^-$ ↓ vitamin E-to-lipid ratio ↓ vitamin C CAN did not raise OS parameters further.	CAN was defined as three or more abnormalities among the HRV indexes.
Thiyagarajan et al., 2012 [82]	Prediabetes	Prediabetes $n = 47$ Controls $n = 76$	Cross-sectional blinded	↑ TBARS ↑ TAC	↓ SDNN ↓ RMSSD ↓ pNN50 ↓ Total power ↓ HFnu ↑ LFnu ↑ LF/HF
Ziegler et al., 2015 [83]	Recently diagnosed type 2 diabetes	Diabetes $n = 69$ Controls $n = 51$	Cross-sectional	↑ Subepidermal SOD2	↓ RMSSD ↑ LF/HF ratio
Al-Saoudi et al., 2022 [84]	Type 1 diabetes	$n = 151$	Cross-sectional	↑ CML ↑ G-H1	↓ SDNN ↑ LF/HF ratio

Abbreviations: ↑ increase; ↓ decrease; ↔ no change; CML, Ne-(carboxymethyl)-lysine; CAN, cardiovascular autonomic neuropathy; COPD, chronic obstructive pulmonary disease; F2-IsoP, F2-isoprostanes; G-H1, glyoxal-derived hydroimidazolone 1; GPX, glutathione peroxidase activity; HF, high frequency; HFnu, high-frequency power normalized; LF, low frequency; LFnu, low-frequency power normalized; MDA, malondialdehyde; PN, polyneuropathy;. RMSSD, root mean square of the successive differences between normal iRRs; SDNN, standard deviation of NN intervals; SOD2, superoxide dismutase 2; TAC, total antioxidant capacity; TBARS, thiobarbituric acid reactive substance.

7. Cardiac Autonomic Dysfunction in Conventional and Electronic Cigarette Smokers and the Role of Oxidative Stress

A key mechanism linking CS to cardiovascular risk is cardiac autonomic dysfunction, which correlates with smoking intensity [85,86]. Our group demonstrated that rats exposed for 30 days to CCS showed systemic changes and autonomic cardiocirculatory

dysfunction, depending on the daily exposure dose. Although basal cardiovascular parameters, spontaneous baroreflex sensitivity, and cardiovascular variabilities were similar among rats exposed to CCS of one or two (CS2) cigarettes/animal/day, CCS exposure progressively blunted the bradycardia response to phenylephrine, increased sympathetic tone and chemoreflex sensitivity, and reduced the intrinsic HR [13].

Other studies have also demonstrated autonomic changes in unanesthetised rats exposed to acute and chronic CCS exposure. Houdi, Dowell, and Diana [87] and Nakamura and Hayashida [88] observed that acute CS exposure reduced the HR and cardiac output and increased the AP, renal sympathetic nerve activity, plasma norepinephrine, and total peripheral resistance in unrestrained conscious rats. On the other hand, Valenti et al. [89] found only mild alterations in autonomic and baroreflex function of rats exposed to CCS for 3 weeks.

In humans, there is consistent evidence that whether acute or chronic, passive or active, smoking alters autonomic function. Data from the literature describes that this disruption is characterized by decreased HRV, parasympathetic modulation and baroreflex sensitivity, and increased sympathetic drive, which increase cardiac morbidity and mortality [90–97]. Of note, there is also evidence in humans demonstrating that the number of cigarettes and the current smoking intensity are crucial for determining the effect of smoking on the ANS [36,38–41]. Freire et al. [86] demonstrated that the frequency and time of passive CCS exposure has a negative influence on the hemodynamic response, ANS, and pulmonary function in humans. Additionally, subjects who smoke more than 10 cigarettes/day or 20 packs/year showed lower HRV [81,94]. Also, the HRV indexes are reduced by more than 9% every 10 g of smoked tobacco daily [92].

Clinical data have shown that CCS exposure promotes cardiac autonomic dysfunction characterized by sympathetic overactivation. The main mechanism involved in this autonomic imbalance is the direct pharmacological action of nicotine in its receptors located in peripheral autonomic ganglia and the adrenal medulla, increasing catecholamine release [90,92,96–106]. Sympathoexcitatory effects may also result from the influence of nicotine on chemoreflexes [107]. Argacha et al. [108] found that nicotine enhances peripheral chemoreflex sensitivity to hypoxia in non-smokers, which increases sympathetic drive.

Non-nicotine components may also cause sympathoexcitation by upregulating nicotine receptors [101] or inhibiting monoamine oxidase to reduce catecholamine degradation [106]. Additionally, non-nicotine smoke inhalation can activate olfactory afferents which activate both sympathetic and parasympathetic responses, affecting cardiovascular and respiratory functions in conscious rats [88]. Of note, Middlekauff, Park, and Moheimani [90] proposed that non-nicotine components, such as fine particulate matter, associated with the direct effect of nicotine, are responsible for sympathoexcitation in smokers by means of a complex mechanism involving OS, inflammation, a decrease in nitric oxide production, and baroreflex suppression. Additionally, there is evidence that chronic sympathetic overactivation also contributes to oxidative stress [109–113], perpetuating a self-sustaining detrimental cycle between sympathetic nerve activity and an increase in ROS production. Thus, increased OS is a crucial mechanism which contributes to sympathoexcitation [90].

One mechanism which can explain this positive feedback between the sympathetic nervous system and OS is the activation of the "splenocardiac axis" or the "neural–hematopoietic inflammatory axis". Libby et al. [114] proposed that this axis is an important pathway connecting the brain, autonomic nervous system, and the hemopoietic system (bone marrow and spleen). The authors hypothesized that this axis is likely to be responsible for the increased risk of future acute ischemic cardiovascular events associated with elevated sympathetic tone in humans. Stress settings that trigger sympathetic outflow from

the central nervous system, such as acute myocardial infarction, pain, and mental stress, mobilize leukocyte progenitors from their bone marrow niche via β3-adrenergic stimulation. These progenitor cells can migrate to the spleen, where they can multiply in response to hematopoietic growth factors, increasing pro-inflammatory cytokines (IL-1β and TNFα) and the methylation of NFκB and STAT3 genes [114,115]. Elevated inflammation can, in turn, further increase OS, creating a positive feedback loop that sustains damaging effects of both inflammation, OS, and sympathetic overactivation [115–118] (Figure 1).

Indeed, Boas et al. [119] demonstrated that smokers showed an increase in metabolic activity in both the spleen and blood vessel wall, indicating that sympathetic overactivation can initiate this axis. Additionally, Ruedisueli et al. [120] observed that the sympathetic nervous system, as activated by the amygdala, plays a role in provoking inflammatory monocyte proliferation and instigating atherosclerotic development. On that account, these findings indicate possible mechanisms by which cigarettes may lead to increased risk of future cardiovascular events.

Furthermore, converging evidence indicated that the ANS is largely involved in the regulation of the "inflammatory reflex" [121]. This reflex is activated by an inflammatory response by means of afferent signals via the vagus nerve to the nucleus of tract solitary. A subsequent efferent signal via the parasympathetic system inhibits pro-inflammatory cytokine synthesis, known as the cholinergic anti-inflammatory pathway. Conversely, the sympathetic nervous system, influenced by this pathway, can increase inflammation in the early stages of inflammation [122,123] (Figure 1). In a meta-analysis of human studies, Williams et al. [124] showed that markers of inflammation were negatively associated with some HRV parameters (SDNN and HF). Hence, the authors proposed that the HRV indices can be used to assess the activity of inflammatory processes. Accordingly, we are proposing that HRV can also be used as an index of OS in CS and e-cig smokers, as inflammatory and oxidative process act as cooperative and synergistic partners in the pathogenesis of cardiovascular diseases [116,125].

In fact, experimental data indicates an association between elevated OS in the brain and autonomic dysfunction in rats exposed to CS. Valenti et al. [89] found that three weeks of side stream CS (SSCS) exposure heightened cardiovascular responses to acute administration of the catalase inhibitor 3-Amino-1,2,4-triazole (AZT) into the 4th ventricle of rats. They observed that AZT injection into the 4th V increased the basal HR more in rats exposed to SSCS and reduced their bradycardic peak compared with fresh air-exposed rats. Furthermore, increased OS in the lungs leads to heightened sympathetic activation by means of the stimulation of lung afferent neurons.

As previously mentioned, several studies showed that cigarette use increases ROS production, such as H_2O_2, $O_2^{\bullet -}$, $^{\bullet}OH$, and $ONOO^-$ [27,126]. Nevertheless, data that associate autonomic dysfunction with OS in tobacco smokers are scanty (Table 2). The study of Trikunakornwong and Suwanprasert [126] was the only one which investigated this relationship by means of HRV assessment and NO and sLOX-1 production during acute smoking in smokers compared with non-smokers. At the basal level, they found a positive correlation between the serum soluble lectin-like oxidized low-density lipoprotein receptor-1 (sLOX-1) and AP and reduced HRV in smokers. After 5 min of smoking, the serum NO level decreased and it was negatively correlated with the LF/HF ratio, indicating an overall sympathetic excitability. These data indicated that the overproduction of sLOX-1 and decreased bioavailability of NO may impair autonomic control in chronic smokers after acute single cigarette smoking, which immediately elevated sympathetic activity and blunted vagal tone.

Although some studies showed that e-cigs may present a lower cardiovascular risk compared with traditional cigarettes, they are not safer and can also pose significant car-

diovascular risks [127,128]. In a recent systematic review, Yacoub et al. [109] observed that both acute and chronic e-cig exposure alters the HR, AP, and cardiovascular autonomic regulation, heightening risks of arrhythmias and MI. Passive exposure to e-cig emissions may also have an effect on cardiac autonomic function. Lee et al. [129] observed that a short-term (<15 min) second-hand exposure to nicotine from e-cigs reduced the HRV parameters (SDNN and ASDNN) and shortened the corrected QT interval in a small group of healthy non-smokers. Additionally, these authors found that longer second-hand e-cig exposure increases cardiac effects, suggesting nicotine may have both immediate and cumulative impacts on the heart.

As in tobacco smoking, studies demonstrated that nicotine is the main instigator of the cardiovascular adverse effects that accompany ENDSs [130–133]. Nguyen et al. [130] demonstrated that higher levels of nicotine acutely delivered by the 4th generation e-cig increased the HR and AP and produced changes in the HRV pattern associated with sympathetic overactivity. Nonetheless, there are data indicating that both nicotine and non-nicotine e-cig components can increase cardiac risk [132]. Nagy et al. [132], by means of a systematic review of case reports, identified cardiovascular complications associated with both nicotine and non-nicotine vaping, with half of the cases resulting in cardiac arrest, likely due to arrhythmias from sympathetic predominance. Conversely, in a systematic review including 19 studies, Garcia, Gornbein, and Middlekauff [131] found that acute vaping increased the HR and AP less than CS. Furthermore, their analysis indicated that nicotine, rather than non-nicotine constituents in e-cig aerosol, was responsible for these sympathoexcitatory effects.

Recently, mice-based model studies evaluated the effects of different formulations of e-cig on HRV. Overall, the e-cig reduces the HR and increases HRV during inhalation exposure, with inverse post-exposure effects [133–136]. Kucera et al. [67] demonstrated that exposure to >2.5% nicotine reduced the HR and augmented the HRV indexes during e-cig exposure, denoting vagal activation. Conversely, after exposure, an increase in the HR and spontaneous ventricular arrhythmias and a decrease in time domain parameters (SDNN and RMSSD) were noted, indicating a shift to sympathetic dominance. Using menthol e-cigs, Ramalingam et al. [135] observed that mice exposed to this formulation showed an increase in the HR and AP, spontaneous ventricular arrhythmias, and in urine epinephrine and a reduction in time domain indices (RMSSD and SDNN). In both studies, pretreatment with atenolol abolished e-cigarette-induced arrhythmias, suggesting the involvement of β1-adrenoceptors in sympathoexcitation. Also, the cardiac autonomic imbalance persisted even after 3 weeks of cessation, as mice exposed to e-cig aerosols showed an altered chronotropic response to acute stress [135].

Evidence that associates autonomic impairment and OS in e-cig smokers is also scarce (Table 2). In humans, Moheimani et al. [128] observed that habitual e-cig users showed a reduced HF and an augmented LF and LF/HF ratio compared with nonuser control participants. These changes indicated a shift in cardiac autonomic balance toward sympathetic predominance and decreased vagal tone, which were also associated with an increase in oxLDL, a measure of OS, in habitual e-cig users. These abnormalities are known mechanisms which increase cardiovascular risk in diverse patient populations with and without known cardiac disease.

In mice, Castellanos et al. [136] showed that 3 weeks of e-cig aerosol exposure altered the HR and HRV within minutes during and after exposure. The authors observed rapid HR reduction and HRV elevation during e-cig events and a decrease in time domain parameters (SDNN, RMSSD, and pNN6) post-exposure. The timing and frequency of e-cig exposure influenced these changes, with marked effects in weeks 2 and 3. Furthermore, e-cig exposure increased hydroxyl radical generation in mouse bronchoalveolar lavage fluid,

which can be a mechanism responsible for mediating autonomic dysfunction. As previously mentioned, autonomic shifting influences ROS production, which may also alter HRV and create a feedback loop that maintains ongoing sympathoexcitation.

Table 2. Key parameters of studies that associated oxidative stress (OS) and autonomic dysfunction, assessed by means of heart rate variability (HRV), with conventional or electronic cigarettes (e-cigs).

Reference	Species	Cigarette Type	Size Sample (*n*)	Study Design	OS Parameters	HRV Parameters
Trikunakornwong and Suwanprasert, 2019 [126]	Human	Conventional cigarette (acute)	Habitual smokers *n* = 30 Non-smokers *n* = 30	Cross-sectional	↓ NO ↑ sLOX-1	↓ SDNN ↓ RMSSD ↓ pNN50 ↓ Total power ↓ HFnu ↑ LFnu ↑ LF/HF ratio
Moheimani et al., 2017 [128]	Human	e-cig (chronic)	Habitual e-cig users *n* = 16 Controls (non-tobacco or e-cig) *n* = 18	Cross-sectional case control	↑ oxLDL ↓ PON-1 ↔ HOI	↓ HFnu ↑ LFnu ↑ LF/HF ratio
Moheimani et al., 2017 [52]	Human	e-cig (acute) with or without nicotine	Healthy volunteers *n* = 33	Open-label, randomized, crossover	↔ HOI ↔ LDL-Ox ↔ PON-1	E-cig with nicotine: ↓ HFnu ↑ LFnu ↑ LF/HF ratio
Castellanos et al., 2025 [136]	Mice	e-cig (post exposure)	C57BL/6J *n* = 6	Experimental	↑ ·OH	↓ SDNN ↓ RMSSD ↓ pNN6 ↑ HF ↑ LF ↑ LF/HF ratio

Abbreviations: ↑ increase; ↓ decrease; ↔ no change; HOI, HDL antioxidant index; HF, high frequency; HFnu, high-frequency power normalized; LF, low frequency; LFnu, low-frequency power normalized; NO, nitric oxide; oxLDL, LDL oxidizability; PON-1, paraoxonase-1 activity; RMSSD, root mean square of the successive differences between normal iRRs; SDNN, standard deviation of NN intervals; sLOX-1, soluble lectin-like oxidized low-density lipoprotein receptor-1.

8. Conclusions

In conclusion, this review indicates that both conventional cigarettes and ENDSs induce cardiac autonomic dysfunction characterized by a shift to sympathetic dominance over parasympathetic, resulting in a global reduction in HRV and poor prognosis. The underlying mechanism is complex and involves the direct and indirect effects of nicotine and non-nicotine components associated with increased ROS production in central and peripheral areas involved in cardiac sympathetic control. Additionally, the neural–hematopoietic inflammatory axis and the inflammatory reflex further increase OS, establishing a feedback mechanism that perpetuates sympathoexcitation and consequently raises the risk of hypertension, arrhythmias, myocardial infarction, heart failure, and stroke. Therefore, this review highlights the correlation between the HRV indices and OS: indices of cardiac sympathetic modulation correlate directly with plasma OS parameters, while parasympathetic indices correlate inversely. This relationship indicates a high cardiovascular risk in conventional cigarette and e-cigarette smokers (Figure 1). Despite comparatively lower levels of toxicants in ENDSs, their regular use still elicits measurable OS changes, posing significant health risks similar to those observed in conventional cigarette users. Although the available literature is limited, the growing popularity of e-cigs, especially among adolescents and young adults, highlights the urgent need for thorough longitudinal studies. Therefore, the present review reinforces the need for public health policies aimed at reducing smok-

ing exposure and highlights the importance of early autonomic assessment as a potential preventative strategy.

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Abbreviations

The following abbreviations are used in this manuscript:

$^{\bullet}OH$	Hydroxyl radicals
8-OHdG	8-hydroxy-2′-deoxyguanosine
ANS	Autonomic Nervous System
AP	Arterial Pressure
ARE	Antioxidant Response Element
AZT	3-Amino-1,2,4-triazole
CAD	Coronary Artery Disease
CCS	Conventional Cigarette Smoking
CS	Cigarette Smoke
CKD	Chronic Kidney Disease
COPD	Chronic Obstructive Pulmonary Disease
DNA	Deoxyribonucleic Acid
e-cig	Electronic cigarette
EC	Electronic cigarette
ECG	Electrocardiogram
ENDS	Electronic nicotine delivery system
eNOS	Endothelial nitric oxide synthase
F2-IsoP	F2-isoprostanes
FMD	Flow-mediated dilation
GPX	Glutathione peroxidase activity
GSH	Glutathione; reduced glutathione
GSSG	Glutathione disulfide; oxidized glutathione
H_2O_2	Hydrogen peroxide
HF	High frequency
HFnu	High-frequency power normalized
HOI	HDL antioxidant index
HR	Heart rate
HRV	Heart rate variability
hs-CRP	High-sensitivity C-reactive Protein

IL-1β	Interleukin-1beta
IL-6	Interleukin-6
iRR	R-R intervals
LDL-Ox	LDL oxidizability
LF	Low Frequency
LFnu	Low-frequency power normalized
LF/HF	LF/HF ratio
MAPK	Mitogen-activated protein kinase
MDA	Malondialdehyde
MI	Myocardial infarction
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NN	Normal-to-normal
NO	Nitric oxide
Nrf2	Nuclear factor erythroid 2-related factor 2
$O_2^{\bullet-}$	Superoxide anions
$ONNO^-$	Peroxynitrite
OR	Odds ratio
OS	Oxidative stress
oxLDL	Low-density lipoprotein oxidizability
PON-1	Paraoxonase-1 activity
RMSSD	Root mean square of the successive differences between normal iRR
ROS	Reactive oxygen species
SDANN	Standard deviation of the mean
SDNN	Standard deviation of NN intervals
sICAM-1	Soluble intercellular adhesion molecule-1
sLOX-1	Soluble lectin-like oxidized low-density lipoprotein receptor-1
sNOX2-dp	Soluble NOX2-derived peptide
SSCS	Side Stream Cigarette Smoke
TAC	Total Antioxidant Capacity
TBRS	Thiobarbituric acid acid reactive substance
TNF-α	Tumour necrosis factor alpha
VLF	Very low frequency

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Review

Nicotinism vs. Glomerulopathies—Smoking as a Risk Factor for Primary Glomerulopathies

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Abstract: Smoking cigarettes affects the human body on many levels—not only are the lungs and heart targeted, but also other organs, directly and through derived alterations. We decided to parallel the impacts most often described in the literature in the hope of better future targeting regarding treatment for smoke-induced renal injury. As a result of our research, it is clear that damage is mostly localized directly in vessels and glomeruli. We perceive it as a connected web, where oxidative stress leads to local inflammation, general inflammation in the form of obesity, or inflammation due to nasopharyngeal infection. It later affects other types of tissues: podocytes, epithelium in both glomeruli, renal tubules, and vessels. We mention major molecules proven to participate in kidney damage that tend to be similar in all disease entities depicted in this study: IgA nephropathy, membranous nephropathy, and minimal change disease. Moreover, as nicotine is a major component of both classic cigarettes and electronic cigarettes, we decided to approximate and summarize the information on its impact on primary glomerulopathies.

Keywords: nicotine; glomerulopathy; IgA nephropathy; minimal change disease; membranous nephropathy; tobacco smoking; cigarette smoking; kidney

1. Introduction

Approximately 1.3 billion people worldwide are cigarette smokers [1,2], and these figures are expected to rise in the coming decades [3]. At the same time, 4.5 million students admit to using tobacco products [4]. Data reported in *The Lancet Public Health* (2021) indicate that the majority of smokers—approximately 82.6%—initiated smoking between the ages of 14 and 25, with nearly one in five (18.5%) becoming regular smokers before turning 15 [5].

While smoking prevalence has decreased notably since 1990 in adults aged 15 years and older, the absolute number of smokers has nevertheless increased, driven by global population growth, from about 0.99 billion in 1990 [3] to about 1.3 billion tobacco users nowadays [2]. Tobacco exposure is a known risk factor for cardiovascular disease (CVD) [6], coronary heart disease (CHD), hypertension, cardiopulmonary dysfunction, myocardial infarction [7–11], and progression of kidney disease [12–15]. There is still uncertainty about whether every kidney disease is equally vulnerable due to cigarette smoking [16]. However, clinical observations suggest that there is a high likelihood that exposure to tobacco smoke may promote the progression of chronic kidney disease (CKD). For example, children exposed to cigarette smoke have higher systolic blood pressure, daytime blood

pressure load, and blood pressure variability compared to children without that kind of exposure [17]. Some studies associate exposure to tobacco smoke with proteinuria [18,19]. Tobacco exposure is also connected with other physiological dysfunctions, e.g., cancer, atherosclerosis, thrombogenesis, and vascular occlusion [20–24].

Even though multiple studies have linked tobacco exposure to CKD, the direct link between smoking and proteinuric glomerulopathies has not been well described. Cigarette smoking is expected to cause approximately 8 million mortalities globally every year, including more than a million non-smokers exposed to second-hand smoke [2]. Smoking stimulates the sympathetic nervous system, alters endothelial activity, induces smooth muscle cell proliferation, and causes dysfunction of vascular tone regulators, which can lead to the onset and progression of arterial damage [25,26].

CKD, characterized by a declining estimated glomerular filtration rate (eGFR) and albuminuria, has become a serious public health issue in recent years, affecting the health of the population [27]. An estimated 10–15% of people worldwide have CKD, making it a serious public health burden [28]. Risk factors for CKD include hypertension [29], diabetes mellitus [30], overweight [31], COVID-19 [32], nephrotoxic drugs, aging [33], and air pollution exposure [34].

Tobacco exposure is recognized as a risk factor for progression of chronic kidney disease and proteinuria in children and adults [18,19,35]. Some reports show that the ratio of nicotine metabolites is negatively correlated with kidney function in adults [36]. Nicotine and its metabolites are known to be directly cytotoxic to glomerular visceral epithelial cells (i.e., podocytes), leading to proteinuria and progression of CKD [37–39]. Glomerular disease may also occur secondary to other causes, including autoimmune diseases, infections, medications, or malignancies [40]. In the literature, examples of alleged correlation between cigarette smoke and progression of primary glomerulopathies are shown in individual studies, with a lack of explanation of the exact mechanism of action on their development.

Although the detrimental impact of smoking on cancer and cardiovascular disease is well established, its role in renal pathology, specifically glomerular injury and the progression of chronic kidney disease, is less widely recognized. According to the popularity of tobacco use, there is a need to study the correlation between components of tobacco smoke and other conditions, such as glomerulopathies.

This review aims to synthesize current knowledge on the impact of smoking and nicotine on kidney diseases, in particular, various types of primary glomerulopathies, including minimal change disease (MCD), IgA nephropathy, and membranous nephropathy (MN). This review focused on how cigarette smoke is a risk factor for glomerulopathies.

2. Methods

A systematic literature review of the link between smoking and various types of glomerulopathies (GN) is presented. A total of 196 papers published between 2020 and 2025 were critically assessed and included in the manuscript. The following Medical Subject Headings terms were used: nephropathy IgA, smoking (PubMed; 16 documents found), nephropathy AND IgA AND smoking OR tobacco OR nicotine (Scopus; 36 documents found), membranous nephropathy AND smoking (PubMed 11, Scopus 40 documents found), minimal change disease AND minimal change nephropathy AND smoking (PubMed 6, Scopus 9 documents found). Prespecified inclusion and exclusion criteria in our research protocol determined whether studies were eligible for full-text analysis. Three reviewers (M.D., A.M., and M.M.) independently screened all manuscripts for relevance to the chosen inclusion criteria. Full-text publications were assessed for definitive inclusion independently by all authors.

3. Glomerulonephritis

The nephron is the foundation of kidney structure. It consists, in general, of glomeruli and tubules. In the glomerulus, the main process is primary filtration, which leads to the creation of primary urine. Bowman's capsule—outer garment of glomerulus—is layered from the inside with glomerular basement membrane (GBM) and with visceral epithelial cells, podocytes, and mesangial cells on the outside. Those comprise filtering barriers for plasma [41].

Glomerulonephritis encompasses a heterogeneous group of renal diseases characterized by injury affecting various components of the glomerulus, including podocytes, slit diaphragms, mesangial cells, the GBM, and the capillary endothelium; in some cases, the tubulointerstitium may also be involved [42]. GN can manifest as a primary kidney disease limited to the glomeruli without evidence of systemic disease, in contrast to secondary GN, which reflects the renal involvement of systemic disorders such as systemic lupus erythematosus (SLE), monoclonal gammopathies, or diabetic nephropathy [43].

Traditionally, GN is classified based on histopathological findings from kidney biopsy, which consider factors such as the location and type of deposits, the degree of cellular proliferation, and fibrosis [44]. Within this system, the primary glomerulopathies include IgA nephropathy (IgAN), minimal change disease (MCD), focal segmental glomerulosclerosis (FSGS), primary membranous nephropathy (PMN), and primary membranoproliferative glomerulonephritis (MPGN) [45].

Advances in immunophenotyping and genetics have revealed that identical histological patterns can arise from distinct underlying diseases and due to different risk factors, each of which may need a different therapeutic approach [44]. Therefore, new classification systems for GN are currently being proposed. Anders et al. [42] and Romagnani et al. [44] suggested that GN requires a classification system based on an immunopathophysiology to better connect with the growing number of available immunomodulating drugs, highlighting five groups: infection-related, autoimmune, alloimmune, autoinflammatory, and monoclonal gammopathy-related GN. For this reason, we aimed to outline especially the underlying pathomechanisms and describe how smoking can influence these processes, and then indicate the specific primary glomerular diseases in which this effect has been observed.

Glomerulonephritis, although relatively rare, remains an important cause of chronic kidney disease (CKD) and end-stage renal disease (ESRD) worldwide. Its incidence varies between 0.2/100,000/year and 2.5/100,000/year [46]. According to most CKD registries, glomerular diseases represent roughly 20–25% of all prevalent cases [47,48], which makes it still a major cause of CKD [49]. In pediatric and young adult populations, glomerular disease frequently leads to irreversible renal damage [48]. GN accounts for approximately 10–25% of ESRD cases in Europe and the US, although its relative contribution varies by region, age, and ethnicity [41].

It is noteworthy that, over the last fifty years, there has been a substantial shift in the epidemiology of glomerular diseases worldwide [50]. Currently, it is well established that IgAN represents the most common and MN the second most common type of GN. Since the 1990s, MN has lost its position as the leading cause of nephrotic syndrome, which has been overtaken by focal segmental glomerulosclerosis (FSGS), the third common GN [50].

Primary GN may manifest as well-defined clinical glomerular syndromes, such as isolated urinary abnormalities, nephritic syndrome, nephrotic syndrome, rapidly progressive glomerulonephritis (RPGN), and chronic kidney disease, which can occasionally progress to ESRD demanding dialysis or transplantation [45]. The hallmark clinical feature is proteinuria, which can range from mild elevations to nephrotic-range levels, frequently accompanied by microscopic or macroscopic hematuria [48]. Additional clinical mani-

festations observed within these syndromes may include varying degrees of azotemia, hypertension, edema, oliguria, fatigue, headache, and malaise [41]. Hypertension is frequently present and can contribute to the progression of kidney injury [51]. Edema, which results from hypoalbuminemia and disordered fluid homeostasis, is particularly common in nephrotic presentations, often first appearing around the periorbital region or lower extremities, and may even be the earliest clinically apparent sign. In rapidly progressive forms, oliguria and acute kidney injury can develop, while systemic symptoms such as fatigue and general malaise tend to be more pronounced in advanced disease stages. Less commonly, hyperlipidemia and thrombotic disease may occur [52].

The severity and constellation of these features largely depend on the underlying histopathological subtype and the balance between disease activity and chronicity, reflecting the pathophysiological process. For instance, in children and young adults, nephrotic syndrome is more likely to be associated with MCD or FSGS; in contrast, in older adults, MN is a more typical cause. Meanwhile, IgAN often presents with episodic macroscopic hematuria, sometimes accompanied by mild edema or hypertension, typically following upper respiratory tract infections [47]. Renal biopsy is essential for confirming GN, assessing the level of ongoing immunologic injury, and evaluating the degree of established, irreversible structural damage [44].

Management of primary glomerulopathies typically combines optimal supportive care, such as rigorous blood pressure control, sodium restriction, and reductions in proteinuria, with disease-specific immunosuppressive or immunomodulatory therapy, tailored according to histological subtype, proteinuria severity, and progression risk as outlined in the Kidney Disease Improving Global Outcomes (KDIGO) 2021 Clinical Practice Guideline [48].

This study concentrates on IgA nephropathy (IgAN), minimal change disease (MCD), and membranous nephropathy (MN), as relatively accessible clinical and pathological data support these. Less common primary glomerulopathies are partially described due to sparse literature and lower prevalence.

3.1. IgA Nephropathy

IgA nephropathy, also called Berger disease [53], is the most common cause of all primary glomerulopathies worldwide [54,55]. IgA is the predominant immunoglobulin of mucosal surfaces, and activation of the mucosal immune system can trigger IgAN production. This activation might be an effect of upper respiratory tract or gastrointestinal infection, major mucous-layered surfaces in the human body [55]. Glomerular inflammation and mesangial proliferation occur due to nephritogenic immune complexes. Then activation of the renin–angiotensin–aldosterone (RAA) and complement system leads to glomerulosclerosis and tubulointerstitial fibrosis, which finally leads to impaired renal function. Risk factors, such as smoking and hypertension, exacerbate disease progression by causing microvascular injury. Glomerulomegaly and maladaptive hyperfiltration related to obesity may also be implicated in the nonimmunological progression of the disease [53].

There are at least four hits needed to induce IgA nephropathy. The initial two depend on increased circulating galactose-deficient IgA [55], which is due to abnormal O-glycosylation of the IgA1 hinge region and production of unique anti-glycan and anti-IgA1 antibodies [45,53]. The first hit is associated with a genetic predisposition to developing a dysregulated immune response [53]. In genetically susceptible people, a precipitating factor, such as infection, can trigger B cells to produce IgA1 missing a galactose molecule at the hinge region. Mucosal infections, chronic exposure to pathogens, or improper handling of gut commensals can trigger the abnormal immune response [53]. Finally, hypoglycosylation enables the aberrant IgA1 molecules to aggregate.

The hallmark is IgA deposition in glomerular mesangium with associated mesangial expansion and/or hypercellularity [45]. The Oxford Classification is used to score the histology according to the degree of mesangial, endocapillary, segmental, and tubule-interstitial involvement, as well as the presence of glomerular crescents [45].

Genetically, the presence of hypoglycosylated IgA1 is a heritable trait. In one fourth of blood relatives of IgAN patients, serum galactose-deficient IgA1 levels are elevated [55]. When it comes to treatment, KDIGO Guidelines for Glomerulonephritis management should focus on best supportive care, reducing proteinuria with the RAA system inhibition, and optimizing blood pressure control [45].

3.2. Membranous Nephropathy

Membranous nephropathy (MN), also known as membranous glomerulopathy, is characterized by the subepithelial deposition of immune complexes (ICs) along the glomerular basement membrane, resulting in diffuse GBM thickening and progressive podocyte injury [56,57]. Histopathologically, MN is distinguished by the presence of granular IgG and complement component C3 deposits along the GBM, manifesting as a strong, finely granular pattern on immunofluorescence microscopy [58]. Electron microscopy reveals electron-dense deposits localized in the subepithelial space, often associated with characteristic basement membrane spikes.

MN is the second most common glomerulopathy after IgAN [59]. It accounts for approximately 30% of nephrotic syndrome cases in adults and represents the leading cause of nephrotic syndrome among white adults [60] and the second worldwide. The global incidence of MN is approximately 1.2 cases per 100,000 annually, predominantly affecting middle-aged and older adults but also occurring, less frequently, in children [46]. Progressing to nephrotic syndrome in approximately 80% of cases [61] and eventually to ESRD in roughly one-third of patients if proteinuria remains uncontrolled.

The first experimental model that provided insights into the disease pathophysiology was developed by Heymann et al. in 1959, who demonstrated in rats that MN could arise from the in situ formation of ICs generated by antibodies directed against antigens located beneath the podocytes [62]. Since then, considerable progress has been made in understanding the underlying mechanisms of MN, particularly with the identification of major autoantigens [59]. Advances in immunology have revealed circulating autoantibodies that recognize several podocyte antigens, including the phospholipase A2 receptor (PLA2R), accounting for >70% of cases [63], thrombospondin type-1 domain-containing 7A (THSD7A) present in 2–5% of MN patients [64], and, more recently, neural epidermal growth factor-like 1 (NELL1). To date, more than 14 target antigens have been identified, collectively explaining approximately 80–90% of MN cases. These discoveries have helped redefine MN as an organ-specific autoimmune disease [65].

MN is traditionally classified into primary (PMN) or idiopathic MN (iMN), which occurs in genetically predisposed individuals, and secondary MN, associated with known etiologies such as infections (hepatitis B and C, HIV, SARS-CoV-2), autoimmune diseases (SLE, rheumatoid arthritis), malignancies, and drug/toxin exposures [65]. Approximately 70–75% of adult cases of MN are classified as PMN, which is characterized by the binding of circulating autoantibodies, most notably of the IgG4 subclass, to podocyte antigens. Although the precise triggers of PMN remain incompletely understood, observational studies suggest potential links with genetic predisposition, infections, and environmental exposures, including components derived from tobacco smoke. Ponticelli et al. [58] indicated that undetected viral infections or ecological pollutants may initiate PMN by exposing podocyte antigens in genetically susceptible individuals, thereby provoking an autoimmune response.

3.3. Minimal Change Disease

Minimal change disease (MCD), or minimal change nephropathy (MCN), is defined as diffuse podocytopathy with nearly normal findings on light microscopy (LM), contrasted by striking podocyte damage visible on electron microscopy (EM), including diffuse effacement of foot processes and disappearance of slit diaphragms, yet notably lacking electron-dense deposits [66]. The GBM is morphologically unremarkable but has decreased charge, which was believed to be responsible for the development of selective proteinuria in MCD [67].

Significantly, MCD represents the leading cause of nephrotic syndrome in the pediatric population, responsible for about 70–90% of cases, and remains an important cause among adults, where it accounts for approximately 10–25% [68]. The cause of MCD is unknown, but many authors believe that immunologic dysregulation and modifications of the podocyte synergize in altering the GBM integrity. It has been suggested that aberrant immune response against podocytes is caused by the T-cell compartment [69]. As to changes in podocyte structure, the foot process effacement is visible by electron microscopy. The research was focused on reasons for disrupted integrity of the glomerular filtration barrier [66].

Some authors implicated that changes in the regulation of the T-cell function are responsible for driving podocyte injury in MCD [70]. Although some abnormalities of T-cell subsets and their activity in vitro have been described, the literature contains many contradictions. These abnormalities are also suggested to be secondary to the nephrotic state rather than primary [71], as MCD has been observed in immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX), a congenital immunodeficiency with severe T regulatory cell (Treg) hypofunction [72].

Meanwhile, animal models and observations in humans suggest that reactive oxygen species (ROS) could also be involved in the pathogenesis of MCN. ROS are first-phase mediators produced by polymorphonuclear cells (PMNs) in response to infectious triggers. Bertelli et al. [73] first demonstrated that PMNs can produce high quantities of ROS in children with MCN during relapses of proteinuria, which they associated with a lack of a negative regulatory circuit that involves anti-inflammatory soluble factors derived from Tregs. Also, oxidant production was negatively correlated with the amount of Treg expression of CD39 antigen [73]—an enzyme in the conversion of ATP to immunomodulatory adenosine, present, among others, in endothelial cells [74]. Indirect evidence for oxidant activity in vivo comes from the observation that a significant part of serum albumin is oxidized in MCN patients. Albumin has only recently been recognized as the major antioxidant in serum that plays a crucial role during infections in humans. Musante et al. [75] characterized serum albumin in children with MCN employing mass spectrometry, and showed chemical modifications of the unique free cysteine residue of the protein sequence to a sulfonic acid (Cys-SO_3H) that is the end product of its oxidation, and therefore represents a surrogate biomarker of oxidative stress [76].

4. Cigarette Smoking Versus the Occurrence of Primary Glomerulopathies

Given the notable prevalence of GN and the widespread exposure to tobacco smoking globally, exploring the potential association is warranted. However, precise incidence data of GN among smokers are insufficient, as GN is a relatively rare disease, and population-based registers rarely capture smoking status. In the NEPTUNE cohort, 371 adults and 192 children with proteinuric glomerulonephritis were recruited. At baseline, 14.6% of adults were active smokers, 29.1% were former smokers, and 4.9% reported passive exposure. Among children, active smoking was rare (0,5%), but 16.7% of children patients reported passive exposure to tobacco smoke. These findings underscore that nearly half

of adult GN patients had a history of active smoking [77], compared with ~20% of adults in the general US population [4]. In contrast, Stengel et al. [78] found that the overall prevalence of smoking among adult men with GN was similar to that of hospital controls (60% vs. 65%), indicating no clear overrepresentation of smokers in GN in that cohort. The authors included 295 adult patients with biopsy-proven primary GN cases (including MN, IgAN, MCD, and FSGS) and 242 matched hospital controls. They demonstrated a significant association between cigarette smoking and the severity of primary GN, reflected by a higher prevalence of chronic renal failure (CRF) among male smokers, particularly those over 40 years of age and with hypertension. A clear dose–response relationship was observed, with greater daily consumption and cumulative exposure correlating with an increased risk of CRF. Interestingly, former smokers showed a higher risk than current smokers, likely due to longer exposure. The effect of smoking was modified by age and hypertension, suggesting its role in disease progression among high-risk male patients. Although causality cannot be confirmed, these findings strengthen the evidence for a potential pathogenic role of smoking in GN. Notably, there was no significant association found in women between smoking and CRF and with the incidence of specific histological GN subtypes.

This section explores the study-proven role of smoking in GN, summarizing clinical data and experimental findings that elucidate its potential impact on disease development and outcomes.

4.1. IgA Nephropathy

Both general research and case reports of patients inform about the noticeable link between the presence of smoking and worsening of the course of IgA nephropathy in already diagnosed people. For example, Wang et al. [79] studied, apart from non-smokers, current and former smokers with primary glomerulonephritis. The indicators that were examined were hypertension (%), systolic blood pressure (SBP) (mmHg), diastolic blood pressure (DBP) (mmHg), serum creatinine (μmol/L), estimated glomerular filtration rate (eGFR) (mL/min per 1.73 m^2), and urine protein (g/24 h). As results showed, IgAN patients with a history of smoking were more likely to have hypertension and renal vascular changes, as well as worse renal outcomes, compared with patients without hypertension who did not smoke [79]. Moreover, current smoking was an independent risk factor for progression of micro- or macroalbuminuria and ESRD among patients with diabetic nephropathy (DN) [79].

In another study, not only was smoking observed solely, but also its possible debuff with hyperuricemia [80]. The authors aimed to identify an association between smoking status and hyperuricemia and renal arteriolar hyalinosis and wall thickening in IgAN patients. Checked indicators were retrieved from renal biopsy and evaluated as grade 0, no hyalinosis of the vessel wall; grade 1, hyalinosis of <25%; grade 2, hyalinosis of 25–50%; and grade 3, hyalinosis of >50% of the vessel wall circumference. The scale used in this study highlights various factors considered to determine the state of the kidneys. The authors showed that smoking and hyperuricemia, but not smoking alone, were significantly associated with an increased risk of higher-grade renal arteriolar wall thickening. Another study showed that male patients with IgAN have more severe renal dysfunction compared with female patients, and smoking is an independent risk factor for crescent formation in the kidneys only in male patients [81].

4.2. Minimal Change Disease

Although many studies were based on MCD patents, very few considered the effect of nicotine on those patients, which brings quite poor literature covering both factors

together. In the research by Zhang et al. [82], MCD patients had higher urine protein, serum creatinine, and cholesterol, and a lower serum albumin compared to healthy people. Seven of those patients were ex-smokers who had stopped smoking for 1 to 5 years.

4.3. Membranous Nephropathy

A multicenter cohort study including patients with PMN examined the impact of cigarette smoking on disease progression. Yamaguchi et al. [83] found that current smoking, higher daily cigarette consumption, and heavy cumulative exposure ($\geq$40 pack-years) were all significantly associated with a 30% decline in eGFR. Notably, the risk increased in a clear dose-dependent manner, and older age and female sex further heightened susceptibility. However, smoking was not associated with achieving complete remission of proteinuria. These findings suggest that cigarette smoking is an important and modifiable risk factor that accelerates renal function decline in PMN [83].

In a retrospective single-center study from China, Jiang et al. [84] compared iMN (PMN) with an increasing number of atypical membranous nephropathy (aMN) cases of unknown etiology. Patients with aMN were significantly younger and had a notably higher prevalence of smoking (37.1% vs. 27%). Although nephrotic syndrome was the main clinical presentation in both groups, aMN patients more frequently also exhibited features of nephritic syndrome. Interestingly, serum anti-PLA2R antibody levels did not distinguish between aMN and iMN. The authors suggest that aMN may represent a distinct clinical and pathological entity, potentially influenced by environmental factors such as smoking, warranting further investigation on a larger scale. Their study highlights the potential role of environmental factors such as smoking in the onset and progression of MN [84].

In a recent study applying deep learning to biopsy slides from patients with PMN, smoking emerged as an independent predictor of poorer response to immunosuppressive therapy. The model demonstrated that patients who smoked had a significantly reduced likelihood of achieving remission, highlighting smoking not only as a potential risk factor for disease progression but also as a modifier of treatment efficacy. Findings of Wei et al. [85] reinforce the clinical importance of smoking cessation in the management of iMN.

5. Proposed Mechanisms of Cigarette-Induced Kidney Damage

These days, it is suspected that smoking affects kidneys in many ways. Here, the most described in the literature are presented. It is evident that damage is primarily localized in vessels and glomeruli, resulting from oxidative stress that leads to local immunological disorders. These disorders subsequently affect various tissues, including podocytes and the epithelium in both glomeruli and vessels, potentially leading to hypertension and other complications. This topic should be considered in future research.

5.1. Role of Oxidative Stress

One of the most considered mechanisms of cigarette smoke in the causation of glomerulonephritis is reactive oxygen species (ROS). It was confirmed that ROS were found in higher amounts in smoking patients than in non-smokers [86]. ROS are derivatives of molecular oxygen (O_2), which can later lead to the creation of superoxide ($O_2\bullet-$), hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), and peroxynitrite/peroxynitrous acid ($ONOO-$/ONOOH) [87]. Those also participate in oxidative stress (Table 1). ROS have a broad action spectrum in organisms. For example, the occurrence of ferroptosis is related to increased production of ROS and other oxides. Ferroptosis is an important, newly discovered form of cell death caused by the deficiency of glutathione peroxidase 4 (GPX4) and associated with oxidation [88].

Table 1. Main factors related to smoking and their impact on glomeruli in GN.

Smoke-Related Factor	Potential Effects	Outcome	Glomerulopathy
Reactive oxygen species	Oxidative stress Aberrant glycosylation of IgA1 Epithelial cells loss Augmentation of pro-inflammatory cytokines	Stiffness of central vessels Rise in arterial pressure Tubular damage	IgA nephropathy Membranous nephropathy
Excessive antibody production	Activation of innate immunity leading to antigen presentation, and those present in dental caries	Damage to podocytes and tubular cells Damage to vessels	Minimal change disease Membranous nephropathy IgA nephropathy
Changes in vasculature	RAA system activation Renal arteriolar hyalinosis Renal atherosclerosis Elevated plasma endothelin levels	Endothelial dysfunction Vascular smooth muscle proliferation	IgA nephropathy Membranous nephropathy

Inflammasomes can be activated by pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) (Figure 1). The most typical mechanism for activating the inflammasome is the production of ROS. Inflammasome consists of NLRP3 (NOD-like receptor family pyrin domain containing 3), apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC), and the pro-caspase-1 [88]. Activated by autocatalytic cleavage, caspase-1 then cleaves pro-interleukin-1β (pro-IL-1β) and pro-interleukin-18 (pro-IL-18) into their biologically active forms IL-1β and IL-18, causing inflammation and tissue damage. NLRP3 inflammasome itself is activated by a priming signal, such as ligands for Toll-like receptors (TLRs), NOD-like receptors (NLRs), or cytokine receptors, which initiate the transcription of nuclear factor-kappa B (NF-κB). NF-κB promotes the expression of NLRP3 and pro-IL-1β. Its activation is well described in the immune cells, but it might also happen in kidney cells, such as podocytes, mesangial cells, and renal tubular epithelium [89].

Studies have shown that the regulation of inflammasomes and autophagy could be the key to the treatment of multiple diseases, including kidney diseases [89]. Huang et al. [90] showed that cisplatin may trigger kidney injury by blocking autophagy and activating NLRP3 inflammasomes. The relationship between the inflammasome and autophagy can play a crucial role in the development of IgAN. In mouse models of progressive IgAN, resveratrol (a type of natural phenol or polyphenol produced by some plants in response to injury) inhibits NLRP3 inflammasome activation by augmenting autophagy and preserving mitochondrial integrity [91].

Based on model evidence, it was also found in induced accelerated progressive IgAN mice that luteolin, which is present in various plants, promoted the nuclear factor erythroid-derived 2-related factor 2 (Nrf2) antioxidant pathway [92]. Nrf2 is a transcription factor that stimulates the expression of antioxidants in response to oxidative damage. During oxidative stress, Nrf2 is transported to the nucleus, where it activates antioxidant response element (ARE)-dependent gene transcription to clear ROS and maintain cellular redox stability. Heme oxygenase-1 (HO-1) is a key antioxidant enzyme regulated by Nrf2. Some studies have confirmed that the upregulation of Nrf2 expression has a protective effect in IgAN animal models. Liang et al. [92] also demonstrated that luteolin significantly improved renal function by inhibiting NLRP3 inflammasome activation, reducing the concentration of pro-inflammatory cytokines and ROS. Based on these findings, it can be concluded

that ROS inhibition may inhibit NLRP3 activation and reduce inflammation in IgAN [89]. In MN, inhibition of ROS reversed complement-induced pyroptosis in podocytes. ROS inhibitors also significantly reduce the expression of key protein molecules in the pyroptosis pathway, including NLRP3, ASC, pro-caspase-1, cleaved caspase-1, pro-IL-1β, and mature IL-1β [93].

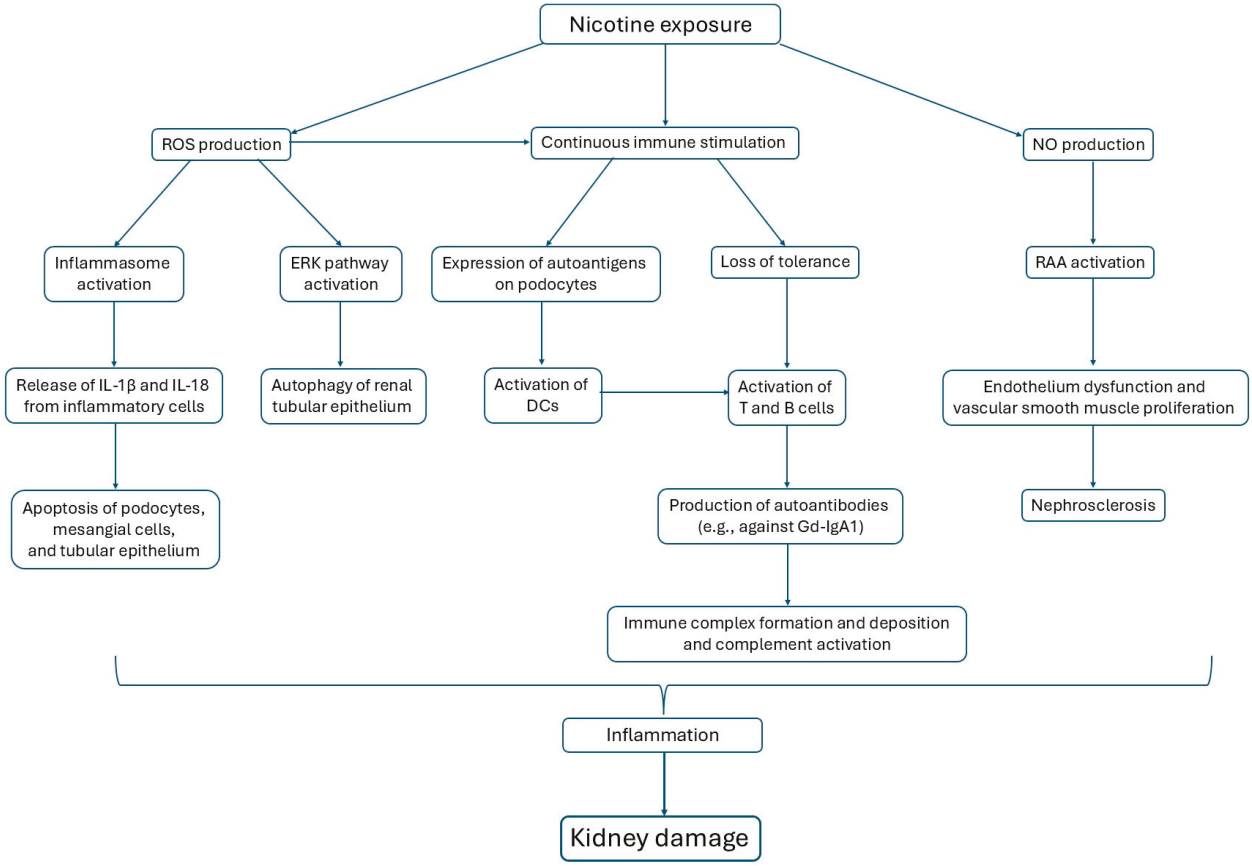

Figure 1. Three main pathways are activated by nicotine exposure, affecting glomerular cells.

As Deng et al. [94] state, in MCD, the ERK pathway in damaged renal tubular epithelial cells might be activated by a ROS-dependent mechanism. ERK, an extracellular signal-regulated protein kinase, is involved in various biological responses, such as cell differentiation and proliferation, cell morphology maintenance, cytoskeletal construction, and cell apoptosis. The ROS-ERK pathway can induce cellular injury and provide an autophagy-associated adaptive response [95]. In MN, a concept appears that a second wave of antibodies targeting superoxide dismutase 2 (SOD2) is generated in autoimmune glomerulonephritis and may negatively influence the clinical outcome. Explaining, knockout mice for SOD2 develop spontaneous proteinuria and focal glomerular damage [95]. Circulating and renal deposits of anti-SOD2 antibodies have been detected in MN patients. In MCD, it was suggested that ROS can modify the GBM selectivity versus circulating proteins by peroxidation of the lipid constituents of the membrane [95].

In IgAN, ROS, as well as chemical exposure to nicotine, may provoke the glomerular mesangial proliferation in the presence of aberrantly glycosylated IgA1 [96], targeting the kidneys directly. What we know is that cigarette smoke induces oxidative stress due to changes in cellular metabolism, which increases the stiffness of central vessels, raises general arterial pressure, and leads to tubular damage [79], associated with a loss of epithelial cells [97]. By contrast, smoking in MN induces oxidative stress and inflammation in the lungs, leading to increased serum levels of pro-inflammatory cytokines such as IL-6

and TNF-α (tumor necrosis factor-alpha), which may promote autoimmune responses, including autoantibody production and immune complex deposition in the kidneys [98]. This comparative data might emphasize different target regions, playing a crucial role in both diseases.

5.2. Role of Inflammation and Immunity

Another often noted impact of cigarette smoke on the kidneys is the augmented production of antibodies. Studies using animal models of MCD showed that a complex interaction of factors and cells exists, starting with activation of innate immunity and continuing with antigen presentation. For example, increased expression of lymphotoxin beta receptor (LTβR) ligands on innate immune cells enhances the NF-κB signaling and enriches LTβR-target gene expression, all in epithelial cells of lungs from mice exposed to chronic cigarette smoke [99]. CD80, responsible for the T-cell activation pathway, and CD40/CD40L antigens essential for the communication between T and B cells were found to be affected in tobacco-consuming oral cancer patients [100]. Distinctive studies propose the possibility that the same antigens are expressed by podocytes under inflammatory stimuli, representing a direct potential mechanism for proteinuria [101,102], as podocytes form the epithelial surface of the glomerulus, where filtration of molecules under 60 kDa takes place [103].

In MN, the most notable autoantigen is located on podocytes as well. For example, while the PLA2R antigen is primarily expressed in the kidney, lung inflammation may trigger or amplify autoimmune mechanisms contributing to MN pathogenesis, even without obvious lung symptoms. There was a case of a 77-year-old patient who suffered from PLA2R antibody-positive MN complicated with IgG4-related disease (IgG4-RD), only affecting the lung, who effectively responded to treatment for IgG4-RD [104]. In their retrospective investigation of 845 patients with IgG4-RD, Tsai et al. [105] suggested that smoking may precipitate the disease.

There are studies connecting kidney dysfunction to oral cavity disturbances in IgAN. Common knowledge is that cigarette smoke reaches tonsils and epipharynx, where nasopharynx-associated lymphoid tissue (NALT) and mucous tissue are present [106]. NALT is a mucosal-associated lymphoid organ implanted in the submucosa of the nasal passage, where inhaled antigens are deposited. During the steady state, conventional dendritic cells (cDCs) within the NALT suppress T-cell responses. [107]. However, in IgA, TLR9 (Toll-like receptor 9) activation in the NALT represents a common pathway by which exogenous antigens engage the mucosal immune system. TLR9 explicitly recognizes unmethylated DNA sequences in bacterial and viral DNA, thus leading to aggravation of renal injury in IgAN. Kano et al.'s study showed that stimulation of TLR9 with CpG oligonucleotides (CpG-ODN) enhanced the production of IgA in the supernatants of cells isolated from the NALT [108]. Moreover, it was shown that TLR9 polymorphisms are associated with disease progression in IgAN patients [109].

Continuous immune stimulation caused by smoking may contribute to the development of IgA nephropathy [106]. Chronic tonsillitis is frequently observed in IgAN patients. Moreover, in chronic tonsillitis, the expression of the J chain in IgA-positive plasma cells decreases in germinal centers (GC) and extrafollicular regions [106]. The J chain (encoded by the *JCHAIN* gene) regulates the polymerization of multimeric IgM and IgA by forming disulfide bonds with the C-termini of heavy chains, which facilitates their transport across mucosal epithelia [109]. In chronic tonsillitis, toxic bacterial factors may stimulate excessive antibody production, thereby contributing to the pathogenesis and progression of IgAN [105]. The exact mechanisms leading to the formation of anti-glycan antibodies are not fully understood. It is hypothesized that in IgAN patients, infection with

bacteria expressing N-acetylgalactosamine (GalNAc) molecules may trigger the production of glycan-specific antibodies that cross-react with galactose-deficient IgA1 (Gd-IgA1) [110].

The presence of high levels of serum Gd-IgA1 is not sufficient to induce glomerular injury. It requires the formation of IgG or IgA1 autoantibodies that recognize the terminal GalNac of Gd-IgA1 as a neoepitope. These autoantibodies, mainly of the IgG isotype, share an unusual sequence in the variable region of their heavy chains that allows binding to galactose-deficient glycans of Gd-IgA1 [110]. Stimulation of NALT can also be an effect of dental caries. As was proven by Nagasawa et al. [106], smoking exacerbates dental caries, leading to activation of antibody production [111].

Other common mechanisms are alterations of the adaptive immune system. This might be due to the role of B and T cells and loss of immune tolerance [64]. It is well known that B cells are essential in humoral immunity to inhaled allergens [112]. Wu et al. [113] proposed a hypothetical model of B-cell involvement in the pathogenesis of MN. B cells recognize local antigens in lung tissue. B-cell surface TLRs and B-cell receptors (BCRs) that are activated during airway inflammation can recognize LPS and PLA2R, respectively, inducing circulating inflammatory responses that affect the phenotype and inhibitory function of regulatory B cells (Breg). Autoreactive B cells can participate in abnormal GC activity, and their entry to GC is supported by B-cell activating factor (BAFF). Autoreactive B cells then present antigens to T follicular helper cells (Tfh) at the light zone and T-B cell border of the GC, thus activating T-cell-dependent responses. Failed positive selection in the light zone and abnormal somatic hypermutation (SHM) and class-switch recombination (CSR) in the dark zone contribute to the formation of abnormal GCs in MN. Memory B cells then enter the circulation and long-lived plasma cells (LLPC) penetrate bone marrow. LLPCs in the bone marrow secrete autoantibodies while showing resistance to anti-CD20 drugs. Finally, autoantibodies bind to podocyte target antigens (e.g., PLA2R), causing glomerular damage and proteinuria [113]. This results in the statement that the lung acts as a critical site for immune activation in MN.

Antigen-presenting cells (APCs), for example, dendritic cells (DCs) in pulmonary tissue, when activated by smoking-induced inflammation, can cause loss of immune tolerance and induce epitope spreading, key steps in autoantibody development [64]. Moreover, DCs take up pathogens in tissues and present pathogen-derived antigens to T cells through MHC (major histocompatibility complexes), which activate naïve T cells. Cigarette smoke extract has been reported to modulate the function of DCs, so they induce Th2 differentiation essential for humoral responses. Also, nicotine has been shown to stimulate the Th2-inducing function of DCs [114]. Additionally, in MN, immune complex formation and deposition, and complement activation can occur.

5.3. Changes in Vasculature

Alternating often noted mechanisms of action in glomerulopathies due to cigarette smoking is known as hypertension. It has long been known that the nicotine content of cigarettes influences blood pressure and heart rate. It causes a rise in blood pressure by vasoconstriction and acceleration of the heart [115]. In vitro studies showed that cigarette smoke extract or isolated components such as nicotine decrease the availability of nitric oxide (NO), which is a powerful vasodilator [116]. In the RAA system, the role of angiotensin-converting enzyme (ACE) is to promote angiotensin I (Ang I) to generate angiotensin II (Ang II), where ACE2 is the specific negative regulatory member of the mechanism after RAA system activation. Yuan [117], in their research, found that expression levels of Ang II in the lung tissues of rats exposed to cigarettes were significantly increased, while ACE2 expression was reduced.

In IgAN, prolonged activation of the RAA system leads to hypertension that is associated with endothelial dysfunction and vascular smooth muscle proliferation [118–120]. Arteriolar wall thickening is a characteristic finding in nephrosclerosis that can lead to renal ischemia. Hypertension in renal arteries is also influenced by renal arteriolar hyalinosis and vasculature, promoting renal atherosclerosis [121]. Particularly, the inflammatory response is an essential component in the initiation and progression of atherosclerosis. Cigarette smoking stimulates the production of multiple pro-inflammatory mediators, especially C-reactive protein (CRP), IL-6, and TNF-α [116]. Progression of renal injury in MN appears to affect the renal vessels, which is associated with elevated plasma endothelin due to smoking.

6. Nicotine as a Main Compound Involved in Pathological Processes in the Kidneys

Among the thousands of compounds present in tobacco, nicotine is considered the most important factor responsible for a variety of biological effects that may play an important role in the pathogenesis of different conditions [118,122–124]. This one of the most stable and active components of cigarette smoke seems to play a key role in the onset and progression of proteinuria, diabetic nephropathy, and subsequently, CKD [120,125–127]. Several studies have proposed mechanisms that may cause nicotine-induced changes in the kidneys.

Hua et al. [128] proved that nicotine aggravates the severity of renal injury in a mouse DN model (db/db mice). Nicotine administered in the drinking water at a concentration of 100 μg/mL to db/db mice for 10 weeks significantly increased urinary protein excretion and caused glomerular hypertrophy. These changes were accompanied by significant increases in NADPH (nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt hydrate) oxidase 4 (~30%) and increased nitrotyrosine expression. Jaimes et al. [37] confirmed that a similar increase in NADPH oxidase activity after nicotine exposure is observed in human podocytes. A similar effect was seen in mesangial cells [19,128], thus demonstrating that NADPH oxidase activity is a main source of ROS in nicotine-exposed kidney cells from both animals and humans. Increase in NADPH oxidase was accompanied not only by ROS generation but also by Akt phosphorylation in human mesangial cells [128]. Akt kinase is known for promoting kidney hypertrophy and extracellular matrix accumulation in an NADPH-dependent mechanism during diabetic nephropathy [129].

Jaimes et al. [130] showed that nicotine increases glomerular cell number in nephritic rats. It also significantly increased the expression of cortical fibronectin, which is a critical matrix component. In another study, Jaimes et al. [37] also confirmed that nicotine binds directly to podocytes via nicotinic acetylcholine receptors (nAChRs) to induce ROS generation and cyclooxygenase (COX-2) expression, increased CD36-mediated oxidized low-density lipoprotein (oxLDL) uptake, reduced podocyte maturity marker expression (i.e., synaptopodin), and induced their apoptosis, causing glomerular injury [37]. Podocyte apoptosis was related to the activation of mitogen-activated protein kinases (MAPKs) and oxidative stress [39]. Interaction of nicotine with proximal tubular cells also resulted in their apoptosis and epithelial–mesenchymal transition (EMT) [131,132].

Further studies confirmed that nAChR subunits are expressed in kidney mesangial cells and tubular cells [130,132]. In rats, the expression of α7-nAChR in the proximal and distal tubules was demonstrated [133]. In humans, high expression of nAChR α5, α6, α7, α10, and β4 was observed in podocytes [37]. At the same time, the expression of nAChR α3, α9, β2, and β3 was relatively lower, and the expression of nAChR α2 and α4 was barely detectable [118,134].

Apart from binding to nicotinic receptors, there is substantial evidence of the direct cytotoxic effects of nicotine and its metabolites on podocytes [38]. Most of the proteinuric diseases, including MCD, are associated with altered podocyte phenotype, reduction in their number, or effacement of foot processes [135]. Zarzecki et al. [136] showed that Sprague-Dawley rats exposed in utero to cigarette smoke extract have significantly reduced glomerular volume and podocyte number, which suggests that prenatal exposure to nicotine negatively influences glomerular development. Singh et al. [38] confirmed that nicotine activates NLRP inflammasome, reduces expression of podocyte maturity markers (i.e., podocin and nephrin), upregulates the expression of caspase 1 and IL-1β, and increases cellular permeability. In mesangial cells, nicotine activates TGF- and Wnt/-catenin pathways, which promote cell growth and production of extracellular matrix [130].

Nicotine increases ROS generation in a dose-dependent manner, where ROS has been incriminated for apoptosis in multiple instances [137]. In some studies, it has also been shown that podocytes, as highly differentiated epithelial cells, are more vulnerable to increased oxidative stress compared with mesangial cells. Nicotine increases ROS generation also in proximal tubule cells. Both active and passive forms of nicotine exposure enhance renal oxidative stress by increasing mitochondrial ROS production, transcriptional activation of the pro-apoptotic and prooxidant p66shc (oxidoreductase that produces ROS in a mitochondria-dependent manner) in renal proximal tubule cells [138], and NLRP3 inflammasome activation [38,139–141]. Oxidative stress steers the inflammatory cascade and renal fibrosis that stimulate chronic kidney injury and lead to ESRD (Figure 2) [38,140,142,143].

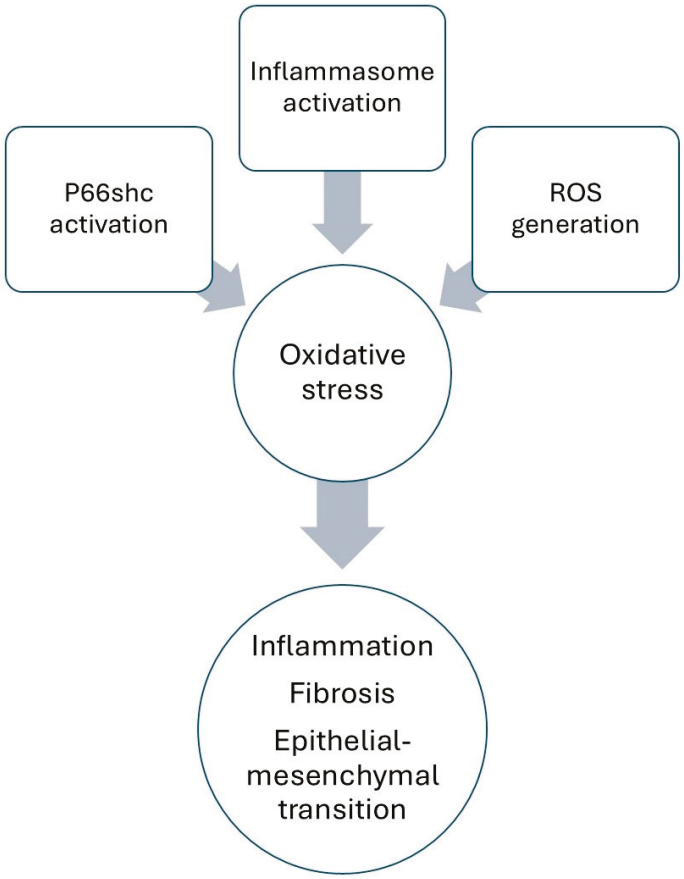

Figure 2. Role of oxidative stress in nicotine-induced kidney damage.

It was also demonstrated that nicotine decreases the expression of nephrin in podocytes [38]. Additionally, nicotine has been demonstrated to increase the proliferation of renal mesangial cells [19], proximal tubular epithelium, renal fibrosis, vimentin,

and fibronectin production [130]. It was examined whether nicotine could affect apoptosis-related proteins, for example, caspase-3, which plays a key role in apoptosis. Studies confirmed that nicotine increases the expression of the cleaved form of caspase-3 and Bax (a pro-apoptotic protein) while decreasing Bcl-2 (an anti-apoptotic protein). Taking everything into consideration, we can say that nicotine increases podocyte apoptosis [39].

Mitogen-activated protein kinases ERK, JNK, and p38 have also been implicated in nicotine-mediated podocyte damage and the progression of GN (Figure 3) [144,145]. Blockade of these kinases with their specific inhibitors significantly reduced nicotine-induced podocyte apoptosis. Kim et al. [145] demonstrated that nicotine-induced oxidative stress enhanced the phosphorylation of the ERK and JNK in renal proximal tubular cells, which stimulated the activation of the NF-κB signaling pathway and led to their apoptosis. In mice, chronic nicotine exposure stimulated the expression of the unphosphorylated signal transducer and activator of transcription-3 (U-STAT3) [132], which is associated with inflammation [146]. Increased expression of U-STAT3 caused transforming growth factor beta 1 (TGF-β1)-dependent transcriptional events that resulted in reorganization of the actin cytoskeleton and increased transcription of monocyte chemotactic protein-1 (MCP-1).

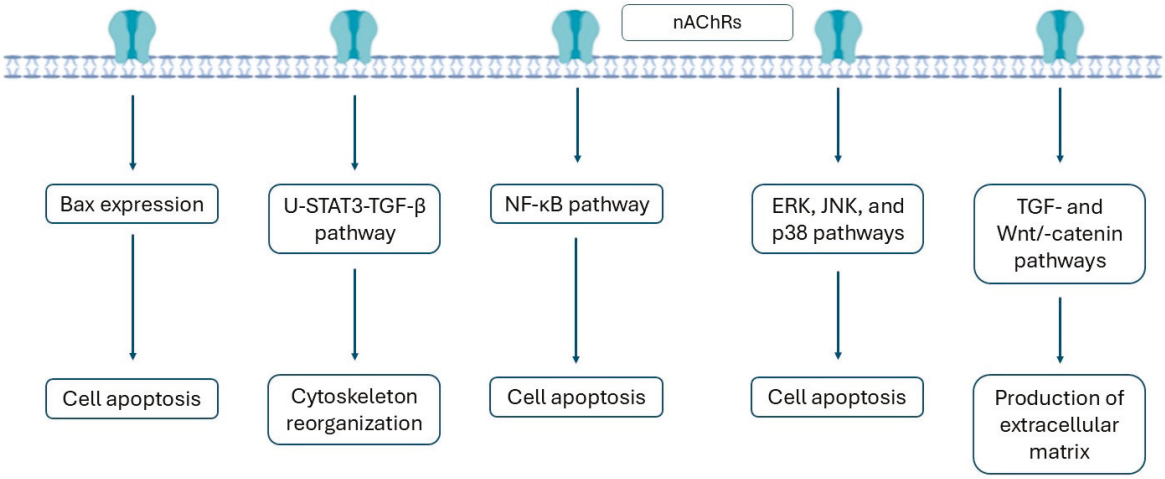

Figure 3. Signaling pathways activated in kidney cells after nicotine exposure.

7. Discussion

In the literature, many reports suggest that cigarette smoking increases the risk of CKD, particularly CKD caused by hypertensive nephropathy and diabetic nephropathy. Clinical reports have demonstrated that smoking both worsens CKD and enhances proteinuria [130,147–151]. Therefore, it is possible that the contents of the tobacco smoke, especially nicotine, directly affect the podocytes. Molecular analysis and immunofluorescent staining revealed the expression of several nAChRs in podocytes and tubular cells. Nicotine was shown to decrease nephrin expression in podocytes, which indicates that it causes cell injury. It also enhances podocyte oxidative stress, resulting in their apoptosis. Nicotine-induced podocyte apoptosis was proven to be regulated by the activation of the kinase pathways (JNK, ERK, and p38). Even though nicotine mediates its effects mainly through the activation of muscle and neuronal nAChRs [130,134,152,153], it can also increase the proliferation of renal mesangial cells [19] and induce apoptosis of podocytes [144]. Inducing ROS generation contributes to the net oxidative stress imposed by cigarette smoking [131,134]. In previously reported studies, Nicotine has also been demonstrated to increase the production of ROS in cultured mesangial cells and stimulate their proliferation and fibronectin production [130].

Many authors say that cigarette smoking disrupts kidney function, with a significant impact on health care outcomes and health care economics. In the last decade, several significant studies using animal models and human subjects demonstrated the role of cigarette smoking in the progression of chronic kidney disease (Figure 4). The effects of smoking, both active and passive, start at an early age and have long-lasting effects. The increasing prevalence of CKD demands stronger measures to halt the progression of the disease. This is particularly important as CKD patients are also at risk for significant cardiovascular diseases, in which smoking cessation provides cardiovascular and renal benefits [14].

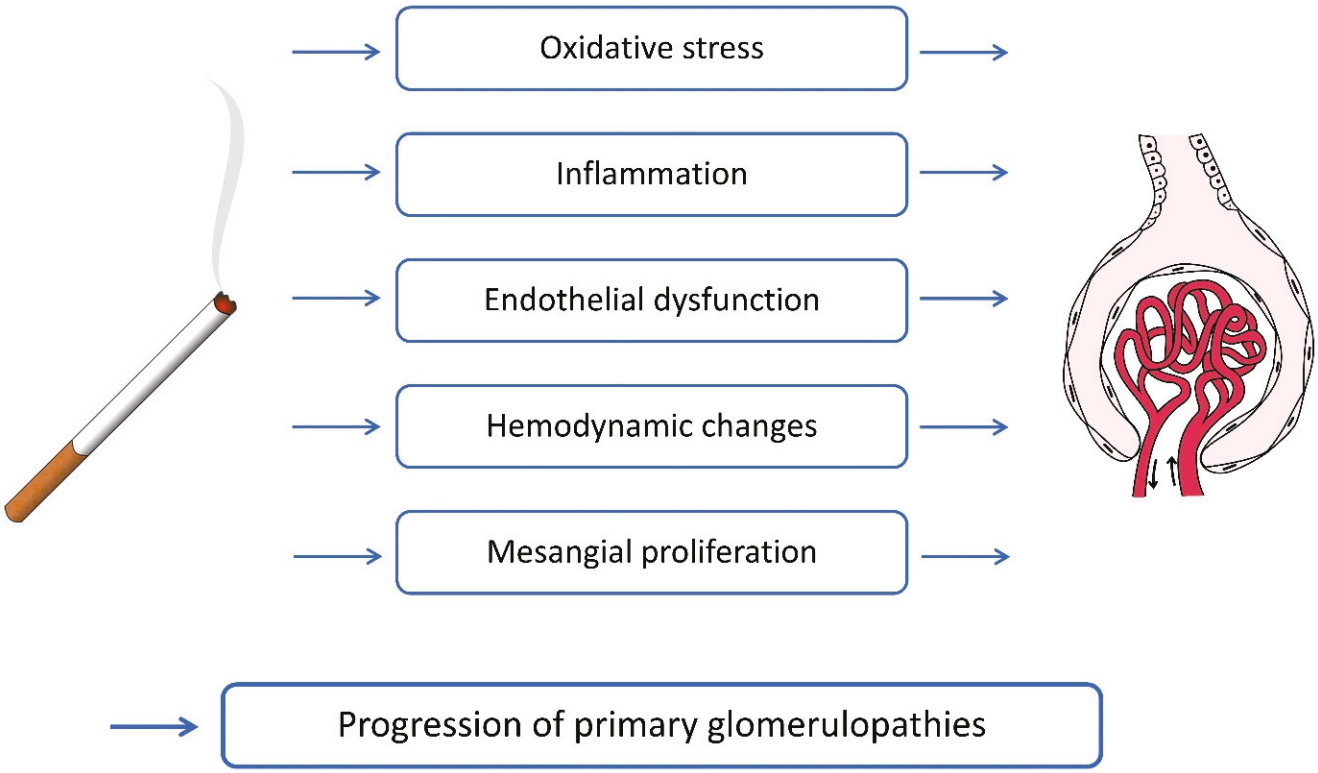

Figure 4. Summary of nicotine action on glomeruli in GN.

Provenzano et al. [154] in their study emphasized that finding more factors impacting kidneys, in general, well-being would allow detection of patients who require stricter monitoring to more efficiently recognize progression of disease. Smoking not only affects the kidneys directly but, more importantly, worsens the course of other chronic diseases, such as cardiovascular disorders and proteinuria. Those morbidities negatively affect the functioning of the kidneys and, together with nicotine, cause CKD to progress more rapidly. Smoking is independently associated with a significantly increased risk for cardiovascular events and mortality in a large cohort of already diagnosed CKD patients. Moreover, any registered data suggests that current smokers are at higher risk for renal end-stage disease and cardiovascular events, compared to both former smokers and never-smokers, who were referred for the study. It highlights the importance of a holistic approach to the treatment of primary glomerulopathies. Patients are under increased risk already having other comorbidities, such as, as mentioned above, hypertension, and smoking might amplify these risks.

8. Conclusions

Tobacco consumption is associated with a broad spectrum of diseases, ranging from malignancies and cardiovascular disorders to chronic kidney disease and autoimmune conditions [155,156]. Notably, tobacco use remains the leading preventable cause of morbidity and mortality worldwide [157], accounting for more than 7 million deaths annually, including approximately 1.6 million deaths from secondhand smoke exposure [2]. Consequently, there is a pressing need to identify, within the population of tobacco users, those patients who are at potentially higher risk of tobacco-related diseases, including renal pathology. It has been proposed that exposure biomarkers with concentrations in biological fluids can vary according to the type and extent of tobacco use. Irrespective of the tobacco form, fluctuations are observed in the levels of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6, IL-8, IL-17, IFN-γ), anti-inflammatory cytokines (IL-10, IL-4, IL-13), growth factors (TGF-β, VEGF, EGF, BDNF), selected biological active molecules (MMP-9, CRP, MCP-1), selected oxidative stress parameters (uric acid, glutathione, glutathione peroxidase, superoxide dismutase, malondialdehyde), and microplastics [1]. Future studies are necessary to explore the mechanisms of nicotine-induced renal injury. Large clinical trials evaluating the effects of smoking in patients with CKD, as well as the effects of smoking cessation on "renal health," may help answer some significant questions, including measures to prevent the progression of kidney diseases.

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Abbreviations

The following abbreviations are used in this manuscript:

CKD	Chronic kidney disease
DN	Diabetic nephropathy
ESRD	End-stage renal disease
FSGS	Focal segmental glomerulosclerosis
GBM	Glomerular basement membrane
GN	Glomerulonephritis
IgAN	IgA nephropathy
MCD	Minimal change disease
MN	Membranous nephropathy
RAA	Renin–angiotensin–aldosterone
ROS	Reactive oxygen species

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Systematic Review

Passive Tobacco Smoke Exposure, Oxidative Stress and Pediatric Allergic and Obstructive Respiratory Diseases: A Systematic Review and a "Second Oxidative Hit" Hypothesis

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Abstract

Pediatric allergic and obstructive respiratory diseases are a leading cause of chronic childhood morbidity. Passive tobacco smoke exposure (TSE) is one of the most prevalent and preventable indoor pollutants affecting children, while oxidative stress is increasingly recognized as a key mechanism linking tobacco smoke exposure to airway inflammation. This systematic review critically evaluated the evidence connecting passive TSE to oxidative stress pathways in pediatric airway disease and integrated it into a "second oxidative hit" hypothesis. Following PRISMA 2020 guidelines, PubMed and MEDLINE were searched to identify studies assessing passive TSE, oxidative or antioxidant biomarkers, and respiratory outcomes in children. Of the 77 records identified, four studies met the inclusion criteria. Across the available evidence, passive TSE was associated with increased lipid peroxidation, NOX2 activation, oxidative–inflammatory signaling, depletion of antioxidant defenses, oxidative DNA damage, and impairment of redox-sensitive corticosteroid-response pathways, although findings were not uniform across studies. Clinically, passive TSE children showed persistent allergic rhinitis, greater wheezing severity, poorer asthma control and reduced corticosteroid responsiveness. Despite limited and heterogeneous evidence, the findings provide a biological rationale for a testable "second oxidative hit" hypothesis, whereby passive TSE, combined with a pre-existing inflammatory environment in the airways associated with the underlying disease, could produce an additional oxidative burden. Further prospective studies integrating standardized oxidative biomarkers and objective exposure assessment are needed to validate this hypothesis, and establish temporal and causal relationships, potentially supporting more targeted preventive and personalized strategies.

Keywords: passive smoke; secondhand smoke; tobacco; oxidative stress; asthma; allergic rhinitis; NOX2; HDAC2; antioxidant defenses; children

1. Introduction

Pediatric allergic and obstructive respiratory diseases, including asthma, allergic rhinitis, recurrent wheezing disorders and sleep-disordered breathing, represent a major cause of chronic morbidity worldwide and are increasingly associated with long-term respiratory and systemic complications [1–5]. Among these conditions, asthma is the most prevalent chronic respiratory disease in children and is characterized by variable airflow obstruction, airway hyperresponsiveness, mucus hypersecretion and chronic airway inflammation [1,2,4,6]. Persistent airway inflammation and remodeling during childhood may impair lung development and predispose to chronic respiratory disease later in life [2].

In parallel with asthma, allergic rhinitis affects up to 40% of children worldwide and is increasingly recognized as a systemic inflammatory disease rather than a localized upper-airway disorder [3], with reported associations with endothelial dysfunction and increased cardiovascular risk in later life [3]. Similarly, sleep-disordered breathing and obstructive sleep apnea syndrome in children are associated with intermittent hypoxia, autonomic dysfunction, vascular abnormalities and systemic inflammatory activation [5]. Collectively, these observations support the concept that pediatric respiratory diseases extend beyond the airways and may contribute to long-term systemic consequences.

Environmental exposures play a pivotal role in the development and exacerbation of pediatric respiratory diseases. Among them, tobacco smoke remains one of the most important and preventable indoor pollutants affecting children [1,7–9]. Epidemiological evidence demonstrates a strong dose-dependent association between passive tobacco smoke exposure (TSE) and asthma onset, wheezing, allergic sensitization and impaired lung function, particularly when exposure occurs during prenatal life or early childhood [10–12]. Maternal smoking during pregnancy may interfere with fetal lung maturation through nicotine-mediated oxidative and inflammatory mechanisms, increasing susceptibility to chronic respiratory disorders [2].

Traditional combustible cigarettes contain thousands of toxic compounds—including aldehydes, hydrocarbons, particulate matter, volatile organic compounds, heavy metals and free radicals—capable of inducing airway injury and systemic inflammation [13]. In recent years, increasing attention has focused on alternative products such as heat-not-burn cigarettes (HNBCs) and electronic cigarettes, whose use is rising rapidly among adolescents [14]. Although marketed as reduced-risk alternatives, both generate aerosols containing nicotine, ultrafine particles and aldehydes capable of promoting epithelial injury, oxidative stress and inflammatory responses [15,16]; passive exposure to these emissions may induce oxidative damage in children comparable to that of traditional cigarette smoke [8,13].

Oxidative stress is increasingly recognized as a key mechanism linking passive TSE to airway inflammation and systemic injury [1,2,4,7]. It results from an imbalance between reactive oxygen species (ROS) and antioxidant defense systems, leading to damage of lipids, proteins, DNA and cellular structures [7]. In pediatric asthma and allergic diseases, activated eosinophils, neutrophils, macrophages and airway epithelial cells are major endogenous sources of ROS [1,4,7], while environmental pollutants, allergens and cigarette smoke directly stimulate ROS generation or amplify inflammatory pathways that further increase oxidative burden [1,2,4,7]. Excessive ROS contribute to epithelial-barrier dysfunction, mucus hypersecretion, airway hyperresponsiveness and remodeling [1,2,4,7], and activates redox-sensitive transcription factors (NF-κB, AP-1) that promote the release of pro-inflammatory cytokines and perpetuate chronic airway inflammation [4,7]. Cigarette smoke also impairs endogenous antioxidant pathways, including Nrf2-mediated responses, am-

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plifying oxidative injury and contributing to corticosteroid resistance in severe asthma [1,7]. NADPH oxidase activation, particularly the NOX2 isoform, appears to be a major molecular pathway linking TSE to ROS overproduction and systemic oxidative damage [3,8,17].

Importantly, the detrimental effects of passive TSE are not confined to the respiratory tract. Chronic airway inflammation and oxidative stress may evolve into a systemic inflammatory condition associated with endothelial dysfunction, platelet activation, vascular injury and increased cardiovascular risk later in life [3,8,13,17].

Despite growing interest, the molecular mechanisms connecting airway inflammation to systemic oxidative injury in passive TSE children remain incompletely understood and poorly defined. The aim of this systematic review is therefore to critically summarize current evidence on the role of passive TSE in pediatric allergic and obstructive respiratory diseases, focusing on the interplay between oxidative stress and airway inflammation, and to integrate the available evidence into a unifying "second oxidative hit" model.

2. Materials and Methods

This systematic review was conducted and reported according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA 2020) guidelines. The review aimed to evaluate the association between passive TSE and oxidative stress pathways in pediatric allergic and obstructive respiratory diseases, with particular focus on oxidative stress biomarkers, inflammatory mechanisms and clinical respiratory outcomes. Because of the substantial heterogeneity in study design, biomarker selection, biological matrices and outcome reporting, a formal quantitative meta-analysis was not considered methodologically appropriate. Observational, cohort, cross-sectional and case–control studies, and translational/mechanistic investigations were included. Among cross-sectional studies, case–controls were defined as patients with respiratory disease versus healthy subjects, while studies were defined as comparative when intra-group patients (TSE versus non-TSE) were considered.

Review articles were excluded from the primary qualitative synthesis; however, they were examined for additional references and contextual interpretation. Studies were eligible if they investigated children or adolescents with allergic or obstructive respiratory conditions, assessed passive exposure to tobacco smoke (second- and thirdhand smoke), encompassed both traditional and heated tobacco products, and analyzed oxidative stress factors.

Exclusion criteria: Adult-only populations; animal or in vitro studies; non-English language studies; studies without oxidative stress-related outcomes; studies lacking pediatric respiratory disease populations; and conference abstracts without sufficient methodological information.

The PICO statement was as follows:

Population (P): Children or adolescents with asthma, recurrent wheezing, allergic rhinitis, obstructive sleep apnea syndrome (OSAS), sleep-disordered breathing (SDB) or related allergic/non-allergic obstructive airway diseases.

Intervention or Exposure (I): Studies evaluating passive TSE, environmental tobacco smoke, secondhand or thirdhand smoke, parental smoking, cotinine/nicotine exposure, or passive exposure to heated tobacco products or electronic cigarettes.

Comparison or control (C): Non-exposed children's cohorts or inter group patients.

Outcome (O): Studies reporting at least one oxidative stress biomarker, antioxidant defense marker, and oxidative–inflammatory pathway, associated with allergic/ non allergic obstructive respiratory diseases.

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2.1. Search Strategy

A comprehensive literature review was conducted utilizing PubMed and MEDLINE databases. The search strategy was implemented in March 2026, employing controlled vocabulary and pertinent keywords related to passive TSE, oxidative stress, pediatric populations, and allergic or obstructive respiratory diseases, within TITLE/ABSTRACT (tiab) fields.

A comprehensive literature search was performed using the following search strategies:

Strategy 1: ("secondhand smoke"[tiab] OR "second-hand smoke"[tiab] OR "environmental tobacco smoke"[tiab] OR "passive smoking"[tiab] OR "passive smoke exposure"[tiab] OR "tobacco smoke exposure"[tiab] OR "tobacco smoke pollution"[tiab] OR cotinine[tiab] OR nicotine[tiab] OR smoking[tiab] OR smoke[tiab] OR "thirdhand smoke"[tiab] OR "third-hand smoke"[tiab]) AND ("oxidative stress"[tiab] OR oxidant*[tiab] OR antioxidant*[tiab] OR "redox"[tiab] OR "reactive oxygen species"[tiab] OR ROS[tiab] OR "8-isoprostane"[tiab] OR isoprostane*[tiab] OR "malondialdehyde"[tiab] OR MDA[tiab] OR glutathione[tiab] OR "superoxide dismutase"[tiab] OR SOD[tiab] OR catalase[tiab] OR "total antioxidant capacity"[tiab] OR "nitric oxide"[tiab] OR nitrosative[tiab]) AND (child*[tiab] OR adolescen*[tiab] OR pediatric*[tiab] OR paediatric*[tiab] OR infant*[tiab] OR preschool*[tiab]) AND(asthma[tiab] OR wheez*[tiab] OR "allergic rhinitis"[tiab] OR rhinitis[tiab] OR "sleep apnea"[tiab] OR "sleep apnoea"[tiab] OR "obstructive sleep apnea"[tiab] OR "obstructive sleep apnoea"[tiab] OR OSAS[tiab] OR OSA[tiab] OR "sleep disordered breathing"[tiab] OR SDB[tiab]).

Filters applied: Adaptive Clinical Trial, Clinical Study, Clinical Trial, Clinical Trial, Phase I, Clinical Trial, Phase II, Clinical Trial, Phase III, Clinical Trial, Phase IV, Collected Work, Comparative Study, Controlled Clinical Trial, Dataset, Electronic Supplementary Materials, Equivalence Trial, Evaluation Study, Introductory Journal Article, Letter, Meta-Analysis, Multicenter Study, Network Meta-Analysis, Observational Study, Pragmatic Clinical Trial, Randomized Controlled Trial, Systematic Review, Validation Study, English, Humans.

Strategy 2: ("Tobacco Smoke Pollution"[Tiab] OR "cotinine" [Tiab] OR "nicotine" [Tiab] OR "tobacco" [Tiab] OR "smoke" [Tiab]) AND ("oxidative stress" [Tiab]) AND ("Child" [Tiab] OR "Adolescent"[Tiab] OR "pediatric*"[Tiab] OR "child" [Tiab] OR "adolescent*"[Tiab] OR "children" [Tiab]) AND ("Asthma"[Tiab] OR "wheez*"[Tiab] OR "Rhinitis, Allergic"[Tiab] OR "allergic rhinitis" [Tiab] OR "Sleep Apnea Syndrome*"[Tiab] OR "obstructive sleep apnea" [Tiab] OR "OSAS" [Tiab] OR "sleep disordered breathing" [Tiab] OR "SDB" [Tiab])".

Reference lists of eligible studies and relevant reviews were screened manually to identify additional studies.

2.2. Study Selection and Data Extraction

Two reviewers (B.L.C. and A.G.) independently screened titles and abstracts; full texts of potentially relevant studies were then assessed, with disagreements resolved by consensus. Data extraction was performed independently by two reviewers using a standardized framework including study and population characteristics, smoke-exposure assessment, oxidative stress biomarkers, and clinical outcomes.

2.3. Risk-of-Bias Assessment

Because the included studies are heterogeneous and mostly observational, the risk of bias was evaluated according to study design. Cross-sectional studies were assessed using the Joanna Briggs Institute (JBI) Critical Appraisal Checklist, while cohort and case–control studies were evaluated with the Newcastle–Ottawa Scale (NOS). Studies were graded as having low, moderate or high risk of bias.

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3. Results

3.1. Study Selection and Characteristics

The systematic literature search identified 77 records through database searching. After removal of duplicates and screening, four studies were considered relevant for qualitative synthesis (Figure 1).

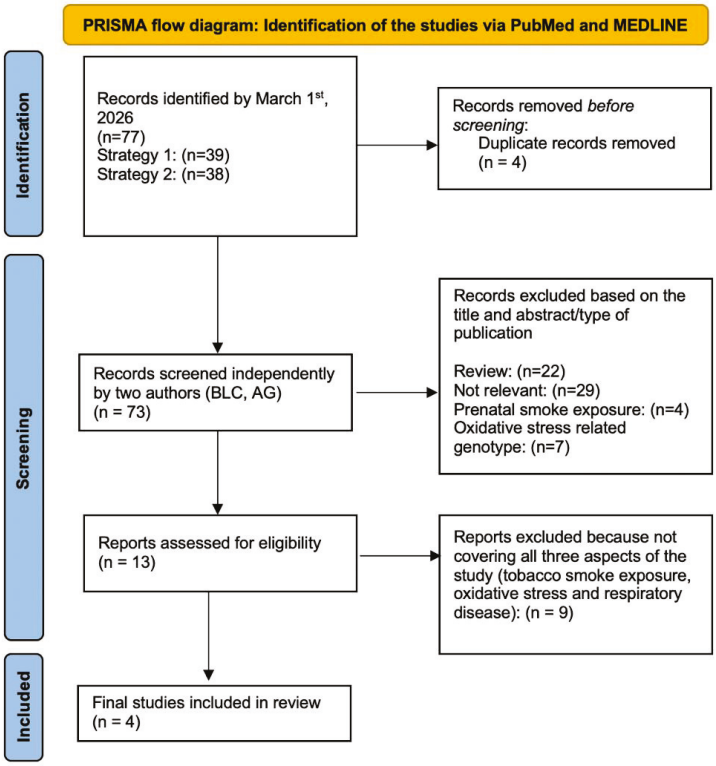

Figure 1. PRISMA flow diagram. Modified from [18].

The included studies were published between 2014 and 2018 and conducted across heterogeneous geographic settings in Europe, North America and Asia. Most adopted observational designs—predominantly cross-sectional or cohort-based—although mechanistic translational and biomarker-focused investigations were also represented. Sample sizes ranged from 19 children in the bronchoalveolar lavage (BAL) mechanistic study to 160 participants in the largest included study. The analyzed pediatric populations included children with asthma of varying severity, recurrent wheezing, and allergic rhinitis.

Smoke-exposure assessment varied substantially: two studies relied on parental questionnaires or household smoking history, whereas the other two incorporated objective biomarkers such as serum/plasma or urinary cotinine, and a subset differentiated low- from high-level passive TSE using predefined cotinine thresholds. Oxidative stress was evaluated in heterogeneous biological matrices including serum, urine, BAL fluid, and nasal lavage. The investigated biomarkers included malondialdehyde (MDA), glutathione, superoxide dismutase (SOD), 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxo-dG), isoprostanes and NOX2-derived peptides, together with inflammatory mediators associated with oxidative signaling such as IL-8, IL-17, myeloperoxidase (MPO) and matrix metalloproteinases. Despite this heterogeneity, three studies consistently reported increased oxidative burden or impaired antioxidant defenses in TSE children with respiratory disease, while a study detecting specific biomarkers on nasal lavage failed to identify a relevant association between oxidative stress, inflammation and severity of respiratory disease in passive TSE versus non-TSE children. The characteristics, biological role and direction of evidence of the included studies are summarized in Table 1.

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Table 1. Characteristics and biological contribution of the included studies ($n = 4$).

Study	REF N.	Design	N Cases and Controls	Population (Disease)	Key Biomarkers of Oxidative Stress and Smoke Metabolites/Type of Sample	Results	Outcome
Loffredo 2018	[3]	Cross-sectional case–control study	65 cases (32 TSE vs. 33 non-TSE) vs. 65 healthy controls	Persistent allergic rhinitis	Serum sNOX2-dp, isoprostanes, NO bioavailability; plasma cotinine	Allergic rhinitis group vs. healthy controls: ↑ serum sNOX2-dp and 8-iso-PGF2a; subgroup analysis in the allergic rhinitis group of TSE vs. non-TSE: ↑ sNOX2-dp ↑ serum 8-iso-PGF2a	Passive TSE was associated with increased NOX2-driven oxidative stress in persistent allergic rhinitis
Kobayashi 2014	[19]	Cross-sectional comparative study; mechanistic	19 subjects (10 not TSE and 9 TSE)	Uncontrolled severe asthma	BAL cell count, MDA, HDAC2 protein/activity, PI3K/Akt, CXCL8; saliva or urine cotinine;	Subjects in the TSE group vs. non-TSE group: in BAL ↑ neutrophils, ↓ eosinophils, ↑ MDA, ↑ CXCL8; positive correlation between CXCL8 concentrations and the percentage of neutrophils; in AMs of TSE BAL, ↓ HDAC2 protein level, ↓ activity of IP-HDAC2, ↑ HDAC2 and Akt1 phosphorylation. Akt1 phosphorylation levels were positively correlated with HDAC2 phosphorylation; Dexamethasone at 10–6 M leads to ↓ TNFa-induced CXCL8 production by 40% in AMs	Passive TSE was associated with increased oxidative stress and inflammatory markers and impairs HDAC2 expression and activity via PI3K/Akt, a pattern consistent with steroid resistance in severe asthma
Yilmaz 2018	[20]	Cross-sectional comparative study	160 subjects (125 TSE-91 High-level TSE and 24 Low-level TSE vs. 35 non-TSE)	Recurrent wheezing	Nasal lavage glutathione, IL-8, IL-17, MMP-9 and TIMP-1; serum cotinine and SP-D	Serum cotinine levels between 3 and 12 ng/mL, and above 12 ng/mL were defined as lower and higher level TSE, respectively. Wheezing symptom scores were higher in TSE children. No differences in the level of biomarkers in the TSE and non-TSE groups and between the two groups of TSE children	Passive TSE was associated with worse wheezing severity; no relevant association between biomarkers of oxidative stress inflammation and severity of respiratory disease.
Topic 2017	[21]	Cross-sectional comparative study	96 subjects (48 TSE in both prenatal and postnatal period vs. 48 non-TSE)	Asthma: 10.4% intermittent, 89.6% persistent (40.6% mild persistent, 49% severe persistent)	Urinary 8-oxo-7,8-dihydro-2-deoxyguanosine; serum MPO and Cu/Zn-superoxide dismutase (SOD)	↑ levels of urinary 8-oxo-7,8-dihydro-2-deoxyguanosine in children with TSE intermittent asthma, in comparison to the non-TSE children with intermittent asthma and to children with mild and severe persistent asthma, regardless of TSE. The level of serum SOD was altered in asthmatic children with eczema under the influence of TSE. Increased urinary 8-oxodG and TSE were identified as independent risk factors associated with intermittent asthma	Passive TSE and increased 8-oxo-dG were linked to intermittent asthma clinical manifestations

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3.2. Passive Smoke Exposure and Lipid Peroxidation Pathways

Evidence from two studies supported an association between passive TSE and increased lipid peroxidation in pediatric airway disease. Investigations demonstrated elevated biomarkers of oxidative membrane injury in TSE children, including increased MDA, isoprostanes and NOX2-related oxidative activity. In children with persistent allergic rhinitis, passive TSE was associated with enhanced NOX2 activation (soluble NOX2-derived peptide) [3]. Similarly, a study in severe asthmatic children showed that MDA in BAL fluid increased in association with TSE [19].

The recurrence of lipid-oxidation biomarkers across heterogeneous biological matrices supports a coherent oxidative response induced by environmental smoke exposure in susceptible children. These results showed a predominantly positive association between passive TSE and lipid-peroxidation markers.

3.3. Oxidative DNA Damage and Systemic Redox Burden

Oxidative nucleic-acid damage emerged as a further pathway. A study evaluating urinary 8-oxo-dG demonstrated associations between TSE and oxidative DNA injury in children with asthma, with increased DNA-damage markers linked to the specific clinical phenotype of intermittent asthma [21]. These observations support the hypothesis that smoke-related oxidative stress in pediatric airway disease extends beyond transient inflammatory responses to contribute to a broader systemic oxidative burden affecting nucleic-acid integrity.

3.4. Oxidative Inflammation and Airway Remodeling

Passive TSE was associated with increased neutrophilic inflammatory signaling and included alterations in IL-8, IL-17, MPO, MMP-9/TIMP-1, CXCL8 and NOX2-related oxidative activation. In recurrent-wheezing children, passive TSE was associated with more severe respiratory symptoms and evidence of airway inflammatory activation, although biomarkers did not show statistically significant between-group differences [20]. A mechanistic study in severe asthma demonstrated enhanced oxidative–inflammatory signaling within BAL macrophages of TSE children, supporting the concept of persistent airway inflammatory activation induced by passive smoking [19].

3.5. Corticosteroid Resistance and Redox-Sensitive Pathways

One of the most mechanistically relevant findings concerned oxidative pathways linked to corticosteroid resistance. In children with severe asthma exposed to passive smoke, reduced histone deacetylase-2 (HDAC2) activity and expression were observed together with activation of PI3K/Akt signaling and increased oxidative stress markers [19]. These molecular abnormalities were associated with impaired corticosteroid responsiveness and poorer asthma control, providing biologically plausible evidence that passive TSE can directly interfere with redox-sensitive anti-inflammatory regulatory pathways and may thereby contribute to severe, treatment-refractory asthma phenotypes.

3.6. Antioxidant-System Impairment

Another finding involved impairment of enzymatic and non-enzymatic antioxidant defense systems. Children with asthma demonstrated significantly lower levels of the antioxidant molecule superoxide dismutase in serum, indicating that passive TSE may contribute not only to oxidant generation but also to depletion or dysregulation of antioxidant defenses, thereby amplifying airway susceptibility to inflammatory injury [21]. A study investigating recurrent wheezing additionally evaluated nasal glutathione in relation to passive TSE; wheezing children exposed to passive smoke generally showed worse

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respiratory symptom severity although biomarkers did not consistently differ by exposure intensity [20].

3.7. Clinical Respiratory Outcomes

Across the included studies, passive TSE was associated with worse respiratory clinical outcomes. TSE children showed persistent allergic rhinitis [3] increased wheezing severity [20], intermittent asthma [21], poorer asthma control and reduced responsiveness to therapy [19]. Taken together, the available evidence supports a clinically meaningful association between passive TSE, oxidative-stress amplification and respiratory disease severity in pediatric allergic and obstructive airway disorders.

3.8. Risk of Bias

The main recurrent limitations across studies included small sample sizes, heterogeneity of oxidative biomarkers, inconsistent smoke-exposure ascertainment, variable adjustment for socioeconomic and environmental confounders, and a general lack of longitudinal oxidative-stress measurements. Nevertheless, several studies strengthened internal validity through objective cotinine measurement, repeated biomarker sampling, mechanistic translational analyses, airway-specific biological matrices and integration of clinical severity outcomes. Preliminary risk-of-bias judgments are summarized in Table 2.

Table 2. Preliminary risk-of-bias appraisal of core studies.

Study	Design	Exposure Assessment	Main Limitation	Preliminary Risk
Loffredo 2018 [3]	Cross-sectional	Cotinine/smoking history	Single-time-point biomarkers.	Moderate
Kobayashi 2014 [19]	Cross-sectional Mechanistic	Cotinine/smoking history	Very small cohort. Single-time-point biomarkers.	Moderate-to-high
Yilmaz 2018 [20]	Cross-sectional	Cotinine	Single-time-point biomarkers.	Moderate
Topic 2017 [21]	Cross-sectional	Questionnaire-based TSE	Self-reported exposure. Single-time-point biomarkers.	Moderate

A complementary study-level assessment of the main methodological limitations, including their likely direction and magnitude and their potential impact on the conclusions of this review, is provided in Table 3. Direction and magnitude of bias and its impact on review conclusions were qualitatively judged according to study design, exposure and outcome assessment, statistical precision, and potential confounding; these ratings do not represent quantitative bias-adjusted estimates.

Table 3. Systematic assessment of limitations of included studies.

Study	Main Source(s) of Bias/Limitation	Likely Direction/Magnitude	Impact on Review Conclusions	Rationale
Loffredo 2018 [3]	Residual confounding; single-time-point assessment; single-center population	Uncertain; moderate	Medium	Cross-sectional assessment limits causal inference, although objective cotinine measurement reduces exposure misclassification and comparison within a clinically defined population improves group comparability. Temporality and cumulative exposure cannot be established.

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Table 3. *Cont.*

Study	Main Source(s) of Bias/Limitation	Likely Direction/Magnitude	Impact on Review Conclusions	Rationale
Kobayashi 2014 [19]	Small, highly selected severe-asthma population; limited control of confounding	Uncertain; potentially large	Medium	The small sample, clinical selection and cross-sectional design limit causal and clinical inference. However, exposed and unexposed children had comparable severe-asthma characteristics, and the study provides detailed mechanistic evidence.
Yilmaz 2018 [20]	Limitations of nasal lavage and single-time-point biomarker assessment; exposure categorization	Mainly toward null for biomarker associations; magnitude uncertain	Medium	Sampling limitations may have reduced sensitivity to detect oxidative differences, although exposure was objectively assessed by cotinine and biomarker outcomes were laboratory-determined. A true null association cannot be excluded.
Topic 2017 [21]	Questionnaire-based TSE assessment; residual confounding; limited subgroup precision	Uncertain; moderate	Medium	Exposure misclassification and subgroup imprecision reduce confidence in specific associations, while assessment of multiple oxidative/antioxidant markers provides complementary evidence on redox alterations.

4. Discussion

This systematic review synthesizes a limited but consistent body of evidence linking passive TSE to oxidative stress and clinical severity in pediatric allergic and obstructive respiratory disease. Despite a low number of scientific articles focused on this topic, marked heterogeneity in design, biomarkers and biological matrices, the included studies demonstrate that passive TSE increases oxidative–inflammatory signaling, depletes antioxidant defenses, and amplifies and interferes with redox-sensitive corticosteroid-response pathways. One of the selected studies failed to find an association between smoking and oxidative stress; however, it is the only study to have analyzed inflammatory and oxidative/antioxidant markers in nasal fluid [20]. These results may have been affected by the potentially low quantity of biomarkers in this biological fluid, making it difficult to establish an association with passive TSE.

Among the diseases considered, asthma with various levels of severity, recurrent wheezing and persistent allergic rhinitis have been analyzed. Although a preliminary study demonstrated increased oxidative stress in children with OSAS [5], no studies have considered smoke exposure as an amplifier of inflammatory response and oxidative stress exacerbation of severity in OSAS.

Analysis of the selected studies identified three mechanisms underlying the proposed approach.

First, continuous exposure to allergens stimulates nasal eosinophils to produce ROS [22]. NADPH oxidase activity represents a source of excessive ROS production and inflammation. Previous studies have shown that NADPH oxidase isoforms NOX1, NOX4, DOX1, and DOX2 are overexpressed in the airway epithelium and mucosa [23,24].

In allergic rhinitis, it was demonstrated that NOX2 activation was exacerbated by passive smoke, with increased soluble NOX2-derived peptide and isoprostanes [3].

Oxidative consequences of passive smoking are not confined to the airway but extend to the endothelium—an early antecedent of later cardiovascular risk. Indeed, Nicotinamide-adenine dinucleotide phosphate (NADPH) oxidase is a primary source of cellular superoxide anion production in humans [25], and studies performed in animals and humans suggest that NADPH oxidase activity modulates arterial tone [26]. Accordingly, it has been shown that a lack of or deficient NADPH oxidase isoform 2 (NOX2) activity is associated with arterial dilation in humans [27]. Children affected by persistent allergic rhinitis and exposed to passive smoking, compared to those who are non-exposed, had significantly

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higher sNOX2-dp, serum 8-iso-PGF2a and also lower FMD, an early sign of endothelial dysfunction [3].

Second, a broad decrease in enzymatic and non-enzymatic antioxidant defenses is observed in asthma, as previously demonstrated [28], and depletion of the enzyme SOD reflects impaired antioxidant defenses, reducing the capacity to counteract cigarette smoke-induced oxidative stress in subjects with asthma and eczema [21].

A nutritional dimension reinforced this mechanism: although it was not possible to include it among the selected studies because oxidative stress markers were not measured, a large cross-sectional analysis showed that a higher dietary antioxidant quality score attenuated the association between passive TSE and asthma outcomes, suggesting that endogenous and dietary antioxidant capacity can modulate susceptibility [29].

The potential involvement of impaired antioxidant defenses is further supported by pediatric observational evidence beyond the studies included in the systematic synthesis. Preston et al. reported lower plasma ascorbate concentrations in children exposed to environmental tobacco smoke, objectively assessed by urinary cotinine, despite comparable vitamin C intake [30]. Similarly, Wilson et al. found inverse associations between serum cotinine and several antioxidant micronutrients, including vitamin C, carotenoids, folate, and vitamin A [31]. These findings provide a biological rationale for investigating whether restoration of antioxidant status may mitigate TSE-associated oxidative alterations. However, they do not establish a therapeutic benefit of supplementation, which requires dedicated pediatric intervention studies assessing efficacy, dosing, and safety.

Third, HDAC2 impairment via PI3K/Akt activation provides a direct molecular bridge between oxidative stress and corticosteroid resistance, offering a possible explanation for the treatment-refractory phenotypes observed in TSE children with severe asthma [19].

Additional experimental evidence suggests that redox-sensitive inflammatory pathways may complement the mechanisms identified in the included studies. Cigarette smoke-induced oxidative injury has been linked to DAMP/RAGE signaling and to TLR4/MyD88/TRAF6-dependent NADPH oxidase activation, with downstream MAPK and NF-κB signaling and pro-inflammatory mediator production [32,33]. These pathways are consistent with the broader evidence linking tobacco smoke-induced oxidant–antioxidant imbalance to airway inflammation [34] and may provide additional mechanistic support to the direct evidence derived from the systematic synthesis. Their relevance to passive TSE in children, however, remains to be specifically investigated.

The leukotriene pathway provides additional clinical insight. Among the studies that were not selected because they did not strictly address the research question, two papers demonstrated that urinary LTE4 identified TSE asthmatic children at higher risk of severe exacerbations requiring emergency department or urgent-care visits, reflecting the high inflammatory state caused by asthma and exacerbated by smoke. Consistent with this, leukotriene-pathway studies showed that passive TSE modifies the relationship between urinary LTE4, treatment response and exacerbation risk—possibly identifying LTE4 as a redox-sensitive susceptibility biomarker with direct therapeutic implications [35–37].

All these findings may be interpreted within a hypothesis-generating framework, the "second oxidative hit" model: repeated passive TSEs, combined with a pre-existing inflammatory environment in the airways associated with the underlying disease, could produce an additional oxidative burden that, in turn, could contribute to less effective disease control and a reduced response to treatment. This hypothesis could be further explored through three complementary research approaches. First, objective exposure to TSE should be assessed repeatedly in prospective longitudinal cohorts, preferably using cotinine-based measurements across different matrices, along with oxidative/antioxidant biomarkers to determine whether cumulative or persistent exposure predicts subsequent changes in

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the redox balance. Second, serial clinical assessments of disease control, exacerbations, and response to corticosteroids should be conducted in the same cohorts to determine whether oxidative alterations precede and predict worsening respiratory outcomes. Third, the reversibility of oxidative damage should be evaluated through lifestyle interventions aimed at reducing exposure or quitting smoking, to determine whether objectively measured reductions in TSE are accompanied by parallel decreases in oxidative burden and improvements in clinical outcomes. Taken together, these study designs would allow for a more rigorous assessment of temporal relationships, dose–response relationships, and reversibility than is possible with the currently available cross-sectional evidence.

Background and contextual studies broaden the framework. Although oxidative stress biomarkers demonstrate the biological impact of TSE, genetic and epigenetic determinants capable of modulating oxidative stress responses provide a biological basis for interindividual susceptibility to tobacco smoke-related respiratory disease.

In this context, the search strategy used to select the previous studies identified an additional 10 studies.

All included studies specifically evaluated passive TSE as the environmental trigger and investigated how oxidative stress-related genetic, epigenetic or antioxidant pathways modify the risk of respiratory disease development or progression.

Eight studies examined genetic susceptibility through polymorphisms in oxidative stress-related genes, whereas two evaluated epigenetic mechanisms associated with prenatal TSE.

Although the magnitude of the observed associations varied among studies, most reported that oxidative stress-related genetic susceptibility became evident primarily following prenatal or postnatal TSE rather than in its absence. Overall, TSE was consistently evaluated as the primary environmental exposure, while oxidative stress-related genetic, epigenetic, or antioxidant pathways were investigated as potential modifiers of individual susceptibility. The molecular pathways most frequently implicated involved the glutathione S-transferase family (GSTM1, GSTT1, and GSTP1) and the antioxidant response regulator NFE2L2, while recent evidence also highlighted the contribution of epigenetic mechanisms, including altered DNA methylation and microRNA expression. Collectively, these molecular alterations were associated with impaired lung development, wheezing, asthma susceptibility, reduced pulmonary function and, in some studies, increased disease severity [38–47].

Taken together, the evidence reviewed supports a coherent pathogenic framework in which oxidative stress represents a central biological mechanism linking TSE during both prenatal and postnatal life to the development and progression of pediatric respiratory diseases. Whether preconception parental or ancestral tobacco exposure may further modify offspring susceptibility through germline epigenetic mechanisms remains an emerging research question requiring specifically designed multigenerational studies.

With respect to emerging products, review-level evidence supports the biological plausibility of e-cigarette- and heat-not-burn cigarette-related oxidative injury [15,16], yet direct pediatric data on passive exposure to electronic and heat-not-burn products are scarce [8]. Unlike conventional cigarettes, e-cigarettes do not involve tobacco combustion and have a different toxicant profile; nevertheless, their aerosols may contain reactive oxygen species, carbonyl compounds, ultrafine particles, and other potentially harmful constituents [48]. Experimental evidence further indicates that aerosol components, including propylene glycol, vegetable glycerin, and flavoring chemicals, are not necessarily biologically inert and may impair airway epithelial function and promote oxidative and inflammatory responses [49–52]. Thus, although conventional tobacco smoke and e-cigarette aerosols differ in composition and toxicant burden, oxidative stress and airway inflammation may

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represent partially convergent biological responses. However, these mechanisms have been characterized predominantly in experimental models and active users, and their relevance to passive exposure in children remains to be established.

4.1. Methodological Considerations

The cross-sectional design of the included studies precludes establishing temporality or causality between passive TSE, oxidative alterations, and respiratory outcomes. Although two studies [3,19] compared TSE and non-TSE children within the same clinically defined respiratory condition, and objective cotinine assessment reduced exposure misclassification in one of the mechanistically informative studies [3], reverse causation cannot be entirely excluded. Accordingly, the proposed "second oxidative hit" model requires prospective longitudinal validation.

A further limitation is the reliance on single-time-point measurements, which cannot capture the dynamic nature of TSE and oxidative/antioxidant balance, and heterogeneity in TSE ascertainment, which varied across parental reports, cotinine measurements, biological matrices, exposure definitions, and thresholds. Questionnaire-based assessment may be affected by recall or social-desirability bias and under-reporting, potentially leading to exposure misclassification, although its direction and magnitude cannot be reliably determined. Conversely, cotinine provides a more objective measure of recent exposure but does not necessarily reflect cumulative TSE. Given the small number of eligible studies and methodological heterogeneity, stratified or sensitivity analyses according to exposure-assessment method were not considered informative.

Residual confounding should also be considered, as socioeconomic factors, co-exposure to other pollutants, dietary antioxidant intake, and other environmental determinants were not consistently accounted for and may have influenced the observed associations. Therefore, TSE should be interpreted as a potential additional oxidative burden within a multifactorial environmental and biological context.

Future longitudinal studies should therefore incorporate repeated within-subject assessments of objective TSE and oxidative/antioxidant balance, while adequately accounting for relevant potential confounders. Assessments should include clinically informative periods such as stable disease, respiratory infections or exacerbations, recovery, and, where feasible, different seasons, while balancing methodological requirements with the feasibility and invasiveness of repeated sampling in pediatric populations.

4.2. Limitations and Strengths

The limitations are substantial and largely inherited from the primary evidence: the number of eligible studies is small, they are cross-sectional with modest samples, longitudinal oxidative measurements are absent, and biomarker methods and exposure ascertainment are heterogeneous.

From a clinical perspective, current evidence does not support the routine use of oxidative stress biomarkers such as 8-oxo-dG or sNOX2-dp for pediatric risk assessment, given the limited and heterogeneous data, lack of standardized pediatric reference ranges and prognostic thresholds, and methodological variability across assays. Further validation is therefore required before their clinical implementation. Conversely, objective TSE assessment by urinary or salivary cotinine is readily available and may provide a more practical tool for identifying and monitoring exposure, particularly in children with severe or difficult-to-control respiratory disease.

Another limitation is that, despite the inclusion of several biomarkers specific to exposure and oxidative stress and the use of two search strategies—one more specific to biomarkers and one more general—they may not have captured all individual tobacco

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smoke constituents or their metabolites potentially relevant to oxidative and respiratory effects. These include acrolein, a highly reactive unsaturated aldehyde abundant in tobacco smoke, which can promote airway epithelial injury, oxidative stress, and inflammatory responses [53], and its major urinary metabolite 3-hydroxypropylmercapturic acid (3-HPMA), an established biomarker of acrolein exposure [54]. Therefore, studies specifically focused on these markers may not have been fully captured.

However, this systematic review has strengths. First, to our knowledge, it is the first systematic review specifically investigating the relationship between passive TSE oxidative stress, and disease severity in pediatric allergic and obstructive respiratory disorders. This study synthesizes clinical, biochemical, and mechanistic evidence, to provide the first comprehensive overview of the biological pathways linking passive TSE to respiratory disease progression in children.

A further strength is that the review clearly identifies a substantial gap in the current literature. Despite the well-established detrimental effects of passive TSE on pediatric respiratory health, only a limited number of studies have specifically investigated oxidative stress as the mechanistic link between passive TSE and disease severity. This finding highlights how underexplored this field remains and emphasizes the need for dedicated research using standardized methodologies and clinically relevant oxidative stress biomarkers.

5. Conclusions

The four studies included in the systematic review provide evidence of associations between passive exposure to TSE, alterations in oxidative/antioxidant pathways, and the clinical characteristics and outcomes of certain pediatric airway diseases. These findings have led us to propose the "second oxidative hit" as a unifying hypothesis, according to which repeated passive exposure to TSE may represent an additional exogenous oxidative insult that overlaps with the pre-existing pro-oxidant environment of inflamed pediatric airways and may, in turn, contribute to less effective disease control and a reduced response to treatment. The oxidative and redox-sensitive pathways identified in the included studies, together with the broader mechanistic, genetic, and epigenetic evidence discussed in this review, support the biological plausibility of this hypothesis. However, this interpretation must be considered in light of the limited number of studies, their predominantly cross-sectional design, the small and heterogeneous populations, and the variability in the assessment of TSE and oxidative biomarkers—factors that preclude drawing definitive causal conclusions.

From a translational perspective, preventing and reducing exposure to secondhand tobacco smoke remains the primary feasible strategy for children with respiratory diseases. Future research should prospectively test the "second oxidative hit" hypothesis and its clinical relevance. Longitudinal cohorts combining repeated objective assessments of TSE, preferably using cotinine-based measurements, with oxidative/antioxidant biomarkers could determine whether cumulative or persistent exposure predicts subsequent changes in the redox balance and whether such alterations precede worsening disease control, exacerbations, or a reduction in or diminished response to corticosteroids. Complementary, family-based interventions aimed at reducing exposure to smoke could assess reversibility by determining whether reductions in objectively measured TSE are accompanied by parallel improvements in oxidative biomarkers and clinical outcomes. These approaches would allow for a more rigorous examination of temporality, dose–response relationships, and partial reversibility than is possible with the currently available cross-sectional evidence. Future studies should also address the issues of individual susceptibility, under-studied pediatric respiratory conditions, and passive exposure to emerging nicotine-containing

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products. Strengthening the evidence in these areas could support the development of more targeted preventive strategies and personalized treatments, while also providing a more solid scientific basis for public health policies aimed at protecting children from secondhand smoke exposure.

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Abbreviations

The following abbreviations are used in this manuscript:

TSE	tobacco smoke exposure
NOX2	NADPH oxidase 2
PGF2a	prostaglandin F2α
HDAC2	histone deacetylase-2
BAL	bronchoalveolar lavage
AM	alveolar macrophages
TIMP-1	tissue inhibitor of metalloproteinases 1
MDA	malondialdehyde
MMP9	matrix metalloproteinase 9
MPO	myeloperoxidase
NO	nitric oxide
SP-D	surfactant protein D

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