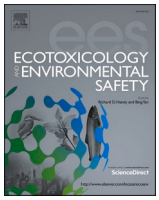
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Genotoxicity and hypomethylation of *LINE-1* induced by electronic cigarettes

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ABSTRACT

Currently, the marketing of electronic cigarettes as a safe alternative to smoking has increased, which is associated with greater use of these devices, especially among young people and smokers interested in quitting tobacco cigarettes. Given the growing use of this type of product, there is a need to determine the consequences of electronic cigarettes on human health, especially since many of the compounds contained in the aerosol and liquid of these devices have a high potential to be carcinogenic and genotoxic. Additionally, many of these compounds' aerosol concentrations exceed the safe limits. We have evaluated the levels of genotoxicity and changes in DNA methylation patterns associated with vaping. We analyzed a total of 90 peripheral blood samples from a population of vapers (n = 32), smokers (n = 18), and controls (n = 32), in which the frequencies of genotoxicity were determined by the cytokinesis-blocking micronuclei (CBMN) assay and the patterns of methylation of the repetitive elements of *LINE-1* through the Quantitative Methylation Specific PCR (qMSP) assay. Here we show an increase in genotoxicity levels associated with vaping habits. Additionally, the group of vapers showed changes at the epigenetic level specifically associated with the loss of methylation of the *LINE-1* elements. These changes in *LINE-1* methylation patterns were reflected in its representative RNA expression detected in vapers.

1. Introduction

Electronic cigarettes are a group of various devices characterized by producing an aerosol after heating a liquid that usually contains solvents, flavorings, nicotine, and other additives that the user will inhale (Polosa et al., 2017). These electronic devices can go by various names such as: "e-cigarettes," "e-cigs," "electronic hookahs," "e-hookahs," "mods," "vapers," "tank systems," Electronic Nicotine Delivery Systems (ENDS) or electronic non-nicotine delivery systems (ENNDS) (U.S. Department of Health and Human Services, 2016). Despite the variety of presentations, design, and complexity, electronic cigarettes have three main components: a battery or power source, a heating element, and a cartridge or reservoir system to contain the liquid (Benowitz and Fraiman, 2017). Approximately 87 chemical compounds have been described as components of these devices, including nicotine, propylene glycol, glycerol, aldehydes, metals, volatile organic compounds, phenolic compounds, and different flavors (Niederbacher et al., 2021). Although the safety of many components such as flavorings in the food industry has been evaluated, their effects when they enter the bloodstream through the respiratory tract are less well known, as are the effects after inhaling solvents such as propylene glycol and glycerol in aerosol form (Stratton et al., 2018). Another concern lies in the secondary products that are generated after heating these compounds to temperatures around 100–250 °C since this can generate pyrolysis of the compounds present in the e-liquid and result in potentially toxic and irritating substances (Niederbacher et al., 2021; Rowell and Tarran, 2015).

Many of the substances present in e-cigarette aerosol have the potential to induce multiple chromosomal alterations, DNA damage, and epigenetic alterations (Martínez et al., 2021). The effect of smoking on

Abbreviations: qMSP, Quantitative Methylation Specific PCR; CBMN, Cytokinesis-blocking micronuclei; LINE-1, long interspersed element-1; MN, micronucleus; NBUDs, nuclear buds; NPB, nucleoplasmic bridges; DEB, -1,3-butadiene diepoxide; PBMCs, peripheral blood mononuclear cells.

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the epigenome has been widely described in humans and has been primarily associated with a reduction in global DNA methylation. The global loss of DNA methylation has been directly associated with the reactivation of latent retrotransposons leading to genomic instability, one of the hallmarks of tumor cells (Lee and Pausova, 2013). Different studies in animal models and cell lines suggest that electronic cigarettes generate transcriptome alteration and increase the probability of developing chronic respiratory diseases similar to tobacco cigarettes (Niederbacher et al., 2021; Shi et al., 2019).

There is very little knowledge regarding the relationship between vaping habits and the genetic and epigenetic impact that they may have. For this reason, this work aimed to evaluate the levels of genotoxicity using the micronucleus biomarker. On the other hand, epigenetic changes were evaluated by quantifying DNA methylation levels in Long interspersed nuclear element 1 (*LINE-1*). Finally, these results were associated with RNA levels expression of *LINE-1*.

2. Materials and methods

2.1. Samples and data collection

The population consisted of 90 volunteer research subjects from Bogotá city, classified as users of Electronic Cigarettes (n = 32), smokers (n = 18), and non-smokers and non-vapers control population research subjects (n = 32). Groups of vapers and controls were matched by age (±1 year) and sex. The group of exclusive smokers was used to compare the genetic and epigenetic impact of using e-cigarettes with conventional tobacco cigarettes. Exclusion criteria for all groups are suspected of having previous exposure to genotoxic agents (heavy metals, fumigants, radiation, oncotherapy drugs), suffering diseases such as COPD and cancer, and having a diagnosis of chromosomal instability syndromes.

The sampling method was non-probabilistic by snowball (Heckathorn, 2011). During the exploration phase, each participant is informed about the research objectives, the possible risks during sample collection, the protection of personal data, and their subsequent authorization by signing the informed consent.

The present study followed The Code of Ethics of the World Medical Association (Declaration of Helsinki), and the Ethics Committee approved all procedures of the Pontificia Universidad Javeriana and the Hospital Universitario San Ignacio (approval no. FM-CIE-0308–20).

Peripheral blood samples were taken from each research subject using the venipuncture procedure. Subsequently, the participants were given a survey in which some demographic, clinical, comorbidities, and consumption variables were evaluated. Once the information and specific samples were collected, each subject was assigned a random code to maintain anonymity throughout the study.

2.2. Serum Cotinine Measurement

Serum was extracted from vapers and smokers from peripheral blood samples obtained in a gel tube (Vacutainer Clot Activator And Gel Tube 5 ml For Serum Separating-1120201). Each tube was centrifuged at 3500 rpm for 15 min, and subsequently, 2 ml of hemolysis-free serum was extracted and frozen (−20 °C). The samples were sent to the service provider (COLCAN-Colombia) to determine the serum cotinine content using the chemiluminescence technique.

2.3. Cytokinesis-blocking micronuclei (CBMN) assay

For the micronucleus test, samples obtained from lymphocyte cultures performed using peripheral blood collected in heparin tubes (BD Vacutainer Plastic Heparin Tubes-367878) were used. Each of the research subjects underwent at least two cultures of lymphocytes. Ten drops of blood were cultured in 5 ml of RPMI 1640 culture medium (Gibco,#31800022) supplemented at 10% with Fetal Bovine Serum (Gibco,#12657029), penicillin/streptomycin (Gibco,#15140122) and 50 μL of phytohemagglutinin (Gibco, #10576015). After 44 h of culture at 37 °C and 5% CO2, the cultures were subjected to the action of cytochalasin-B (6 ug/ml) (Sigma, C6762–5 mg) for 28 h. After this culture, the cells were harvested, subjected to hypotonic shock for 10 min with a hypotonic solution of 0.075 M KCL, and fixed with a fixative solution (methanol: glacial acetic acid, 6:1). After fixation and extension on a slide, the cells were stained with Giemsa stain (Merck, 109204–0500) for 10 min.

The slides were evaluated double-blind by a single observer in a light microscope with an immersion objective with a total magnification of 1000X. Micronucleus frequency results are shown as the micronucleus (MN) count per 1000 binucleate cells counted, and genotoxicity frequency is defined as the count of MNs, nuclear buds (NBUDs), and nucleoplasmic bridges (NPB) in 1000 binucleate cells following the analysis criteria suggested by Fenech et al.(Fenech, 2007).

2.4. Treatment with DEB

Lymphocyte cultures were made from 5 research subjects, between vapers and controls, to whom, after 24 h of culture, (DEB)− 1,3-butadiene diepoxide (Sigma, #202533–5 g) was applied at a concentration final 100 ng/ml following that described by Arleen et al. (Auerbach, 2015). DEB is a DNA clastogenic chemical agent; therefore, the slides obtained after DEB treatment functioned as a positive internal control for the analysis of the CBMN assay.

2.5. Genomic DNA extraction and bisulfite conversion

Peripheral blood samples from vapers (n = 32),controls (n = 32), and smokers (n = 18) were collected in the EDTA anticoagulant tube (BD Vacutainer® EDTA Tubes 00382903678563). An erythrocyte lysis process was initially performed to separate the leukocyte fraction from the sample to extract genomic DNA. Each blood sample was added to 8 ml of red blood cell lysis buffer and centrifuged at 3000 rpm for 15 min. The extraction protocol was performed with the Quick-DNA™ Miniprep Plus kit (ZYMO Research, D4069), starting with 200 μL of the buffy coat obtained and following the manufacturer's specifications. Bisulfite treatment was performed using 500 ng of genomic DNA extracted following the manufacturer's instructions for the EZ DNA Methylation kit (ZYMO Research, #D5002).

2.6. Quantitative Methylation-Specific PCR (qMSP)

The bisulfite-converted DNA was used as a template in a methylation-specific real-time PCR performed on the Roche Instrument Cobas z 480 analyzer, using oligos explicitly designed for the methylated condition of *LINE-1*: forward 5′-CGCGAGTCGAAGTAGGGC-3 ', reverse 5 ´-ACCCGATTTTCCAAATACGACCG-3′. Specific β-*ACTIN* oligos were used as initial endogenous control of the *"input DNA"*: forward 5 ´-TGGTGATGGAGGAGGTTTAGTAAGT-3′, reverse 5 ´ -AACCAA-TAAAACCACTCCTCCCTTAA-3′. qMSP reactions were performed with a final volume of 10 μL and implemented FastStart SYBR Green Master (Roche, #4673484001), methylated *LINE-1* specific primers, and 2 μL bisulfite modified genomic DNA. The qMSP assays were validated using the methylated and non-methylated control set (Human Methylated and Non-methylated DNA set D5014-Zymo research). CT data were normalized using poor primers in CG sites of the *β-ACTIN* gene by the ΔCT method and compared against the methylated control by the 2-ΔΔCT method.

2.7. Isolation of PBMCs by Ficoll-Paque density gradient centrifugation

For the LINE-1 expression analysis, 50% of the subjects from both groups were selected and isolated their peripheral blood mononuclear cells (PBMCs). PBMCs were isolated by density gradient using Ficoll-

Paque PLUS (Cytiva, #10303396), following the manufacturer's instructions. Briefly, the blood EDTA anticoagulant tube (BD Vacutainer® EDTA Tubes 00382903678563) was centrifuged at 3000 rpm for 10 min, and the upper layer containing plasma was removed. The remaining blood was diluted with an equal volume of phosphate-buffered saline (PBS). 7 ml of diluted blood was placed on 3 ml of Ficoll-Paque PLUS (Cytiva). The gradients were formed by centrifugation at 3000 rpm for 30 min at 15 °C. The PBMCs interface was carefully removed by pipetting and washed twice with PBS by centrifugation at 2000 rpm for 10 min. The supernatant was removed, and the PBMCs were frozen at −80 °C in TRIzol for further RNA extraction (Life Technologies).

2.8. Quantitative real-time PCR (qPCR)

LINE-1 activity can be measured at various points in its life cycle, including RNA expression through the quantification of transcripts encoding its two open reading frames, *ORF1* and *ORF2*. From mononuclear cells isolated from vapers (n = 16), smokers (n = 16), and controls (n = 16), total RNA extraction was performed with TRIzol (Life Technologies) according to the manufacturer's protocol. An equal amount of each sample (2 μg) was used for reverse transcription with the ProtoScript First Strand cDNA Synthesis Kit (New England Biolabs). The results were analyzed by the ΔCt method for relative quantification using GAPDH as a reference gene (housekeeping) fw: 5 ´ -AGAAGGCTGGGGCTCATTTG-3′ and rev: 5 ´ -AGGGGCCTCCA-CAGTCTTC-3′. The sequences of the primers used to amplify *ORF1* were fw: 5 ´ -AGGAAAGCCCATCAGACTAACAGT-3′ and rev: 5 ´ -GGCCTGGTGGTGACAAAATCT-3′, and for *ORF2*, they were fw: 5′-TCATAAAGCAAGTCCTCAGTGACC-3′y rev: 5′ -GGGGTGGA-GAGTTCTGTAGATGTC- 3 ´. Since a lower ΔCt indicates a minor difference with a high expressing gene and thus a higher expression level, the Y-axis in the mRNA expression plots is inverted for a more straightforward interpretation.. The results of the expression are plotted as the median.

2.9. Statistical analysis

Shapiro-Wilk test was performed, which revealed that the data do not follow a normal distribution; for this reason, non-parametric statistical tests were used. To determine differences between demographic, clinical, and consumption variables between each group (vapers and controls), the Mann-Whitney U test was used to compare two independent groups, and the Kruskal-Wallis test when three were compared independent groups. In the micronucleus frequency and genotoxicity analyses between the control and vaping groups, the non-parametric Mann-Whitney Wilcoxon test was implemented. Simple logistic regression analyses were performed to identify the determining factors for high genotoxicity or low levels of *LINE-1* methylation. All logistic regression analyses were performed with this study's two objective outcome variables: 1) High Genotoxicity frequency, defined as the research subjects who presented the highest levels of genotoxicity frequency and who, when distributing the genotoxicity frequency data, correspond to the *Q3-Q4* quartiles. 2) Low *LINE-1* Methylation levels are defined as the 25% of subjects with the lowest *LINE-1* methylation levels (lowest 2-ΔΔCT results) when apportioning the expression data by quartiles corresponding to the *Q1* quartile. All statistical analyses and variable associations were performed in Stata statistical software: Version 16 (StataCorp LLC.). GraphPad Prism version 8.01 (GraphPad Software) was used to create graphs and figures. In all figures, error bars are represented as median and 95% CI; $*, P < 0.05$; $**, p < 0.01$; $***, p < 0.001$, $****, p < 0.0001$.

3. Results

3.1. Characteristics of the study population

The characteristics of the selected population, classified into vapers, smokers, and controls, are shown in Table 1. The median and age range of the vapers was 23 (18−59), of the smokers 24 (19−65), and controls 22 (18−59). The most relevant clinical variables were associated with comorbidities such as asthma, heart disease, bronchitis, and pneumonia, without finding statistically significant differences. The median and range of years of e-cigarette use for the group of vapers was 3.5 (1−10) for exclusive and 2 (0.25–5) for mixed vapers. The median and range of years of smokers' consumption was 9 (3−50). Serum cotinine biomarker levels were quantified to determine the extent of nicotine exposure from e-cigarette aerosol and conventional cigarette smoke. Serum cotinine was assessed to determine nicotine exposure status because, in humans, approximately 80% of absorbed nicotine is metabolized to cotinine (Hukkanen et al., 2005). Both mixed and exclusive vapers presented higher cotinine levels compared to smokers. Some studies have determined plasma and saliva cotinine concentrations in e-cigarette users, with measured concentrations ranging from 0 to 548 ng/ml after multiple puffs (Marsot and Simon, 2016). Rapp J. et al. identified that the levels of cotinine and nicotine present in users of electronic cigarettes are proportional and even higher than those presented by users of conventional cigarettes, demonstrating the potential for nicotine addiction that these devices have (Rapp et al., 2020).

Vapers were given a survey associated with consumption variables regarding electronic cigarettes. According to the type of consumption of electronic cigarettes, the classification identified that 20 of the vapers correspond to exclusive users and 12 to mixed users. Other relevant variables, such as the average number of puffs/day and days of weekly use, were summarized in Table 2. Likewise, essential characteristics of the devices used by vapers were determined, inquiring about the power in watts (W), the type of vape or electronic cigarette, the user's preferred flavorings, and the nicotine presence in the devices. It is important to note that of the total number of vapers included in this study, 96.8% use electronic devices that contain nicotine. However, vapers also alternate their consumption with different device types, such as disposable devices, pre-filled cartridges, tank systems, or modified systems. Finally, it is worth noting that the research subjects belonging to this group have a 46.9% preference for fruit-type flavors, followed by menthol and vanilla flavors.

Table 1
Characteristics of the population associated with clinical and consumption variables.

Variable	Controls (n = 32)	Vapers (n = 32)		Smokers (n = 18)
		Exclusives (n = 20)	Mixed (n = 12)	
Age (Years)[a]	22 (18–59)	23 (20–34)	22 (18–59)	24 (19 – 65)
Sex				
Women	8	6	2	8
Men	24	14	10	11
Comorbidities				
Asthma	1	1	1	-
Heart disease	2	1	-	-
Bronchitis	-	-	1	-
Pneumonia	-	-	1	-
Years of Consumption[a]	-	3.5 (1–10)	2 (0.25–5)	9 (3 – 50)
Serum Cotinine (ng/ml)[a]	-	174 (9–445)	251.5 (9–501)	6,65 (1–33.8)
Pack-year index (PYI)[a]	-	-	1 (0.01 – 30)	2.5 (0.2–57.5)

[a] Results expressed as median (range).

Table 2
Variables associated with consumption in vapers.

Variable	N (%)
Consumption type	
Exclusives	20 (62.5%)
Mixed	12 (37.5%)
Puffs/day	
< 250	19 (59.4%)
250–499	7 (22%)
500–749	3 (9.3%)
750–1000	3 (9.3%)
Days of use	
< 7	10 (31.2%)
7	22 (68.8%)
Watt Device (W)	
< 50	22 (68.7%)
50–100	7 (22%)
> 100	3 (9.3%)
Device Type	
Disposable	14
Capsules or cartridges	19
Tank System	17
Modifed system	12
Flavorings	
Mentholated	11 (34.5%)
Fruit	15 (46.9%)
Vainilla	3 (9.3%)
Others	3 (9.3%)
Use of nicotine	31 (96.8%)

3.2. The increased frequency of genotoxicity is associated with the use of electronic cigarettes

To evaluate the frequency of genotoxicity associated with the habit of vaping, the cytokinesis-blocking micronucleus assay (CBMN) was performed, in which the score of the degree of genomic instability was determined by analyzing the presence of micronuclei (MNs), nucleoplasmic bridges (NPBs), and nuclear buds (NBUDs). Fig. 1A- 1 H corresponds to the images obtained by microscopy that show the most representative nuclear anomalies and findings in the analyzed slides.

Mononuclear cells (Fig. 1 A) have an intact cytoplasm and normal nuclear morphology. Binucleated cells (Fig. 1B) are characterized by having two nuclei bounded by intact nuclear membranes, which are located within the same cytoplasmic boundary and are of equivalent size (Fenech, 2007). MNs have a morphology like the nuclei present in binucleated cells; however, they are characterized by being smaller in size. The MNs should not be connected to or overlapping the main nuclei and should present the same staining intensity as the main nuclei (Fig. 1 C). Some findings include more than one micronucleus per binucleated cell (Fig. 1D). NBUDs are characterized by structures connected to some of the nuclei in a similar way to a protuberance or budding of the nuclear structure (Fig. 1E) (Fenech, 2007). NPBs are continuous structures that join the nuclei of a binucleate cell and have the same staining characteristics as the main nuclei (Fig. 1 F,1 G) (Fenech, 2007).

Considering each characteristic alteration in the CBMN assay, the reading and recognition of MNs, NBUDs, and NPBs were validated using the clastogenic agent diepoxybutane (DEB). DEB significantly increased MN formation and genomic instability markers approximately 2-fold over their untreated controls ($p < 0.05$) (Supplementary Figure 1).

Fig. 1I-1 J shows micronucleus and genotoxicity frequencies in controls, vapers, and smokers. The analyses using the Kruskal-Wallis test showed statistically significant differences between the groups ($p = 0.0021$). Specifically, when comparing groups of vapers and controls, the MN frequency is higher in the group of vapers ($p < 0.05$) (Fig. 1I). Similarly, the genotoxicity frequency also increases in the group of vapers compared to controls not exposed to vaping electronic cigarette aerosol ($p < 0.01$) (Fig. 1 J). To determine if the increases in genotoxicity levels in the group of vapers were associated with concomitant use of conventional cigarettes, we compared the frequency of micronuclei and levels of genotoxicity between mixed and exclusive vapers (Supplementary Figure 2A). No statistically significant differences were found when comparing the genotoxicity levels between the mixed and exclusive vapers groups. When comparing the results between the groups of smokers and controls, statistically significant ($p = 0.0003$) differences are evident, showing higher levels of genotoxicity in the group of smokers. The median and mean total values of the DNA damage parameters obtained for each of the groups by the CBMN assay are shown in Table 3. The median MN result in controls was 11; for vapers, it was 13, and for smokers, it was 16. In addition, median and mean indicators were established for the remaining parameters of the CBMN assay, such as NBUD and NPB.

To identify some factors that increase the probability of presenting high levels of genotoxicity, logistic regression analyses were performed with some clinical and consumption variables. These categorical variables were obtained from the survey results on the vapers group. Each of these categories was established by dividing the data by quartiles. The results in Q3 and Q4 were defined as the high condition according to each variable; these categories are specified in Table 4.

The simple logistic regression analysis results indicate that being an active vaper increases the probability of presenting high genotoxicity by 3.69 times more than non-vapers. Similarly, through bivariate analysis, it was found that the variables of adding nicotine to the devices increased the probability of presenting a high frequency of genotoxicity in the CBMN assay (Table 4).

3.3. Hypomethylation of LINE-1 repeat elements in vapers

To determine LINE-1 methylation percentage, qMSP assays were performed; the percentages of *LINE-1* methylation levels in vapers, smokers, and controls were compared to the methylated control (M Control) to assess whether there are epigenetic changes due to exposure to the aerosol of electronic cigarettes. There are statistically significant differences ($p < 0.01$) in the percentages of methylation of *LINE-1* in the group of vapers compared to the methylated control, observing a decrease in the percentages of methylation of *LINE-1* for this group. In contrast, no significant differences with the methylation control were detected in research subjects designated as controls and smokers. On the other hand, when making a comparison between the group of controls and vapers, *LINE-1* methylation levels in vapers are lower compared to controls with statistically significant differences ($p < 0.05$) (Fig. 2). Moreover, no significant changes were observed in *LINE-1* methylation levels when comparing the group of controls and smokers. These results demonstrate that DNA methylation in *LINE-1* elements is lower in research subjects with a vaping habit. To determine if these changes in the level of DNA methylation evidenced in the group of vapers are a product of the mixed use of these devices with conventional cigarettes, we wanted to compare the methylation levels between mixed and exclusive vapers (Supplementary Figure 2B). These results show no statistically significant differences in the percentages of methylation of *LINE-1* between these two groups of vapers. Both mixed and exclusive vapers show decreased DNA methylation in repetitive elements compared to the methylated control.

Beginning at the analysis of the results of the LINE-1 methylation patterns in the group of vapers, we sought to identify which factors increase the probability of presenting low levels of LINE-1 methylation; for this, logistic regression analyses were performed with some clinical and consumption variables (Table 5). The simple logistic regression analysis results indicate that being a vaper increases the probability of presenting low levels of *LINE-1* methylation by 5.44 times more than non-vapers. Similarly, it was found that those vapers that add nicotine to the devices increase the probability of presenting low levels of methylation of the *LINE-1* elements (Table 5). Finally, a new categorical variable defined as high genotoxicity (CBMN assay score $\geq$18/1000 BN cells) was added; this category's logistic regression analyses showed that having

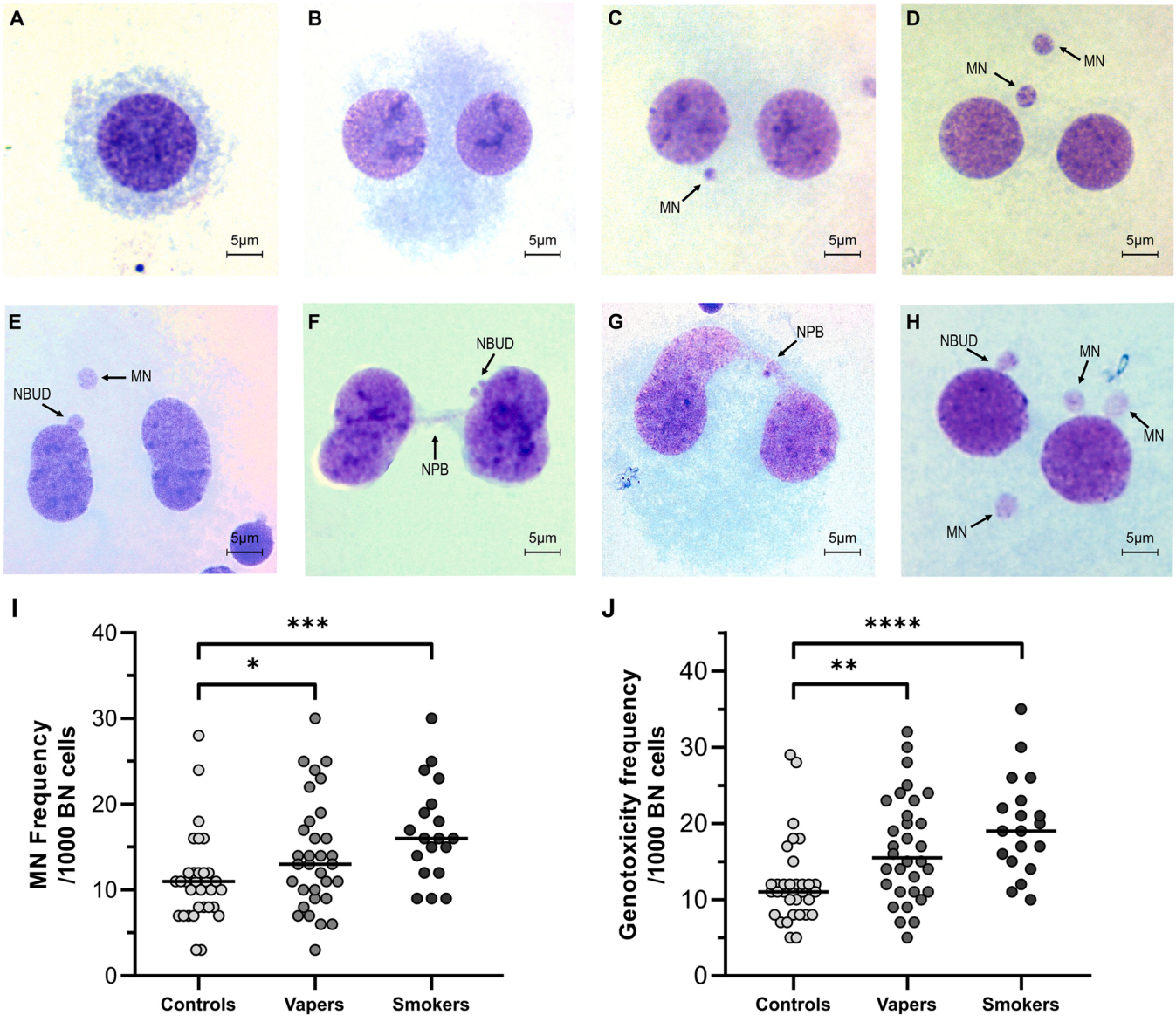


Fig. 1. Frequency of MN and genotoxicity in lymphocytes isolated from controls, vapers and smokers. A-H) Main findings in the CBMN assay in lymphocytes isolated from peripheral blood. The images correspond to cell smears made on slides stained with Giemsa stain. The images were obtained from a light microscope with a total magnification of 1000X with an immersion objective. The cells without evidence of damage at the DNA level include A) Mononuclear cells and B) Binuclear cells. Biomarkers of DNA damage include: C) Binucleated cell with one micronucleus, D) Binucleated cell with two micronuclei, E) Binucleated cell with nuclear bud and one micronucleus, F) Binucleated cell with nucleoplasmic bridge and nuclear buds, G) Binucleated cell with nucleoplasmic bridge, H) Binucleate cell with multiple micronuclei and nuclear buds. I) MN frequency expressed as the number of micronuclei per 1000 binucleated cells, J) Genotoxicity frequency expressed as the number of MN, NBUDs, and NPBs per 1000 binucleated cells. Significance *p-values* are shown as: $*p < 0.05$; $**p < 0.01$; $***p < 0.001$; $****p < 0.0001$. *MN: micronucleus NBUD:nuclear buds NPB: nucleoplasmic bridge.*

Table 3
Total count of CBMN assay parameters in controls, vapers, and smokers.

Category	MNi		NBUDs		NPBs	
	Mean±SD	Median (Range)	Mean±SD	Median (Range)	Mean ± SD	Median (Range)
Controls (n = 32)	11.16 ± 5.19	11 (3–28)	1.72 ± 1.61	1 (0–5)	0.09 ± 0.296	0 (0–1)
Vapers (n = 32)	14.06 ± 6.48	13 (3–28)	1.97 ± 1.94	2 (0 –7)	0.09 ± 0.39	0 (0–2)
Smokers (n = 18)	16.79 ± 5.74	16 (9–30)	2.78 ± 1.5	3 (0–5)	0.1 ± 0.31	0 (0–1)

Results are shown as mean ± deviation and median (range) values for each assay parameter (CBMN) to approximate the total number of micronuclei, nucleoplasmic bridges, and nuclear buds per 1000 cells. MN: micronucleus NBUD: nuclear buds NPB: nucleoplasmic bridges.

Table 4
Bivariate logistic regression analysis of determining factors to present high levels of Genotoxicity.

Variable	Simple Logistic Regression		
	OR	IC 95%	*Valor p*
Active Vaper	3.69	1.13–12.10	**0.031** *
Mixed Vaper	3.86	0.87–17.16	0.076
Device with Nicotine	3.73	1.12–12.39	**0.031** *
Daily consumption Vape	0.31	0.07–1.47	0.140
Cotinine (≥347 ng/ml)	0.39	0.07–2.36	0.308
Puffs/day (≥ 400)	2.34	0.49–11.27	0.288
Years of use (≥5)	0.63	0.09–4.18	0.631
Watts (≥ 80)	0.88	0.14–5.58	0.888

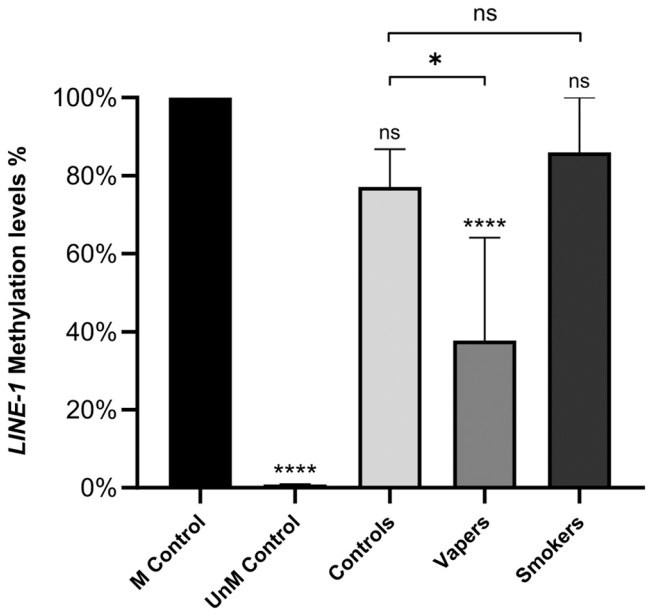


Fig. 2. Percentage of LINE-1 methylation levels in research subjects than controls, vapers, and smokers. Methylation of *LINE-1* relative to the methylated control (M Control). Significance *p-values* are shown as: *$p < 0.05$; * ** *$p < 0.0001$. *ns: Not significant.*

Table 5
Bivariate logistic regression analysis of determining factors for presenting low levels of *LINE-1* methylation.

Variable	Simple Logistic Regression		
	OR	IC 95%	*Valor p*
Active Vaper	5.44	1.55–19.18	**0.008** *
Mixed Vaper	3.5	0.71–17.22	0.123
Device with Nicotine	4.28	1.30–14.14	**0.017** *
Daily consumption Vape	0.69	0.15–3.11	0.632
Cotinine (≥347 ng/ml)	1.4	0.28–6.98	0.681
Puffs/day (≥ 400)	0.55	0.11–2.72	0.460
Years of use (≥5)	1.4	0.28–6.98	0.681
Watts (≥ 80)	0.61	0.12–3.16	0.557
High Genotoxicity (≥18)	11.88	0.38–3.72	0.768

high genotoxicity is not a determining factor for low levels of *LINE-1* methylation.

3.4. LINE1-ORF1 Over-expression in users of electronic cigarettes

A full-length human *LINE1* is 6 kb long and contains a bi-directional promoter in the 5′UTR; two open reading frames, *ORF1* and *ORF2,* and a 3′UTR. *ORF1* encodes a 40KD protein with the chaperone activity to stabilize a new *LINE1* copy (Protasova et al., 2021). *ORF2* encodes 150KD protein with endonuclease and retrotransposase activity (Protasova et al., 2021). In order to evaluate changes RNAm expression of *LINE1* in vaper users, qPCR assays were performed with specific primers in the gene regions ORF1 and ORF2.

As shown in Fig. 3A, *ORF1* mRNA expression significantly increased for the vapers group versus the controls and smokers' group. Thus, it is evident that electronic cigarette users increase the expression of the *ORF1* transcript compared to the expression levels of the control group (non-smokers, non-vapers). These results are like what was found at the level of DNA methylation, where hypomethylation was evidenced in vapers compared to smokers and controls. To identify whether these changes at the *ORF1* expression level were related to being a mixed user of electronic and conventional cigarettes, we compared the expression at the *ORF1* mRNA level between mixed and exclusive vapers and found no statistically significant differences (Supplementary Figure 2C). Regarding the expression analysis of the *ORF2* transcript, the results show that the expression does not show a significant change between controls and vapers (Fig. 3B). However, it is essential to note that results show increased RNAm expression in the vapers group.

4. Discussion

The rapid growth of the electronic cigarette industry results from the various promotional strategies used when referring to these devices. E-cigarettes are seen as a smoking cessation strategy and an engaging user experience. They allow for personalization and choice within the vast catalog of flavors and device variants. Given the growing use of this product, especially among the young population, there is a need to determine the effects of electronic cigarette exposure consequences on health. Many of the compounds in the aerosol have a high genotoxic potential, and exposure concentrations to these compounds normally exceed the limits established as safe for occupational exposure (Ebersole et al., 2020).

In the case of tobacco smoke, it is known that this corresponds to a mixture of chemical substances, among which are carcinogenic agents, many of which induce DNA damage. As a result of this damage and a concomitant failure in the repair, mechanisms generate alterations in genes whose mutation facilitates tumor establishment and progression. These somatic mutations are known as type *"Drivers,"* which explain one of the reasons why smoking has been linked to approximately 17 different types of cancer (Alexandrov et al., 2016; Yamaguchi, 2019). However, many of these chemical agents induce cancer through other molecular mechanisms.Some chemical substances have been called non-genotoxic carcinogens, corresponding to diverse compounds associated with tumor pathologies. Their mechanism of action is mainly linked to epigenetic mechanisms (Ravegnini et al., 2015). Among the epigenetic changes, the most related to smoking is DNA methylation; due to this exposure, states of global hypomethylation have been described. Global hypomethylation facilitates genomic instability, increases susceptibility to mutations, and is associated with retro-transposition element reactivation and oncogene activation (Koturbash et al., 2011). As a first approximation to determine the effects of aerosol exposure from electronic cigarettes, one can take it as a starting point to assess the known effects of conventional tobacco cigarettes at genetic and epigenetic levels.

Our results show that subjects exposed to e-cigarette aerosol have a higher MN frequency and genotoxicity marks (MN, NBUDs, NPBs) than unexposed controls. Some findings observed in the animal model of rats exposed to the aerosol of electronic cigarettes showed oxidative damage to DNA in the lungs through the identification of 8-hydroxy-2′-deoxyguanosine (8-OHdG). Likewise, using the comet and micronucleus assay, the damage at the peripheral blood level was evaluated in these animal models, observing an increase in genotoxic damage in the rats exposed to the aerosol of these devices (Canistro et al., 2017). In an in vitro study on HaCaT (Keratinocytes), UMSCC10B (Laryngeal Squamous Cell

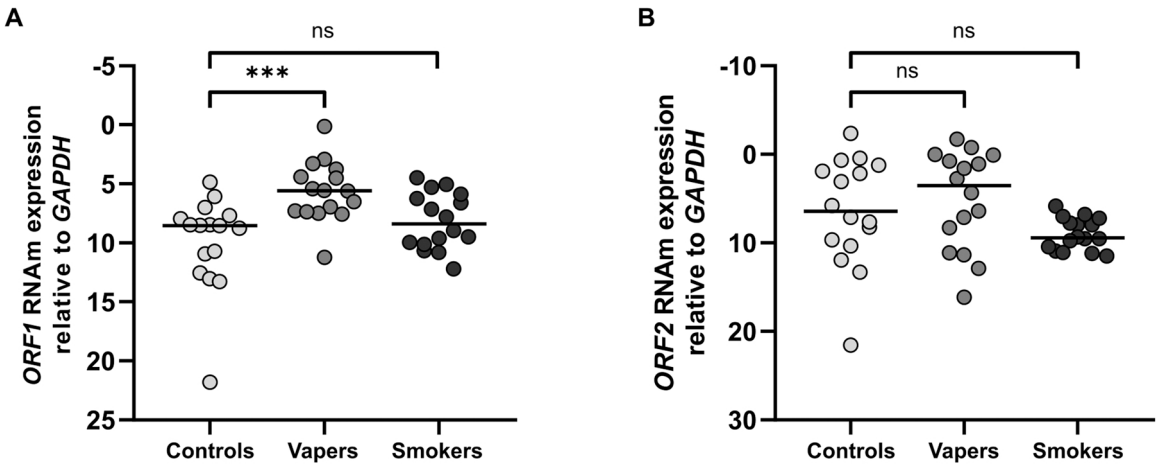


Fig. 3. Quantitative expression of *ORF1* and *ORF2* transcripts. mRNA expression comparison of A) *ORF1* and B) *ORF2* transcripts relative to *GAPDH* between controls, vapers and smokers. The lines represent the median expression of each analyzed group. Significance *p-values* are shown as: * **$p < 0.001$. *ns: Not significant.*

Carcinoma) and HN30 (Pharyngeal Squamous Cell Carcinoma and Oral Cavity) cell lines exposed to aerosol extracts from nicotine- and non-nicotine-containing devices, revealed the existence of high levels of genotoxicity through the comet assay and the detection of histone variant γ-H2AX, indicating increased DNA breaks (Yu et al., 2016).

One of the reasons for an increase in DNA damage is directly focused on the chemical substances and compounds present in the aerosol of these devices. Some of the e-cigarette compounds described as genotoxic agents include formaldehyde, metals, tobacco-specific nitrosamines (TNSAs), volatile organic compounds (VOCs), phenolics, polycyclic aromatic hydrocarbons (PAHs), tobacco flavors, and minor alkaloids. Formaldehyde can induce N-hydroxymethyl monoadducts and N-methylene crosslinks between adjacent purines in DNA. These crosslinks correspond to types of DNA damage potentially severe for cell survival, especially if not repaired by the nucleotide excision repair (NER) pathway (Kawanishi et al., 2014). The metals present in the heating coil of electronic cigarettes have been associated with the generation of monoadducts and single-strand breaks. Furthermore, organic compounds such as nitrosamines and polycyclic aromatic hydrocarbons (PAHs) are characterized by adduct formation on DNA (Barros, 2002) PAHs themselves are biologically inert, and their genotoxic effects require the metabolic activation of biologically reactive intermediates, which covalently modify DNA and are responsible for forming adducts (Huang et al., 2013).

Some compounds present in flavorings, such as cinnamaldehyde, have been associated with a genotoxic potential due to unsaturated α and β aldehydes in their structure; these aldehydes are electrophilic and can react with macromolecules such as DNA. Studies have shown that cinnamaldehyde shows positive results in both *in vitro* and *in vivo* genotoxicity tests (Honma et al., 2021). ROS plays a critical role in generating oxidative damage, DNA double-strand breaks (DSBs), single-strand breaks, and an exacerbated inflammatory response in studies of exposed human and mouse epithelial cells to the aerosol and liquid of electronic cigarettes (Lerner et al., 2015).

Our results show that those research subjects exposed to the aerosol from electronic devices have higher genotoxicity levels than unexposed people. These differences are probably because the consumption of electronic cigarettes increases exposure to each of these chemical agents with genotoxic potential (Hiemstra, 2012).

Our results in the vaping subjects showed a significant loss of DNA methylation levels in the *LINE-1* repetitive elements compared to controls. Considering that repetitive elements in DNA represent almost 50% of the human genome, they turn out to be an indicator of more than a third of global methylation levels (Yang et al., 2004). DNA methylation plays a role in protecting the integrity of the genome by repressing the activity of mobile transposable elements. These transposable elements usually are hypermethylated, which results in their transcriptional silencing. Since the transposition machinery requires enzymes encoded within transposable elements, transcriptional repression of these elements effectively prevents translocation and deletions events (Lee and Pausova, 2013). These events have indeed been observed in several human cancers. A second consequence of DNA hypomethylation of repetitive sequences could be increased mitotic recombination which requires the open chromatin structure usually associated with hypomethylated DNA (Florl et al., 1999).

The results obtained in this work support the idea that environmental exposure to e-cigarette aerosol and its associated chemical products correlates with aberrant changes in the epigenome, inducing a loss of methylation in the *LINE-1* repetitive elements. Some studies have shown changes in DNA methylation patterns after exposure to low doses of compounds such as benzene, also present in electronic cigarette aerosol, revealing a significant reduction in *LINE-1* methylation (Bollati et al., 2007). Furthermore, some *in vitro* and animal model studies have also confirmed that carcinogens induce changes in DNA methylation levels in normal tissues similar to those found in tumor cells (Chen et al., 2004).

A study conducted by Caliri et al. provided the first evidence of the association between vaping and DNA methylation loss in humans from peripheral blood samples of vapers, smokers, and non-smokers; it was found to analyze methylation levels (5-mC) in the *LINE − 1* elements by Elisa assays. The results showed a significant decrease in methylation (5-mC) in the *LINE-1* elements of vapers by 18% and smokers by 13%, compared to control non-smokers, with no significant difference between vapers and smokers. Additionally, demethylation was quantified by global DNA hydroxymethylation (5-hmC) quantification, showing a decrease in both vapers (66%) and tobacco smokers (88%) (Caliri et al., 2020).

We also detected an increase in mRNA expression of *ORF1* and *ORF2* in samples of the vapers group, being significant in the expression levels of *ORF1*. Potential biological implications of these results are currently under extensive investigation by our group, as the increase of *ORF1* and *ORF2* may impact its activation and may be associated with various pathologies, such as COPD (chronic obstructive pulmonary disease), cancer and some autoimmune and neuropsychiatric disease (Protasova et al., 2021). Previous reports in the literature have established that the translation of ORF1 and ORF2 proteins are fundamental for the phenomenon of transposition in the genome to occur, and that this event is closely related to genomic instability processes. In this study, we only evaluate changes in mRNA expression , and for future studies it would be interesting to evaluate protein expression.

Interestingly, in the group of smokers, the results showed a high

percentage of genotoxicity represented in a significant increase in the number of micronuclei. However, qMSP DNA methylation assays for *LINE-1* showed no loss in methylation levels. We also analyzed mRNA expression of ORF1 and ORF2 in samples of the smokers' group ,and we have not found significant changes in the expression levels regarding control group. Our findings may implicate the epigenetic silencing of *LINE-1* in smokers. However, our results should be considered exploratory at this stage due to the relatively small number of persons who were analyzed. Previous reports examining the blood of smokers have reported differential methylation of loci within the aryl hydrocarbon receptor repressor (AHRR) gene, a tumor suppressor which mediates the detoxification of products in cigarettes, and the coagulation factor II (thrombin) receptor-like 3 (F2RL3) gene, implicated in blood clotting (Barrow et al., 2017).

The results shown in this work show, for the methylation analyses, the impact of electronic cigarette use at the epigenetic level, showing a possible influence of the aerosol of these devices on the epigenetic mechanisms associated with DNA methylation.

5. Conclusion

Currently, very few studies determine the biological impact of electronic cigarettes; however, the reports in the literature that aim to estimate the magnitude of the health consequences of these devices are based mainly on animal models and studies of cell lines. Our experiments are based on peripheral blood samples from research subjects corresponding to vapers, smokers, and control groups. Genotoxicity analyses were carried out through the CBMN assay and DNA methylation analysis in the *LINE-1* regions. Therefore, these results correspond to a first approximation of electronic cigarettes' genetic and epigenetic impact.

We have shown an increase in the levels of genotoxicity due to the use of electronic devices compared to the control research subjects, demonstrating that vaping corresponds to a risk factor for high levels of genotoxicity. Additionally, due to exposure to the aerosol of electronic cigarettes, significant epigenetic alterations are generated, especially those related to the loss of methylation of the *LINE-1* elements in the population of vapers. These epigenetic alterations could explain the increases in mRNA expression levels detected for *ORF1* and *ORF2* transcripts in vape users.

Our findings highlight the importance of studying the genetic and epigenetic effects of vaping to determine the health consequences of e-cigarette use. This work provides scientific evidence that electronic cigarettes are not entirely harmless and that despite their diversity of characteristics, they share potential risks.

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Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Adriana Rojas reports financial support was provided by Asociación Colombiana de neumología y cirugía de tórax-ASONEUMOCITO.

Data Availability

Data will be made available on request.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2023.114900.

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