Vaping-induced metabolomic signatures in the circulation of mice are driven by device type, e-liquid, exposure duration and sex

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To the Editor:

Electronic (e)-cigarette devices have evolved rapidly since the modern version was introduced in 2007 [1, 2]. Inhalation of aerosols generated by these devices has been tied to multiple lung diseases, and diseases and pathology outside the lungs, while the underlying mechanisms driving e-cigarette-associated pathology remain unknown [3–7]. With over a hundred vaping devices and thousands of e-liquids on the market, e-cigarettes introduce numerous chemicals first into the lung and then rapidly into the circulation due to the high permeability of alveoli. While e-cigarettes can deliver the same amount of or more nicotine than conventional tobacco [8], and nicotine is known to induce numerous inflammatory, carcinogenic and epigenetic changes, it is unknown what impact the other 30–120 chemicals in e-cigarette aerosols have on the body [6]. Because changes in metabolite profiles in the circulation have been associated with downstream health effects, plasma level changes in metabolites caused by e-cigarette inhalants may have profound effects on long-term health and disease risk. Utilising metabolomic data to assess the effects of different e-cigarette devices in biological systems may illuminate what downstream pathology is likely to occur secondary to chronic inhalation of e-cigarette aerosols.

E-cigarette devices were purchased from popular online stores (Kanger Mini-protank glassomizers, 1.5 Ω coils, Kanger eVOD Variable Voltage 1000-mA·h battery). For vape pens, 50/50 propylene glycol (PG)/glycerine (Gly) with 24 $mg \cdot mL^{-1}$ nicotine e-liquids were used (Xtreme Vaping). For Mods, 70/30 PG/Gly with 6 $mg \cdot mL^{-1}$ nicotine was made (Sigma). JUUL Mango and Mint (the most popular flavours in 2018–2019; 30/70 PG/Gly, 59 $mg \cdot mL^{-1}$ nicotinic salts) were bought from the manufacturer.

C57BL/6 male and female 6–8-week-old mice (Harlan) were placed in the inExpose system (Scireq) for 60 min once daily or 20 min three times daily. Power calculations were performed to identify sample size and found 82% power to detect a 50% increase in bronchoalveolar lavage cellularity (SD 33%; the primary outcome for the original study) with a group size of n=6. Thus, groups for all exposures and controls were designed with six mice apiece. Because we also wanted to define differential metabolites across different exposures, such as exposure pattern, e-device type and nicotine exposure, a secondary outcome of metabolomic differences across groups was pursued. Additional power calculation was conducted, following the pipeline suggested by Charan *et al.* [9], and determined the power to be 93.3% for the sample sizes used for these metabolomic analyses. As previously described, 4-s e-cigarette aerosol puffs were generated every 20 s, across all devices [10–13]. Mice underwent intra-aortic blood collection, and plasma was aliquoted and stored at −80°C. All studies were conducted with UCSD Institutional Animal Care and Use Committee approval.

Small polar, lipophilic bioactive metabolites were extracted from plasma samples using organic solvent followed by offline solid-phase extraction as previously described [14, 15]. Metabolites were chromatographically separated (Thermo Vanquish UPLC system, Phenomenex Kinetex C18 (1.7 µm, 100×2.1 mm) column) and mass spectra acquired (Thermo QExactive orbitrap). Metabolites were identified by matching accurate mass, retention time and tandem mass spectrometry fragmentation patterns to an in-house library of commercially available standards [14, 15].

Shareable abstract (@ERSpublications)
Each type of vaping device (vape pen, box Mod and JUUL), as well as nicotine and flavourings, induces a disparate metabolite profile or signature, such that each device and liquid is likely to lead to its own set of health effects https://bit.ly/3eExKzi

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All detected metabolites, including 9163 untargeted metabolites and 14 deuterated molecules as internal standards, were firstly standardised by ((*x*-mean)/SD). A simple unsupervised method, principal component analysis, was then used to evaluate and visualise the batch effect by untargeted metabolites and internal standards markers separately. The coefficient of variation of each internal standards marker was also calculated for batch effect evaluation. Unpaired student t-tests were used to evaluate the difference of metabolites between two candidate groups. p-values were two-sided and <0.05 was considered statistically significant because of the small sample size in each group. Pearson's correlation coefficient was used to measure the strength of the association between two variables in the global pattern of metabolites. All statistical analyses were performed using R version 3.5.2.

We utilised a nontargeted approach to detect potential small polar, lipophilic bioactive metabolites in the plasma of e-cigarette-aerosol exposed mice and controls, and assay a total of 9177 metabolites, including 14 internal standards markers. All samples were grouped together, indicating an absence of significant batch effects. Because each vaping device has different characteristics, in some cases including differences in e-liquids, we first assessed the metabolites within the plasma from mice exposed to individual e-device types.

18 unique metabolites were identified in the plasma of vape pen aerosol-exposed mice. 17 were lower in e-cigarette mice, with one oxylipin/eicosanoid-like metabolite (EIC_356) being elevated. Inhalation of nicotine-free (vehicle) aerosols from Mod devices for 12 weeks led to six unique metabolites, while nicotine containing aerosols (EV) led to seven unique metabolites (figure 1a), with the majority being higher relative to controls. Intriguingly, comparison of EV to vehicle identified 23 significantly different metabolites, with 22 being lower in EV, suggesting a preponderance of nicotine-independent effects. After Bonferroni correction among the three groups, there still existed 12 unique metabolites (the p-value set for significance post-Bonferroni correction is 0.05/3 or p<0.017).

Subacute exposure of 4 weeks led to 17 unique metabolites in vehicle and 23 in EV, with the majority being decreased, compared to controls (figure 1b). EV and vehicle mice had 11 metabolites in common (figure 1b). By merging comparisons from 4- and 12-week exposures, we identified 22 of 23 target metabolites specific for nicotine *versus* vehicle, and 10 of 22 were specific for nicotine at 4 weeks of exposure. When assessing all metabolites, 270 of 345 metabolites were specific for 12 week exposure to nicotine and 113 of 272 were specific for 4 week exposure to nicotine.

Comparing mice exposed to EV generated from Mods for 4 *versus* 12 weeks, there were significant differences in 47 metabolites, with 44 having higher levels at 12 weeks. When vehicles were compared, 48 of 49 metabolites had higher levels at 12 weeks. Interestingly, only one metabolite was shared across the two comparisons (figure 1c).

Mice that inhaled aerosols from JUUL mint *versus* JUUL mango were found to share one metabolite in common, putative β-HC (figure 1d). JUUL Mango was found to have three specific metabolites in the plasma not found in JUUL mint or air controls: Eicosanoid_12,13, Putative_1-Stearoyl-2-hydroxy-sn-glycero-3-phosphoethanolamine and FFA_Heptadecaenoic Acid. When intranasally challenged with lipopolysaccharide (LPS), air mice had 52 differentially targeted metabolites (52 out of 271). Mice exposed daily to JUUL mint prior to LPS challenge had 26 differentially targeted metabolites (26 out of 271), while those exposed to JUUL mango had 52 (52 out of 271). Mint–LPS had six unique metabolites, while mango–LPS had 26 unique metabolites, and the two groups shared five metabolites (figure 1e). Particularly, four differential metabolites still exerted significance with the Bonferroni correction (p<0.05/3). These data suggest that daily inhalation of JUUL aerosols leads to immunomodulation such that the host's inflammatory response to a common clinical challenge is altered.

From the metabolites identified in the plasma of mice exposed to aerosols made by vape pens, Mod and JUUL devices, which were not found in air controls, there were very few differentially targeted metabolites shared by all groups (figure 1f). This indicated that metabolites were group specific. Globally, there was a similar pattern, with no differential metabolites in all groups. There was a high correlation in the metabolic profile of mice exposed to aerosols once daily *versus* three times daily (r=0.93–0.99). This suggests that total aerosol murine exposures have a similar effect on the metabolite profile in the blood, whether the aerosols are administered all at once or spread out over the day.

In female mice who were exposed to e-cigarette aerosols, 18 unique metabolites were identified, while males had 25 unique metabolites. Comparing males to females led to only one sex-independent metabolite (figure 1g). This metabolite was present at low levels in both sexes: Putative_1-Hexadecanoyl-sn-glycero-3-phosphoethanolamine.

FIGURE 1 Changes in circulating metabolites induced by daily, chronic inhalation of aerosols generated from multiple e-devices and e-liquids, and for varying exposure durations. Mice were placed in the inExpose system (Scireq) for 1 h daily. Box Mod, vape pen and pod-based e-devices (JUUL) were activated and negative pressure applied to generate fresh e-cigarette aerosols every 20 s, with puff duration of 4 s across all devices. Plasma

isolated on the day of harvest underwent comprehensive nontargeted, liquid chromatography–mass spectrometry-based metabolomics to assay thousands of circulating bioactive molecules. a) Venn diagram of significant metabolites ($p<0.05$) identified in the plasma of mice that inhaled aerosols generated from box Mod e-cigarette devices. No-nicotine aerosols (vehicle) compared to air controls, nicotine-containing aerosols (EV) *versus* air controls and vehicle *versus* EV after 12 weeks of daily 1-h exposures. b) Venn diagram of significant metabolites ($p<0.05$) identified in comparisons among vehicle *versus* air, EV *versus* air and vehicle *versus* EV after 4 weeks of daily box Mod aerosol exposures. c) Venn diagram of unique and shared metabolites across different time exposures (4 *versus* 12 weeks) for vehicle *versus* EV. d) Metabolites detected in JUUL mango *versus* air, JUUL mint *versus* air and JUUL mango *versus* JUUL mint after 4 weeks of exposure. The red circle indicates the sole metabolite shared between JUUL mango and JUUL mint exposures: putative β-hydrocortisone (HC). e) Venn diagram of unique and shared metabolites in the setting of lipopolysaccharide (LPS) challenge across air, JUUL mango and JUUL mint exposures. f) Identification of different metabolites across all device types and exposure durations with no overlapping metabolites across e-cigarette exposures. Inhalation of JUUL aerosols caused the smallest metabolite signatures, relative to box Mod and vape pen aerosols. The reduced metabolite signatures may be due to Mod and JUUL devices applying different temperatures and wattages to e-liquids. g) Sex effects on metabolite changes induced by 4 weeks of e-cigarette aerosol exposure, with males and females only having one shared metabolite induced by e-cigarette exposure. Plots were developed using the *VennDiagram* package in R.

These data highlight some key points that may assist in the design of future research studies, as well as the development of tobacco policies. They also give insight into the molecular impact of these popular nicotine delivery devices. We found that each type of e-cigarette exposure led to unique metabolite profiles within the circulation of exposed mice. Unique profiles can reveal specific metabolomic signatures that are associated with disease risks; these signatures have been associated with cardiovascular disease and serve as predictors of chronic kidney disease [16, 17].

Specifically, JUUL mango and vape pen mice had different plasma levels of eicosanoids, biological molecules that act as activators and suppressors of inflammation [18]. Pro- and anti-inflammatory eicosanoids have been associated with rheumatologic diseases. More recently, eicosanoid storms have been found to potentially play a role in severe COVID-19 [19]. Though extensive research is still needed, the involvement of eicosanoids in inflammatory physiological processes is concerning, as the immune system constantly balances between an inflammatory and anti-inflammatory state; any disruption of this balance, in either direction, is known to lead to pathology and thus disease.

Chronic inhalation of e-cigarette aerosols induced specific metabolomic signatures in the circulation depending on e-device used, nicotine content, flavourings, sex and duration of exposure. Notably, spacing of exposures had no impact on the chemical signature produced while sex played a major role, with disparate systemic metabolite profiles in males *versus* females exposed to e-cigarette aerosols. Daily inhalation of JUUL aerosols led to numerous metabolite changes occurring in a flavour-specific manner when mice were challenged with inhaled LPS as a model of Gram-negative pneumonia, demonstrating that use of these popular pod-based devices leads to immunomodulation. Finally, the composition of each e-cigarette device and e-liquid, as well as temperature and wattage applied to the e-liquid in the process of vaping and aerosolisation, played a role in the chemical profile produced, which in turn impacted the metabolomic profile of the host.

Thus, these data demonstrate that e-device type, chemical components, duration of exposure and sex all play critical roles in altering metabolomic profiles. This is concerning as unique profiles can reveal specific metabolomic signatures associated with disease risks; these signatures have been associated with cardiovascular disease and serve as predictors of chronic kidney disease [16, 17]. Future work is needed to fully understand the physiological and pathophysiological effects of these e-cigarette aerosol exposure-related metabolomic changes within humans.

Alexander Moshensky[1,2], Mulong Du[3], John Shin[1,2], Ira Advani[1,2], Deepti Gunge[1,2], Denzil Mathew[1,2], Rita Alkolla[1,2], Ashley Du[1,2], Christian Javier[1,2], Lauren Ma[1,2], Albert Tran[1,2], Nicholas Nguyen[1,2], Jarod Olay[1,2], Sedtavut Nilaad[1,2], Jeffrey Ding[4], Mahan Najhawan[4], Jeramie D. Watrous[4], Christine M. Bojanowksi[1,2], Mohit Jain[4], David C. Christiani[3,5] and Laura E. Crotty Alexander[1,2]

[1]Pulmonary Critical Care Section, VA San Diego Healthcare System, La Jolla, CA, USA. [2]Division of Pulmonary, Critical Care and Sleep Medicine, Dept of Medicine, University of California San Diego (UCSD), La Jolla, CA, USA. [3]Dept of Environmental Health, Harvard T.H. Chan School of Public Health, Boston, MA, USA. [4]Depts of Medicine and Pharmacology, UCSD, La Jolla, CA, USA. [5]Division of

Pulmonary and Critical Care Medicine, Dept of Medicine, Massachusetts General Hospital, Boston, MA, USA.

Corresponding author: Laura E. Crotty Alexander (lca@ucsd.edu)

Author contributions: Conception and design of the experiments: A. Moshnesky, M. Du, J. Shin, D. Mathew, J. Ding, M. Najhawan, J.D. Watrous, C.M. Bojanowski, M. Jain, D.C. Christiani and L.E. Crotty Alexander. Acquisition, analysis and interpretation of data: A. Moshensky, M. Du, J. Shin, D. Mathew, R. Alkolla, A. Du, C. Javier, L. Ma, A. Tran, N. Nguyen, J. Olay, S. Nilaad, J. Ding, M. Najhawan, J.D. Watrous, C.M. Bojanowski, M. Jain, D.C. Christiani and L.E. Crotty Alexander. Manuscript composition: A. Moshensky, M. Du, M. Jain, D.C. Christiani and L.E. Crotty Alexander. All authors reviewed, contributed to and approved the manuscript.

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