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Effect of electronic cigarette aerosols on cisplatin resistance in head and neck cancer cells: a collaborative replication study

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Abstract

Background Cisplatin chemoresistance is a critical challenge in treating head and neck squamous cell carcinoma (HNSCC). Previous research from Manyanga et al. (2021) suggested that e-cigarette (e-cig) aerosol, including formulations with and without nicotine, might increase cisplatin resistance in oral cancer cells. This multicenter replication study aimed to validate the findings and evaluate the oncologic impacts of e-cigarette use during chemotherapy.

Methods This in vitro study utilized standardized and harmonized protocols across international laboratories to examine the effects of cigarette smoke (1R6F) and e-cig aerosols with different concentrations of nicotine (0, 12, and 20 mg/ml nicotine) on cisplatin sensitivity in HNSCC cell lines (SCC-25, FaDu, and UM-SCC-1). Aqueous extracts from 1R6F smoke and e-cig vapor were collected using a smoking and a vaping machine, respectively, following ISO20778:2018 and ISO20768:2018 puffing regimes. The smoke and vapor were collected in PBS and diluted to 10 puffs/5L for HNSCC cell treatment. Chemosensitivity, clonogenicity, DNA repair gene expression related to cisplatin-induced damage, and gene/protein expression of cisplatin transporters, were assessed by MTS, NRU, trypan blue exclusion, qRT-PCR, and Western blot assays, respectively.

Results Contrary to previous findings, exposure to e-cig aerosols did not significantly modulate cisplatin sensitivity in all cell lines. IC50 values, cytotoxicity assays, and clonogenic survival rates remained similar between cells treated with e-cig aerosols and those exposed to cisplatin alone. Analysis of gene and protein expression revealed occasional variations in the levels of cisplatin transporters and DNA repair mechanisms, but these changes were inconsistent.

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Conclusions This study did not fully substantiate previous claims that aerosols generated from e-cigarette, with and without nicotine, increase cisplatin resistance. The variability in gene and protein expression among different cell lines underscores the need for cautious interpretation and further investigation of the role of e-cigarette components in cancer treatment. These findings provide a critical perspective for shaping public health policies and clinical practices regarding e-cigarette use during chemotherapy.

Keywords Cisplatin resistance, Nicotine, Electronic cigarette, Chemosensitivity, Head and neck squamous cell carcinoma (HNSCC)

Background

Cisplatin-based chemotherapy remains a cornerstone in the treatment of head and neck squamous cell carcinoma (HNSCC) [1], a prevalent malignancy characterized by poor survival rates due to late-stage diagnosis and therapy resistance. Since cisplatin was introduced as a treatment for head and neck cancer in the 1970 s, it has remained a crucial component of therapy. Now, it is linked with other kinds of treatments that make it more specific and stronger, while also boosting its healing ability and success rate [2]. Yet, various environmental factors and genes contribute to develop resistance to cisplatin. This risk harms how well the treatment works and makes the problems linked with HNSCC worse [3].

Using tobacco is still the top environmental risk factor for head and neck cancer, making up around 75% of all cases in a study from Western Europe [4]. Smoking after a cancer diagnosis is associated with reduced treatment efficacy, higher recurrence risk, and impaired quality of life [5, 6]. Independent of tumor location and stage, continued smoking has been shown to negatively impact survival outcomes in cancer patients [7, 8]. Multiple studies have linked continued tobacco use in cancer patients with increased treatment-related toxicity, greater risk of recurrence, and reduced overall survival [5, 8–10]. Since it is difficult for these people to quit smoking, using an alternative plan may make the therapy work better and increase the chances of recovery. An alternative available for about a decade is the electronic cigarette, which gives smokers the possibility of continuing to take nicotine from a cleaner source, i.e. from an aerosol, without having to take in the by-products of tobacco combustion. Several reports have discussed the growing use of e-cigarettes among cancer patients seeking alternatives to smoking, yet the long-term oncologic effects of e-cig exposure remain uncertain [11, 12].

The usage of electronic cigarettes, or "e-cigarettes" (e-cig), which are promoted as a safer alternative to traditional smoking, has increased exponentially in recent years due to changes in nicotine use habits. However, the safety of e-cigarettes, particularly in the context of cancer treatment, remains under scrutiny. The possibility that e-cigarette aerosols could alter the effectiveness of chemotherapy, especially by enhancing cisplatin resistance in oral cancer cells, has been questioned by preliminary research, including the groundbreaking study conducted by Manyanga and colleagues [13].

The study by Manyanga et al. [13] provided critical insights into how e-cigarette exposure alters the expression of cisplatin transporters, thereby increasing drug resistance. Mechanisms implicated in this resistance include changes in drug influx and efflux transporters, independent of nicotine content [14]. These findings underscore the urgent need for evidence-based evaluations of e-cigarette use, particularly among cancer patients undergoing chemotherapy.

Since the spotlight was turned on the replicability crisis about a decade ago, many studies have been organized to validate previously reported findings [15]. This collaborative replication study aimed to verify findings from Manyanga et al. under harmonized and standardized conditions, addressing previously reported limitations such as non-standardized exposure systems, to enhance data reproducibility and comparability [16–18]. By confirming or challenging these results, our work seeks to contribute to the growing body of literature assessing the oncological implications of e-cigarette use, with the ultimate goal of informing clinical practices and public health policies.

Material and methods

Study design and harmonization process

This research work represents an in vitro interlaboratory study conducted as part of the new phase of the REPLICA 2.0 project. The collaborative network for this initiative involves laboratories based in Italy (LAB-A), which serves as the coordinating center, as well as in the United States (LAB-B), Indonesia (LAB-C) and Serbia (LAB-D). In line with the guidelines for promoting transparency and openness (TOP) proposed by the Center for Open Science (https://www.cos.io/initiatives/top-guidelines), experimental protocols were harmonized among all laboratories through the development of standardized operating procedures (SOPs) for each experimental phase. To ensure consistency, all participating centers used the same cell line, exposure systems and analytical methods for endpoint assessment. LAB-A organized a harmonization meeting aimed at training the international teams and unifying the SOPs. These procedures were designed to closely mirror the original study protocols, with the

exception of the aerosol generation method. In particular, non-standardized exposure conditions, which had been reported as a limitation in Manyanga et al. [13], were addressed using standardized smoking and vaping machines (LM1, LM4E/LM5E; Korber—Hamburg, Germany). Another difference from the original work is the replacement of two cell lines, WSU-HN6 (tongue) and WSU-HN30 (pharynx), as they were not commercially available. These two cell lines were replaced with SCC-25 (tongue) and FaDu (pharynx), respectively. While these cell lines represent similar anatomical sites, we acknowledge that differences in genetic background, growth characteristics, and treatment sensitivity could influence the experimental outcomes. LAB-A, LAB-B, and LAB-C conducted experiments using all the three cell lines. Whereas LAB-D conducted the experiments using only the SCC-25 cell line. We also performed the MTS assay instead of the MTT analog and added cell viability assessment by Neutral Red Uptake (NRU) assay. Protein expression assessment by in-cell western was also added, which was conducted only by LAB-C. All experiments were performed in two independent experimental sessions, with technical triplicates for each condition to ensure reproducibility and accuracy, except for the experiments conducted by LAB-A on UM-SCC-1, which were conducted in triplicate for one experimental session due to delays in cell line acquisition. The in-cell western assay by LAB-C was conducted in quadruplicate for one experimental session.

Cell culture

Following the indicated cell lines utilized by Manyanga et al. [13], human epithelial cancer cell lines from different head and neck regions were chosen for this project. SCC-25 cells (tongue; ATCC: CRL-1628) were cultured in Dulbecco's modified Eagle's Medium (DMEM): F-12 (ATCC: 30–2006) supplemented with 10% fetal bovine serum (FBS), 1% Penicillin/Streptomycin, and 400 ng/mL hydrocortisone. FaDu cells (pharyngeal; ATCC: HTB-43) were cultured in Eagle's Minimum Essential Medium (EMEM) (ATCC: 30–2003) supplemented with 10% FBS, and 1% Penicillin/Streptomycin. UM-SCC-1 cells (floor of mouth; Millipore: SCC070) were cultured in DMEM with 4500 mg/L glucose (Millipore: SLM-021-B) supplemented with 10% FBS, 1% Penicillin/Streptomycin, 1% Non-Essential Amino Acids (Millipore: TMS-001-C), and 1% L-Glutamine (Millipore: G7513-100ML). All the cell cultures were maintained in a humidified atmosphere at 37 °C and 5% CO_2. The media used to prepare all the experimental conditions were supplemented with HEPES buffer (20 mM) to prevent pH variation.

Test products for smoke and aerosol generation

Smoke and aerosol were generated using a standardized experimental tobacco reference cigarette, 1R6F (Center of Tobacco Reference Products, University of Kentucky), and a commercially available e-cigarette (Joyetech eGo AIR), respectively. The eGo AIR device features a built-in 650mAh battery, an integrated 1.0-Ω coil, and a maximum power output of 12 watts, with a tank capacity of 2 ml. Aerosol generation was carried out using three commercial e-liquids with nicotine concentrations of 20, 12, and 0 mg/ml (Watson Reserve, Fashion Vape E-liquid), flavored (labelled as "aroma", with no specific flavor disclosed by the manufacturer), with a glycerin to propylene glycol ratio of 50:50. Nicotine (CAS 54–11-5) was present in freebase form, with no declared acidifying agents, suggesting the absence of nicotine salts.

Preparation of smoke and aerosol Aqueous Extracts (AqEs)

The LM1 smoking machine (Korber AG) was used for the 1R6F cigarette smoke exposure. The LM1 is a linear one-port mechanical syringe-based smoking machine that delivers undiluted smoke. The 1R6F reference cigarettes were conditioned before use for at least 48 h at 22 ± 1 °C and 60 ± 3% relative humidity, in accordance with ISO 3402:2023 [19]. Subsequently, 1R6F was smoked following the Health Canada Intense (HCI) regimen (55 ml puff volume, 2 s puff duration, 30 s puff frequency, with bell-shaped profile, 27.5 ml/s puff velocity, and the hole vents blocked), accredited under ISO 20778:2018 [20]. Laboratory conditions were monitored using temperature and humidity sensors before and during the exposure, ensuring a test atmosphere with 60% (± 5%) relative humidity and a temperature of 22 °C (± 2 °C), also following ISO 3402:2023 [19].

E-cigs were used according to the manufacturer's instructions. Before each exposure, they were fully charged, cleaned, and loaded with fresh consumables. The LM4E/LM5E vaping machine (Korber AG) was used for the aerosol exposure. The three different e-liquids loaded into the e-cigs were vaped according to the "CORESTA Reference Method n. 81" (CRM81) regimen (55 ml puff volume, 3 s puff duration, 30 s puff frequency, with square shaped profile, and 18.3 ml/s puff velocity), accredited into ISO 20768:2018 [21]. During the exposure, a relative humidity of 40–70% and a temperature of 15–25 °C (± 2 °C) were maintained in the laboratory, also in accordance with ISO 20768:2018.

Aqueous extracts (AqEs) were prepared by bubbling 9 puffs of smoke or aerosol into 45 ml of phosphate-buffered saline (PBS) contained in an impinger, resulting in a concentration of 0.2 puffs/ml. The stock AqEs were diluted 1:100 (in cell culture medium supplemented with HEPES) in order to achieve a final concentration of 0.002 puffs/ml (equivalent to 10 puffs/5L), which corresponds

to the concentration used in Manyanga's study [13]. This dilution was chosen by Manyanga and colleagues to maintain a nicotine range between 0 and 39 ng/ml, similar to that observed in plasma from vapers [22]. In our study, we maintained the same dilution of aerosol and smoke, although we found higher nicotine concentrations in our extracts (Table S1), likely due to the efficiency of our vaping machine and the standardized vaping regimens used [20, 21], as well as the different e-cig device. Exploring higher or cumulative doses could be an important future research goal. Nicotine was quantified in each stock AqE by reversed-phase HPLC as reported in the supplementary materials, and results were reported in table S1.

The pH of the PBS was measured before exposure and immediately after each exposure (freshly prepared AqE) using a calibrated pH meter (Eutech pH 700). The pH values are shown in Table S2. Since AqEs were diluted into HEPES-buffered culture medium for cell treatment, slight pH variations were not expected to substantially affect the final exposure conditions in the wells.

Chemosensitivity assay by MTS and NRU

MTS and the NRU assays were used for determining the cell viability after treatment with AqE of 1R6F smoke and e-cigs aerosol, in presence and absence of Cisplatin at different concentrations. Cells were seeded 24 h before treatment in 96-well plates at a density of 5×10^3 (SCC-25), 3×10^3 (FaDu), and 1.25×10^3 (UM-SCC-1) cells per well. The next day, the cells were treated with AqEs (from 1R6F smoke and e-cigs aerosol with 20, 12 and 0 mg/ml of Nicotine) at a concentration of 0.002 puffs/ml. After 48 h, the treatment was replaced with new AqEs (0.002 puffs/ml) in combination with different concentrations of Cisplatin (100, 10, 1, 0.1, 0.01 µM). After 48 h, the MTS and NRU assays were performed following the manufacturer's instructions. After every treatment, a microplate sealing membrane (Diversified Biotech Breath-EASIER BERM-2000) was applied to the plate.

MTS assay

CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega: G3580) was used by LAB-A, LAB-B, and LAB-D. In brief, MTS assay was performed by replacing all the treatments with fresh medium, and by adding 20 µL of MTS Solution to each well. The plates were incubated at 37 °C for 3 h in a humidified atmosphere of 5% CO_2. A microplate reader was used to detect the optical density of the MTS solution (490 nm filter). CellTiter-Glo® Luminescent Cell Viability Assay (Promega: G7572) was used by LAB-C. A volume of CellTiter-Glo® Reagent equal to the volume of the treatment present in each well was added. After 2 min on an orbital shaker, the plates were incubated at room temperature for 10 min. The generated luminescent signal was recorded using a luminometer microplate reader.

NRU assay

The NRU vital dye staining by Sigma Aldrich (#N2889) was used by all the laboratories. The day before the NRU assay, the NR solution was prepared in the medium at a ratio of 1:65 (0.05 g/L) plus HEPES buffer at 20 mM and placed in an incubator at 37 °C 5% CO_2. On the day of the NRU assay, the NR solution was filtered prior to use (0.2 µm filter). After treatment removal, the cells were washed with PBS. Then, 150 µL of the filtered NR solution was added to each well. The plates were incubated at 37 °C for 3 h in a humidified atmosphere of 5% CO_2. After incubation, the NRU solution was removed, and the cells were washed with PBS. The NR Destain Solution was prepared immediately before the use (Ratio: [ethanol: glacial acetic acid: distilled water] [50: 1: 49]) and added to each well. After 10 min of incubation on a plate shaker, the optical density was detected by a microplate reader with a filter of 540 nm and a background filter of 630 nm.

Trypan blue exclusion assay

Cell viability after treatment with 1R6F or e-cig AqEs, both with and without Cisplatin, was also assessed using the Trypan blue exclusion assay. Cells were seeded 24 h before treatment in 48-well plates at a density of 10×10^3 (SCC-25), 6×10^3 (FaDu), and 20×10^3 (UM-SCC-1) cells per well. The day after, the cells were treated with 1R6F or e-cig (20, 12 and 0 mg/ml of Nicotine) AqEs at a concentration of 0.002 puffs/ml. After 48 h, the treatment was replaced with fresh AqEs (0.002 puffs/mL), with or without 2.5 µM Cisplatin. After another 48 h, floating cells were collected, and adherent cells were harvested using trypsin. The two cell populations were then mixed, pelleted, stained with 0.4% Trypan blue (Gibco) at a 1:1 ratio, and counted using a Neubauer chamber.

Clonogenic survival assay

Cell seeding and treatment procedures were carried out as previously described for the Trypan blue exclusion assay. Afterward, to assess colony survival, adherent cells were trypsinized, pelleted, counted, and seeded into 6-well plates at a density of 10^3 (SCC-25, FaDu, and UM-SCC-1) cells per well. Cells were cultured for 7–15 days to allow colony formation, with the media changed every 2–3 days. When colony growth was sufficient, methanol was added to each well for 15 min in order to fix the cells. Subsequently, the cells were stained with 0.5% Crystal Violet (Sigma) in 25% methanol – 75% distilled H_2O for 10 min. The colonies were then washed 4 times with distilled H_2O and air-dried. Colonies formed by 50 or more cells were counted under a microscope. Plating efficacy

(PE) and the surviving fraction (SF) values were calculated as follow [23]:

$$\mathrm{PE} = (\text{n. of colonies formed/n. of cells seeded}) \times 100.$$

$$\mathrm{SF} = \text{n. of colonies formed after treatment/n. of cells seeded} \times \mathrm{PE}.$$

Protein expression by western blot and in-cell western

The protein expression of Cisplatin transporters after cell treatment with AqEs (PBS bubbled with 1R6F smoke and e-cigs aerosol with 20, 12 and 0 mg/ml of Nicotine) was assessed by Western Blot analysis (LAB-A, LAB-B, and LAB-D) and by In-cell Western (LAB-C).

Western Blot (LAB-A and LAB-D)

The day prior to the treatment, the cells were seeded in T-25 flasks at a density of 2×10^5 (SCC-25), 1×10^6 (FaDu), and 3×10^5 (UM-SCC-1) cells per flask. After 24 h, the cells were treated with the different AqEs at a concentration of 0.002 puffs/ml. 48 h after exposure to AqEs, cells were trypsinized, pelleted, washed with ice-cold PBS, and then resuspended in radioimmunoprecipitation assay (RIPA) buffer (Sigma-Aldrich: R0278-50ML) supplemented with 1X Protein Inhibitor Cocktail (Sigma-Aldrich). The protein quantification was performed with the Protein Assay Dye Reagent Concentrate (Biorad) using bovine serum albumin (BSA) as standard. The total protein content was quantified and an amount of 30–50 µg for each sample was denatured for 10 min at 70 °C in an appropriate loading buffer composed of NuPAGE™ LDS Sample Buffer (Invitrogen) and Bolt™ Sample Reducing Agent (Invitrogen). The separation of proteins was performed by electrophoresis using a 4-12% polyacrylamide gel (Bolt™ Bis–Tris Plus Mini Protein Gels, 4-12%, 1.0 mm, WedgeWell™ format) followed by the electro-transfer of proteins onto the nitrocellulose membrane (Power Blotter Select Transfer Stacks). Subsequently, the membranes were blocked with 5% BSA solution for 1 h at room temperature. After blocking the nonspecific sites, the membranes were incubated overnight at room temperature with the following primary antibodies resuspended in 5% BSA solution: anti-ATP7A (1:100; Santa Cruz Biotechnologies, Texas, USA, sc-376467), anti-CTR1 (1:40.000; Abcam, Massachusetts, USA, #ab129067), anti-ABCG2 (1:100; Cell Signaling Technology, Massachusetts, USA, #42,078), and β-actin as a loading control (1:20.000; Cell Signaling Technology, Massachusetts, USA, #8457). After incubation, the membranes were washed three times with Tris-buffered saline with 0.1% Tween 20 (TBS-T), and subsequently incubated for 1 h at room temperature with the following secondary antibodies resuspended in 5% BSA solution: rabbit anti-mouse IgG (1:10.000; Abcam, Massachusetts, USA, #ab6728) and goat anti-Rabbit IgG (1:10.000; Cell Signaling Technology, Massachusetts, USA, #7074). The membranes were then washed four times with TBS-T. Finally, the protein bands were visualized using the Clarity Max Western ECL Substrate (Biorad: 1,705,062) and detected by Li-Cor C-DiGit® Chemiluminescent Western Blot Scanner. The protein levels were quantified by densitometric analysis using the ImageJ software, while data were normalized on β-actin expression levels.

Western Blot (LAB-B)

Western blot analysis was performed as previously described [24]. Briefly, cells were seeded in T-75 flasks and then treated with AqEs (0.002 puffs/ml) for 48 h before reaching 80–90% confluence. Cell pellets were lysed in ice-cold whole-cell lysis buffer (50 mM Tris–Cl, pH 7.4, 5 mM EDTA, 250 mM NaCl, 50 mM NaF, 0.1 mM Na3VO4, 0.1% Triton X-100) with protease inhibitors (Sigma-Aldrich 539,136-1SET). Protein concentration was measured using the Bradford assay. Equal amounts of protein (20–50 µg) were boiled with Laemmli buffer (10% β-mercaptoethanol) at 95–100 °C for 5 min and cooled on ice. Proteins were separated on Bio-Rad 4–15% gradient gels and wet transferred onto PVDF membranes overnight at 4 °C. The successful transfer was confirmed using Ponceau S staining before the membranes were blocked by 5% non-fat milk diluted in TBST for 1 h at room temperature and incubated overnight at 4 °C with primary antibodies diluted in 3% non-fat milk (ATP7A, Santa Cruz SC376467, 1:100; ABCG2, Millipore Sigma SAB5700094, 1:100; CTR1, Abcam Ab129067, 1:200; β-actin, Cell Signaling D6A8, 1:500). After washing with TBST, membranes were incubated with HRP-conjugated secondary antibodies (1:2000–1:10,000). Protein bands were detected using ECL substrate (Bio-Rad 1,705,062) and visualized using an imaging system.

In-cell Western (LAB-C)

SCC-25, FaDu, and UM-SCC-1 cells were seeded at a density of 5×10^3, 3×10^3, and 1.25×10^3 cells/well, respectively, in a 96-well black plate (Thermo Scientific, USA) and incubated at 37 °C in a 5% CO_2. After 24 h of incubation, the cells were treated with the different conditions of AqE (1R6F smoke and e-cig aerosol with 20, 12, and 0 mg/ml of Nicotine) at a concentration of 0.002 puff/ml, for 24 h. After incubation, the cells were fixed with 3,7% formaldehyde (150 µl) (Merck Cat No. 1040031000, Germany) for 20 min at room temperature. The cells were then permeabilized using 0.1% Triton X-100 in TBS (150 µl) for 20 min, by gently agitating on a microplate shaker. Then, the cells were blocked with the Intercept Blocking Buffer (LICORbio, P/N: 927–70,001) for 30 min. After blocking buffer removal, the cells were incubated with 50 µl of the following primary antibodies: CTR1 (#13,086), ATP7A (Santa Cruz Biotechnology), ABCG2

(D5V2K Rabbit mAb #42,078), and ß-actin (D6A8 Rabbit mAb #8457). The antibodies were prepared in PBS-Tween with 0,1 mg of BSA at 1:1000 dilution. The plate was incubated overnight at 4 °C in the dark. After incubation, the primary antibodies were removed from the wells. All the wells were washed four times with washing solution (150 µl) at room temperature by gently agitating with microplate shaker. Subsequently, the plate was dried and incubated with 50 µl of secondary antibodies: IRDye® 800 CW Goat anti-Rabbit IgG Secondary Antibody 925–32,211, IRDye® 800 CW Goat anti-Mouse IgG Secondary Antibody 925–32,210, and IRDye® 680 RD Goat anti-Rabbit IgG Secondary Antibody 925–68,071. The plate was incubated in the dark for 1 h at room temperature. The secondary antibodies were removed, and the wells were washed four times. The protein expression was evaluated with Odyssey® CLx Imaging System (Licor Biosciences, US) with 169 µm resolutions, Lowest Quality, 4.0 mm Focus offset, and 800 wavelength Intensity. For ß-actin detection, 680 wavelength Intensity was used. Untreated cell monolayers were used for subtracting unspecific and/or background fluorescent signals. All the data were acquired by Licor Image Studio Software (version 3.1), while recorded values were then exported into Excel (Microsoft, US, version 2010).

Gene expression by Real-Time PCR

Real-time PCR was employed to evaluate the gene expression of membrane transport proteins involved in Cisplatin uptake and efflux in cells exposed to cigarette smoke and e-cig aerosol (Table S3). The day prior to the treatment with AqEs, cells were seeded in 12-well plates at a density of 50×10^3 (SCC-25), 30×10^3 (FaDu), and 40×10^3 (UM-SCC-1) cells/well. After 24 h, cells were treated with AqE from 1R6F smoke and e-cigs aerosol with 20, 12 and 0 mg/ml of Nicotine, at a concentration of 0.002 puff/ml, and then incubated for 48 h. After incubation, cells were detached and collected by using the RLT lysis buffer (Qiagen) supplemented with β-mercaptoethanol, and the RNA was then extracted by RNeasy Mini Kit (Qiagen), following the manufacturer's instructions. RNA content and related purity were evaluated by spectrophotometric absorbance (A260/A280 nm ratio). The cDNA synthesis via reverse transcription from the isolated RNA was carried out with the QuantiTect Reverse Transcription Kit (Qiagen) according to the manufacturer's protocol. Quantitative Real-Time PCR was performed using the KAPA SYBR FAST Universal (KAPA Biosystems), while the obtained mRNA expression levels were normalized with β-actin as housekeeping gene, by using a comparative $2^{-\Delta\Delta Ct}$ method.

Statistics

All raw data were organized and processed using Microsoft Excel. Data were analyzed following predefined criteria to identify outliers or anomalous results. Some experiments were excluded from the analysis under the following parameters: if an entire condition showed an anomalous result compared with the other experimental replicates and if the technical cause was not identified. Outlier detection was performed using the ROUT (robust regression-based outlier rejection) test. To assess the normality or skewness of the data distribution, the Shapiro–Wilk test was applied. Symmetrical data were expressed as mean ± standard error of the mean (SEM), while skewed data were represented as median (95% confidence interval—95% CI). The intraclass correlation coefficient (ICC) was calculated using a two-way mixed-effects model with absolute agreement to assess the consistency and repeatability of intrasession measurements across laboratories. ICC calculations were performed with R software, version 4.2.3 (2023–03–15). To identify statistically significant differences between study groups, either one-way ANOVA or the Kruskal–Wallis test was used. Further comparisons between groups were conducted using post-hoc tests, including Tukey's test or Dunn's test, which account for multiple comparisons within each analysis. All pairwise comparisons were evaluated. For clarity, only statistically significant comparisons are reported in the figures. When multiple significant comparisons involved control groups, results versus the vehicle control are shown, unless otherwise stated. IC50 values for the tested products were determined using GraphPad Prism 8 software, by fitting a sigmoidal dose–response curve with a variable slope, using nonlinear regression analysis to identify the best parameters and compare the IC50 values. Statistical significance was set at $p < 0.05$ for all analyses. GraphPad Prism 8 software was used for data analysis and generation of graphs unless otherwise stated.

Results

Reproducibility across all laboratories

To assess the reproducibility of our results, we calculated the intraclass correlation coefficient (ICC) for cell viability, cell death, chemosensitivity, clonogenic, gene expression and western blot results from the different laboratories. The ICC values, together with their 95% confidence intervals and corresponding p values, are given in the supplementary materials (Tables S4-S9).

Cell viability data (MTS and NRU)

The reproducibility of cytotoxicity assays was evaluated in multiple laboratories using MTS and NRU assays. ICC

values indicated moderate to high agreement depending on cell line and treatment (Table S4). For MTS data, reproducibility was highest for SCC-25 with 1R6F treatment (ICC = 0.884, $p < 0.0001$), but decreased significantly for e-cig treatments (ICC = 0.556, $p < 0.0001$). Similar trends were observed for the other cell lines, with moderate ICCs for FaDu (0.609 for 1R6F; 0.506 for e-cig) and UM-SCC-1 (0.598 for 1R6F; 0.574 for e-cig). For NRU data, higher ICC values were observed for FaDu in both treatments (ICC = 0.932 for 1R6F; 0.906 for e-cig, both $p < 0.0001$), while SCC-25 and UM-SCC-1 showed moderate reproducibility, with ICC values ranging from 0.500 to 0.665.

These results highlight that the NRU assay generally provided better interlaboratory reproducibility than the MTS assay, particularly for FaDu cells.

Trypan blue data

Trypan Blue assay results revealed varying levels of reproducibility, with the lowest ICCs observed for the SCC-25 cell line (Table S5). For data on this cell line, the ICC values were 0.154 (1R6F) and 0.119 (e-cig), reflecting poor if significant agreement ($p = 0.0022$ and $p = 0.0005$, respectively). For the FaDu cell line, reproducibility improved significantly, with ICC of 0.534 (1R6F) and 0.648 (e-cig) (both $p < 0.0001$). Data from UM-SCC-1 showed moderate to high agreement, with ICC values of 0.634 (1R6F) and 0.826 (e-cig), both statistically significant ($p = 0.0005$ and $p < 0.0001$, respectively).

The Trypan Blue assay showed weaker reproducibility for SCC-25 than FaDu and UM-SCC-1, suggesting that this assay is more sensitive to experimental conditions. While the Trypan Blue exclusion assay is a reliable method for validating results from MTS or NRU assays in small-scale experiments, its manual implementation at a larger scale becomes labor-intensive and time-consuming. This not only complicates the process but also makes it challenging to complete the assay within a defined timeframe, potentially compromising the results.

Chemosensitivity data

Chemosensitivity assays using the MTS and NRU assays showed excellent interlaboratory reproducibility (Table S6). For MTS data, SCC-25 revealed an ICC of 0.943 (1R6F) and 0.895 (e-cig), both indicating near-perfect agreement ($p < 0.0001$). Similar trends were observed for FaDu (ICC = 0.899 for 1R6F; 0.882 for e-cig) and UM-SCC-1 (ICC = 0.867 for 1R6F; 0.796 for e-cig). For NRU data, even higher ICC values were observed, particularly for FaDu (ICC = 0.942 for 1R6F; 0.949 for e-cig) and UM-SCC-1 (ICC = 0.885 for 1R6F; 0.894 for e-cig).

The high ICCs suggest strong reproducibility of chemosensitivity assays, especially with NRU protocols, among all cell lines.

Clonogenic data

The clonogenic assay showed variable reproducibility depending on treatment and cell line (Table S7). SCC-25 showed poor concordance, with ICC of 0.154 (1R6F) and 0.119 (e-cig) although this was significant ($p = 0.0022$ and $p = 0.0005$, respectively). Data from FaDu presented moderate reproducibility for both 1R6F (ICC = 0.534, $p < 0.0001$) and e-cig aerosols (ICC = 0.648, $p < 0.0001$).

UM-SCC-1 showed better reproducibility, with ICC of 0.634 (1R6F) and 0.826 (e-cig) (both $p < 0.0001$).

These results indicate that the clonogenic assay was more reproducible for FaDu and UM-SCC-1, while the reproducibility of SCC-25 remains limited.

Gene expression data

The reproducibility of gene expression measurements was evaluated for major cisplatin transporter genes, including XPA, MMS19, ERCC1, and ATP7A. Reproducibility was not evaluated for the other genes because their expression was conducted only by LAB-A. ICC values for gene expression data varied widely (Table S8). For SCC-25, ICC was higher for MMS19 (ICC = 0.360, $p < 0.0001$) and poor for ERCC1 (ICC = 0.171, $p = 0.0187$), but very low for XPA (ICC = 0.0163, $p = 0.357$).

FaDu showed very low agreement for MMS19 and XPA (negative ICC values), while the reproducibility of ERCC1 remained low (ICC = 0.283, $p = 0.0162$). In UM-SCC-1, ICC values were low overall, with the highest agreement observed for ERCC1 (ICC = 0.151, $p = 0.0511$).

Gene expression assays showed inconsistent reproducibility, particularly for FaDu and UM-SCC-1, highlighting the very high sensitivity of this assay to experimental variables.

Western blot data

The reproducibility of protein expression data from Western blot analysis was generally low (Table S9). For SCC-25 data, ICC values were poor for ATP7A (ICC = 0.132, $p = 0.0751$) and CTR1 (ICC = 0.120, $p = 0.159$). Negative ICC values were observed for ABCG2, reflecting substantial interlaboratory variability. Western blot analysis demonstrated poor overall reproducibility, supporting the reputation of this assay as a difficult and nonreproducible technique [25–27].

Evaluation of cell viability and death induced by cisplatin in combination with 1R6F and e-cigarette aerosol

As shown in Figs. 1, 2 and 3, our findings across all three oral cancer cell lines – SCC-25 (Fig. 1), FaDu (Fig. 2) and UM-SCC-1 (Fig. 3) – indicate that the addition of 1R6F cigarette smoke extract or e-cig aerosols at varying nicotine concentrations did not impact the cytotoxicity or cell death induced by cisplatin treatment. Unlike the results reported by Manyanga et al. [13], we observed no

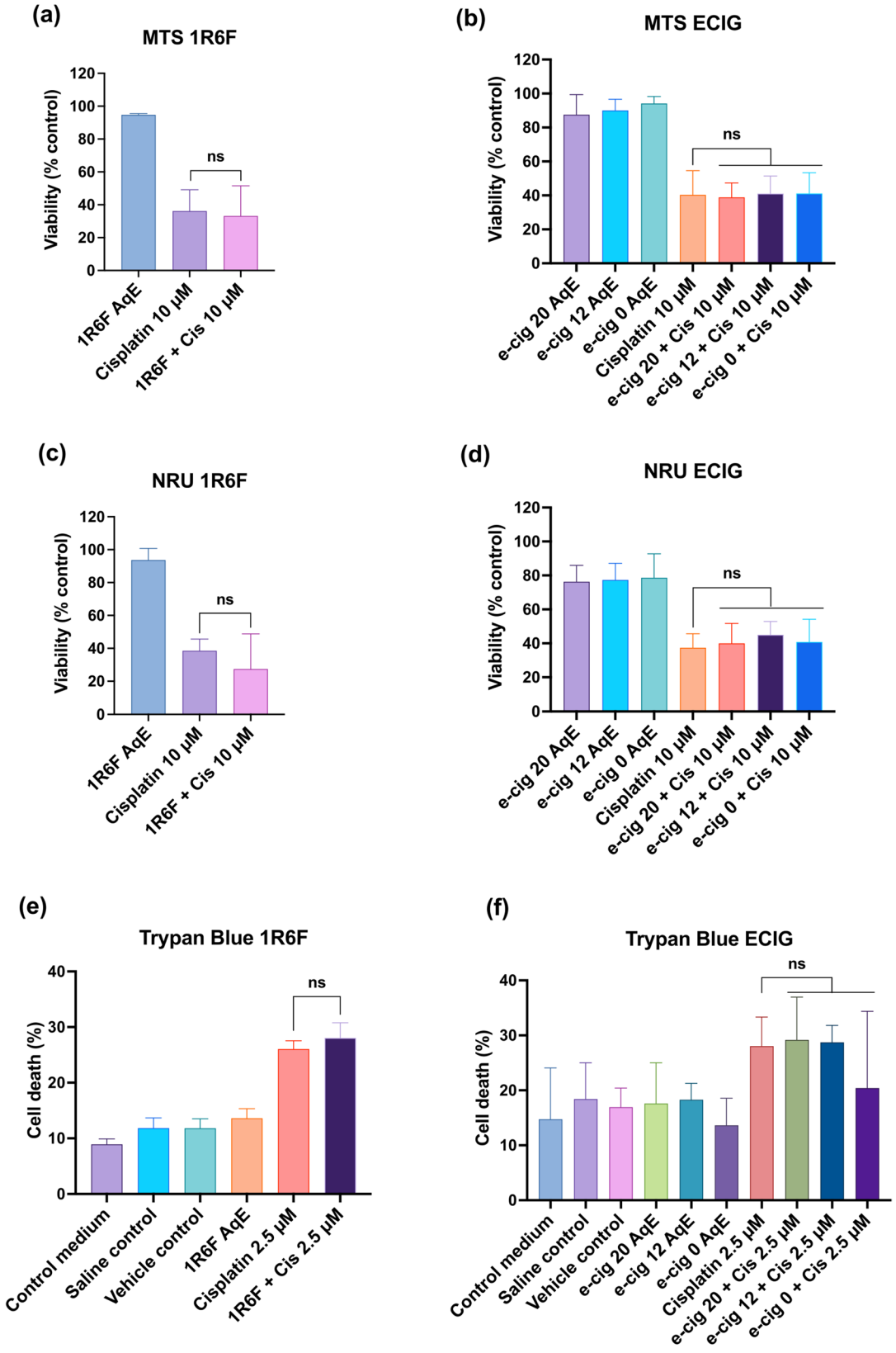

Fig. 1 Evaluation of cell viability (MTS and NRU) and cell mortality (Trypan Blue exclusion assay) in SCC-25 cell line in response to cisplatin treatment and its combination with cigarette smoke extract (1R6F) and electronic cigarette (e-cig) aerosols. Data in the panel **a**, **b**, **c**, **d**, and **f** were presented as median and CI 95% and were analyzed by using the Kruskal–Wallis test followed by Dunn's test for multiple comparisons. Data in the panel **e** were presented as mean + SEM, and were analyzed by using one-way ANOVA followed by Tukey's test for multiple comparisons. Cis: cisplatin; ns: not significant

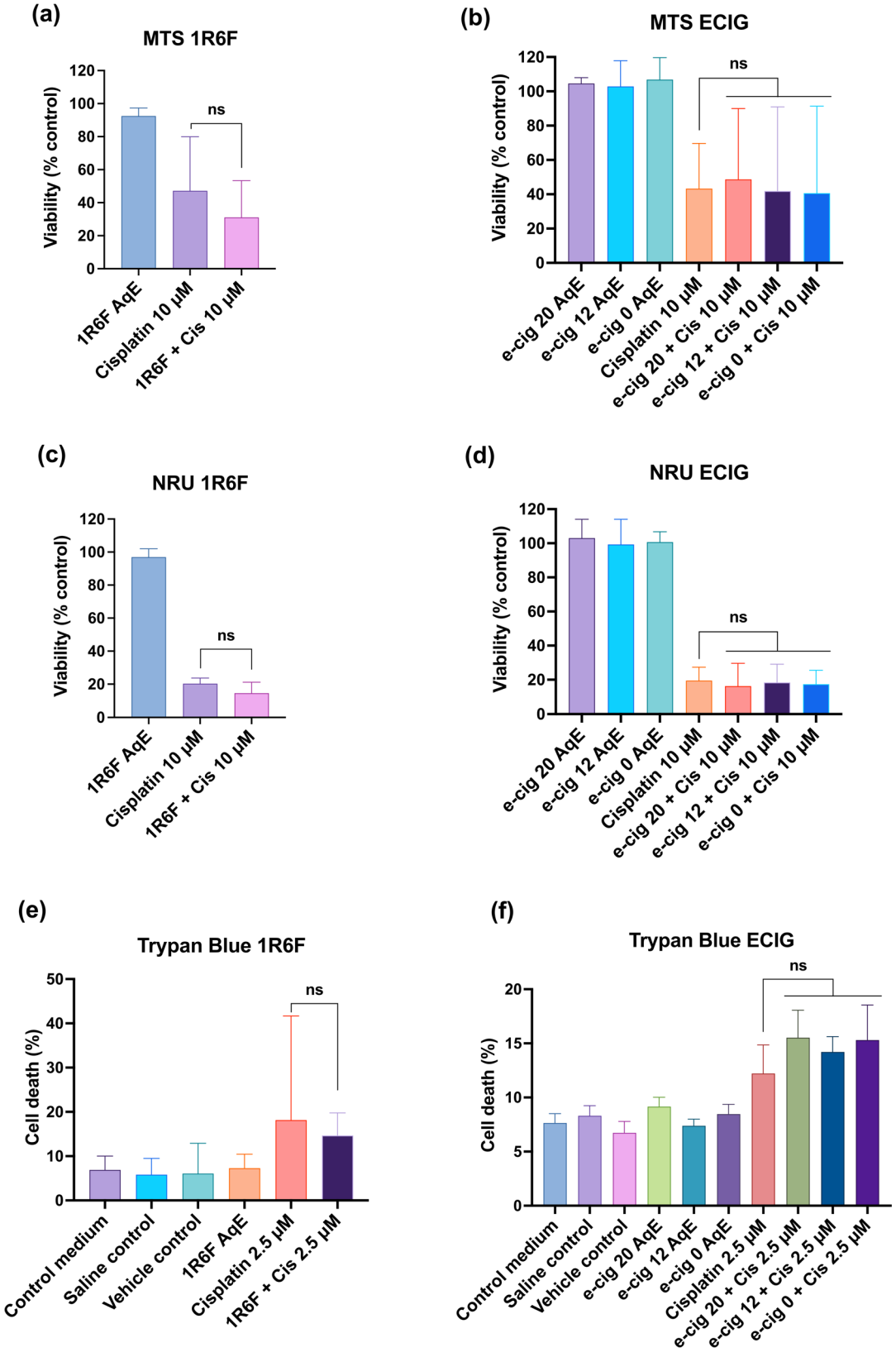

Fig. 2 Evaluation of cell viability (MTS and NRU) and cell mortality (Trypan Blue exclusion assay) in FaDu cell line in response to cisplatin treatment and its combination with cigarette smoke extract (1R6F) and electronic cigarette (e-cig) aerosols. Data in the panel **a**, **b**, **c**, **d**, and **e** were presented as median and CI 95% and were analyzed by using the Kruskal–Wallis test followed by Dunn's test for multiple comparisons. Data in the panel **f** were presented as mean + SEM, and were analyzed by using one-way ANOVA followed by Tukey's test for multiple comparisons. Cis: cisplatin; ns: not significant

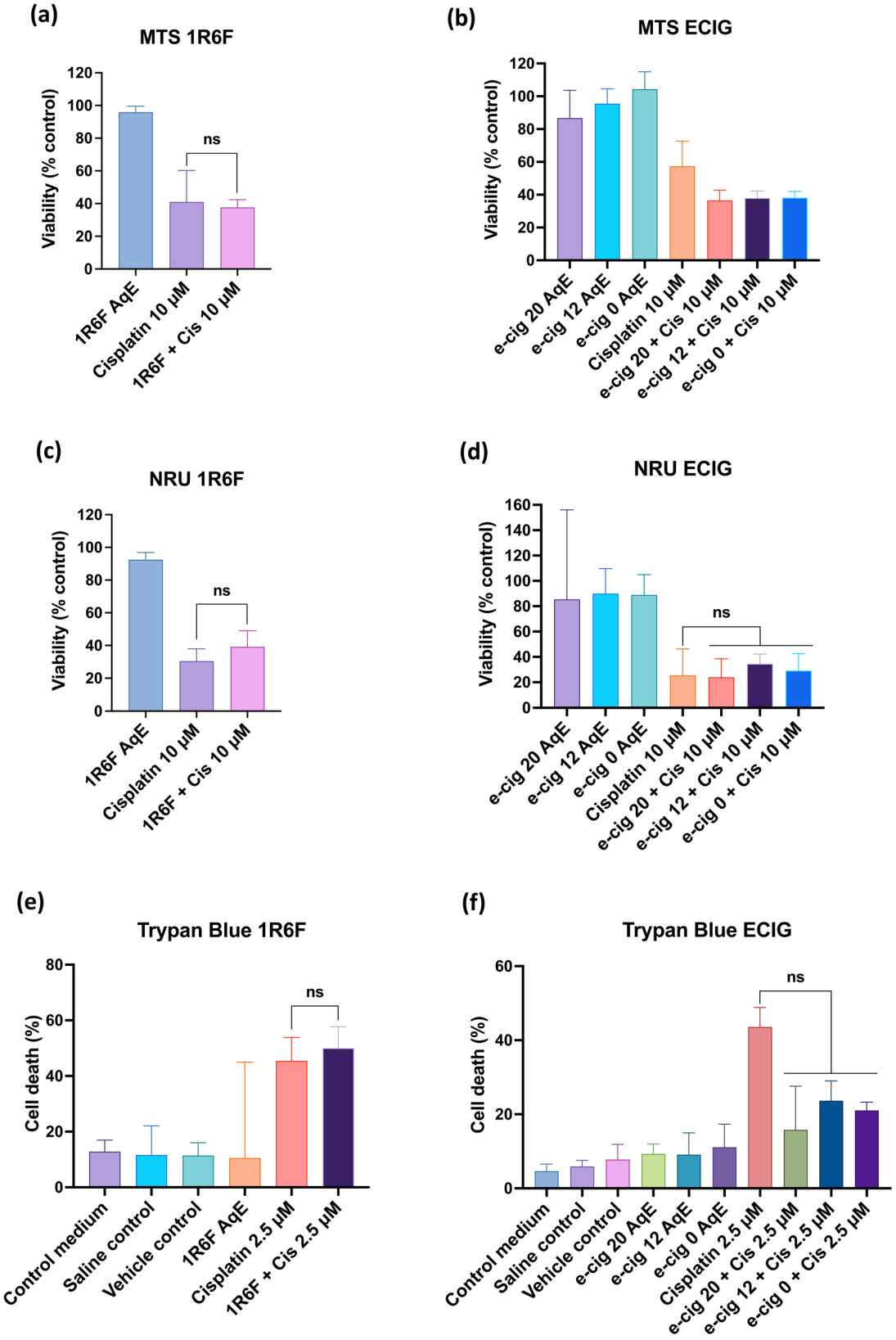

Fig. 3 Evaluation of cell viability (MTS and NRU) and cell mortality (Trypan Blue exclusion assay) in UM-SCC-1 cell line in response to cisplatin treatment and its combination with cigarette smoke extract (1R6F) and electronic cigarette (e-cig) aerosols. Data in the panel **a**, **b**, **d**, **e** and **f** were presented as median and CI 95% and were analyzed by using the Kruskal–Wallis test followed by Dunn's test for multiple comparisons. Data in the panel **c** were presented as mean + SEM, and were analyzed by using one-way ANOVA followed by Tukey's test for multiple comparisons. Cis: cisplatin; ns: not significant

significant modulation of cisplatin sensitivity in response to these exposures. Although some conditions appear to show variability with respect to cisplatin, as with trypan blue in UM-SCC-1 cells, these variations are not statistically significant.

Evaluation of cisplatin required to induce a 50% reduction in cell growth (IC50) in combination with 1R6F and e-cigarette aerosol

The chemosensitivity of SCC-25, FaDu, and UM-SCC-1 cell lines to cisplatin was evaluated using the MTS and NRU assays. All the IC50 values were reported in supplementary materials (Tables S10-S13). The MTS assay results revealed significant differences in drug efficacy in FaDu and UM-SCC-1 (Fig. 4). For the SCC-25 cell line, the IC50 values for cisplatin and 1R6F indicated a trend toward increased sensitivity with 1R6F ($p = 0.0589$), although this difference did not reach statistical significance. FaDu cells showed a marked reduction of IC50 for 1R6F ($p = 0.0007$) compared to cisplatin alone, whereas exposure to e-cig did not significantly alter IC50 values (p = 0.183). In UM-SCC-1 cells, no significant difference was detected for 1R6F exposure ($p = 0.387$), but e-cig exposure led to significant decrease in IC50 values compared to cisplatin alone ($p < 0.0001$). Similar trends were observed in the NRU assay (Fig. 5), with FaDu cells exhibiting decreased IC50 values for 1R6F ($p = 0.015$), while no significant differences were detected in the other treatments.

Clonogenic survival after cisplatin treatment in combination with 1R6F and e-cigarette aerosol

The results of the clonogenic assay are shown in Fig. 6 for the three different cell lines: SCC-25 (Fig. 6 a and b), FaDu (Fig. 6 c and d) and UM-SCC-1 (Fig. 6 e and f). In contrast to the work of Manyanga et al. [13], our data indicate no interaction between cisplatin alone and cisplatin combined with 1R6F and e-cigarette extracts in the formation of colonies. No or few colonies were observed with cisplatin treatment alone and in combination with 1R6F and e-cig extracts (Fig. S1- S3). Instead, the exposure to 1R6F and e-cig extracts without the cisplatin cotreatment did not affect the reproductive viability in terms of clonogenic survival for all tested cancer cell lines.

mRNA expression of genes for the repair of cisplatin-induced DNA damage

We investigated whether cigarette and e-cigarette aerosol exposures affect the mRNA expression of XPA, MMS19, and ERCC1, which are critical genes for the repair of cisplatin-induced DNA damage (Fig. 7). Compared to the original study, notable differences emerged in our results, particularly regarding the magnitude and direction of gene expression changes.

Across all cell lines (SCC-25, FaDu, UM-SCC-1), no significant differences were observed between treatments, indicating that XPA mRNA expression remained relatively stable irrespective of exposure condition (Fig. 7 a, b, c). MMS19 mRNA expression was significantly downregulated only for SCC-25 following exposure to e-cig 12 nic compared to vehicle control ($p = 0.047$) and greater following exposure to e-cig 0 nic compared to control medium ($p = 0.011$) and vehicle control ($p < 0.0001$) (Fig. 7 d). No significant differences in MMS19 mRNA expression were observed for FaDu and UM-SCC-1 cells (Fig. 7 e, f). ERCC1 expression levels remained essentially unchanged in all cell lines and under all conditions (Fig. 7 g, h, i), with only slight downregulation observed in SCC-25 for e-cig 0 compared to vehicle control alone ($p = 0.039$) and in FaDu for e-cig 20 and e-cig 0 compared to vehicle control alone ($p = 0.012$ and $p = 0.001$, respectively).

Our replication study agrees with Manyanga's findings on the stability of XPA expression, but diverges significantly on the effects of e-cigarette aerosol on MMS19 and ERCC1. While Manyanga et al. [13]reported widespread and significant downregulation of MMS19 and ERCC1, our replication study found that these effects are sporadic and limited to specific conditions and cell lines, suggesting no nicotine-dependent effect.

Expression of drug influx and efflux transporter *mRNA expression results*

The gene expression assessment of CTR1, ABCG2, ATP7B, ABCC2, ABCA1, and ABCC1 was performed only by LAB-A in two independent experimental sessions for SCC-25 and FaDu, and one experimental session for UM-SCC-1. The ATP7A gene expression assessment was performed by LAB-A and LAB-B in triplicate in two independent experimental sessions, except for the UM-SCC-1 with one experimental session by LAB-A and two experimental sessions by LAB-B. Results regarding the expression of drug efflux and influx transporter genes are shown in Figs. 8 and 9. CTR1 expression levels remained stable for SCC-25 only, with no significant differences, but slight downregulation was observed for 1R6F compared to vehicle control in FaDu ($p = 0.039$) and for e-cig 20 nic compared to control medium in UM-SCC-1. Unlike Manyanga et al. [13] who reported significant upregulation of ATP7A in all cell lines analyzed, we observed no significant change in SCC-25, FaDu and UM-SCC-1. Evaluation of ABCG2 expression showed downregulation following exposure with e-cig 20 nic compared to controls in SCC-25 ($p < 0.05$), e-cig 12 nic compared to control medium in FaDu ($p = 0.021$), and e-cig 0 nic compared to control

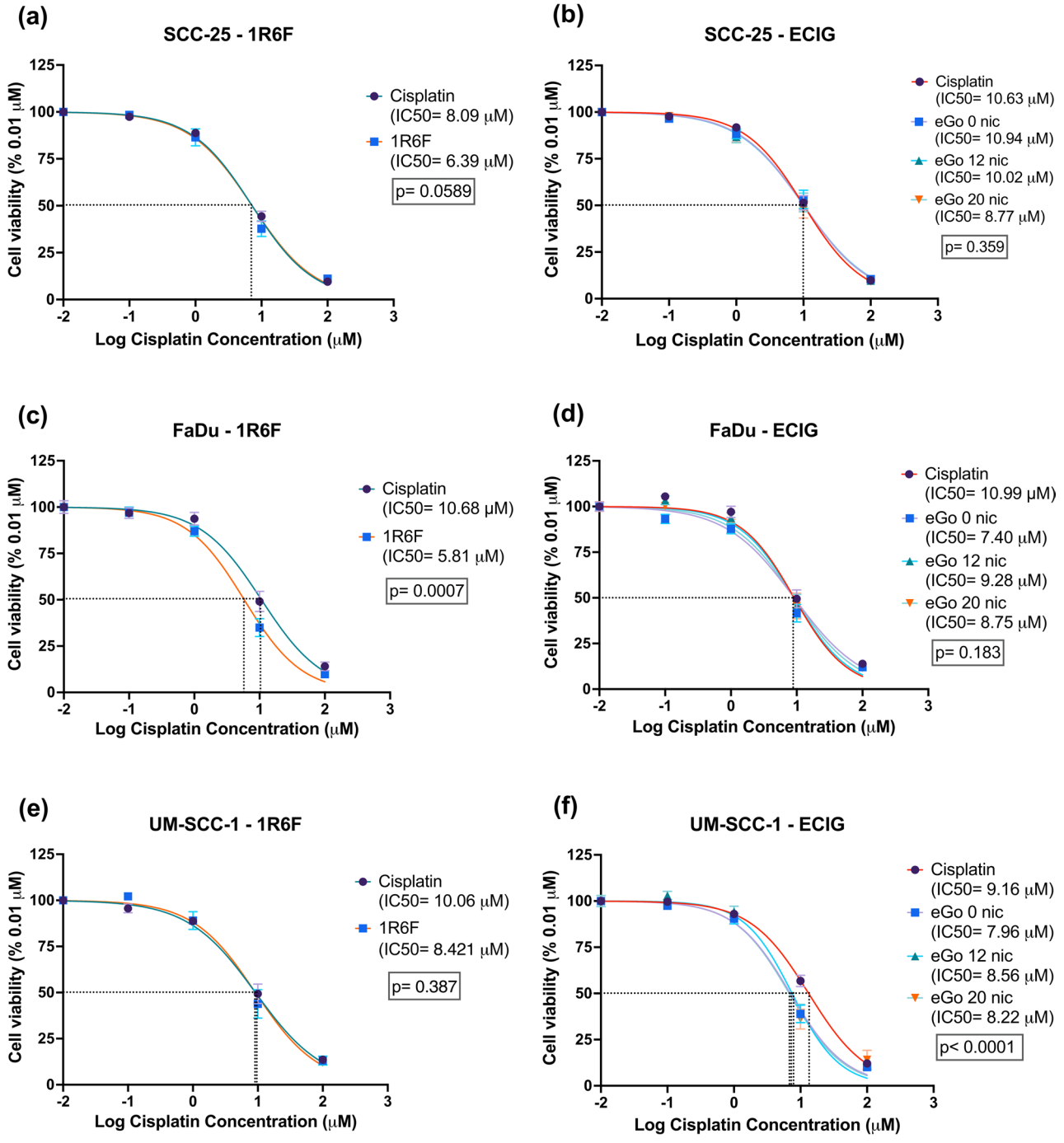

Fig. 4 The chemosensitivity of SCC-25, FaDu, and UM-SCC-1 cell lines to cisplatin, alone or in combination with 1R6F or e-cigarette aerosols, was evaluated using the MTS assay. Panels **a, c, e** show 1R6F exposure, while panels **b, d, f** show e-cigarette aerosol exposure in SCC-25, FaDu, and UM-SCC-1 cell lines, respectively

medium in UM-SCC-1 ($p = 0.01$). This result contrasts with that reported by Manyanga et al. [13]who found significant upregulation of ABCG2 in all cell lines tested. ATP7B expression levels were found to be increased for e-cig 20 nic compared to controls only in SCC-25 ($p < 0.05$) and decreased for e-cig 0 nic compared to control medium in UM-SCC-1. No significant changes were observed in FaDu. Unlike Manyanga's results, the expression levels of ABCC2, ABCA1, and ABCC1 remained unchanged in all cell lines under all conditions.

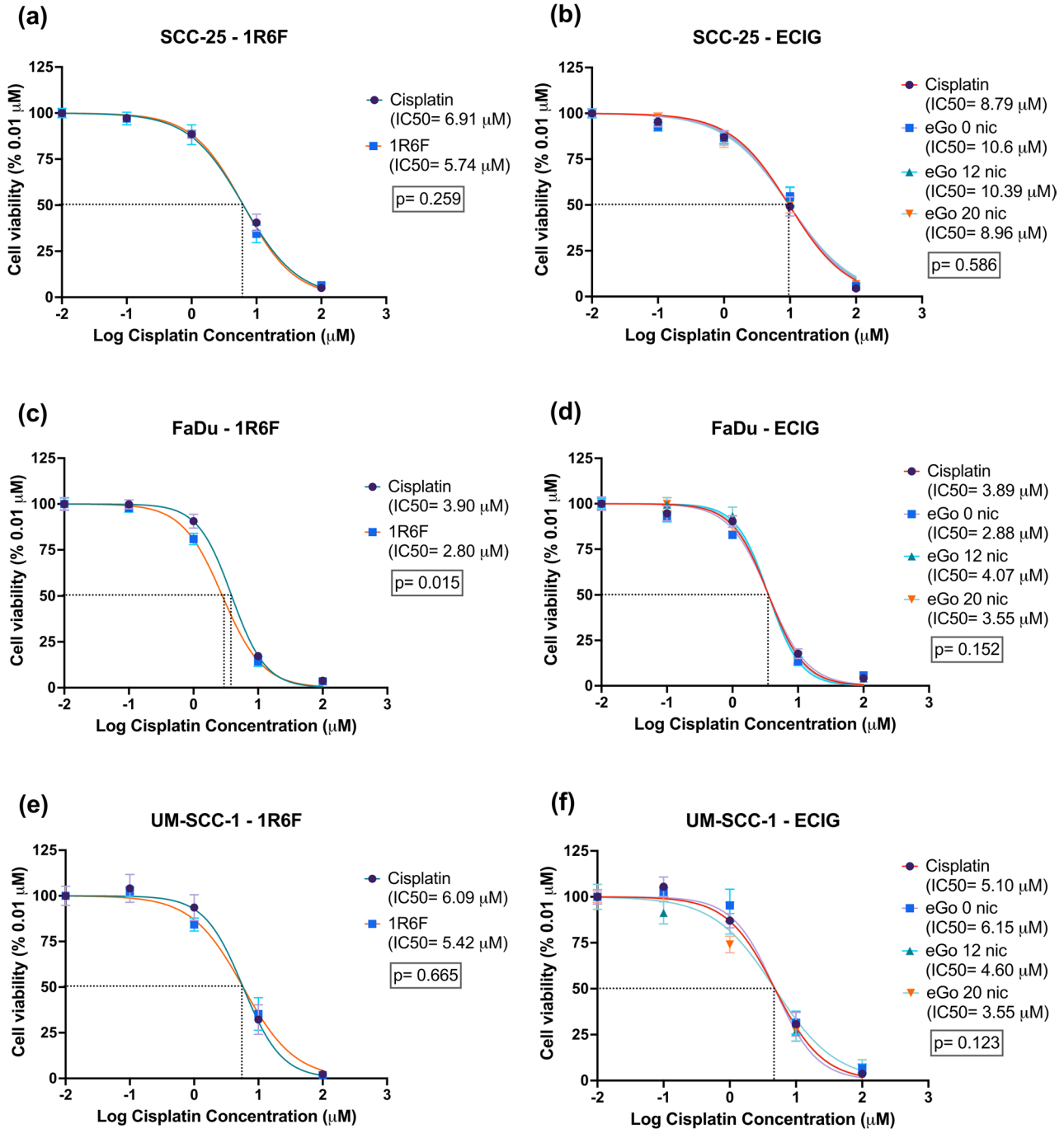

Fig. 5 The chemosensitivity of SCC-25, FaDu, and UM-SCC-1 cell lines to cisplatin, alone or in combination with 1R6F or e-cigarette aerosols, was evaluated using the NRU assay. Panels **a, c, e** show 1R6F exposure, while panels **b, d, f** show e-cigarette aerosol exposure in SCC-25, FaDu, and UM-SCC-1 cell lines, respectively

Western blot results

Western blot was performed by LAB-A, LAB-B, and LAB-D. LAB-A produced quantifiable results for SCC-25 and UM-SCC-1; LAB-B produced quantifiable results for FaDu (only ABCG2 and CTR1); LAB-D produced results for SCC-25. As reported by Manyanga et al. [13], we investigated the protein expression of efflux (ATP7A and ABCG2) and influx (CTR1) transporter associated with cisplatin resistance in SCC-25, FaDu and UM-SCC-1 cell lines. For SCC-25 three independent experiments were performed by LAB-A and LAB-D. Whereas, two independent experiments for FaDu and UM-SCC-1 were performed by LAB-B and LAB-A, respectively. All the unprocessed blot and the corresponding red ponceau

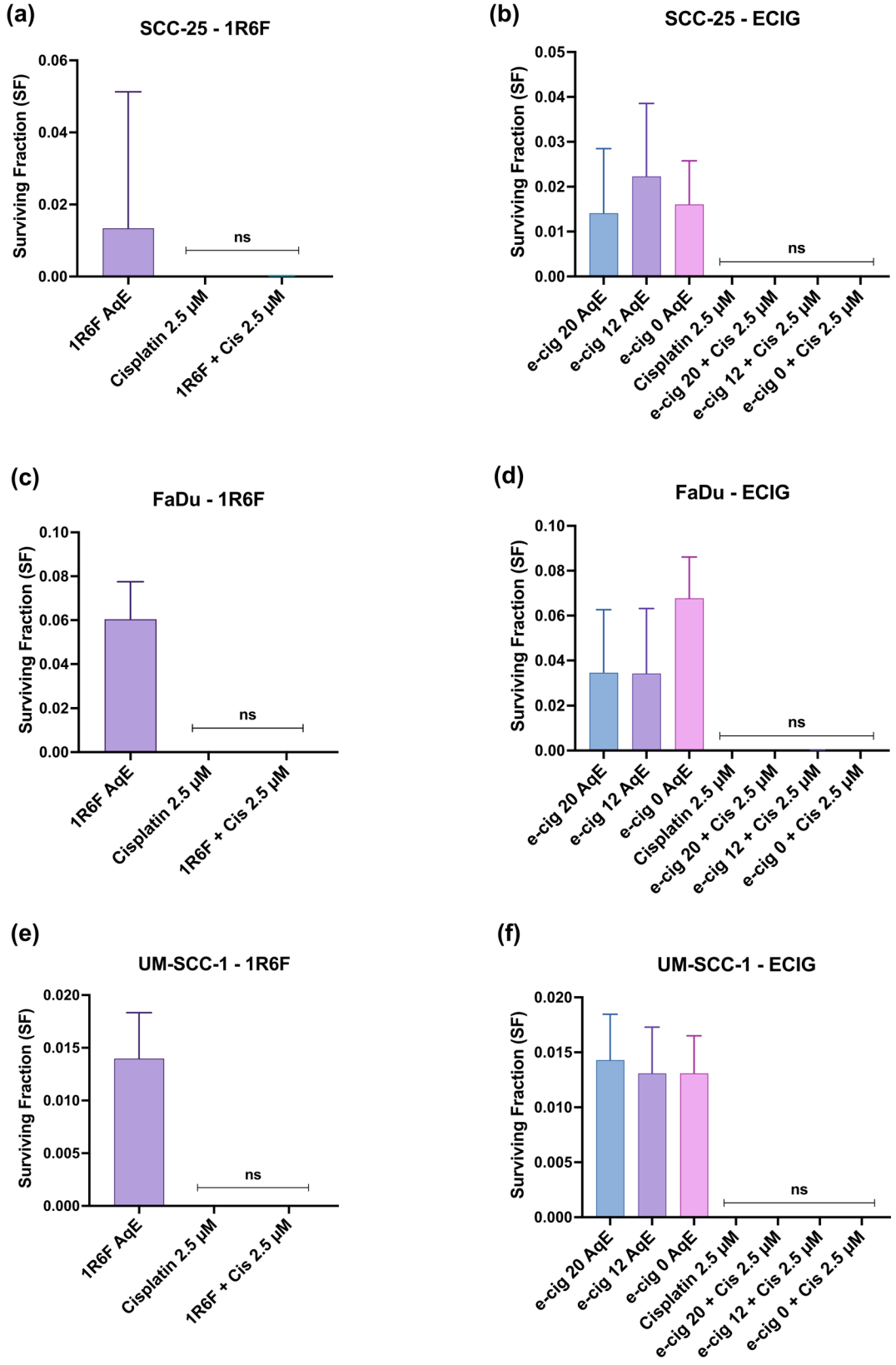

Fig. 6 The survival fraction (SF) was measured after treatment with 1R6F aqueous extract (AqE), cisplatin (2.5 µM), and combinations of e-cigarette extracts (20, 12, and 0 mg/ml nicotine) with cisplatin. Panels **a, c, e** show 1R6F exposure, while panels **b, d, f** show e-cigarette aerosol exposure in SCC-25, FaDu, and UM-SCC-1 cell lines, respectively. Cis: cisplatin; ns: not significant

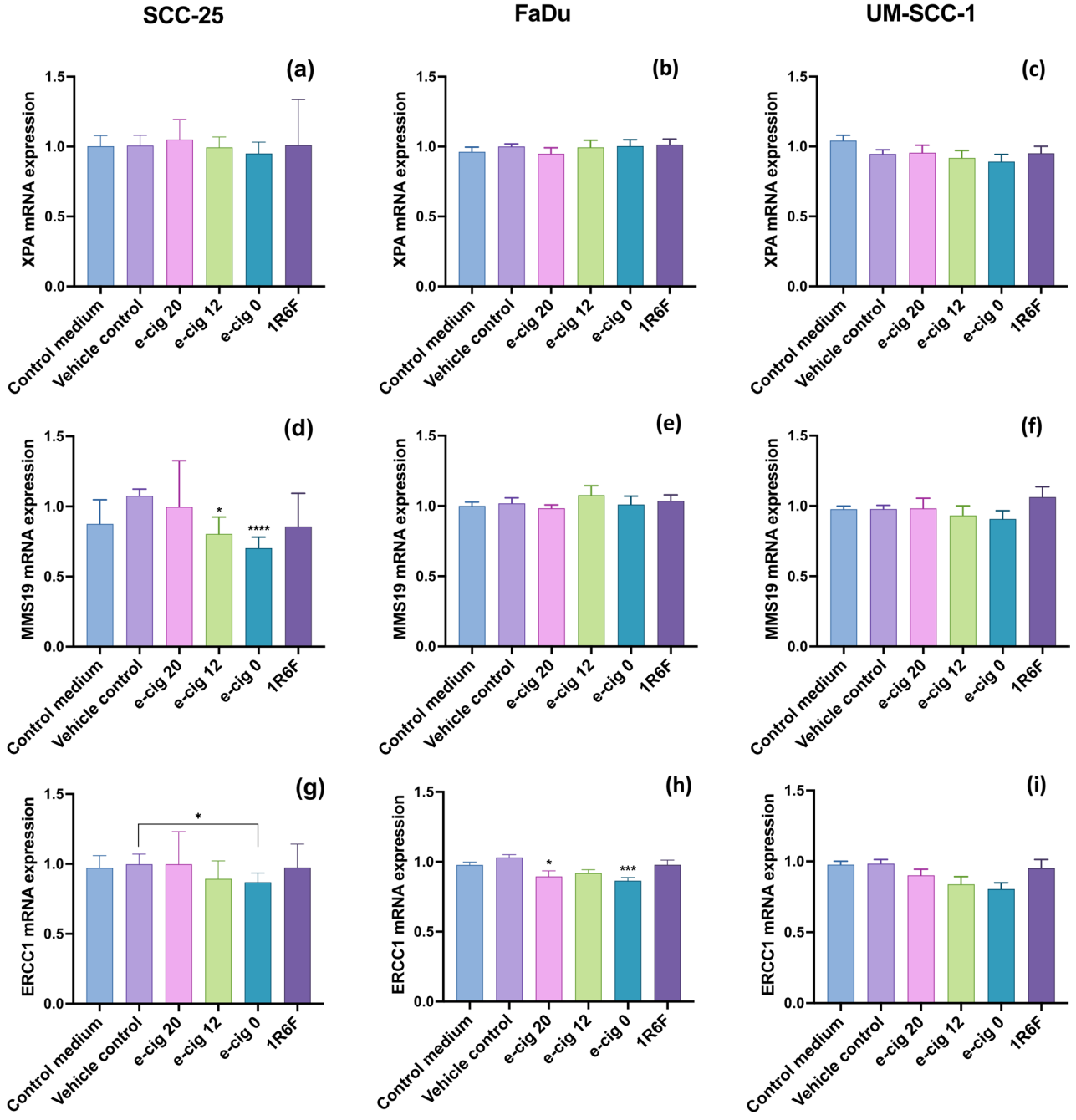

Fig. 7 Expression of gene repair of cisplatin-induced DNA damage XPA (**a**, **b**, **c**), MMS19 (**d**, **e, f**), and ERCC1 (**g**, **h**, **i**) in SCC-25, FaDu, and UM-SCC-1. Data in the panel **a**, **d**, and **g** were presented as median and CI 95% and were analyzed by using the Kruskal–Wallis test followed by Dunn's test for multiple comparisons. Data in the panel **b**, **c**, **e**, **f**, **h**, and **i** were presented as mean + SEM, and were analyzed by using one-way ANOVA followed by Tukey's test for multiple comparisons. Asterisks indicate statistically significant differences. Brackets denote specific pairwise comparisons; in their absence, comparisons are versus vehicle control. *p < 0.05; ***p < 0.001; ****p < 0.0001

are reported in figure S4, S5, S6 and S7 of supplementary material. For the SCC-25 cells, LAB-A obtained protein expression results for ATP7A and CTR1 (Fig. S4); while LAB-D obtained protein expression results for ATP7A, ABCG2 and CTR1 (Fig. S5). For FaDu cells, LAB-B obtained protein expression results for ABCG2 and CTR1 (Fig. S6). For UM-SCC-1 cells, LAB-A obtained protein expression results for ATP7A, ABCG2 and CTR1 (Fig. S7).

No significant differences in ATP7A, ABCG2, or CTR1 protein expression were observed across treatments in SCC-25 cells (Fig. 10 a-c). In FaDu cells, ABCG2 and CTR1 levels remained unchanged across all conditions (Fig. 10 d, e), while ATP7A protein was not detected

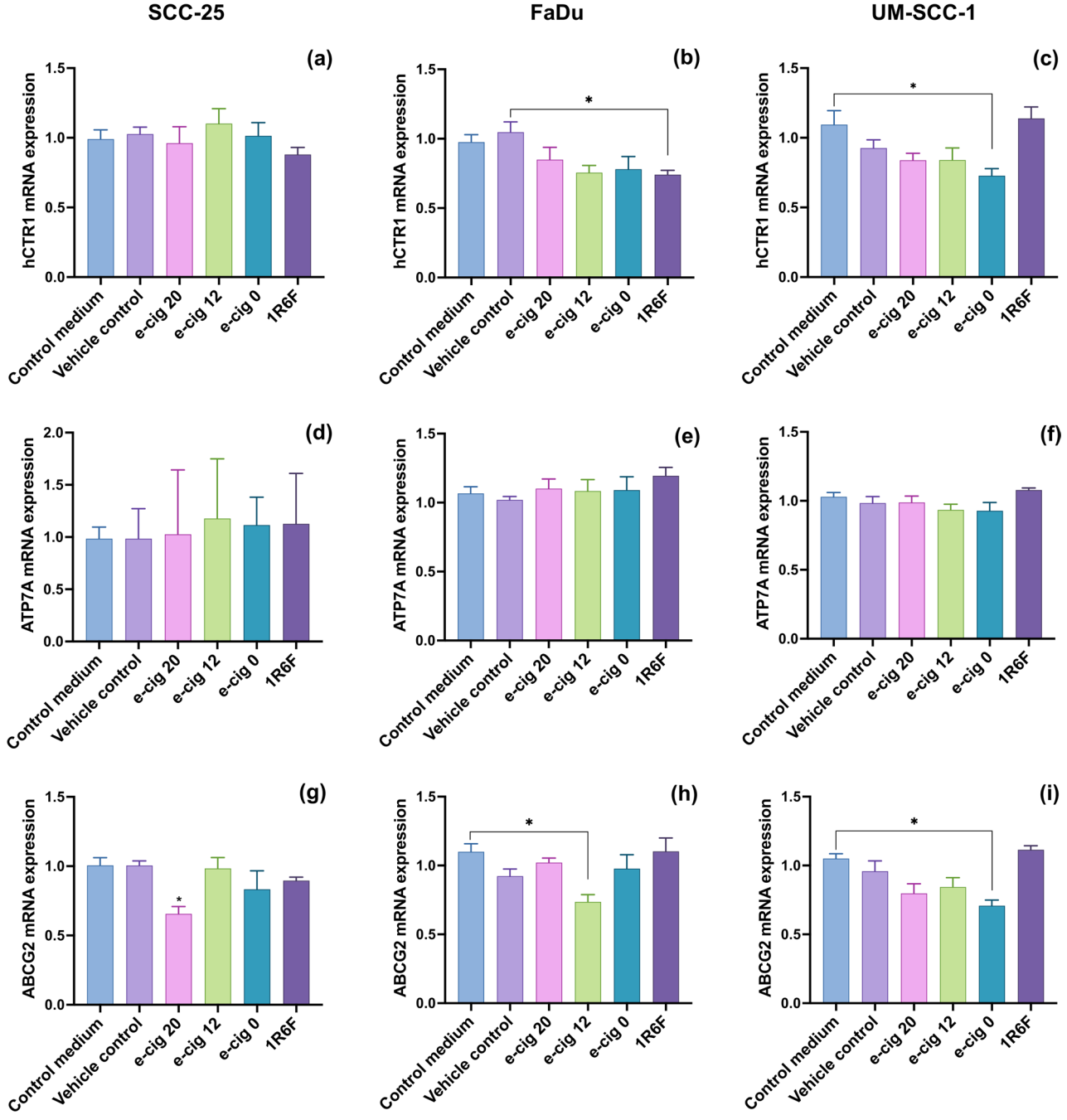

Fig. 8 mRNA expression of drug influx and efflux transporter gene CTR1 (**a**, **b**, **c**), ATP7A (**d**, **e f**), and ABCG2 (**g**, **h**, **i**) in SCC-25, FaDu, and UM-SCC-1 cell lines. Data were presented as mean + SEM, and were analyzed using one-way ANOVA followed by Tukey's test for multiple comparisons. Asterisks indicate statistically significant differences. Brackets denote specific pairwise comparisons; in their absence, comparisons are versus both controls. *p < *0.05*

despite multiple attempts. For UM-SCC-1, results showed selective changes in ATP7A protein expression (Fig. 10f). Notably, significant downregulation of ATP7A was observed in e-cigarette aerosol treatments compared with the control group. Specifically, e-cig 20 nic exposure led to moderate downregulation ($p = 0.0207$), while greater downregulation was observed with e-cig 12 nic ($p = 0.0072$) and e-cig 0 nic ($p = 0.0032$). In contrast, ABCG2 and CTR1 protein levels remained unchanged across all conditions (Fig. 10 g, h).

Compared with Manyanga et al. [13], who reported consistent upregulation of ATP7A, downregulation of CTR1, and robust upregulation of ABCG2 in all cell lines, our study revealed distinct differences: ATP7A

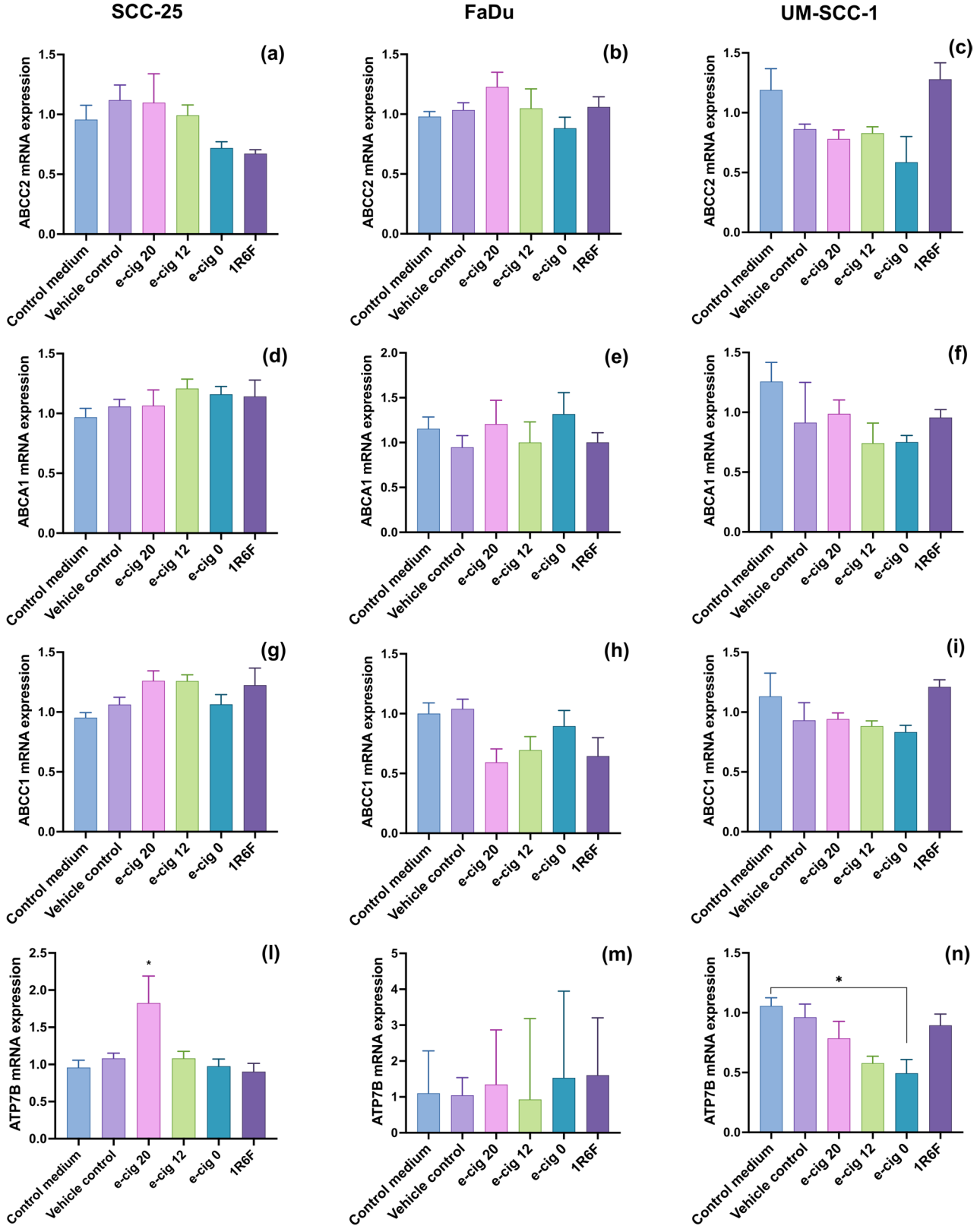

Fig. 9 mRNA expression of drug influx and efflux transporter gene ABCC2 (**a**, **b**, **c**), ABCA1 (**d**, **e**, **f**), ABCC1 (**g**, **h**, **i**), and ATP7B (**l**, **m**, **n**) in SCC-25, FaDu, and UM-SCC-1 cell lines. Data were presented as mean + SEM and were analyzed using one-way ANOVA followed by Tukey's test for multiple comparisons. Asterisks indicate statistically significant differences. Brackets denote specific pairwise comparisons; in their absence, comparisons are versus both controls. * $p < 0.05$

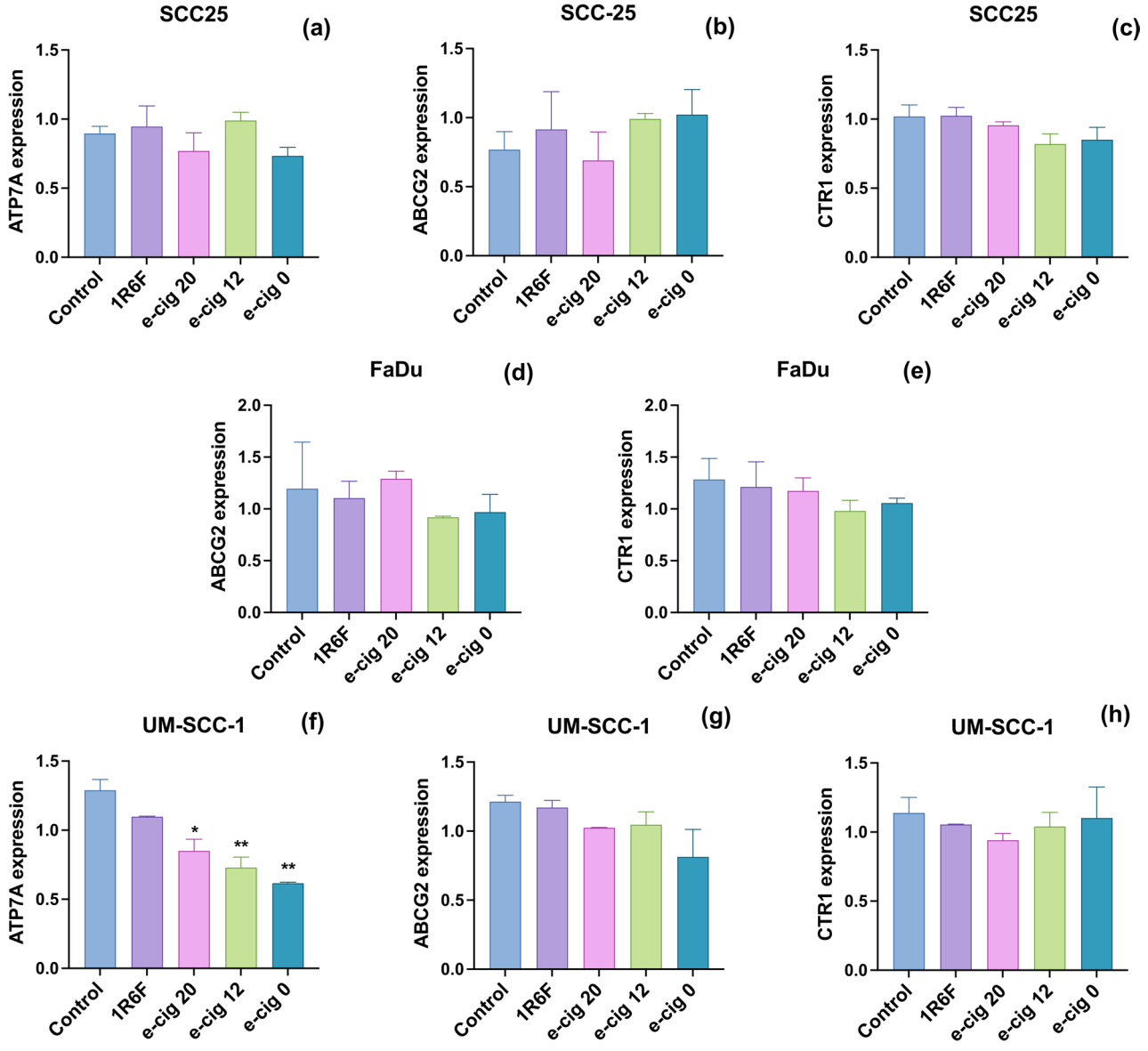

Fig. 10 Protein expression of drug influx and efflux transporter in SCC-25 (**a**, **b**, **c**), FaDu (**d**, **e**), and UM-SCC-1 (**f**, **g**, **h**) cells exposed to 1R6F and e-cigarette at different nicotine concentrations assessed by western blot. Data were presented as mean + SEM, and were analyzed used one-way ANOVA followed by Tukey's test for multiple comparisons. Statistically significant differences are shown versus control. * $p < 0.05$; ** $p < 0.01$

was significantly downregulated in UM-SCC-1 and unchanged in SCC-25, expression of CTR1 remained stable in both cell lines, and ABCG2 showed no significant changes in all conditions.

In cell western blot results

Evaluation by in cell western was performed only by LAB-C. The images of the plate used for quantification are included in the supplementary materials (Fig. S8-S16).

ATP7A expression levels remained stable under all exposure conditions, with no significant differences between treatments in all three cell lines, indicating the stability of efflux transporter levels in this cell line (Fig. 11). A significant increase in ABCG2 protein expression was observed in SCC-25 s following e-cig 12 nic exposure compared with controls ($p < 0.05$). In FaDu, ABCG2 expression was significantly increased in response to aerosols of 1R6F and e-cigarettes at all concentrations compared with controls ($p < 0.05$). In addition, a significant increase was found following exposure to 1R6F ($p < 0.05$). In contrast, no significant changes in ABCG2 expression were observed for UM-SCC-1 under any treatment condition. No significant differences in CTR1 protein expression were observed in SCC-25 and FaDu under any of the experimental conditions.

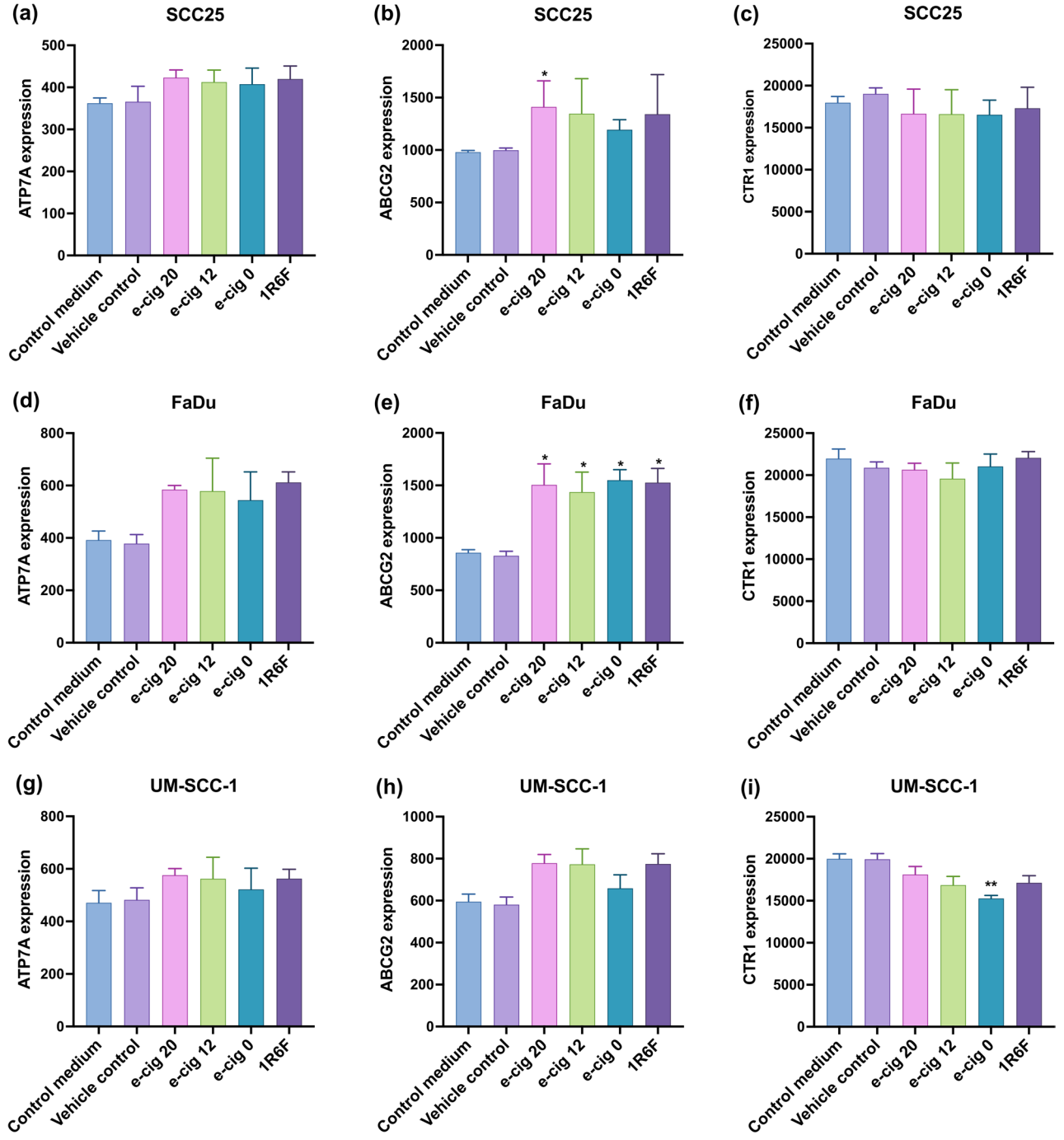

Fig. 11 Protein expression of drug influx and efflux transporter in SCC-25 (**a**, **b**, **c**), FaDu (**d**, **e**, **f**), and UM-SCC-1 (**g**, **h**, **i**) cells exposed to 1R6F and e-cigarette at different nicotine concentrations assessed by in-cell western blot. Data were presented as mean + SEM, and were analyzed using one-way ANOVA followed by Tukey's test for multiple comparisons. Statistically significant differences are shown versus the vehicle control. *$p < 0.05$; **$p < 0.01$

Expression levels remained constant in all treatments, including e-cigarette aerosols and 1R6F. In contrast, significant downregulation of CTR1 was noted in cells exposed to e-cigarette aerosol at 0 mg/ml nicotine compared with controls ($p < 0.01$).

Discussion

Our multicenter study aimed to replicate the study conducted by Manyanga et al. [13] on the effects of both cigarette smoke and e-cig aerosol on cisplatin resistance in head and neck cancer cell lines. Contrary to their

observations, we found no consistent modulation of cisplatin sensitivity in all three cell lines (SCC-25, FaDu, and UM-SCC-1) in response to exposure to e-cigarette aerosol and cigarette smoke (1R6F), although occasional statistically significant differences were observed under specific conditions. The increase in colony formation by e-cigs and 1R6F observed in the original work was also not found in our results. We have also partially replicated some results regarding the expression of genes and proteins associated with drug transporters and DNA repair pathways. While Manyanga et al. [13] reported substantial alterations in gene and protein expression, our results indicate that these effects are sporadic, cell line-specific and limited to selected experimental conditions, without a clear dose–response pattern across the nicotine concentrations tested. Since dose responsiveness is an important hallmark in toxicology, these findings do not support a consistent causal effect of e-cigarette aerosol exposure on cisplatin resistance-related pathways.

Nicotine has been studied as a potential inducer of chemotherapy resistance, as it has been observed that smokers may show increased resistance to treatment [7]. However, interactions between cancer therapies and smoking remain highly complex and not yet fully elucidated [28]. Several papers have investigated how nicotine may influence cell survival and chemotherapy drug resistance through activation of specific signaling pathways and interaction with nicotinic receptors, emphasizing the importance of considering nicotine exposure in patients undergoing chemotherapy. Hsu et al. investigated the effect of nicotine on cell survival and cisplatin resistance in oral carcinoma by highlighting the role of α7-nicotinic acetylcholine receptors. The results showed that nicotine at concentrations ≥ 1 µM increases oral carcinoma cell survival, accelerates cell cycle progression and significantly reduces cisplatin-induced apoptosis [29]. In another study, 1 µM nicotine increased cisplatin resistance through activation of specific signaling pathways [30]. Other studies have also associated nicotine with increased chemotherapy resistance in several tumor types. In nasal carcinoma, it seems to reduce drug-induced apoptosis, impairing treatment efficacy [31]. In lung carcinoma, it appears to modulate mitochondrial signaling and promote cell survival, as well as reduce Bcl-2 ubiquitination and degradation [32, 33]. However, it is important to consider that most of these studies used high and unphysiologically relevant concentrations of nicotine for smokers and e-cigarette users. While concentrations above 1 µM are relevant for determining mechanistic effects in vitro, they do not necessarily reflect physiological exposures. Indeed, clinical studies reported nicotine plasma levels in smokers and vapers ranged from 0.025 to 0.44 µM (4 to 72 ng/ml) [34, 35]. In their work, Manyanga et al. reported nicotine concentrations similar to that observed in the plasma of vapers (5.1—39 ng/ml/0.031 µM—0.241 µM) [13]. Instead, our AqEs had nicotine concentrations between 0.44 and 0.63 µM (72–102.2 ng/ml). These concentrations are slightly higher than those in Manyanga et al.'s study, suggesting more intense or acute exposure to the compounds tested but still plausible for simulating normal to intense exposure scenarios. The main reason for the difference between the two studies lies in the standardization of AqE generation. Unlike Manyanga et al., in our study we used standardized smoking or vaping machines and regimens that provide greater control over experimental conditions. These differences in the exposure methods account for the different nicotine levels obtained between the two studies and underscore the importance of tightly controlled experimental conditions to ensure consistency and reproducibility of results. A further factor that may have contributed to the observed difference in nicotine levels is the use of PBS as trapping solution, rather than the unbuffered saline used in the original study. Unlike saline, PBS maintains a stable pH, whereas unbuffered solutions may undergo pH shifts during aerosol bubbling, potentially influencing nicotine speciation through changes in its protonated and non-protonated forms. PBS was selected as the collection medium to avoid potential confounding effects of serum or other nutrients present in culture medium and to ensure consistency with the method used by Manyanga et al. While consistent with the exposure model of Manyanga et al. (2021), the selected concentration may not encompass the entire variability of realistic human exposure conditions experienced by human airway cells. Importantly, our model relies on an acute, continuous in vitro exposure lasting 24–48 h, which does not fully reproduce the episodic nature of inhalation exposure in humans. In vivo, inhaled aerosol constituents must first penetrate the airway surface liquid, including the mucus layers, which are continuously transported by mucociliary clearance. This dynamic barrier limits both the concentration and the residence time of inhaled constituents at the epithelial surface. In this regard, Bennett et al. [36] reported that acute inhalation of e-cigarette aerosols accelerated mucociliary clearance in a small group of e-cigarette users, a mechanism that would further reduce epithelial contact time. Collectively, these considerations suggest that continuous in vitro exposure may overestimate the duration of direct cellular contact compared to real-world exposure. Accordingly, the dose used in this study was chosen for methodological consistency and reproducibility, but future studies should investigate a broader range of exposure conditions.

Beyond the effect of nicotine alone, it is important to note that cigarette smoke and e-cigarette aerosols are complex mixtures of different substances. Therefore,

it is crucial to assess their health effects by considering the interactions among all aerosolized components, as Manyanga and colleagues have done, albeit with some limitations in the AqE generation that led to substantial differences in our results.

In our study, the exposure to 1R6F or e-cigarette aerosol showed no significant impact on cisplatin-induced cytotoxicity in the three cell lines analyzed, as shown through MTS, NRU and Trypan Blue exclusion assays. Despite some observed variations, as in the case of Trypan Blue staining in UM-SCC-1 cells, the data were not statistically significant. This is in contrast to the results of Manyanga et al. who showed an increase in cisplatin resistance in the presence of e-cigarette aerosol. Like Manyanga's work, Simon and colleagues observed that cigarette smoke condensate reduced the efficacy of cisplatin in head and neck cancer cells (PiCa and FaDu), showing increased cell viability and proliferation. However, this increase was not very pronounced and, as in our work, they did not observe an increase in cell viability for FaDu cells [37]. Moreover, the evaluation of the IC50 values of cisplatin obtained in combination with 1R6F and e-cigarette aerosol was not replicated in our work. Overall, we did not observe a reduction in cisplatin sensitivity in the three head and neck cancer cell lines. For the FaDu line, exposure to 1R6F resulted in increased sensitivity to cisplatin, with significantly lower IC50 values, suggesting a more sensitive response to the combination. However, this could be due to the cytotoxic effect of the combination of cisplatin and combustion byproducts. Also, exposure to e-cigarette aerosol did not produce significant changes in IC50 value in all cell lines except for the MTS evaluation in UM-SCC-1, where a significant decrease in IC50 values was observed compared to cisplatin alone. As observed in Manyanga studies [13, 38], we confirmed that neither e-cigarette extracts alone, nor corresponding nicotine concentrations increased cell proliferation. In fact, both nicotine-containing and nicotine-free e-cigarette extracts produced comparable effects on cisplatin-induced cell death and IC50 values. These results contrast with previous studies that reported increased tumor cell proliferation in response to nicotine exposure [29, 39]. The use of the NRU test in our work deserves special recognition, as it increases the consistency of results in our multicenter study. This test is often used in the evaluation of tobacco products because of its greater sensitivity in detecting cell viability than other commonly used viability tests [40, 41]. Moreover, our results showed high reproducibility in cytotoxicity and chemosensitivity assays, with better performance of NRU than MTS. This high reproducibility is particularly important to emphasize the reliability of these results.

The clonogenic survival assay is a widely used method to assess the ability of cells to proliferate and form colonies after exposure to harmful treatments, such as chemotherapeutic agents or exposure to toxic substances. This technique provides information on the reproductive capacity of cells and is a key indicator of resistance or sensitivity to anticancer treatments [23]. In our study, the clonogenic assay was used to analyze the effect of cisplatin in combination with traditional cigarette (1R6F) and e-cigarette extracts on three HNSCC cell lines. Consistent with the results obtained from cytotoxicity and chemosensitivity assays, the clonogenic results indicate that, in all three cell lines, there was no significant interaction between cisplatin alone and cisplatin combined with 1R6F extracts and e-cigarette aerosol. Furthermore, exposure to 1R6F and e-cigarette aerosol extracts, in the absence of cisplatin, did not significantly alter the clonogenic capacity of the cells. These results differ from those reported by Manyanga et al. [13], who observed that nicotine in e-cigarette aerosols can increase clonogenic survival after cisplatin treatment in HNSCC cell lines. In our study, no such effect was found, even for the UM-SCC-1 line derived from a smoking patient. Unfortunately, there are no other data in the literature investigating the effects of e-cigarette aerosols on colony-forming ability in combination with cisplatin. Our results suggest that the use of e-cigarettes during cisplatin chemotherapy may not increase the risk of clonogenic survival of HNSCC tumor cells. The clonogenic assay reproducibility results indicated that our data are reasonably consistent among the cell lines, particularly for FaDu and UM-SCC-1, where moderate to high reproducibility was observed. While the reproducibility for SCC-25 was more limited, the statistical significance of the ICC values supports the reliability of our results. This reinforces the validity of our conclusions and underscores scores the robustness of our experimental approach. However, discrepancies with Manyanga's results underscore the need for further studies to clarify the effect of nicotine and other components of e-cigarette aerosols on different tumor lines and under varying experimental conditions.

Cisplatin is an intercalating agent known for its ability to bind to purine bases in DNA, generating damage that interferes with repair mechanisms and inducing cell cycle arrest and apoptosis [42]. However, cisplatin resistance is a complex phenomenon, mediated by genetic and epigenetic modifications involving several pathways, including those for DNA repair [43]. Replicating Manyanga's study [13], we evaluated whether exposure to aerosols from cigarettes and e-cigarettes affects the expression of three genes critical for cisplatin-induced DNA damage repair: XPA, MMS19 and ERCC1. Again, our results showed significant differences from Manyanga et al.'s work [13], especially regarding the magnitude and direction of changes in gene expression, particularly for MMS19 and ERCC1. Our results showed sporadic downregulation of

MMS19, limited to the SCC-25 cell line and independent of nicotine concentration, and slight downregulation of ERCC1 under some conditions (SCC-25 exposed to e-cig 0 nic and in FaDu with e-cig 20 nic), but globally stable. Consistent with the results of Manyanga et al. [13], XPA expression was not significantly altered under any of the conditions tested. This finding confirms that the expression of this gene remains stable, even with exposure to cigarette aerosols and e-cigarettes, suggesting that the role of XPA in cisplatin-induced DNA repair may not be directly modulated by these treatments. Unlike Manyanga et al. who reported more pronounced and widespread effects, our results suggest a less uniform influence of aerosols on DNA repair, with variations dependent on cell line and type of exposure. Variability remains a critical factor in reproducibility data, especially for cell lines such as FaDu and UM-SCC-1. However, although reproducibility is not ideal, results showing good to moderate concordance for MMS19 and ERCC1 gene expression are indicative of higher reliability. Moreover, given our rigorous and standardized approach to reduce systematic experimental errors, the variability observed in the FaDu and UM-SCC-1 cell lines could be due to their intrinsic biological response to aerosols, rather than methodological flaws [44].

Membrane transporters play a crucial role in determining the efficacy of cisplatin therapy, influencing both the entry and efflux of the drug from tumor cells. In this study, CTR1 represents the main active transport system for cisplatin entrance into cells, and its expression is directly correlated with intracellular accumulation of the drug and, consequently, with the efficacy of therapy [45, 46]. The efflux transporters ATP7A and ATP7B are P-type ATPases involved in the transport of copper and platinum. In particular, ATP7A plays a key role in the expulsion of cisplatin from cells, and its increased expression has been correlated with reduced response to therapy [47]. Among various members of ABC (ATP-Binding Cassette) family of transporter proteins, ABCG2 seems to be mainly involved in cisplatin resistance associated with tobacco smoke exposure [37, 48]. Regarding mRNA expression of membrane transporters, our study revealed substantially different results from Manyanga et al. While they observed significant upregulation of ATP7A in all cell lines, we found no significant changes in the expression of this gene in either SCC-25, FaDu or UM-SCC-1 cells. For ABCG2, our results show downregulation in all cell lines, in contrast to the significant upregulation reported by Manyanga [13]. Furthermore, unlike Manyanga et al., which observed significant increases in ABCC2, ABCA1 and ABCC1 in at least one cell line, our results showed no alterations in the expression of these transporters under any of the conditions tested. Despite some technical issues associated with signal optimization, Western blot analysis was performed to evaluate the protein expression of the selected cisplatin transporters (ATP7A, ABCG2, and CTR1). The main limitation occurred during the analysis was due to the requirement to perform all the experiments according to the specific materials employed in the original paper. This aspect forced all the LABs involved into the analysis to adapt and align their own methods in order to obtain reliable results to be comparable to original data. Although employing the same specific antibodies and the related chemiluminescence method, the obtained results showed fundamental differences from Manyanga data. ATP7A was downregulated in UM-SCC-1 with e-cigarette treatment and unchanged in SCC-25, while its detection was not noticeable in FaDu cells, probably due to a low basal expression in the pharyngeal cancer cell line. CTR1 levels remained stable in all the cell lines as well as ABCG2 expression showed no significant changes, in contrast to the upregulation reported by Manyanga for all three transporters. Also, in-cell Western blot results performed by LAB-C showed variability among cell lines. Only ABCG2 was upregulated in SCC-25 for e-cig 20 nic and FaDu for all e-cigs but not in UM-SCC-1. Meanwhile, CTR1 was downregulated only for e-cig 0 nic in UM-SCC-1. Our results highlight the variability in transporter regulation among cell lines and suggest that cisplatin resistance is not entirely determined by environmental factors, such as exposure to e-cigarette components. This indicates the need to consider intrinsic cellular factors, such as genetic or metabolic differences, to understand and overcome cisplatin resistance. The strengths of this study lie in its rigorous multicenter design and harmonization of experimental protocols among different international laboratories, which increases the consistency of our results. The use of technical triplicates and multiple experimental sessions contribute to the robustness of the data. In addition, by using standardized operating procedures (SOPs), our research ensured consistency in the experimental process. However, some limitations should be considered. The late acquisition of the UM-SCC-1 cell line by LAB-A resulted in experiments conducted in a single session, reducing the data sample analyzed. The limited application of the in-cell western assay by only one laboratory also reduces the consistency of these results. The replacement of WSU-HN6 and WSU-HN30 cell lines with other alternatives may limit direct comparison with Manyanga's study. The discrepancies observed in our results compared to the original study using WSU-HN6 cells may be explained by significant molecular differences between the two cell lines. SCC-25 cells exhibit lower expression of oncogenes such as EZH2 and TRIM24, reduced invasive potential, and increased intrinsic sensitivity to chemotherapeutic agents like cisplatin, in contrast to WSU-HN6 cells, which are

characterized by a more aggressive phenotype and higher drug resistance [49–51]. FaDu cells, derived from hypopharyngeal carcinoma, exhibit higher invasive potential, stronger expression of immunosuppressive markers such as ITGA5, and a more aggressive tumor phenotype compared to the relatively less invasive WSU-HN30 line [52]. These features may contribute to a differential drug response and underscore the importance of cell line selection in the context of replication studies. Moreover, while the dilution factor was kept identical, our analysis revealed higher nicotine concentrations in the resulting AqEs (ranging from 72 to 103 ng/mL; see Table S1), likely due to the improved efficiency of our vaping machine, the use of a standardized puffing regimen, and differences in device generation and liquid formulation. Importantly, although the original justification by Manyanga et al. for this dilution was to achieve a plasma-relevant nicotine range (0–39 ng/mL), our findings suggest that the real nicotine dose delivered to cells in this model significantly exceeds physiologically realistic concentrations observed in human plasma. Therefore, while the protocol was retained for comparability with the original study, the resulting exposure conditions likely represent a high-dose scenario. Rather than undermining the model, this highlights the importance of cautious dose–response interpretation when aiming for translational relevance.

Finally, acute in vitro exposure does not recapitulate the temporal complexity and cumulative effects of chronic human exposure.

Conclusions

In conclusion, our multicenter data suggest that exposure to e-cigarette aerosols, with and without nicotine, does not consistently promote cisplatin resistance in HNSCC models. The comparable effects observed across nicotine-containing and nicotine-free aerosol extracts further suggest the absence of a clear nicotine-dependent effect in this context. These findings highlight the importance of standardized exposure protocols to ensure reproducibility and underscore the need for further systematic investigations into the impact of e-cigarette use during chemotherapy, with potential implications for clinical guidance. Notably, the variability observed in gene expression responses underscores the relevance of cell-specific effects when evaluating the interaction between aerosol exposure and treatment outcomes. Although our study did not replicate the increase in cisplatin resistance observed in previous studies, it contributes valuable data to the ongoing debate about the nicotine use and cancer therapy, suggesting that more comprehensive investigations are needed to better understand these effects and their clinical implications.

Abbreviations

ABC	ATP-Binding Cassette
AqEs	Aqueous Extracts
BSA	Bovine serum albumin
Cis	cisplatin
CRM81	CORESTA Reference Method n. 81
DMEM	Dulbecco's modified Eagle's Medium
E-cig	E-cigarette
EMEM	Eagle's Minimum Essential Medium
FBS	Fetal bovine serum
HCI	Health Canada Intense
HNSCC	Head and neck squamous cell carcinoma
ICC	Intraclass correlation coefficient
NRU	Neutral Red Uptake
PBS	Phosphate-buffered saline
PE	Plating efficacy
RIPA	Radioimmunoprecipitation assay
ROUT	Robust regression-based outlier rejection test
SEM	Standard error of the mean
SF	Surviving fraction
SOPs	Standardized operating procedures
TBS-T	Tris-buffered saline with 0.1% Tween 20
TOP	Transparency and openness
1R6F	Cigarette smoke

Supplementary Information

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Supplementary Material 1.

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Authors' contributions

R.E.: Writing—Original Draft, Writing—Review & Editing, Methodology, Investigation, Data Curation, Formal analysis, Validation, Visualization; G.C.: Investigation, Writing—Original Draft; K.P.: Writing—Original Draft, Investigation; S.R.: Project administration; A.S.: Supervision, Writing—Review & Editing, Investigation, Data Curation, Validation; A.G.: Supervision, Writing—Review & Editing; V.V.: Supervision, Writing—Review & Editing; R.L.: Supervision, Writing—Review & Editing; H.G.: Investigation, Writing—Review & Editing; M.I.B.: Investigation, Writing—Review & Editing; A.A.: Investigation, Writing—Review & Editing; N.K.: Investigation; B.S.: Investigation; C.G.: Investigation; M.Ca.: Investigation; A.P.: Investigation; M.W.S.: Investigation; A.M.A.: Investigation, Writing—Review & Editing, R.P.: Supervision, Funding acquisition, Writing—Review & Editing; M.C.: Conceptualization, Methodology, Supervision, Writing—Original Draft, Writing—Review & Editing; G.L.V.: Conceptualization, Methodology, Supervision, Writing—Review & Editing.

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Data availability

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

Declarations

Ethics approval and consent to participate
Not applicable.

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Not applicable.

Competing interests
R.P. 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. He has received grants from U-BIOPRED and AIR-PROM, Integral Rheumatology & Immunology Specialists Network (IRIS), Foundation for a Smoke Free World, Pfizer, GlaxoSmithKline, CV Therapeutics, NeuroSearch A/S, Sandoz, Merk Sharp & Dohme, Boehringer Ingelheim, Novartis, Arbi Group Srl., Duska Therapeutics, Forest Laboratories, Ministero dell Università e della Ricerca (MUR) Bando PNRR 3277/2021 (CUP E63C22000900006) and 341/2022 (CUP E63C22002080006), funded by NextGenerationEU of the European Union (EU), and the ministerial grant PON REACT-EU 2021 GREEN- Bando 3411/2021 by Ministero dell Università e della Ricerca (MUR) – PNRR EU Community. He is 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. He receives consultancy fees from Pfizer, Boehringer Ingelheim, Duska Therapeutics, Forest Laboratories, CV Therapeutics, Sermo Inc., GRG Health, Clarivate Analytics, Guidepoint Expert Network, and GLG Group. He receives textbooks royalties from Elsevier. He is also involved in a patent application for ECLAT Srl. He is a pro bono scientific advisor for Lega Italiana Anti Fumo (LIAF) and the International Network of Nicotine Consumers Organizations (INNCO); and he is Chair of the European Technical Committee for Standardization on "Requirements and test methods for emissions of electronic cigarettes" (CEN/TC 437; WG4); and scientific advisor of the non-profit Foundation RIDE2Med. G.LV. is currently elected Director of the Center of Excellence for the acceleration of HArm Reduction (CoEHAR). The other authors have no relevant financial interests to disclose.

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