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Comparative assessment of electronic nicotine delivery systems aerosol and cigarette smoke on endothelial cell migration: The Replica Project

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Abstract

Cigarette smoking is associated with impairment of repair mechanisms necessary for vascular endothelium homeostasis. Reducing the exposure to smoke toxicants may result in the mitigation of the harmful effect on the endothelium and cardiovascular disease development. Previous investigations evaluated in vitro the effect of electronic cigarette (EC) compared with cigarette smoke demonstrating a significant reduction in human umbilical vein endothelial cells (HUVECs) migration inhibition following EC aerosol exposure. In the present study, we replicated one of these studies, evaluating the effects of cigarette smoke on endothelial cell migration compared with aerosol from EC and heated tobacco products (HTPs). We performed an in vitro

Abbreviations: AqE, aqueous extract; CRM81, CORESTA Recommended Method no. 81; EC, electronic cigarettes; ENDS, electronic nicotine delivery systems; HCI, Health Canada Intensive; HCS, high content screening; HTPs, heated tobacco products; ISO, International Organization for Standardization; THPs, tobacco heating products.

Massimo Caruso and Rosalia Emma contributed equally to this work.

circumstances shall be regarded as reflecting the positions of the Foundation for a Smoke-Free World, Inc. ECLAT srl. is a research based company that delivers solutions to global health problems with special emphasis on harm minimization and technological innovation.

scratch wound assay on endothelial cells with a multi-center approach (ring-study) to verify the robustness and reliability of the results obtained in the replicated study, also testing the effect of aerosol from two HTPs on endothelial cells. Consistently with the original study, we observed a substantial reduction of the effects of aerosol from EC and HTPs on endothelial cell migration compared with cigarette smoke. While cigarette smoke reduced endothelial wound healing ability already at low concentrations (12.5%) and in a concentration-dependent manner, EC and HTPs aerosol showed no effect on endothelial cells until 80%–100% concentrations. In conclusion, our study further confirms the importance of EC and tobacco heated products as a possible harm reduction strategy for cardiovascular diseases development in smokers.

KEYWORDS
cigarette smoke, e-cigarette, endothelial cells, ENDS, wound healing

1 | INTRODUCTION

Cigarette smoking is a risk factor for many pathological conditions, including cardiovascular diseases (CVD).[1] According to the World Health Organization,[2] more than 8 million people die each year due to the consumption of tobacco products, making smoking the leading cause of preventable deaths worldwide. Cigarette smoking is strictly related to endothelial dysfunction and structural damage to the endothelium,[3] which leads to the impairment of vascular repair mechanisms, such as the inhibition of endothelial cell migration.[4–6] The ability to maintain the endothelium integrity is one of the most critical functions of the endothelial cells. Following endothelial injury, there is an increased risk of developing vascular diseases such as atherosclerosis[7] and an utmost need to rapidly restore the endothelial continuity. Forthwith, platelets and inflammatory cells adhere to the lesion to heal the wound triggering the migration and subsequent proliferation of medial smooth muscle cells in the neointima and thus concurring to the development of occlusive vascular lesions.[8] Epidemiologic studies evidenced a strong correlation between the incidence of several atherosclerotic syndromes and cigarette smoking.[9] Moreover, the Canadian Coronary Atherosclerosis Intervention trial identified cigarette smoking as an independent predictor of new coronary lesion formation.[10] So, it is obvious that the cessation of smoking and its toxicants significantly reduces this risk.[9] Different studies investigated in vitro the detrimental effects of smoking-related harm to clarify the mechanisms and key events associated with the development of atherosclerosis, including cell migration inhibition.[11,12] Alternatively, reducing the exposure to these toxicants therefore may represent a possible strategy to reduce the harmful effect on the endothelium and, consequently, the effect of cigarette smoke on cardiovascular diseases.[9,13] Fearon et al. have shown that altering smoke toxicant yields changes endpoints in cardiovascular diseases in vitro models by examining the effects of particulate matter (PM) derived from a cigarette with blend and filter modifications to reduce the machine yields of a number of cigarette smoke toxicants. The inhibitory effects on endothelial migration of PM derived from this cigarette were significantly lower than those seen when using PM from a reference cigarette (3R4F). These data support the concept that altering cigarette smoke toxicant levels may modify biological responses to cigarette smoke.[13] In particular, a study by Taylor et al.[14] showed a significantly reduced inhibition of endothelial cell migration in vitro by electronic cigarette (EC) aerosol exposure when compared with cigarette (3R4F) smoke. EC is a non-combustible technology able to deliver nicotine to users with a lower toxicants content than smoke.[15] Similarly, tobacco heated products (THPs) heat, without combustion, an element named "HeatStick" (Heet) including a tobacco plug into a hollow acetate tube, a polymer-film filter, a cellulose-acetate mouthpiece filter, and mouth-end papers.[16] The HeatStick heated with a working temperature within 350°C provides users with an aerosol containing nicotine with an aroma similar to that of a cigarette, but with a lower content of combustion toxicants.[17] These products are generally referred to as electronic nicotine delivery systems (ENDS) and are often proposed as reduced-risk alternatives to the classic cigarette. However, the scientific debate is still open[18] and warrants further studies with particular regards to prolonged exposure to the aerosols released by ENDS.

The aim of the present study was to perform a multi-center replication study (ring study) to verify the results of Taylor and colleagues[14] on the reduced ability of EC aerosol to inhibit in vitro endothelial migration compared with cigarette smoke. In the last decade, the issue of the reproducibility and replicability crisis of the original scientific studies has emerged.[19,20] We started the "Replica Project" 3 years ago with the aim of independently replicating relevant in vitro studies in this area. This is a very complex and often difficult work,[21] and we substantiate our results by working in ring with other experienced laboratories following harmonized protocols, from the exposure of cells to the smoke/aerosols, up to the evaluation of biological parameters, such as in this case the width of the wound.

2 | METHODS

2.1 | Recruitment of laboratories

International laboratories experienced in maintaining HUVEC cultures were invited to participate in the inter-laboratory Replica study based on predefined criteria. An online questionnaire was administered to the international laboratories participating to the study that listed skills and knowledge pertaining to the core activities of this in vitro research to assess levels of proficiency in general and in relation to specific area of this research, including experience in assessments of endothelial cell migration and laboratory compliance with the Routine Analytical Cigarette-Smoking Machine — Definitions and Standard Conditions ISO3308:2012,[22] European Good Laboratory Practice, and US Environmental Protection Agency Good Laboratory Practice Standards guidelines.[23]

The selected laboratories were provided with workshops, hands-on training, and on-site assessments of laboratory capacity and personnel expertise, with follow-up by virtual sessions, if necessary. Scientists received previous formal training in smoke and aerosol exposure procedures[23] along with the standard operating procedures (SOPs) for use of smoking/vaping machines, cell-exposure systems, and scratch wound assay. Four selected laboratories in academic establishments joined this study: one from each of Italy (LAB-A; leading center), Greece (LAB-B), the United States (LAB-C), and Serbia (LAB-D).

2.2 | Harmonization process

Laboratory protocols were harmonized across study sites with SOPs defined for each experimental step and use of the same cell lines, cell-exposure equipment, and methods to assess endpoints, as suggested by the Center for Open Science transparency and openness promotion guidelines (https://www.cos.io/initiatives/top-guidelines). A kick-off meeting held by LAB-A to introduce the SOPs and personnel training was provided as previously reported.[23] The SOPs for cell exposure to cigarette smoke and ENDS aerosol Aqueous Extract (AqE), cell culture, and scratch wound assay were drawn up using the information contained in the original study[14] and manufacturers' instructions and adapted by the principal investigator sites according to laboratory capacity, equipment, and test products, ensuring they met the ISO3308:2012 guidelines.[22]

Detailed recording of technical data and deviation communication forms were collected by each laboratory partner by template spreadsheets (Microsoft Excel; version 16.43, 2011, Microsoft, Redmond, WA, USA) and shared with the leading laboratory, as previously stated.[23] To maximize assay standardization of cell growth and assessments, a list of key consumables was shared with all laboratories, and these were obtained from the same lot when possible. A SOP was distributed for thawing, freezing, and subculturing of the cell line, including testing for mycoplasma contamination with the Plasmotest™ kit (InvivoGen, San Diego, CA, USA) before freezing the cells, to allow the laboratory partners to generate their own working cell bank.

2.3 | Original study

The study from Taylor and colleagues[14] conducted a comparison study between the effects of two commercial e-cigarette (EC) products (Vype ePen and Vype eStick) and a scientific reference cigarette (3R4F) on endothelial migration in vitro. Here, we replicated the same study comparing the effects of three commercial ENDS (Vype ePen 3, Glo™ Pro, and IQOS 3 DUO) and a scientific reference cigarette (1R6F). Vype eStick has been withdrawn from the market in many countries, and the experimental 3R4F cigarette has been replaced with the 1R6F by the manufacturer (Center for Tobacco Reference Products, University of Kentucky). We measured the scratch wound area at different time points in order to quantify the migration over the time and the percentage wound area of the initial wound and the time-by-time wound area, for each test product. Moreover, comparisons among each product response were reported.

2.4 | Test products

The following products were used for this study: (1) 1R6F reference cigarette (Center for Tobacco Reference Products, University of Kentucky); (2) Vype e-Pen 3 electronic cigarette (British American Tobacco); (3) Glo™ Pro (British American Tobacco); (4) IQOS 3 Duo (Philip Morris International SA). The 1R6F cigarette has a tar yield of 29.1 mg/cigarette (Health Canada Intense [HCI] regime) and a nicotine content of 1.896 mg/cigarette. Before use, 1R6F cigarettes were conditioned for a minimum of 48 h at 22 ± 1°C and 60 ± 3% relative humidity, according to ISO 3402:1999.[24] The Vype e-Pen 3 is an electronic cigarette with a "closed-modular" system consisting of two modules: a built-in 650 mAh rechargeable lithium battery section and a replaceable "e-liquid" cartridge ("cartomizer"). The pods contain a reservoir of 2 ml of pre-filled liquid, and the "Master Blend" (18 mg/ml nicotine) flavored variant was used for the experiment. Glo™ Pro and IQOS Duo are tobacco heating products (THPs). The THPs are devices that heat tobacco to generate a nicotine-containing aerosol with a tobacco taste inhaled by users. Glo™ Pro device was used with "Ultramarine" Neostick, instead IQOS device was used with "Sienna Selection" Heatsticks. Each of these products, the Neosticks and the Heatsticks, can be called heated tobacco products (HTPs), which are small cigarettes specifically prepared to be heated and not burned. All devices have been fully charged and checked before use and cleaned before and after use.

2.5 | Preparation of aqueous aerosol extracts (AqE)

All the regimes used in this study were described in Table 1. Whole smoke from 1R6F cigarette was generated on a LM1 smoking machine (Borgwaldt KC GmbH, Hamburg, Germany). 1R6F cigarette was smoked for nine puffs, following the HCI puffing regime (55 ml, 2 s duration bell shape profile, puff every 30 s with filter vent

TABLE 1 Puffing regime description for each product

Product	Puffing regime	Puff volume (ml)	Puff frequency (s)	Puff duration (s)	Puff profile	Vent blocking	Pre-activation (s)
1R6F	HCI	55	30	2	Bell	100%	NA
Vype ePen3	CRM81	55	30	3	Square	NA	0
IQOS Duo	HCI	55	30	2	Bell	0%	30
Glo™ Pro	HCI	55	30	2	Bell	0%	20

Abbreviations: CRM81, Coresta Recommended Method no. 81; HCI, Health Canada Intense; NA, not applicable.

TABLE 2 AqE exposure concentration range for each test product

1R6F cigarette AqE (%)	Electronic cigarette (ePen3) AqE (%)	THPs (IQOS duo and Glo™ pro) AqE (%)
30	100	100
25	90	90
20	80	80
15	70	70
12.5	60	60
10	50	50
5	40	40

blocked). Vype e-Pen 3 and the THPs were machine-puffed on a LM4E vaping machine (Borgwaldt KC GmbH, Hamburg, Germany). Vype ePen 3 was vaped for 10 puffs following the CORESTA Reference Method 81 (CRM81) regime (55 ml puff volume, drawn over 3 s, once every 30 s with square shape profile), accredited into ISO 20768:2018.[25] IQOS 3 Duo and GLO™ Pro were vaped using the Health Canada Intense (HCI) regime, accredited under ISO/TR 19478-2:2015,[26] without blocking the filter vents, for 8 (1 Neostick) and 12 (1 Heatstick) puffs, respectively. The AqEs from 1R6F cigarette smoke, EC, and THP aerosol were generated by bubbling through 20 ml of AqE capture media (VascuLife® media with added supplements and 0.1% of FBS). This procedure provided the AqE 100% stocks, which were diluted with appropriate volumes of VascuLife® media to produce the AqE concentrations for in vitro exposures. A range of concentration from 5 to 30% was used for the 1R6F cigarette. Instead, a range from 40 to 100% was used to test Vype ePen 3, IQOS 3 Duo, and Glo™ Pro (Table 2).

2.6 | Nicotine dosimetry

Nicotine dosimetry was carried out only by LAB-A on 100% diluted AqEs samples, collected after exposure for each product. A blank sample and six calibration standards, prepared in the same matrix at concentrations between 1 and 50 μg/ml (1, 2, 5, 10, 20, and 50 μg/ml), were analyzed too. Aliquots of 0.1 ml of each sample and each calibration standards were transferred to vial with a 250 μl conical insert, added with nicotine-(methyl-d_3) solution—used as internal standard at 10 μg/ml—and 0.1 ml of acetonitrile. Nicotine was determined by UPLC-ESI-TQD (Waters Acquity) with an Acquity UPLC® HSS T3 1.8 μm – 2.1 × 100 mm column, operating in Multiple Reaction Monitoring (MRM) and positive ion mode. Ion transitions for nicotine and nicotine-(methyl-d_3) are reported in Table 3. Isocratic elution (80% water and 20% acetonitrile, both added at 0.1% with formic acid) was performed. The mass spectrometry settings were as follows: capillary energy at 2.5 kV, source temperature at 150°C, column temperature at 40°C, desolvation temperature at 500°C, desolvation gas at 1000 L/h, and cone gas at 100 L/h. The injection volume was 1 μl. To calculate nicotine concentrations of each AqE dilution, a linear proportion was applied.

2.7 | Endothelial cell culture

Normal human umbilical vein endothelial cells (HUVECs; Lifeline Cell Technology, California, USA) were cultured as described by Taylor and colleagues.[14] Briefly, HUVECs have grown at 37°C and 5% CO_2 in complete VascuLife VEGF Medium (Lifeline Cell Technology, California, USA), containing vascular endothelial growth factor (5 ng/ml), epidermal growth factor (5 ng/ml), basic fibroblast growth factor (5 ng/ml), insulin-like growth factor 1 (15 ng/ml), ascorbic acid (50 μg/ml), L-glutamine (10 mM), hydrocortisone hemisuccinate (1 μg/ml), heparin sulfate (0.75 units/ml), fetal bovine serum (FBS) (2%), penicillin (10,000 units/ml), streptomycin (10,000 μg/ml), and amphotericin B (25 μg/ml). When the cells reached confluence, they were detached with trypsin–EDTA solution (0.05%) and replated in new flasks or into 24-well plates and used in experiments. Cultured HUVECs maintain their normal appearance for 15 population doublings.[27,28] Then, we discarded HUVECs after four passage cycles. The cells were received by the vendor in a frozen vial, and we considered the thawed cells to be "step 1."[29]

2.8 | Endothelial cell scratch wound assay

HUVECs were seeded in 24-well plates at a density of 2×10^5 cells/well in complete VascuLife VEGF Medium and incubated at 37°C and 5% CO_2 until they reached the total confluency (24–48 h prior to performing the assay). When the HUVECs were ready to perform the scratch wound assay, the complete VascuLife VEGF Medium was replaced with AqE capture media (VascuLife® media containing 0.1%

TABLE 3 MRM mode: Ion transitions (m/z) and relative cone and collision voltages

Analyte	MRM (m/z)	Cone (volts)	Collision energy (eV)
Nicotine	163.0 → 117.0	40	25
	163.0 → 132.0	40	15
Nicotine-(methyl-d_3)	165.8 → 116.8	40	20
	165.8 → 129.7	40	20

FBS), and then cells were incubated for 6 h. Linear scratch wounds were created manually by using sterile 10 μl pipette tips. Immediately after wounding, the medium with detached cells was removed, and a washing step with PBS was performed. Next, cells were exposed with 1 ml of each test product AqE in triplicate. A negative control with not exposed AqE capture media and a positive control with cytochalasin D (2 μM) were entered for each plate. One laboratory (LAB-A) used the Operetta CLS™ high-content analysis system to read the experimental plates by using a ×5 objective to acquire the images. Instead, the other laboratories (LAB-B, LAB-C, and LAB-D) incubated the plates into an incubator at 37°C and 5% CO_2. Then the cells were taken out of the incubator at established time-points ranging from 0 to 48 h (T0–T48) and placed under a microscope with ×5 objective. Two pictures per well of fixed positions in the wounds were taken with a digital camera mounted on the microscope.[30]

2.9 | Statistical analysis

The scratch wound area (μm^2) was measured at each time point using the open-source software imageJ/Fiji®. In order to quantify the migration over the time, the percentage wound area of the initial wound area was calculated by the following formula: A (Tn)% = A (Tn)/A(T0) *100, where A (Tn) is the wound area at time Tn and A(T0) is its initial area. Data distribution was assessed with the Shapiro–Wilk test. Pearson's correlation (symmetric data) and Spearman's Rank correlation (skewed data) analyses were performed to assess the relationship of scratch data provided from each laboratory. Bland–Altman plotting was performed for the laboratory difference evaluation and to describe the level of agreement between laboratories results. Comparisons among the tested concentrations were analyzed by fitting a repeated measure mixed model followed by Dunnett's test to perform multiple comparisons with the untreated cellular response. Moreover, comparisons among each product response were analyzed by fitting a repeated measure mixed model followed by Tukey's test adjustment for multiple comparisons. Data were expressed as mean ± standard error (SE). All analyses were considered significant with a p value of less than 5%. R version 3.4.3 (2017-11-30) was used for correlations analyses and generation of Bland–Altman plots, whereas GraphPad Prism version 8 (GraphPad Software, San Diego, California, USA, www.graphpad.com) was used to perform comparisons among the tested concentrations.

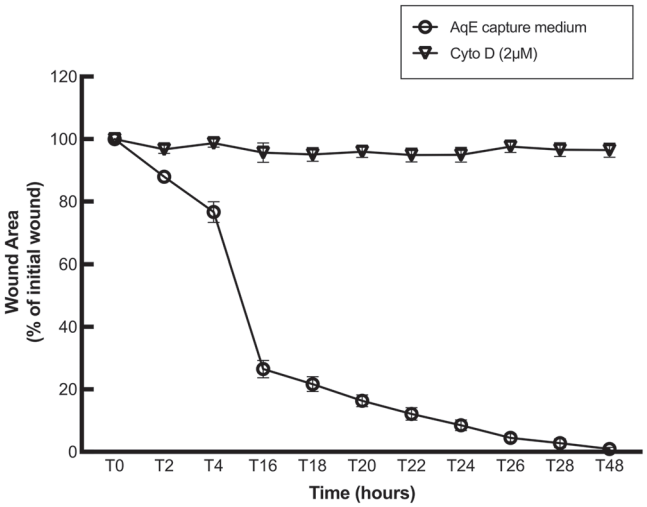

FIGURE 1 The incubation of HUVECs with aqueous extract (AqE) capture media allowed the closure of the wound with a time-dependent reduction of wound area over 48 h. Cytochalasin D (Cyto D - 2 μM) inhibited HUVECs migration. Data were shown as mean ± standard error (SE) of wound area (μm^2) percentage from duplicate wells of three independent experiments.

3 | RESULTS

3.1 | Analyzed data and their reproducibility among laboratories

Not all the images produced during the scratch assay were suitable for the analysis. Particularly, some 1R6F data (5%, 12.5%, 20%, 30%, and AqE capture medium), some ePen data (50%, 90%, and AqE capture medium), and all IQOS and Glo data from LAB-D were excluded from the analyses due to bad quality of images. Also, ePen data at 100% from LAB-B were excluded for bad quality of images. Correlation and Bland–Altman results were reported in the supporting information (Figures S1–S36).

3.2 | Preparation of extracts (AqE) and HUVEC migration baseline

Analysis on 100% diluted AqE samples showed nicotine concentrations of 12.8 μg/ml for 1R6F, 4.2 μg/ml for Vype ePen, 8.4 μg/ml for

IQOS, and 4.5 μg/ml for Glo, respectively. The calculated concentrations of nicotine for each tested AqE dilution were reported in Table S4 (in supporting information).

For each scratch wound assay, a negative control with AqE capture media and a positive control with cytochalasin D (2 μM) were evaluated to determine the baseline and the maximal inhibition of HUVEC migration, respectively. The untreated HUVECs incubated with AqE capture media showed a time-dependent closure of wound area (Figure 1). We observed a mean wound area percentage under 20% starting from T-20 (A(T20)% = 16.33 ± 1.85%) until a complete wound closure at T-48 (A(T48)% = 0.91 ± 0.5%). However, the incubation with cytochalasin D totally inhibited the closure of the wound during the 48 h exposure period (Figure 1). Representative images of wound healing after AqE capture medium and cytochalasin D treatments were shown in Figure 2.

3.3 | HUVEC migration after exposure to cigarette smoke, EC, and THPs AqE

Cigarette (1R6F) smoke AqE on HUVEC migration was assessed across concentrations ranging from 5 to 30% 1R6F AqE, which represent exposure to 0.64–3.84 μg/ml of nicotine. Exposure to 1R6F AqEs inhibited HUVEC migration in a concentration-dependent manner, confirming findings by Taylor and colleagues.[14] In particular, we observed a significant inhibition of wound area closure for concentrations ranging from 12.5% to 30% compared with AqE capture media treatment ($p < 0.05$). Only the 5% 1R6F AqE allows a complete wound closure at 48 h. Complete inhibition of endothelial migration was observed when the cells were exposed to 25% and 30% 1R6F AqE (Figure 3).

Vype ePen 3 AqE wound repair effect was assessed with a range from 40 to 100%. These AqE concentrations contained a 1.68–

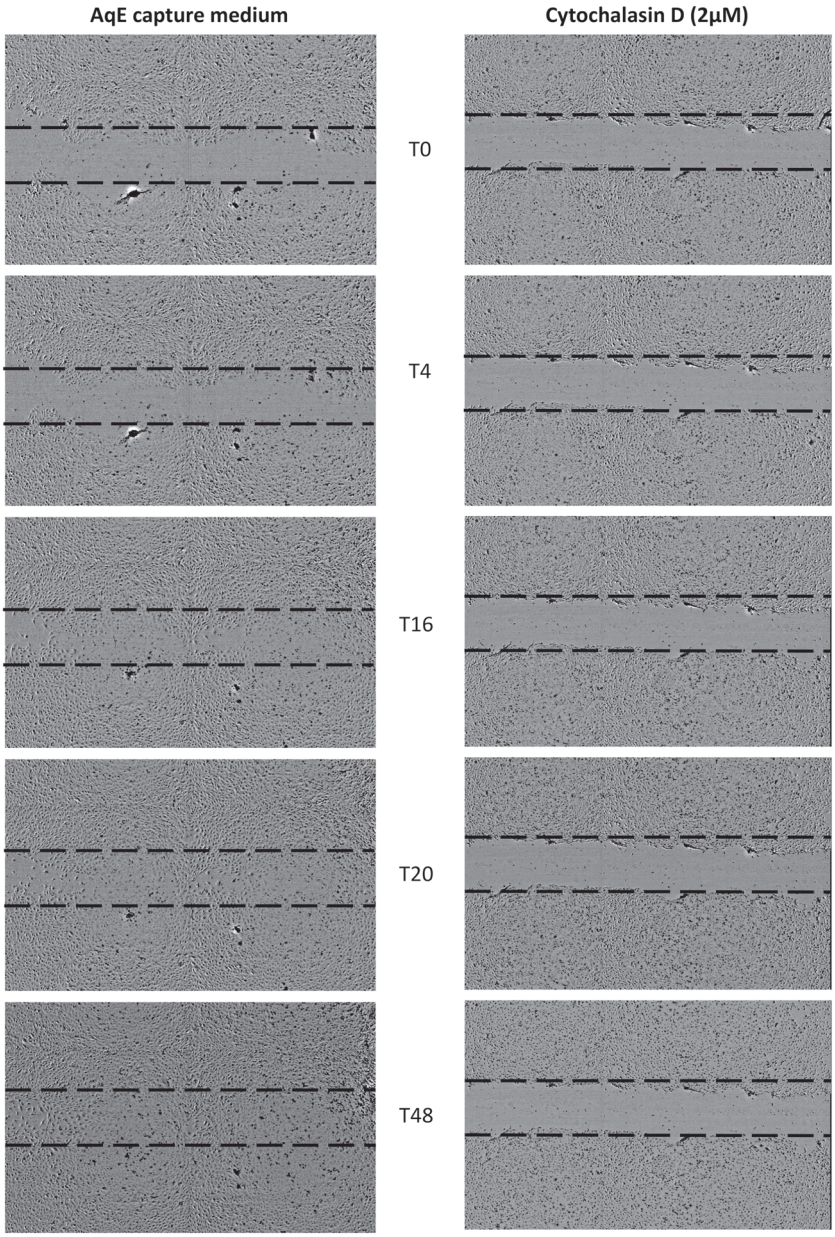

FIGURE 2 Representative images of wound healing in the presence of aqueous extract (AqE) capture medium and Cytochalasin D at time 0 h, 4 h, 16 h, 20 h, and 48 h (T0, T4, T16, T20, and T48)

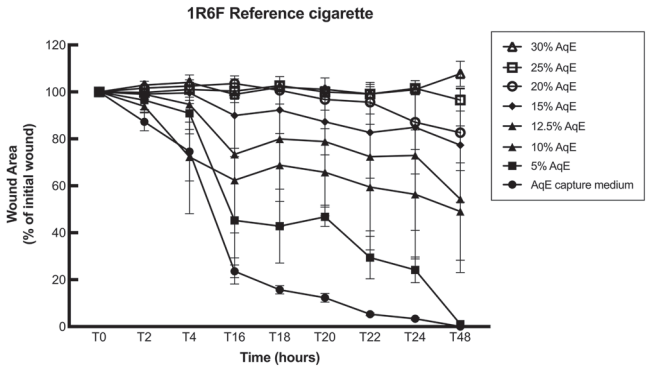

FIGURE 3 The HUVEC wound healing was inhibited by 1R6F aqueous extract (AqE) in a concentration dependent manner. Data from 0 h (T0) to 48 h (T48) are reported as mean ± standard error (SE) of wound area (μm^2) percentage from triplicate wells of four independent experiments.

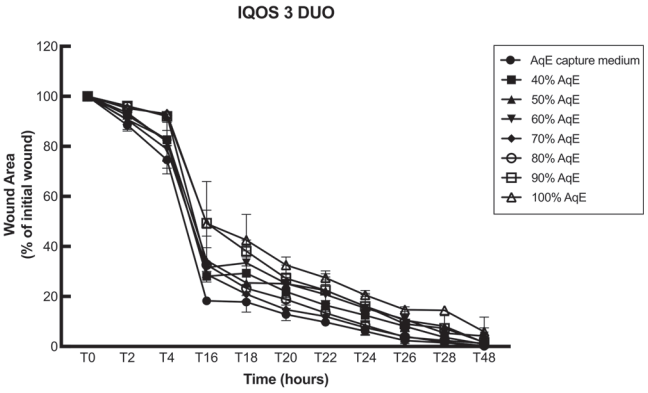

FIGURE 5 IQOS duo aqueous extract (AqE) effect on HUVEC migration. Data from 0 h (T0) to 48 h (T48) are reported as mean wound widths as mean ± standard error (SE) of wound area (μm^2) percentage from triplicate wells of three independent experiments.

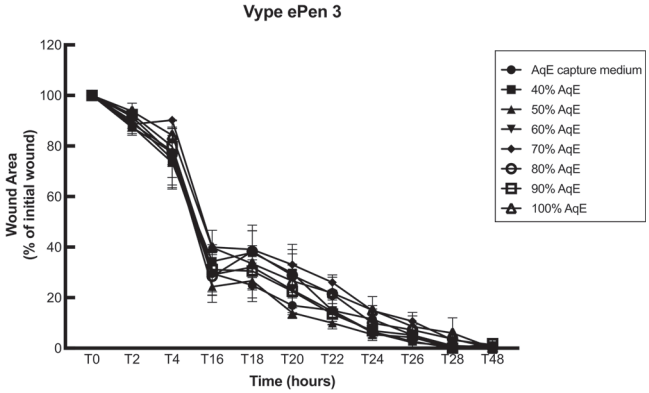

FIGURE 4 Vype ePen3 aqueous extract (AqE) did not inhibit HUVEC migration. Data from 0 h (T0) to 48 h (T48) are reported as mean wound widths as mean ± standard error (SE) of wound area (μm^2) percentage from triplicate wells of four independent experiments.

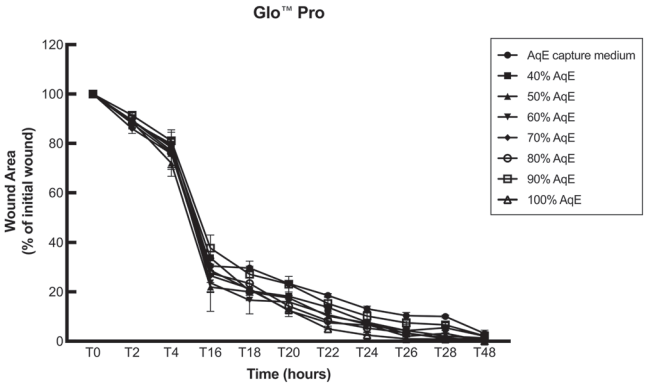

FIGURE 6 Glo™ pro aqueous extract (AqE) effect on HUVEC migration. Data from 0 h (T0) to 48 h (T48) are reported as mean wound widths as mean ± standard error (SE) of wound area (μm^2) percentage from triplicate wells of three independent experiments.

4.2 μg/ml nicotine (Table S4; supporting information). All the Vype ePen 3 AqE concentrations did not produce any significant reduction of wound area closure compared with AqE capture media treatment (Figure 4).

Both THPs AqEs (IQOS Duo and Glo™ Pro) were tested with concentration ranging from 40 to 100%. This AqE concentration range contained 3.36–8.4 μg/ml nicotine for the IQOS Duo and 1.8–4.5 μg/ml nicotine for the Glo™ Pro. IQOS Duo AqEs with concentrations ranging from 40% to 80% did not affect the HUVEC migration compared with AqE capture media ($p > 0.05$). Instead, significant differences were observed for 90% ($p = 0.009$) and 100% ($p = 0.002$) IQOS Duo AqEs when compared with AqE capture media (Figure 5), despite the complete closure of wound at T48. All the Glo™ Pro AqE concentrations did not reduce the HUVEC migration compared with AqE capture media (Figure 6).

3.4 | Comparison among tobacco cigarette, EC, and THPs exposures

A comparison of HUVEC migration was made among all the tested products at the higher concentration (Figure 7). Significant differences were observed for Vype ePen 3 100%, IQOS Duo 100%, and Glo™ Pro 100% AqEs compared with 1R6F 30% AqE ($p < 0.0001$). Moreover, a slight significant difference was shown between IQOS Duo 100% and Glo™ Pro 100% AqEs ($p = 0.039$). No differences were observed between Vype ePen 3 100% and THPs 100% AqEs ($p > 0.05$). Even comparing the migration of endothelial cells between all alternative products with equal or greater amounts of nicotine released (Figure 8) by 1R6F cigarette at higher concentration (3.84 μg/ml), we observed significant differences. Finally, comparison of the migration of endothelial cells exposed to the maximal concentration of each product AqE (Figure 6) highlighted a significant difference between ENDS and 1R6F cigarette AqE.

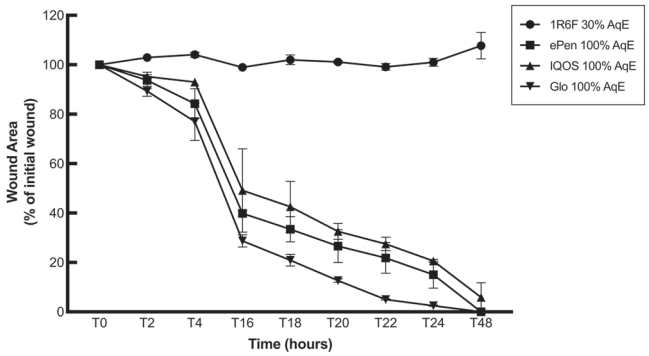

FIGURE 7 Comparisons among all the test products at the higher concentrations. Data from 0 h (T0) to 48 h (T48) are reported as mean wound widths as mean ± standard error (SE) of wound area (μm^2) percentage from triplicate wells of four independent experiments.

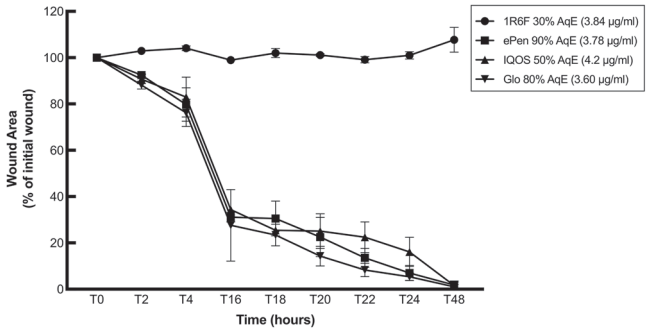

FIGURE 8 Comparisons among all the test products at similar nicotine concentrations. Data from 0 h (T0) to 48 h (T48) are reported as mean wound widths as mean ± standard error (SE) of wound area (μm^2) percentage from triplicate wells of four independent experiments.

4 | DISCUSSION

The detrimental effects of tobacco smoking to the cardiovascular system are well established,[1] although the exact molecular mechanisms are yet to be fully defined. Taylor and colleagues[14] investigated the ability of both cigarette smoke and EC aerosol to affect vascular endothelial cells wound healing, one of the key processes in atherosclerotic disease initiation and progression in the cardiovascular system. The authors used an in vitro model of endothelial cells (HUVECs) exposed to AqE, the water-soluble fraction of tobacco smoke or EC aerosols, to resemble the in vivo exposure of endothelial cells to toxicants. The results from Taylor and colleagues demonstrated that cigarette smoke AqE resulted in a concentration-dependent inhibitory effect on endothelial cell migration, but no significant inhibition of endothelial cell migration following exposure to EC AqEs was observed. Furthermore, they hypothesized that chemical species present in the smoke AqE were responsible for the inhibition of endothelial cell migration and that these chemicals are absent, or present in insufficient concentrations, in the EC AqEs to elicit any significant response in the wound healing assay. In the present study, we replicated the paper by Taylor and colleagues in a multicenter study. The choice of a multicenter approach was used to verify the robustness and reliability of the results obtained in the original study. However, some methodological issues need to be clarified to fully explain our results. In particular, we used an updated version of the EC device compared with that used in the original study, the Vype ePen 3 in place of the Vype ePen device, and we included two THPs, IQOS 3 Duo and Glo™ Pro. Additionally, the tobacco cigarette used in our study (1R6F) was different from that used in the original study (3R4F) since this has been out of production in recent years and the manufacturer recommends the 1R6F as a replacement for the 3R4F.

The results on the ability of endothelial cells to heal the wound following exposure to cigarette smoke and EC aerosol from the four laboratories involved in this ring-study confirmed the results reported in the original study, despite the difference in the method used to assess the size of the wound at each time-point. However, Correlations and Bland–Altman plots demonstrated significant variability among 1R6F scratch results among laboratories. Instead, good reproducibility was reported for scratch results of ePen, IQOS, and Glo exposures. In our previous study from Replica Project,[23] we observed the greatest interlaboratory variability for the 1R6F exposures, indicating an operator-dependent effect. Another possible gap that influenced the 1R6F results is the possible loss of cell viability when cells are out of the incubator for images caption for a long time. Indeed, Taylor and colleagues used the IncuCyte live-cell imaging system to measure wound width as the measurement parameter for wound healing at each time-point and for each treatment. In our multicenter study, one laboratory used an automated live-cell imaging system to acquire the scratch images, whereas the other three participating laboratories obtained scratch imaging manually with a digital camera mounted on the microscope. This step could be negatively influenced the 1R6F results due to the sum of smoke toxicity plus the cell distress outside the incubator. Despite Taylor and colleagues, all the centers of the Replica Project analyzed each image manually, using the same software to measure the wound area. Furthermore, we found a higher nicotine concentration in 1R6F AqE compared with that reported by Taylor and colleagues.[14] This difference could be due to the different smoking machines used in our study. Despite the observed variability and the difference in nicotine concentrations in cigarette AqEs, the exposure concentration range for the EC AqE included an equivalent nicotine dose to the 30% 1R6F AqE. As reported in the previous paper from Taylor et al.,[14] we observed no significant variations in endothelial migration rates following exposure to different concentrations of Vype ePen 3 AqE, differently from what observed with the1R6F smoke AqEs, which showed a consistent concentration-dependent effect. The different migration response of endothelial cells is significant only for the cigarette AqE, thus supporting the results by Taylor and colleagues,[14] namely, that the inhibitory effect on cell migration and wound repair must be exerted by other chemicals contained in the smoke and absent, or scarcely present, in the ENDS aerosol, and not by nicotine. Cigarette smoke contains free radicals and others chemical species able to induce cellular free radical generation by cellular enzyme systems, concurrently decreasing the

antioxidant protection. It is well known that ROS may regulate endothelial migration[31] promoting the initial processes of the atherogenesis. AqE can enhance the activity of NADPH oxidase[32] and modulate the mitochondrial function[33] elevating ROS production in cultured endothelial cells. Although the hypothesis that increased oxidative stress affects endothelial dysfunction seems to be one of the most shared, Fearon and colleagues in their aforementioned study[13] seem to exclude that this is the main reason that affects the wound repair capacity of the endothelium, suggesting a main role for other toxicants contained in cigarette smoke. In a prospective, randomized control trial with a parallel nonrandomized preference cohort and blinded endpoint conducted on smokers ≥18 years of age who had smoked ≥15 cigarettes/day for ≥2 years, George and colleagues observed that tobacco smokers switching to EC showed a significant improvement in endothelial function within 1 month by measuring the flow-mediated dilation (FMD) and vascular stiffness, as predictors of atherosclerosis and cardiovascular risk.[34] On the other hand, different studies have demonstrated that nicotine may induce migration and proliferation of vascular cells by binding to specific nicotinic acetylcholine receptors[35,36] and by increasing proangiogenic VEGF.[37] However, nicotine use appears not to accelerate atherogenesis but may contribute to acute cardiovascular events in the presence of cardiovascular disease.[38] Another conceivable hypothesis on the detrimental effects of smoke on migration and proliferation of endothelial cells could be the high quantity of oxidative species present in cigarette smoke. These chemicals induce endothelial dysfunction through oxidative damage to membrane constituents, mitochondria and DNA[39] inducing both apoptosis and necrosis,[40] as a result from oxidative damage to mitochondria or DNA. Differently from smoke, aerosol from EC contains a substantially reduced number and quantity of such chemical species[41,42] suggesting a possible explanation for EC aerosol reduced endothelial cell toxicity. The hypothesis on the involvement of oxidative stress as a key phenomenon underlying the effect of smoking on endothelial function is still to be confirmed. Fearon et al.[11] found that reducing the amount of toxicants in the smoke, but not the oxidative species, resulted in a reduced ability to interfere with migratory capacity of endothelial cells. These findings could highlight the hypothesis that other constituents of cigarette smoke may be responsible for the impairment to endothelial cells functionality.

We also studied vascular wound repair following the aerosol exposure of two commercially available THPs, IQOS 3 duo and Glo™ Pro. These set of experiments showed no significant variation in wound healing for all the tested concentrations of Glo AqE. However, we only observed a slight inhibitory effect in wound closure for the 90% and 100% IQOS AqEs compared with media control, however significantly less than that observed with the 30% dilution of 1R6F AqE. Surprisingly, there have been few in vitro studies on aerosol from THPs and its effect on vascular endothelial wound repair. To this regard, in 2017, Breheny and colleagues[43] performed an in vitro screening of a commercial and prototype THPs, showing an inhibitory effect on HUVEC wound healing for the AqE from the prototype THP at higher concentrations. Interestingly, this effect was not as evident as the inhibition observed with the AqE from cigarette. In another study by British American Tobacco, the AqE from THP1.0 (GLO™ heating device with KENT Neosticks) did not affect HUVEC wound closure. Consistently, the highest concentration of 100%, the relative density of wound closure has a similar trend to media control.[44] Finally, in 2015, van der Toorn and colleagues showed that AqE from Tobacco Heating System 2.2 (THS2.2, commercialized under the brand name IQOS®) affect the integrity of human coronary artery endothelial cell (HCAEC) monolayer but in a reduced manner compared with AqE from cigarettes.[45]

5 | CONCLUSIONS

In conclusion, our data confirmed the results from Taylor and colleagues[14] showing that AqEs from aerosol of a commercially available EC (Vype ePen 3) does not induce the inhibition of endothelial cell migration in vitro as compared with cigarette smoke AqE. We additionally demonstrated a product-specific response on HUVEC migration for THP aerosol. Particularly, it appears that aerosol from IQOS Heatsticks possesses the potential to induce adverse cellular effects on the cardiovascular system, however such effects are much less pronounced than those observed with cigarette smoke. Our results provide useful scientific information in support of the decision-making process of regulating these products in order to develop evidence-based harm reduction strategies and policy decisions by governments.

CONFLICT OF INTEREST

Riccardo Polosa is full tenured professor of Internal Medicine at the University of Catania (Italy) and Medical Director of the Institute for Internal Medicine and Clinical Immunology at the same University. In relation to his recent work in the area of respiratory diseases, clinical immunology, and tobacco control, Riccardo Polosa has received lecture fees and research funding from Pfizer, GlaxoSmithKline, CV Therapeutics, NeuroSearch A/S, Sandoz, MSD, Boehringer Ingelheim, Novartis, Duska Therapeutics, and Forest Laboratories. Lecture fees from a number of European EC industry and trade associations (including FIVAPE in France and FIESEL in Italy) were directly donated to vaper advocacy non-profit organisations. Riccardo Polosa has also received grants from European Commission initiatives (U-BIOPRED and AIRPROM) and from the Integral Rheumatology & Immunology Specialists Network (IRIS) initiative. He has also served as a consultant for Pfizer, Global Health Alliance for treatment of tobacco dependence, CV Therapeutics, Boehringer Ingelheim, Novartis, Duska Therapeutics, ECITA (Electronic Cigarette Industry Trade Association, in the UK), Arbi Group Srl., Health Diplomats, and Sermo Inc. Riccardo Polosa has served on the Medical and Scientific Advisory Board of Cordex Pharma, Inc., CV Therapeutics, Duska Therapeutics Inc, Pfizer, and PharmaCielo. RP is also founder of the Center for Tobacco prevention and treatment (CPCT) at the University of Catania and of the Center of Excellence for the acceleration of Harm Reduction (CoEHAR) at the same University, which has received support from Foundation for a Smoke Free World to conduct eight independent investigator-initiated research projects on harm reduction. Riccardo

Polosa is currently involved in a patent application concerning an app tracker for smoking behavior developed for ECLAT Srl. RP is also currently involved in the following pro bono activities: scientific advisor for LIAF, Lega Italiana Anti Fumo (Italian acronym for Italian Anti-Smoking League), the Consumer Advocates for Smoke-free Alternatives (CASAA) and the International Network of Nicotine Consumers Organizations (INNCO); Chair of the European Technical Committee for standardization on "Requirements and test methods for emissions of electronic cigarettes" (CEN/TC 437; WG4). Konstantinos Poulas has received service grants and research funding from a number of Vaping Companies. He is the Head of the Institute of Research and Innovations, which has received a grant from the Foundation for a Smoke Free World. Giovanni Li Volti is currently elected Director of the Center of Excellence for the acceleration of HArm Reduction. The other authors have no relevant financial interests to disclose.

AUTHOR CONTRIBUTIONS

Conceptualization, M.C. and G.L.V.; methodology, A.D., R.E., and P.Z.; software, R.E.; validation, M.C., G.L.V., and M.F.; formal analysis, R.E. and P.Z.; investigation, M.C., A.D., K.M., S.B., A.A., G.K., R.Pu., A.I., A.C., and P.Z.; data curation, R.E. and A.D.; project administration, S.R.; writing – original draft preparation, M.C., A.D., and R.E.; writing – review and editing, M.C., K.P., A.G., V.V., S.B., M.F., and G.L.V.; supervision, G.L.V., M.C., and R.Po. All authors have read and agreed to the published version of the manuscript.

DATA AVAILABILITY STATEMENT

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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