

Dysregulated Metabolites Serve as Novel Biomarkers for Metabolic Diseases Caused by Vaping and Cigarette Smoking

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Short running title: Metabolites as biomarkers by vaping and smoking

Abstract

Metabolites are essential intermediate products in metabolism, and metabolism dysregulation indicates different types of diseases. Previous studies have shown that cigarette smoke dysregulated metabolites; however, limited information is available with electronic cigarette (E-cig) vaping. We hypothesized that E-cig vaping and cigarette smoking altered systemic metabolites, and we propose to understand the specific metabolic signature between E-cig users and cigarette smokers. Plasma from non-smoker controls, cigarette smokers, and e-cig users were collected, and metabolites were identified by UPLC–MS (Ultraperformance liquid chromatography-mass spectrometer). Nicotine degradation was activated by e-cig vaping and cigarette smoking with increased concentrations of cotinine, cotinine N-oxide, (S)-nicotine, and (R)-6-hydroxynicotine. Additionally, we found significant decreased concentrations in metabolites associated with tricarboxylic acid (TCA) cycle pathways in e-cig users versus cigarette smokers, such as: D-glucose, (2R,3S)-2,3-dimethylmalate, (R)-2-hydroxyglutarate, O-phosphoethanolamine, malathion, D-threo-isocitrate, malic acid, and 4-acetamidobutanoic acid. Cigarette smoking significantly up-regulated sphingolipid metabolites, such as D-sphingosine, ceramide, N-(octadecanoyl)-sphing-4-enine, N-(9Z-octadecenoyl)-sphing-4-enine, and N-[(13Z)-docosenoyl]sphingosine, versus e-cig vaping. Overall, e-cig vaping dysregulated TCA cycle related metabolites while cigarette smoking altered sphingolipid metabolites. Both e-cig and cigarette smoke increased nicotinic metabolites. Therefore, specific metabolic signature altered by e-cig vaping and cigarette smoking could serve as potential systemic biomarkers for early cardiopulmonary diseases.

Keywords: Metabolome, TCA, Lipids, e-cigarette, cigarette, biomarkers

Introduction

E-cig vaping has been increasing rapidly in the United States during recent decades since e-cig is considered a relatively safer alternative to help quit smoking ¹. The e-cig devices deliver aerosolized e-liquid with different concentrations of nicotine. The constituents from e-cig liquid, which are usually propylene glycol (PG) and vegetable glycerin (VG), which are Generally Recognized as Safe (GRAS). Although PG and VG are GRAS, the aerosolized constituents have proven to be toxicants ². It has been known that e-cig delivers more nicotine than cigarette smoke ^{3,4}. Furthermore, we have shown that e-cig vapor contained various chemical constituents that can affect the downstream metabolism ⁵. Cigarette smoke is known to contain thousands of toxic chemicals ⁶. The chemicals generated from e-cig or cigarette smoking as xenobiotic chemicals in human organisms could dysregulate metabolomics profiles ⁷⁻¹⁰ and increase the risk of lung diseases, even lung cancers ¹¹. Commonly, cotinine is one of the significant metabolites during nicotine degradation, which has been used to identify the smoker or e-cig user ¹². We have shown circulating biomarkers are increased from e-cig users or cigarette smokers, predicting the risk of lung and heart diseases ^{13,14}. In this study, we have found e-cig vaping to be more associated with bioenergy synthesis (TCA cycle) than cigarette smoking, while cigarette smoking leads more active the sphingolipid pathway.

Bioenergy synthesis, including gluconeogenesis, glycolysis, and TCA cycle, is one of the major metabolic reactions in mitochondrion for generating energy among all the organs/tissues. Previous studies reported that e-cig vaping and cigarette smoking inhibited bioenergy synthesis and induced mitochondrial dysfunction ^{15,16}.

Mitochondrial metabolism alternation in lungs was followed by cigarette smoke exposure ^{15,16}; e-cig exposure induced mitochondrial-oxidative stress and DNA damage ^{17,18}. Interestingly, a previous study explained that circulated PG would be metabolized into lactic acid in the liver and go through the TCA cycle ¹⁹. However, no study is available to show the bioenergy synthesis-related circulating metabolites in e-cig users and cigarette smokers compared to healthy controls.

Sphingolipids are lipids that contained sphingoid structures and major constituents of plasma membrane^{20, 21}. Recent studies have shown that sphingolipid metabolites regulate pulmonary inflammatory responses, and they are essential mediators in lung cancer^{20, 22}. Cigarette smoke-induced accumulation of sphingolipid metabolites in the lungs is mediated with mitophagy, necroptosis, autophagy, and oxidative stress²²⁻²⁵. Interestingly, previous reports have described dysregulated plasma sphingolipids associated with lung cancer and Chronic Obstructive Pulmonary Disease (COPD) phenotypes^{26, 27}. In this study, we determined the dysregulation of sphingolipid metabolites in plasma from cigarette smokers or e-cig users.

We collected plasma from healthy controls, e-cig users, and cigarette smokers for metabolites analysis. Our results showed that metabolites related to nicotine degradations are both dysregulated in the plasma from e-cig users and cigarette smokers. TCA cycle-related metabolites showed alternation only in the plasma of e-cig users, while sphingolipid metabolites presented dysregulation only in cigarette smokers' plasma.

Results

Global metabolic profiling of plasma from healthy controls, e-cig users, and cigarette smokers analyzed by UPLC–MS.

We performed global metabolites profiling based on negative and positive ion modes to identify dysregulated metabolites in plasma from cigarette smokers and e-cig users through UPLC-MS (**Figure 1. A&B**). During the profiling, a total of 1018 metabolite features were detected in negative ion mode, and 7244 metabolite features were detected in positive ion mode. To determine the significance of metabolomics profiling, we have applied multivariate statistical analysis via the PCA model (**Figure 1. C&D**). In the negative ion mode UPLC-MS measurement, the absolute value of metabolites between the control and cigarette smoking groups are majorly overlapped, while metabolites in the e-cig group show significantly different metabolites distribution (**Figure 1C**). Interestingly, we have found an overlapped metabolites distribution in control and e-cig users' plasma from positive ion mode, and cigarette smokers showed a significant difference in dysregulated metabolites (**Figure 1D**).

We also screened and identified the dysregulated metabolic pathways in cigarette smoke and e-cig groups (**Table 1**). Metabolic pathways, including nicotine degradation III, serotonin degradation, and gluconeogenesis, were altered in both cigarette smoke and e-cig groups. Interestingly, the TCA cycle, D-galactose degradation, and UDP-N-acetyl-D-galactosamine biosynthesis II were found to have dysregulation in the e-cig group, while nicotine degradation IV was altered in the cigarette smoke group.

Figure.1

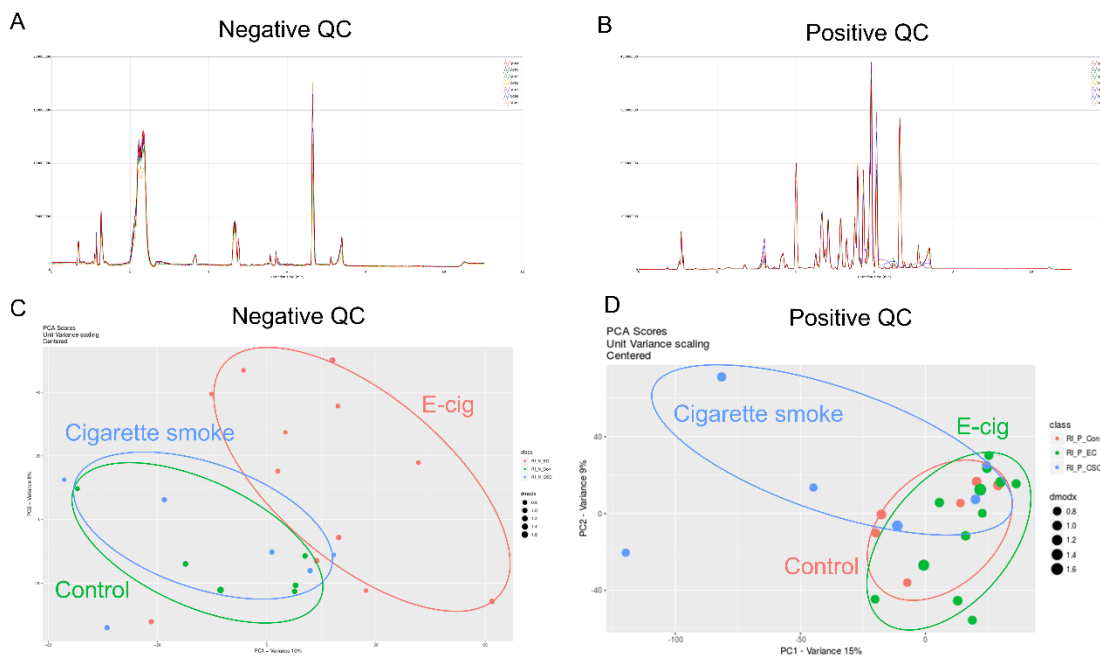


Figure 1. Metabolites from plasma were analyzed from ultra-performance-liquid-chromatography mass spectrometry (UPLC-MS). Spectrums from UPLC-MS measured from (A) negative and (B) positive ion modes were used to identify individual metabolites. Score plots including all samples from principal component analysis (PCA) based on (C) negative and (D) positive ion modes presented dysregulated metabolomics affected by e-cig vaping and cigarette smoking.

Table 1 Metabolic pathway dysregulation among non-smokers, e-cig users, and cigarette smokers

Control vs E-cigarette				Control vs Cigarette smoke			
Pathways	Overlap size	pathway size	p-value	Pathways	Overlap size	pathway size	p-value
Nicotine degradation III	7	17	0.00361	Nicotine degradation III	6	17	0.00042
Serotonin degradation	5	7	0.00106	Serotonin degradation	3	7	0.00106
Gluconeogenesis	5	9	0.00138	Gluconeogenesis	4	9	0.00094
TCA cycle	8	9	0.00086	nicotine degradation IV	4	15	0.0016
D-galactose degradation V	6	6	0.00087				
UDP-N-acetyl-D-galactosamine biosynthesis II	6	7	0.00088				

Nicotine degradation-related metabolites increased in both plasma from cigarette smokers and e-cig users.

Nicotine degradation is commonly seen after cigarette smoking and e-cig (with nicotine) vaping²⁸. As expected, we have shown increased metabolites related to nicotine degradation in plasma from both e-cig users and cigarette smokers (**Figure 2**). Significantly increased metabolites were cotinine, cotinine N-oxide, L-nor-nicotine, (S)-nicotine, trans-3-hydroxycotinine, and (R)-6-hydroxynicotine (**Figure 2**). However, there was no significant difference between e-cig and cigarette smoke groups (**Figure 2**). L-Nor-nicotine, (R)-6-hydroxynicotine, and cotinine are the metabolic products converted from (S)-nicotine or nicotine; trans-3-hydroxycotinine and cotinine N-oxide are the downstream metabolites converted from cotinine.

Figure.2.

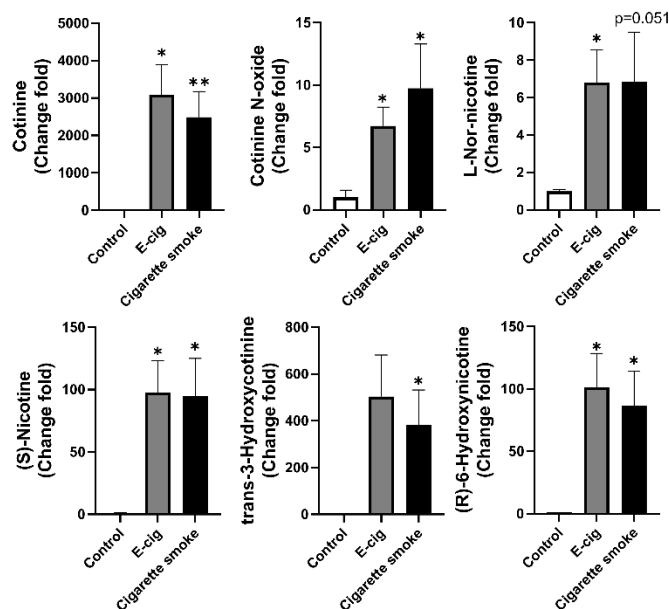


Figure 2. Metabolites from plasma analyzed from UPLC-MS from positive ion mode identified dysregulated nicotine degradation related metabolites in e-cig users and cigarette smokers. Change folds were calculated based on the normalized area from UPLC-MS spectrums and used control group as base line. Data was showed as mean \pm SEM (n=6 for non-smoking control and cigarette smoke groups, n=12 for e-cig group; *p<0.05 vs control non-smokers).

Metabolites associated with TCA cycles dysregulated in plasma from e-cig users.

Metabolites screened from the negative ion mode UPLC-MS were further identified, and we have shown that significant amounts of metabolites related to TCA cycles are statistically dysregulated in e-cig users' plasma compared to cigarette smokers' plasma (**Figure 3**). TCA cycle-related metabolites, such as (2R,3S)-2, 3-dimethylmalate, D-glucose, (R)-2-hydroxyglutarate, O-phosphorylethanolamine, malathion, D-threo-isocitrate, malic acid, and 4-acetamidobutanoic acid, are significantly decreased in e-cig users' plasma compared to healthy control or cigarette smokers (**Figure 3**). There was no change among groups for the concentration of cis-aconitic acid (**Figure 3**), and an increased plasma concentration of 2-oxoglutarate was found in e-cig users compared to control and cigarette smokers (**Figure 3**).

Figure.3.

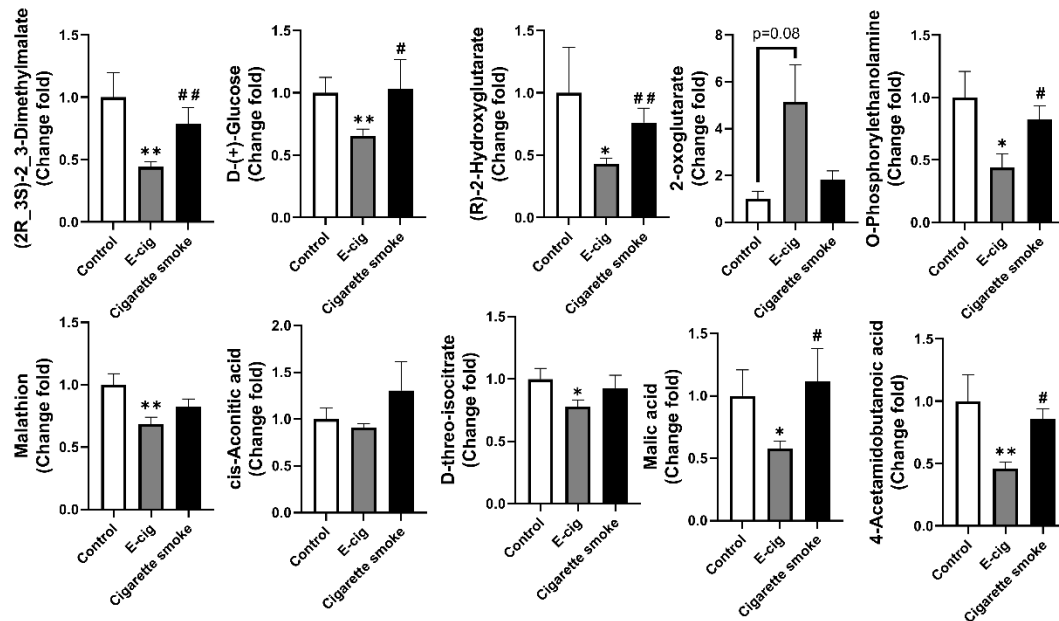


Figure 3. Metabolites from plasma analyzed from UPLC-MS from negative ion mode identified dysregulated TCA cycle related metabolites in e-cig users. Change folds were calculated based on the normalized area from UPLC-MS spectrums and used non-smoking control group as base line. Data was showed as mean \pm SEM (n=6 for non-smoking control and cigarette smoke groups, n=12 for e-cig group; *p<0.05, **p<0.01 vs non-smoking control; #p<0.05, ## p<0.01 vs E-cig).

Dysregulated sphingolipid metabolites found in plasma from cigarette smokers.

From the positive ion UPLC-MS analysis of screened metabolites, we have further showed that sphingolipid metabolites are dysregulated in cigarette smokers' plasma compared to e-cig users or healthy controls (**Figure 4**). We found the concentrations of D-Sphingosine, N-(octadecanoyl)-sphing-4-enine, N-(9Z-octadecenoyl)-sphing-4-enine, ceramide, and N-[(13Z)-docosenoyl]sphingosine, increased significantly in plasma from cigarette smokers compared to e-cig users and healthy controls (**Figure 4**). The concentration of N-acetylsphingosine showed an increasing trend, but not a significant difference between the cigarette smoke and control groups (**Figure 4**).

Figure 4.

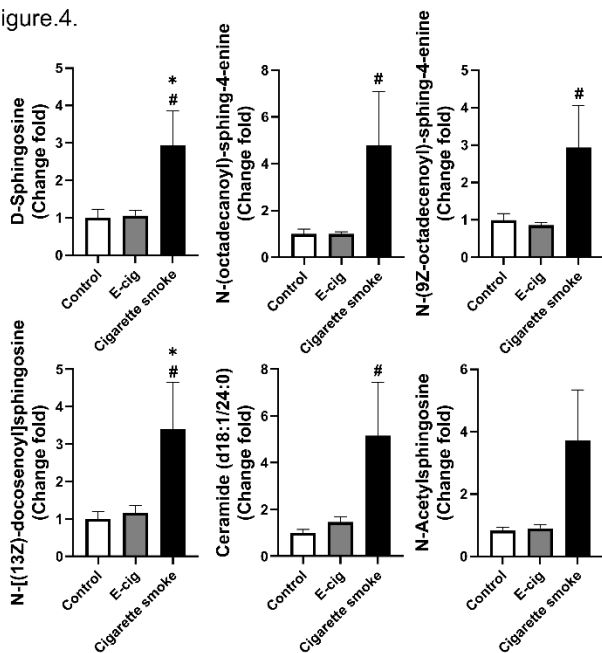


Figure 4. Metabolites from plasma analyzed from UPLC-MS from positive ion mode identified dysregulated sphingolipid metabolites in e-cig users. Change folds were calculated based on the normalized area from UPLC-MS spectrums and used non-smoking control group as base line. Data was showed as mean \pm SEM (n=6 for non-smoking control and cigarette smoke groups, n=12 for e-cig group; *p<0.05 vs non-smoking control; #p<0.05 vs E-cig).

Other dysregulated metabolites in plasma from e-cig users or cigarette smokers.

In addition to the TCA cycle or sphingolipid metabolites, we also identified other significantly dysregulated metabolites (**Figures 5-6**). Among the metabolites significantly dysregulated in e-cig user's plasma, we observed increased jasmonic acid in e-cig users compared to cigarette smokers and healthy controls (**Figure 5**). Most of the metabolites we identified, such as (R)-2-hydroxyglutarate, DL-4-hydroxyphenyllactic acid, 4-acetamidobutanoic acid, S-(3-oxo-3-carboxy-n-propyl)cysteine, and (2R_3S)-2_3-dimethyl-malate, were decreased in e-cig users compared to cigarette smokers and healthy controls (**Figure 5**).

Figure 5

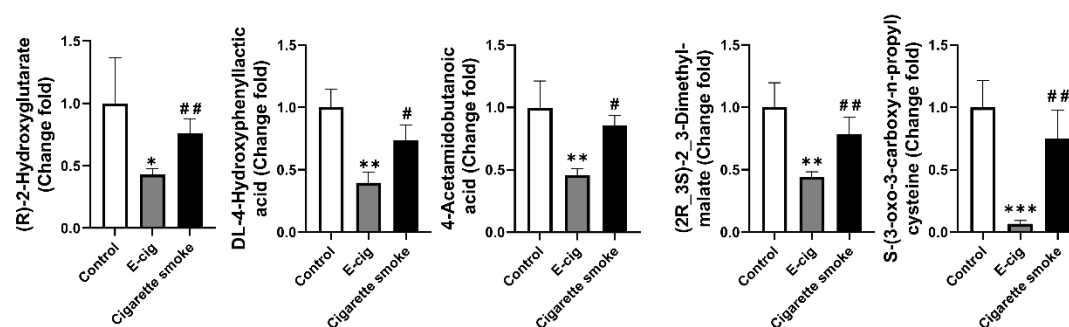


Figure 5. Metabolites from plasma analyzed from UPLC-MS from both negative and positive ion mode identified dysregulated metabolites only in e-cig users. Change folds were calculated based on the normalized area from UPLC-MS spectrums and used non-smoking control group as base line. Data was showed as mean \pm SEM (n=6 for non-smoking control and cigarette smoke groups, n=12 for e-cig group; *p<0.05, **p<0.01 vs non-smoking control; #p<0.05, ##p<0.01 vs E-cig).

We also detected significantly dysregulated metabolites from cigarette smokers' plasma compared to e-cig users and healthy controls (**Figure 6**). We found significantly increased metabolites such as glycolic acid, 6-hydroxy-2-naphthoic acid, 2-beta-D-glucosyle anthranilate, and budesonide, as well as significantly downregulated metabolites such as L(-)-methionine, 2-methylthiazolidine, 4-(stearoylamino)butanoic acid, and 3-methylsulfolene (**Figure 6**).

Figure 6.

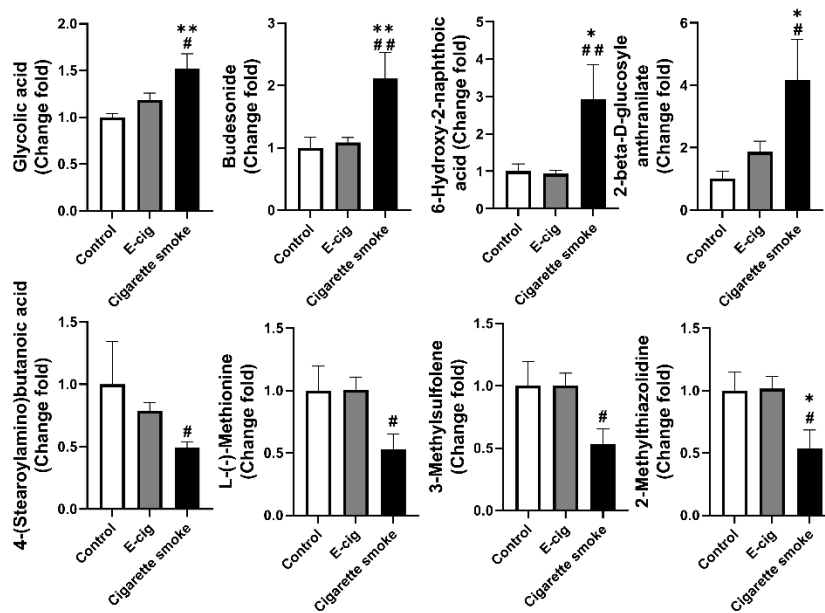


Figure 6. Metabolites from plasma analyzed from UPLC-MS from both negative and positive ion mode identified dysregulated metabolites only in cigarette smokers. Change folds were calculated based on the normalized area from UPLC-MS spectrums and used control group as base line. Data was showed as mean \pm SEM (n=6 for non-smoking control and cigarette smoke groups, n=12 for e-cig group; *p<0.05, **p<0.01 vs non-smoking control; #p<0.05, ## p<0.01 vs E-cig).

Discussion

E-cig vaping has rapidly increased since it has been presumed as a safe alternative to cigarette smoke, evoking public concerns about the health risks of e-cig vaping²⁹. Our previous studies have proven that both acute and chronic e-cig exposure can induce pulmonary inflammation and oxidative stress^{30, 31}. Many studies have shown about cigarette smoking-induced metabolic disease with dysregulated metabolites^{9, 32}; however, limited studies have elucidated the effects of e-cig on metabolic disorders which in turn to identify promising metabolite biomarkers related to potential diseases^{32, 33}. In this study, we have successfully identified dysregulated metabolites from the plasma of e-cig users and cigarette smokers related to nicotine

degradation, TCA cycle, and sphingolipid metabolism, as well as some other metabolites introduced by e-cig aerosol and cigarette smoke.

Nicotine degradation pathways were the most commonly activated metabolic responses in cigarette smokers and e-cig users (nicotine contained e-cig vaping). When nicotine from cigarette and e-cig aerosol was inhaled into the human body, a number of metabolites are metabolized from nicotine²⁸. The most important and commonly used metabolite to identify nicotine degradation is cotinine, which will be converted from 70%–80% of nicotine introduced into the human body²⁸. The other cotinine-associated metabolites identified from our study, including cotinine N-oxide and trans-3-hydroxycotinine. Around 35%~42% of the total cotinine will be transformed to cotinine N-oxide and trans-3-hydroxycotinine²⁸. Nicotine-related metabolites, such as nor-nicotine and 6-hydroxynicotine, will be converted from nicotine²⁸. About 10% of the nicotine will not be metabolized, and we have detected it as (S)-nicotine, and²⁸. From our and other previous studies cotinine has been used as a biomarker to identify nicotine degradation, which is the commonly activated metabolism after smoking and nicotine vaping^{30,34}. Furthermore, nicotine, cotinine, cotinine N-oxide, and trans-3-hydroxycotinine are considered to be primary metabolites in total nicotine equivalent (TNE), which have been used as standards to validate nicotine intake³⁵. Other metabolites such as nor-nicotine and 6-hydroxynicotine are less concentrated (<2%) and lower in abundance compared to TNE metabolites²⁸. Hence, they are not considered as regular biomarkers for the characterization of nicotine inhalation^{28,35,36}. A previous study has identified that nor-nicotine preserves a longer half-life compared to either nicotine or cotinine³⁷, and nor-nicotine was highly relevant to TNE in smokers' urine compared to health control³⁸. Consistent with these data, our results confirm that although nor-nicotine or 6-hydroxynicotine are low abundance in body fluids, they are still sufficient to serve as biomarkers to identify smoking status as well as an indicator for nicotine degradation pathway activation.

The TCA cycle is a series of biochemical conversions with the generation of bioenergy, which usually occurs in mitochondrion with the products from glycolysis. A previous study has shown that either PG or PG/VG inhibited the glucose metabolism

and ATP generation in airway epithelium³⁹. The aerosolized PG/VG inhaled into lungs were unlikely deposited and accumulated in the bloodstream since the half-life for PG is ~4h; PG will be converted to lactic acid via alcohol dehydrogenase in the liver and then merged in the TCA cycle¹⁹. Our previous studies described that e-cig exposure is capable of inducing oxidative stress in the mitochondrion and dysregulation of mitochondrial complexes in lung fibroblasts⁴⁰. Furthermore, e-cig exposure causes an increased amount of damaged mitochondrial DNA in plasma, as well as increases the risk of cardiovascular diseases⁴¹. In this study, we showed that most of the TCA cycle-related metabolites are downregulated in e-cig users while there were no changes in the cigarette smokers compared to the healthy control. This is the first study to report that a series of metabolites associated with the TCA cycle are altered in e-cig users since former studies are focused on nicotine-related metabolites identified from e-cig users. Surprisingly, we did not find significant difference between the cigarette smokers and the healthy control about the TCA cycle metabolites in plasma. It is well-known that cigarette smoke inhibits mitochondrial respiratory function and dysregulates TCA cycle¹⁵. The dysregulated TCA cycle-related metabolites identified from the e-cig group provide information that vaping might associate with synthetic bioenergy metabolism. Therefore, a larger sample size is needed for the future study.

Sphingolipid metabolites are associated with lung inflammation, emphysema, and COPD^{22, 27, 42}. Among all the known sphingolipid metabolites, sphingosine-1-phosphate (S1P) and ceramide are well-studied²⁰. Increased ceramide levels found in the elastase-induced mouse emphysema model and ceramides inhibitors were capable of attenuating elastase caused airspace enlargement^{42, 43}. We found that the cigarette smoke group showed significantly higher plasma levels of ceramide and sphingosine compared to e-cig users and the healthy control group. Since chronic cigarette smoking is shown to cause COPD/emphysema, our results are indirectly in agreement with previous studies⁴²⁻⁴⁴. Additionally, ceramide accumulation and the disproportion of sphingolipids were identified from the lungs of COPD/emphysema patients and smokers⁴⁵. We have observed increased sphingosine as well, which can be converted from S1P, which is one of the downstream products of ceramide. Both ceramide and S1P were involved in the pathogenesis of various lung diseases^{22, 46} which allows

sphingosine great potentiality as a biomarker for lung disorders associated with cigarette smoke. Other sphingolipid metabolites, such as N-(octadecanoyl)-sphing-4-enine, N-(9Z-octadecenoyl)-sphing-4-enine, and N-[(13Z)-docosenoyl]sphingosine were also promising biomarkers for lung injury induced by cigarette smoke.

We have also identified other metabolites dysregulated in either e-cig users or cigarette smokers. We have showed a decreased (R)-2-Hydroxyglutarate ((R)-2HG) in e-cig users; (R)-2HG has been proved to exhibit as an onco-metabolite which is capable of inhibiting tumor growth ⁴⁷. E-cig vaping downregulated the level of (R)-2HG in plasma indirectly reveals the risk of carcinogenesis associated with vaping, and (R)-2HG can serve as a biomarker for identifying e-cig vaping and cancers. We found a lower plasma level of (2R_3S)-2_3-Dimethyl-malate in e-cig users as compared to cigarette smokers and healthy controls, and the (2R_3S)-2_3-Dimethyl-malate can serve as a precursor of pyruvate, which is a basic substrate for the TCA cycle. A decreased level of (2R,3S)-2,3-dimethylmalate is in line with the TCA cycle substrates we have discussed above, and it is a promising biomarker for reflecting e-cig vaping inducing inhibition of bioenergy synthesis and mitochondrial respiration. In addition, a decreased level of L-(-)-Methionine was found in cigarette smokers' plasma as compared to e-cig users and healthy controls. A lower level of L-(-)-Methionine was identified with increased metabolic rates and weight loss, which have also been showed in cigarette smokers ⁴⁸, ⁴⁹. The dysregulated metabolites from cigarette smokers or e-cig users are all capable of serving as promising biomarkers for various diseases.

In conclusion, various dysregulated metabolites were identified from e-cig users or cigarette smokers when compared to healthy controls/non-smokers. Dysregulated metabolites from both e-cig users and cigarette smokers were correlated with nicotine degradation, which has been shown previously. Dysregulated metabolites related to the TCA cycle were found only in e-cig users, and altered sphingolipid metabolites were shown only in cigarette smokers; specific dysregulated metabolites identified in different groups preserve the potential as novel biomarkers for vaping and smoking associated with metabolic diseases. Further biochemical measurements of altered metabolites are required to confirm our findings in a larger cohort.

Methods and Materials

Human Subjects

Participants in this study have provided information including age, sex, gender, and ethnicity. Detailed information about cigarette smoking, e-cig vaping, and health control allowed us to categorize the condition groups as described previously ⁵⁰.

Institutional Review Board (IRB) Statement

This study was conducted at general clinical research center of the University of Rochester Medical Center with IRB approval (RSRB00064337). Participants in this study have provided information including age, sex, gender, and ethnicity. Detailed information about cigarette smoking, e-cig vaping, and health control allowed us to categorize the condition groups as described previously ⁵⁰.

Plasma samples collection

Blood samples were centrifuged at 1000 rpm for 5 min at room temperature, and plasma was collected and stored at -80°C until UPLC–MS analysis.

Chemicals

LC–MS-grade methanol, LC–MS-grade 2-propanol, LC–MS-grade acetonitrile, LC–MS-grade water, formic acid (99.5+%), ammonium acetate, and ammonium hydroxide were purchased from Fisher Chemical (Fisher Scientific International, Inc. Pittsburgh, PA) and used to prepare mobile phases and solutions.

UPLC–MS Analysis

UPLC–MS Analyses were performed at the Mass Spectrometry Core Facility at Georgia Institute of Technology according to protocols previously described (PMID: 31290664). Chromatography was performed with an Ultimate 3000 UPLC (Thermo Fisher Scientific, Inc., Waltham, MA) system equipped with a Waters ACQUITY UPLC BEH C18, 2.1 × 50 mm, 1.7 μm particle column or a Waters ACQUITY UPLC BEH HILIC, 2.1 × 75 mm, 1.7 μm particle column. A Q-Exactive HF Orbitrap mass spectrometer (Thermo Fisher Scientific, Inc., Waltham, MA) was used in all cases. For reverse-phase (RP)

separations, mobile phase A was water/acetonitrile (40:60 v/v), and mobile phase B was acetonitrile/2-propanol (10:90 v/v). Both mobile phases included 10 mM ammonium formate and 0.1% formic acid additives to improve peak shape and ionization efficiency. For hydrophilic interaction chromatography (HILIC) separations, mobile phase A was water/acetonitrile (95:5 v/v), 10 mM ammonium acetate, and 0.05% ammonium hydroxide. Mobile phase B was acetonitrile with 0.05% ammonium hydroxide. The column temperature was 55 °C, while samples were maintained at 5 °C in the autosampler. Injection volumes of 5 and 2 µL were used in RP and HILIC methods, respectively. RP and HILIC chromatographies were performed both in positive and negative ion modes.

For metabolite identification purposes, the top five data-dependent acquisition (DDA) experiments were used to collect MS/MS spectra using stepped normalized collision energy (NCE) of 10, 30, and 50 V. For compounds missed by DDA, parallel reaction monitoring (PRM) experiments were performed at collision energies ranging from 10 to 40 V to obtain fragmentation data for identification purposes.

Data Processing

Spectral features were extracted from the raw data using Compound Discoverer v2.1 software (Thermo Fisher Scientific, Inc., Waltham, MA) and XCMS software. This procedure included chromatographic alignment, peak picking, peak area integration, and QC-based compound area normalization. Features that eluted with the chromatographic solvent front with retention times <0.5 min in RP data sets and <0.9 min in HILIC data sets were considered unreliable due to potential ion suppression effects (PMID: 12816898). The screening criteria for differential metabolic indicators include $p < 0.05$, fold change > 2 , or < 0.5 . Further filtering was carried out by removing features that were not present in 50% of at least one of the plasma sample groups at 10 times the baseline abundance, defined as the peak area of the sample blank run. Welch's t-test with a Benjamini Hochberg correction was applied to cigarette smoke vs Control, and E-cig user vs control. A further selection of dysregulated metabolic pathways was based on overlap size (> 6).

Change fold of metabolite was calculated based on the normalized area from positive or negative mode spectrums. In brief, normalized areas from the control group will be averaged and used as the baseline. The individual normalized area from different samples will be divided by the averaged normalized area from the control group as change folds compared to the baseline.

Statistical analysis

One-way ANOVA and student's t-test were used here to determine the significant difference in the change fold of metabolites among groups through GraphPad Prism Software version 8.0 (La Jolla, CA). Data were presented as mean \pm SEM, and $p < 0.05$ was considered as a statistical difference.

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Author Contributions

QW and IR. Conceived and designed the experiments; QW and XJ. Conducted experiments; QW and XJ. Analyzed the data; QW, XJ and IR. Wrote and revised/edited the manuscript.

Conflicts of Interest

The authors have declared that no competing interests exist.

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