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Electronic cigarettes containing nicotine increase endothelial and platelet derived extracellular vesicles in healthy volunteers

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**HIGHLIGHTS**

\begin{itemize}
  \item The vascular effects of e-cigarettes with and without nicotine were investigated in healthy volunteers.
  \item Circulating extracellular vesicles (EVs) of endothelial and platelet origin were assessed.
  \item Thirty puffs of nicotine-containing e-cigarette vapor caused an increase in EVs of endothelial and platelet origin.
  \item Following exposure to e-cigarettes without nicotine only platelet derived EVs expressing CD40 ligand were increased.
  \item E-cigarettes cause stress to endothelial and platelet cells similar to what has been observed following cigarette smoking.
  \item This effect seems mainly to be driven by the addition of nicotine in the e-cigarette vapor.
\end{itemize}

**ARTICLE INFO**

*Keywords:* Electronic cigarette e-cigarette Extracellular vesicles Endothelial microvesicles Platelet microvesicles Microparticles

**ABSTRACT**

**Background and aims:** E-cigarette use is increasingly common. Whether e-cigarettes are harmful to human health is an intensely debated subject. In order to investigate whether e-cigarettes with and without nicotine cause different vascular responses, we obtained blood samples from healthy young volunteers who performed brief active e-cigarette inhalations. Extracellular vesicles (EVs) of endothelial and platelet origin were measured to determine vascular changes.

**Methods:** Using a randomized, double-blind, crossover design, 17 healthy occasional smokers inhaled 30 puffs of e-cigarette vapor during 30 min. Blood samples were collected at baseline, as well as at 0, 2, 4 and 6 h post-exposure. EVs from platelets and endothelial cells were measured by flow cytometry.

**Results:** Platelet and endothelial derived EVs were significantly increased with peak levels seen at 4 h following exposure to active inhalation of e-cigarette vapor with nicotine. Moreover, platelet derived EVs, expressing platelet activation marker P-selectin and the inflammation marker, CD40 ligand, were also significantly increased following inhalation of e-cigarette vapor with nicotine. In addition, platelet derived EVs expressing CD40 ligand was increased after inhalation of e-cigarette vapor without nicotine.

**Conclusion:** As few as 30 puffs of nicotine-containing e-cigarette vapor caused an increase in levels of circulating EVs of endothelial and platelet origin, which may signify underlying vascular changes. Although e-cigarette vapor without nicotine caused an increase in platelet EVs expressing CD40 ligand, nicotine, as a component in the vapor, seems to have a more compelling effect on extracellular vesicle formation and protein composition.

1. **Introduction**

It has long been acknowledged that conventional cigarette smoking is attributed to several adverse health effects including respiratory and cardiovascular disease. Following the 2003 patent of the modern electronic cigarette (e-cigarette) it was quickly launched on a global scale. This new product was vigorously marketed as a safer alternative to conventional cigarette smoking, as well as a tool for smoking cessation.
However, its efficacy as a smoking cessation aid is highly debated and the risk of dual use of both cigarettes and e-cigarettes has been proven to be elevated [1–3].

E-cigarettes all consist of a mouthpiece, a cartridge (liquid tank) and a battery powered heating atomizer. In the third and fourth generation models, these systems come with various adjustable settings in order to change the efficacy and characteristics of the e-cigarette vapor. There are thousands of different e-liquids on the market, however they share a similar base, which primarily consists of a mixture of propylene glycol and vegetable glycerin. The atomizer vaporizes this base liquid (e-liquid), which can contain varying amounts of nicotine and numerous flavorings. The e-cigarette user inhales the vapor from the e-cigarette (vaping) in a comparable manner as during conventional cigarette smoking [4]. E-cigarette usage is increasing in popularity worldwide, with high sales margins that are estimated to exceed the conventional cigarette industry [5]. E-cigarettes have proven particularly popular among adolescents, with marketing campaigns as well as flavorings designed to appeal to a very young crowd [6].

E-cigarette usage has been linked to various acute adverse physiological changes in humans. These include increased airway inflammation, increased airway obstruction as well as increased levels of endothelial progenitor cells (indicative of vascular changes) and arterial stiffness (an important independent risk factor for future cardiovascular disease) [7–11].

Extracellular vesicles (EVs), also known as microparticles or microvesicles are membrane bound vesicles that are released into the circulation upon cell activation and/or apoptosis [12]. EVs can expose and harbour biologically active molecules and seem actively involved in the homeostatic regulation [13,14]. All types of cells, such as endothelial cells, leukocytes as well as platelets, release EVs upon stimulation [15].

The majority (roughly 60%) of the EVs in the blood of healthy individuals, consist of platelet derived EVs, with elevated levels found in patients with stroke, acute coronary syndrome and peripheral arterial disease [16].

Endothelial cell (EC) derived EVs are considered an emerging biomarker for endothelial dysfunction and are pivotal in vascular injury and angiogenesis as well as thrombosis [17]. Increased levels of EC derived EVs are not only associated with endothelial dysfunction, but also increased arterial stiffness as well as a variety of vascular diseases including acute coronary syndrome and severe hypertension with end organ damage [9,17]. Moreover, long term conventional cigarette smoking has been linked to increased levels of EC derived EVs and the elevated levels are indicative of early lung destruction in otherwise healthy cigarette smokers [18].

In the present study we investigate whether e-cigarette use may cause similar vascular effects as demonstrated following cigarette smoking, as we and other groups have previously demonstrated [19,20]. Moreover, we investigate if nicotine is the main component for the adverse vascular effects. Briefly, seventeen healthy volunteers inhaled 30 e-cigarette puffs with or without nicotine. Extracellular vesicles of platelet and endothelial cell origin was measured by flow cytometry in blood samples taken at various timepoints both before and after each exposure.

2. Materials and methods

2.1. Study design and subjects

Seventeen healthy young occasional smokers (maximum of 10 cigarettes per month) where included in the present study. The participants inhaled e-cigarette vapor with or without nicotine for 30 min with a randomized, double-blind, crossover design (Fig. 1). Exposures and measurements were performed on two separate occasions, with a wash out period of one week. Volunteers had to abstain from caffeine and alcohol for 12 h prior to the study and from heavy exercise for at least 24 h. Nicotine containing products (including cigarettes, e-cigarettes or Swedish snus) were not allowed 14 days prior to the study. All volunteers underwent a clinical examination upon inclusion, including dynamic spirometry, blood pressure, ECG, pregnancy test and routine blood tests (electrolytes, full blood count, apolipoproteins, creatinine, HbA1c, aPTT and PT). The exclusion criteria were any form of respiratory, cardiovascular, systemic or chronic disease, symptoms of infection or inflammation within two weeks prior to study start, pregnancy or BMI ≥ 30.

2.2. E-cigarette exposure

The E-liquid (Valeo laboratories GmbH, Germany) constituted a mix of propylene glycol (49.4%), glycerin (44.4%) and ethanol (5%) without added flavors. Pre-mixed e-liquids with and without nicotine were used (19 mg/ml and 0 mg/ml resp.). A third-generation e-cigarette with adjustable settings was used (eVic-VT, Shenzhen Joyetech Co., Ltd., China). In all exposures, the same settings were applied (temperature 230 °C, effect 32 W, resistance 0.20 Ω). An atomizer with dual nickel coils was used. All exposures took place in the same well ventilated, temperature-controlled room. For 30 min, all volunteers inhaled 30 puffs from the e-cigarette, with each puff lasting approximately 3 s.

2.3. Blood sampling

Blood samples were taken at baseline and again at 0, 2, 4 and 6 h following exposures. Briefly, all blood samples at inclusion were obtained at the same time in the morning (after fasting over night). Blood samples were drawn into citrated tubes with a 17G needle (without tourniquet), after discarding 5 ml blood prior to collecting the sample. Blood samples were then centrifuged within 1 h at 2 000g for 20 min at room temperature (RT) and subsequently stored at −80 °C as platelet-poor plasma (PPP). Macroscopically, no samples showed signs of haemolysis.

2.4. Cotinine measurement

Baseline cotinine levels in blood were measured using a commercially available ELISA method (Calbiotech, Spring valley, CA, US) in accordance with the description of the manufacturer.

2.5. Measurement of extracellular vesicles

PPP was thawed in a water bath (37 °C) for roughly 5 min and centrifuged at 2 000g for 20 min at RT. The supernatant was re-centrifuged at 13 000g for 2 min. Twenty μL of the supernatant was incubated for 20 min together with 5 μl lactadherin-FITC (Haematologic Technologies, Essex Junction, VT, USA) and with 5 μl CD41-PE (platelet activation marker, abcam, Cambridge, UK). In addition, platelet derived EVs were also labelled with 5 μl CD62P-APC (P-selectin; platelet activation marker, abcam, Cambridge, UK) and 5 μl CD154-PC7 (CD40 ligand; platelet activation marker, abcam, Cambridge, UK).

EVs were measured using flow cytometry (Beckman Gallius instrument, Beckman Coulter, Brea, CA, USA). Using Megamix beads (BioCytex, Marseille, France) EV-gate was determined (0.3, 0.5 and 0.9 μm beads) (Fig. 2A). Particles less than 0.9 μm in size and positive for lactadherin (i.e. phosphatidylserine (PS) positive) as well as the antibodies described above, were defined as EVs. Lactadherin was used to detect EVs exposing phosphatidylserine (PS) and has been commonly used as a general EV marker (i.e. EVs from all cell origin). In the present study, all EV data presented are solely performed on PS positive EVs. In order to define background noise of the cytometric analysis, conjugate isotype-matched immunoglobulin (IgG1-FITC, IgG1-PE, IgG1-APC and IgG1-PC7) without reactivity against human antigens was used as a
negative control. In addition, control experiments were performed with Triton X100 (0.1–0.5%) to ensure that Lactadherin binds specifically to PS exposed on vesicles and not cellular debris (data not shown).

Results are presented as EVs/μl plasma, processed from the 20 μl sample prepared for the flow cytometric analysis. The intra- and interassay coefficients of the flow cytometric analysis were less than 9.0% respectively. Representative dot-plots of platelet derived EVs and EC derived EVs are shown in at baseline and 4 h are shown in Fig. 2B.
2.6. Statistical analysis

Statistical analyses were performed with GraphPad Prism 8.0 (GraphPad Software Inc., CA, US) and JMP v14.1 (SAS Institute Inc., NC, US). Data was checked for normality visually and by Shapiro-Wilk test prior to the analysis. Two-way ANOVA was performed between time point 0 h and 6 h. Baseline value is omitted from the statistical analysis as the EVs concentration before e-cigarette exposure could differ due to circumstance not related to e-cigarettes. However, the baseline value is shown in all figures. p-values of < 0.05 were considered statistically significant. All statistical analyses were performed by a single blinded investigator. The study had approval from the local Ethics Review Board in Umeå and was performed in accordance to the Declaration of Helsinki with the written informed consent of all participants.

3. Results

Due to detected cotinine values at baseline, two volunteers were excluded from further analysis. Fifteen occasional cigarette smokers (6 males, 9 females, with a mean age of 26 ± 3 years) were included into the analysis. Prior to the study, blood pressure, heart rate, BMI and waist circumference were measured and routine blood samples were collected. Subject characteristics are shown in Table 1.

### 3.1. Extracellular vesicles

EVs were analysed by flow cytometry and phenotyped according to protein expression as described in methods. Results are presented both in Table 2 as concentration as well as Figs (3-4). Briefly, EC derived EVs (PS + CD62E+) and platelet derived EVs (PS + CD41+) increased significantly with peak values at 4 h, but only following exposure to e-cigarettes containing nicotine (Fig. 3A–B). Moreover, platelet derived EVs exposing P-selectin (PS + CD41+ + CD62P) or CD40 ligand (PS + CD41+ + CD154) increased significantly as well, again with peak values at 4 h (Fig. 4). Following exposure to nicotine-free e-cigarettes, no changes could be observed in EC and platelet derived EV concentrations, with exception of platelet derived EVs exposing CD40 ligand which was significant (Fig. 4).

Two-way, multiple measures ANOVA were significant for nicotine exposure (p = 0.0018 and p = 0.0010) as well as for no nicotine exposure (p = 0.0434).

### Table 1

<table>
<thead>
<tr>
<th>Subject characteristics</th>
<th>Mean</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age [years]</td>
<td>26</td>
<td>3</td>
</tr>
<tr>
<td>Waist [cm]</td>
<td>82.2</td>
<td>8.0</td>
</tr>
<tr>
<td>BMI [kg/m²]</td>
<td>24.4</td>
<td>3.0</td>
</tr>
<tr>
<td>Systolic blood pressure</td>
<td>109.4</td>
<td>9.5</td>
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<tr>
<td>Diastolic blood pressure</td>
<td>70.3</td>
<td>5.7</td>
</tr>
<tr>
<td>Heart rate</td>
<td>65.4</td>
<td>8.5</td>
</tr>
<tr>
<td>Hb [g/L]</td>
<td>136</td>
<td>14</td>
</tr>
<tr>
<td>WBC [x 109/L]</td>
<td>6.2</td>
<td>1.8</td>
</tr>
<tr>
<td>Platelets [x 109/L]</td>
<td>280</td>
<td>53</td>
</tr>
<tr>
<td>K [mmol/L]</td>
<td>4.0</td>
<td>0.2</td>
</tr>
<tr>
<td>Na [mmol/L]</td>
<td>141</td>
<td>1</td>
</tr>
<tr>
<td>Creatinine [μmol/L]</td>
<td>73</td>
<td>14</td>
</tr>
<tr>
<td>HBA1C [mmol/mol]</td>
<td>35</td>
<td>3</td>
</tr>
<tr>
<td>ApoA [g/L]</td>
<td>1.54</td>
<td>0.28</td>
</tr>
<tr>
<td>ApoB [g/L]</td>
<td>0.83</td>
<td>0.19</td>
</tr>
<tr>
<td>ApoB/ApoA ratio</td>
<td>0.55</td>
<td>0.16</td>
</tr>
<tr>
<td>PT/INR</td>
<td>1.0</td>
<td>0.1</td>
</tr>
<tr>
<td>aPPT [s]</td>
<td>29.4</td>
<td>1.7</td>
</tr>
</tbody>
</table>

4. Discussion

In this study, we demonstrated that brief inhalation of e-cigarette vapor containing nicotine caused an increase in extracellular vesicles of platelet and endothelial origin. Increased levels of EC derived EVs and platelet derived EVs are associated with vascular disease including acute coronary syndrome and stroke [21,22]. Furthermore, we observed a significant increase in CD40 ligand expressing platelet derived EVs following e-cigarette inhalation without nicotine.

We have previously demonstrated that brief exposure to conventional cigarette smoke caused increased levels of endothelial progenitor cells (EPC), a stem cell that is mobilized into the circulation upon vascular injury [13]. The smoking exposure also caused increased levels of circulating EVs of leukocyte, endothelial and platelet origin indicating vascular damage, increased thrombosis and inflammation. In order to investigate whether exposure to e-cigarettes induced a similar response, we performed a study where healthy volunteers, very briefly (only 10 puffs), inhaled e-cigarette vapor containing nicotine [8]. This vaporing exposure caused an increase in EPCs in the blood of the same magnitude as previously demonstrated following conventional cigarette smoking. CD62E expressing EC derived EVs increased slightly, but other EVs remained unaffected [8]. We concluded that this may be due to e-cigarette vapor not having the same toxic potential as conventional cigarette smoke. However, we could not exclude that the exposure level we had chosen was too low, considering that the normal exposure in a regular e-cigarette user is around 150–250 puffs per day [23,24]. Furthermore, due to the absence of a nicotine-free control, we could not establish if the observed effect was caused by the nicotine content or another substance in the base-liquid.

Recently, Kerr et al. demonstrated that brief use of nicotine-containing e-cigarettes caused increased levels of EC derived EVs and platelet derived EVs [19]. However, this study did not highlight whether the increase was due to the nicotine content, since no nicotine free control was included. In line with our findings, a small study with 10 volunteers exposed to only 10 puffs of e-cigarette vapor demonstrated increased levels of EC derived EVs following e-cigarette inhalation with nicotine, but not without [20]. Our study confirms this observation and expands that not only the endothelium but also platelets are activated upon inhalation of nicotine.

Chronically elevated levels of EC derived EVs are observed in daily cigarette smokers and smoking cessation leads to a decrease in EC derived EVs in otherwise healthy individuals [25]. Platelet derived EV concentrations, on the other hand, seem to be decreased in cigarette smokers compared to non-smoking controls [26,27]. It is possible that chronic exposure with consecutive repeated platelet derived EV mobilization leads to a depletion of circulating platelet derived EV concentrations and may therefore be seen as a risk factor for a pro-coagulative state. Based on these findings, it is difficult to posit to what degree these effects are attributed to inhaled nicotine and has to be evaluated further in future studies.

Whether nicotine is a harmful substance is heavily debated. Several studies have suggested that nicotine may be directly involved in the development of atherosclerosis, yet some have indicated that nicotine is a rather harmless substance if used by healthy individuals without established cardiovascular disease [28,29]. However, beyond the known sympathomimetic effects, nicotine is also known to cause increased inflammation and arterial stiffness, impair endothelial function, as well as promote atherogenesis and insulin resistance [30–35]. Nicotine may exhibit those effects through mobilization or production of reactive oxygen species in the endothelial wall, resulting in decreased NO bioavailability [36,37]. In a recently published study, we demonstrated that brief e-cigarette inhalation containing nicotine caused increased arterial stiffness, further strengthening the argument that nicotine may have a significant impact on vascular integrity [9].

Investigating other tobacco products such as Swedish snus may be of interest in this query, as it contains high amounts of nicotine and...
smaller amounts of other health hazardous compounds that are associated to combustible tobacco [38]. Hergens et al. demonstrated that Swedish snus users had higher mortality rates following a myocardial infarction or stroke [39,40]. Discontinuation of Swedish snus use following a myocardial infarction was associated with a risk reduction in mortality of almost 50%, a risk reduction that was of the same

Table 2
Extracellular vesicles concentration (EVs/μl) from endothelial and platelet origin following exposure to e-cigarette inhalation with and without nicotine. Results are presented as mean and standard variation (SD).

<table>
<thead>
<tr>
<th>Extracellular vesicles (EVs/μl)</th>
<th>Nicotine (n = 13)</th>
<th>No Nicotine (n = 15)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline 0 h 2 h 4 h 6 h</td>
<td>Baseline 0 h 2 h 4 h 6 h</td>
</tr>
<tr>
<td>EC derived EVs (PS + CD62E +)</td>
<td>26.1 19.9 32.7 48.0 22.4 30.3 20.7 24.9 28.6 25.5</td>
<td></td>
</tr>
<tr>
<td>SD</td>
<td>7.0 4.8 7.7 12.1 5.7 11.8 6.7 8.2 9.1 6.6</td>
<td></td>
</tr>
<tr>
<td>Platelet derived EVs (PS + CD41 +)</td>
<td>239.0 252.2 565.8 961.0 859.4 212.4 393.3 382.6 440.5 532.2</td>
<td></td>
</tr>
<tr>
<td>SD</td>
<td>142.2 168.8 362.7 657.3 608.4 162.9 536.9 371.3 398.5 330.4</td>
<td></td>
</tr>
<tr>
<td>Platelet derived EVs + P-selectin (PS + CD41 + + CD62P)</td>
<td>70.6 96.9 209.4 222.2 195.3 53.8 64.4 80.9 80.7 110.9</td>
<td></td>
</tr>
<tr>
<td>SD</td>
<td>47.3 139.4 289.6 130.0 139.7 47.6 37.5 58.8 46.6 65.7</td>
<td></td>
</tr>
<tr>
<td>Platelet derived EVs + CD40 L (PS + CD41 + + CD154)</td>
<td>45.0 52.5 89.8 132.8 94.6 38.6 39.9 60.6 62.6 83.5</td>
<td></td>
</tr>
<tr>
<td>SD</td>
<td>30.5 46.3 62.0 67.2 27.4 38.7 24.2 45.7 45.0 41.4</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 3. Extracellular vesicles of endothelial (A) and platelet origin (B) (PS + CD62E and PS + CD41) following exposure to e-cigarette inhalation with and without nicotine.

Two-way, multiple measures ANOVA were significant for nicotine exposure ($p = 0.0001$ and $p = 0.0011$).
magnitude as with smoking cessation [41]. Another Scandinavian study demonstrated that Swedish snus use was associated with a significantly increased risk of developing type 2 diabetes [42]. Snus use causes high levels of nicotine in the blood, and the authors suggested that e-cigarettes may very well be the next major health concern, considering the high nicotine exposure associated with e-cigarette use.

Interestingly, the use of nicotine replacement therapy (NRT) seems safe, even in patients with cardiovascular disease [43]. This is most likely due to the relatively slow uptake of nicotine when administered orally or through a dermal patch [44,45]. Beyond what is taken up in the oral mucosa, most of the nicotine in nicotine chewing gum reaches the gastrointestinal tract, where very little is absorbed. Therefore, blood levels of nicotine following intake of NRT increases slowly [44]. During conventional cigarette smoking, nicotine is efficiently absorbed in the respiratory tract causing a high and very rapid increase in blood levels [45,46]. Due to the development of increasingly efficient atomizers in the newer generations of e-cigarette devices as well as the addition of nicotine salts in some base liquids, the swiftness and magnitude of nicotine levels now counterparts what is seen following conventional cigarette smoking [47]. Moreover, in an in vitro model of a respiratory tract, Zhang et al. demonstrated that the emission deposition pattern of e-cigarette vapor was similar to that of conventional cigarette smoking [48].

A slight increase in platelet derived EVs expressing CD40 ligand reached significance also following e-cigarette inhalation without nicotine. There are several components besides nicotine in the e-cigarette fluid and vapor that may cause adverse and toxic effects, including acetaldehydes, polycyclic aromatic hydrocarbons (PAHs), reactive oxygen species (ROS), metals, volatile organic compounds as well as fine and ultrafine particles [49]. These pro-inflammatory and carcinogenic toxicants have all been found to be present in the e-cigarette vapor and fluid, although mostly in a lesser degree as compared to conventional cigarette smoke [50,51]. Ultrafine particles are well known air pollutants with the ability to reach the periphery of the bronchial tree and even the systemic circulation where they may cause oxidative stress and inflammation [52]. Today, it is well established that exposure to particulate air pollution is associated with several adverse vascular effects including increased thrombosis and arterial stiffness as well as endothelial dysfunction. Furthermore, a lower threshold level for where these effects cease to exist has yet to be established [53]. Therefore, it is not surprising to see that e-cigarette inhalation without nicotine also has an adverse physiological response.
in the volunteers, but to a much lesser extent than following exposure with nicotine. As results demonstrate, only platelet derived EVs expressing CD40 ligand was affected and not platelet derived EVs expressing P-selectin. P-selectin is a specific marker of platelet activation as compared to CD40 ligand which can also be expressed by t-lymphocytes, although the major source are platelets [54]. As such, future studies including EVs originating from leukocytes should be of interest.

We have previously demonstrated that a very brief exposure to e-cigarette inhalation caused a small increase in EVs of EC. In the present study, with a higher exposure of inhaled nicotine in terms of longer duration and a higher nicotine content, volunteers displayed a more pronounced increased in EC derived EVs, but also in platelet derived EVs. This is analogous to the acute increase in EC and platelet derived EVs associated with brief exposure to conventional cigarette smoke (both active and passive). As of today, there is a lack of data regarding the long-term health effects of e-cigarette use and we can only speculate on the possible effects of chronic e-cigarette use following short-term exposure studies like the present. Therefore, caution should be warranted in advocating this relatively novel product, especially as a smoke cessation aid.

4.1. Limitations

All of the participants of this study were young occasional smokers (maximum of 10 cigarettes per month) who refrained from smoking a minimum of 14 days prior to each study day. However, although the cumulative exposure to cigarette smoking was judged as low, it cannot be excluded that cigarette smoking may have affected baseline-values of the present measurements.

4.2. Conclusions

The current study investigates the acute vascular effects of e-cigarette use, with and without added nicotine, in healthy volunteers, using measurements of extracellular vesicles of endothelial and platelet origin. The findings of this study suggest that the increase in EVs of endothelial and platelet origin as seen following e-cigarette vapor inhalation, can mainly be attributed to the content of added nicotine in the e-liquid. Even at the relatively low exposure employed in the current study, e-cigarette vapor with nicotine seems to cause stress on the endothelial cells and platelets. Therefore, we encourage health professionals to be cautious if and when advocating the use of e-cigarettes, particularly containing nicotine, as smoking cessation aid.

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Declaration of competing interest

The authors declared they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

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