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Redox Metabolomics of Menthol in Children's Plasma with Second-Hand Cigarette and Electronic Cigarette Exposures

Matthew Ryan Smith[a,b,*], **Zachery R. Jarrell**[a], **Ken H Liu**[c], **Choon-Myung Lee**[a,d], **Edward T Morgan**[d], **Young-Mi Go**[a,1,*], **Dean P. Jones**[a,1,*]

[a]Division of Pulmonary, Allergy, Critical Care and Sleep Medicine, Department of Medicine, Emory University, Atlanta, GA 30322, USA

[b]Atlanta VA Healthcare System, Decatur, GA, 30033, USA

[c]Department of Chemistry, Emory University, 1515 Dickey Drive NE, Atlanta, Georgia, 30322, USA

[d]Department of Pharmacology and Chemical Biology, Emory University, Atlanta, GA, 30322, USA

Abstract

Background: Cigarettes and electronic cigarettes generate many redox-active materials which could impact children's health through second-hand exposures. High-resolution metabolomics methods enable use of non-targeted mass spectrometry of plasma to test for redox consequences of second-hand exposures.

Objectives: Our objectives were to test for oxidative stress metabolites and altered metabolic pathways associated with second-hand exposure to redox-active flavorants and flavorant metabolites in plasma of infants and children.

Methods: Untargeted plasma metabolomics data for infants and children in a population known to include individuals with second-hand exposures to cigarettes and electronic cigarettes were analyzed for cotinine and metabolites of flavorants. A metabolome-wide association study

[*]Correspondence authors at: Whitehead Biomedical Research Building, 615 Michael St, Room 225, Atlanta, GA, 30322, USA matthew.ryan.smith@emory.edu (M.R. Smith), ygo@emory.edu (Y.-M. Go), dpjones@emory.edu (D.P. Jones).
[1]Drs. Young-Mi Go and Dean P. Jones share equal senior authorship in this collaborative research.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

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CRediT authorship contribution statement

Matthew Ryan Smith: Writing – review & editing, Writing – original draft, Visualization, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Zachery R. Jarrell:** Writing – review & editing, Writing – original draft, Visualization, Funding acquisition. **Ken H Liu:** Writing – review & editing, Writing – original draft, Methodology, Formal analysis. **Choon-Myung Lee:** Methodology, Formal analysis, Data curation. **Edward T Morgan:** Writing – review & editing, Writing – original draft, Conceptualization. **Young-Mi Go:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Conceptualization. **Dean P. Jones:** Writing – review & editing, Writing – original draft, Funding acquisition, Conceptualization.

(MWAS) was performed separately for cotinine and menthol glucuronide, derived from the redox-active flavorant, menthol. Pathway enrichment analysis was used to identify metabolic pathways, and xMWAS was used to detect metabolic communities associated with flavorant metabolites.

Results: Menthol glucuronide was one of several flavorant metabolites positively correlated with cotinine. MWAS and pathway enrichment analysis revealed that some pathways associated with both menthol glucuronide and cotinine, while others only associated with menthol glucuronide, including sphingolipid, glycerophospholipid, antioxidant, N-glycan and mitochondrial energy metabolism. 4-hydroxynonenal and other oxidized lipids positively correlated with menthol glucuronide.

Discussion: The results show that flavorants from second-hand electronic cigarette and cigarette exposures in infants and children are associated with changes in redox metabolism which are known to associate with human lung diseases.

Keywords

Flavoring agents; High-resolution metabolomics; Metabolic disruption; Oxidative stress

Introduction

Environmental health risks to children from second-hand cigarette smoke are well-recognized [1–3], and potential risks of second-hand exposure to electronic cigarettes are becoming evident. Both traditional and more recently, electronic cigarettes, produce acrolein and other toxic chemicals during aerosol generation [4] and contain numerous redox-active flavorants, some present at relatively high concentrations. Use of flavoring agents have come under criticism because the smell is attractive to children and youth, potentially contributing to the rapid rise in electronic cigarette use in younger populations [5]. Of concern is that toxicology testing for flavorants is limited. For instance, commonly used flavorants, menthol, vanillin, cinnamon, maltol and ethyl maltol [6–8] are generally regarded as safe (GRAS) [9,10], but few have been studied for potential adverse effects on lung epithelia or metabolic effects in vivo. Recent studies of menthol show that menthol both reduced [11,12] and enhanced oxidative stress [6,13], depending on its concentration and in conditions of inhalation, enhanced pulmonary injury [14]. Lipid peroxidation products have been well-documented in traditional cigarette smoke [15–17], and recent studies analyzing flavor aldehyde acetals found in electronic cigarettes observed higher cytotoxicity than their parent aldehydes [18]. Additionally, flavorants commonly used in electronic cigarettes have also been identified to perpetuate lipid peroxidation of arachidonic acid [19].

Previous research using high-resolution metabolomics (HRM) of an airway epithelial cell line, BEAS-2B, showed that a commonly used flavorant, vanillin, caused changes in metabolism at doses found in electronic cigarette use [20]. A metabolome-wide association study (MWAS) showed that vanillin perturbed specific energy, amino acid, antioxidant and sphingolipid metabolic pathways previously associated with human disease [20,21]. Additionally, the study [20] showed that these pathways varied with vanillin levels in infants and children exposed to second-hand electronic cigarettes and cigarette smoke. Prior MWAS of human amniotic fluid also showed that energy, amino acid and antioxidant pathways vary

with cotinine from low-level second-hand cigarette smoke exposure [22], but these studies did not test for possible metabolic effects of flavorants.

To test for metabolic effects of flavorants and flavorant metabolites, we used human liver S9 fraction to generate metabolites of menthol, cinnamon, ethyl maltol and furaneol, and characterized products with LC-HRMS [23]. We used these to identify metabolites correlated with the major nicotine metabolite, cotinine, in plasma of infants and children that we previously studied for vanillin metabolites [20]. Because menthol glucuronide was very commonly detected and is known to be redox active, we used menthol glucuronide for subsequent MWAS, with targeted searches for known oxidative stress metabolites [4-hydroxynonenal (4-HNE), 4-oxo-2-nonenal (4-ONE)] and pathway enrichment analysis to look for metabolic pathways previously found in human research to associate with idiopathic pulmonary fibrosis [24], asthma [25] and acute respiratory distress [26]. We also applied a data-driven network analysis tool to identify metabolic communities associated with flavorant metabolites. The results showed distinct metabolic pathway associations for different flavorant metabolites from second-hand exposures in infants and children and suggest that this redox metabolomics approach could be useful to characterize human responses to flavorants and other GRAS substances for which toxicologic evaluations are not available.

Materials and methods

Human metabolomics data

Untargeted mass spectral feature table and associated metabolite table for infants and children with cystic fibrosis and/or second-hand exposures to electronic cigarette and cigarette smoke along with their respective controls were used as previously described [20]. Briefly, data were downloaded from the NIH Common Fund's National Metabolomics Data Repository (NMDR) website, the Metabolomics Workbench, https://www.metabolomicsworkbench.org, where it had Project ID PR000722. The data along with details for liquid chromatography-mass spectrometry (LC-MS) analysis and associated clinical data had Project DOI: 10.21228/M8BT3D.

Metabolite annotation and identification

Downloaded LCMS data had associated metabolite data with Level 1 identification [27] of amino acids and other endogenous metabolites, including cotinine and metabolites in associated metabolic pathways [28]. Annotations of flavorants and flavorant metabolites were obtained using xMSannotator [29] based upon in-house generation of flavorant metabolites (see below), KEGG, (Kyoto Encyclopedia of Genes and Genomes) [30], HMDB (Human Metabolome Database) [31], T3DB (Toxin and Toxin Target Database) [32], and Lipid Maps [33] databases at 5 ppm tolerance. xMSannotator uses a multistage clustering algorithm to assign annotation confidence levels, and data reported with high or medium confidence in xMSannotator (≥ 4) had M-H/M + H adducts detected in the negative/positive electrospray ionization (ESI) mode, respectively.

Flavorant metabolite generation and identification

Commercial sources of authentic standards for flavorant metabolites were not found so we used a human liver S-9 system [23] to generate oxidation products as well as glucuronide, sulfate, glutathione, methyl and acetyl derivatives. To accomplish this, 0.5 mL aliquots of pooled human lung or liver S9 fractions (mixed gender, H0610.PS9; 20 mg/mL protein), stored at − 80 °C, were thawed at room temperature and used to enzymatically generate flavorant metabolites. Mint oil was diluted to 40 ppm with water before addition to fractions. An NADPH regenerating system (K5000–10) from Sekisui Xenotech (Kansas City, KS) was reconstituted with addition of 3.5 mL of water to make a final volume of 5 mL. Cofactors were combined to form a 4X cofactor stock prior to addition into the reaction mixture, and then a 3 μl/mL addition of the following phase 2 xenobiotic metabolism co-substrates were added: 10 mM UDP-glucuronic acid and 1 mg/mL of alamethicin, a channel-forming peptide antibiotic to enhance UDP-glucuronosyl transferase reactions, 2 mM GSH, 2 mg/mL phosphoadenosyl-phosphosulfate (PAPS), 0.1 mM acetyl-Coenzyme A, and NADPH regenerating system (1 mM NADP, 5 mM glucose-6-phosphate, 1 unit glucose-6-phosphate dehydrogenase). Reactions with mint oil were carried out at 30 °C on 96-well plates. S9 fractions were diluted 10-fold in water immediately before mixing with 0.2 M Tris-Cl, pH 7.5/ 2 mM $MgCl_2$ and 0.3 mM of the xenobiotic solution in a 1:1:1 ratio (15 μL each) and incubated at 30 °C for 5 min. To start the reaction, an equal volume (15 μL) of 4X cofactor stock was added, and incubation was carried out at 30 °C for the indicated times. To terminate the reaction, we added a three-fold volume of acetonitrile, covered the plate with parafilm, vortexed, and froze at −20 °C to precipitate insoluble materials such as protein. After centrifugation of the incubation plate, the supernatants were transferred into assay vials, which were stored at −20 °C until LC/MS/MS analysis. Confidence in metabolite identification utilized principles from Liu et al. [28], which showed that co-occurrence of multiple metabolites in 80 % of at least one of the comparator groups from a xenobiotic precursor and/or correlation of intensities for related metabolic products were useful for metabolite identification when intensities of signals were too low for MS/MS analysis or when authentic standards were not available.

Samples from the xenobiotic metabolite generation system above were prepared for LC-MS analysis by addition of 100 +μL of acetonitrile (LCMS grade; Sigma Aldrich) per 50 μL incubation volume [34,35] and centrifugation (20,817 × rcf at 4 °C for 10 min). Extracts were analyzed using a High-Field Q-Exactive Orbitrap instrument (ThermoFisher; Waltham MA) coupled to a Thermo Dionex Ultimate 3000 liquid chromatography system. Dual column chromatography was used, with the HILIC column (hydrophilic interaction liquid chromatography; ThermoFisher Scientific, Accucore, 50 × 2.1 mm, 2.6μm) operated in parallel to a reverse phase column (C18; Higgins Analytical, 50 × 2.1 mm, 2.6 μm) for simultaneous analytical separation on one column while flushing of the other. Positive electrospray ionization (ESI) was used for analysis with the HILIC column (HILIC+), and negative ESI was used with the C18 reverse phase column (C18-). A sample volume of 10 μL was injected for analysis on each column. The flow rate of the HILIC column was maintained at 0.35 mL/min until 1.5 min, increased to 0.4 mL/min at 4 min and held for 1 min, resulting in a total analytical run time of 5 min. Mobile Phases A and B were LCMS grade water and acetonitrile, respectively. Mobile phase C was composed of 2 % formic acid

(v/v) in water. Mobile phase conditions consisted of 22.5 % A, 75 % B, 2.5 % C which was held for 1.5 min, with a linear gradient to 77.5 % A, 20 % B, 2.5 % C at 4 min, and held for 1 min. The HILIC column was then flushed for 5 min with a wash solution of 77.5 % A, 20 % B, 2.5 % C. For the reverse-phase C18 column, the flow rate was maintained at 0.4 mL/min for 1.5 min and was then increased to 0.5 mL/min at 2 min and held constant for 3 min. Mobile Phases A and B were LCMS grade water and acetonitrile, respectively. Mobile phase C was 10 mM ammonium acetate in water. Mobile phase conditions for the C18 column were 60 % A, 35 % B, 5 % C for 0.5 min, with a linear gradient to 0 % A, 95 % B, 5 % C starting at 1.5 min, and held for 3.5 min, resulting in a 5 min run. The reverse phase column was flushed with 0 % A, 95 % B, 5 % C for 2.5 min, followed by an equilibration solution of 60 % A, 35 % B, 5 % C for the remaining 2.5 min. Collected mass spectral data were analyzed for flavorant metabolites using Xcaliber (ThermoFisher; Waltham MA).

Data processing and selection of metabolic features

The metabolomics data for infants and children were filtered to remove some samples due to repeated measures from the same individuals, keeping only the baseline samples ($n = 73$). Spectral intensities were log2 transformed and quantile normalized for statistical analyses. Features were retained if they were detected in 80% of the assigned groups, as well as 20% across the entire dataset. Selection of differentially expressed *m/z* features (hereafter termed metabolites) between those with class label, second-hand exposed and non-exposed (controls), as well as metabolites associated with flavorants were performed based on one-way ANOVA (*limma())* and with Spearman rank correlations using linear regression (*lmreg())* packages in R respectively. Benjamini-Hochberg false discovery method [36] was used for multiple hypothesis testing correction (FDR <0.2). Patterns of selected metabolites as well as pattern similarity in samples were determined and visualized in an unsupervised two-way hierarchal clustering analysis (HCA) plot using *hclust()* function in R. Principal component analysis (PCA) was performed using the *pca*() function implemented in the R package *pcaMethods.* Post hoc comparisons of ANOVA results were performed using Tukey's honest significant difference in R.

Estimation of cotinine concentration

Reference standardization was used to calculate the plasma concentration of cotinine, a major metabolite of nicotine, based upon the concentration of cotinine in the pooled reference material in the Metabolomics Workbench data [22,35,37,38]. Cotinine is isobaric with serotonin; confidence in signal identification as cotinine was provided by co-occurrence of hydroxycotinine, norcotinine and cotinine glucuronide. For additional details in validation and use of this approach, see [22,35,37,38].

Metabolic pathway enrichment analysis

Pathway enrichment analysis was performed on selected mass spectral features using *mummichog 1.10* [39]. Filtering thresholds for pathway enrichment analyses included an *m/z* drift tolerance of 5 ppm, as well as permutation testing using 1000 iterations for increased accuracy. Additionally, pathway inclusions were limited to those with ≥4 annotated metabolites. The Metabolomics Workbench dataset included metabolites found

in the *mummichog* analysis for most of the pathways identified, thereby confirming the pathway identifications for all pathways except phytanic acid, limonene, ubiquinone, which lacked additional confirmation.

xMWAS

To investigate potential interactions of flavorants and flavorant metabolites with metabolic responses, data-driven analysis was performed using xMWAS [40]. For this, flavorant-related annotations (73 samples × 108 metabolic features annotated as flavorant-related metabolites) were integrated with the remaining metabolome data (73 samples × 10,398 metabolic features) using the partial least-squares (PLS) regression method for data integration. Community detection was obtained with xMWAS through the multilevel community detection algorithm which identifies groups of nodes that are heavily connected with other nodes in the same community but have sparse connections with the rest of the network. The threshold criteria were set to $|r| > 0.60$ and $P < 0.05$ as determined by Student's *t*-test.

Bubble plots

Bubble plots of associated metabolic pathways were generated using *corrplot()* in R. Using the metabolic features selected by the parameters from xMWAS, *mummichog* was used to identify relevant pathways associated with top flavorant metabolites independently, and significant pathways were selected using the criteria described above. Both the size as well as the color of the bubble represent the pathway significance level based on the $-log_{10}$ P value.

Data sharing

Datasets corresponding to figures and metabolomics datasets used for xMWAS analysis, and full xMWAS network membership, are available upon reasonable request.

Results

Quantification of cotinine levels for second-hand exposure in children

A summary of information available for the 73 individuals studied is provided in Table 1. The population was relatively balanced for sex and reported smoke exposure versus the non-exposed. Ages ranged from 4 months to 10 years, with more children (58 %) than infants, and most (78 %) had cystic fibrosis. Because of the large effect size of nicotine exposure on metabolism [41–45]. Due to the small number of subjects in the study, and unknown differences in the extent of exposures to flavorants in different subpopulations with respect to age or CF status, we performed analyses without attempting to adjust for potentially confounding issues.

In a metabolome-wide association study (MWAS) to test for differences between the groups labeled as exposed and non-exposed groups, 25 metabolites differed at FDR 0.2, but 2-way hierarchical cluster analysis showed that these metabolites did not clearly separate the groups (Fig. 1A). Although one of the three subclusters consisted mostly of individuals without reported exposure, the results were sufficiently heterogeneous to suggest that

exposures within the hours preceding sampling may not be effectively captured by the class designation for reported smoke exposure. We therefore examined cotinine concentrations in the plasma of the “exposed” and “non-exposed” groups and found no difference in cotinine levels, with 1.36 ng/mL compared to 1.38 ng/mL (Fig. 1B). In the distributions of values, the non-exposed group had increased very low values as well as increased relatively high values, but the average was the same as the entire population (Table 2). Overall, these concentrations were substantially below concentrations found in the literature for first-hand smokers, and less than 3 ng/mL, a value consistent with second-hand exposure [46]. Because the assigned group-wise differences between the second-hand exposed group and their controls did not have differences in measured cotinine values, we concluded that measured cotinine values were more suitable as an objective reference to determine metabolic effects associated with secondhand exposures to nicotine product use and second-hand exposures to flavorants in nicotine-containing products. This sequential analysis also provided an opportunity to test for possible metabolic associations with flavorants which differ from metabolic associations with cotinine.

Metabolome-Wide association study (MWAS) of cotinine

To examine metabolic associations with cotinine, we performed MWAS to test for associated metabolic pathways and possible flavoring agents (Fig. 2). After data extraction and quality filtering for HILIC+, 10,477 metabolic features remained for statistical analyses. Linear regression analysis showed that 5093 features were associated with cotinine at FDR<0.2 (Fig. 2A). These associated features included accurate mass *m/z* matches to hydroxycotinine, norcotinine and cotinine-glucuronide. Eleven of the 25 differentially expressed metabolites from class-wise comparison of second-hand exposure to non-exposed individuals (Fig. 1) were also associated with cotinine. For a complete list of metabolites associated with cotinine, please refer to Supplemental Table 1.

In a 2-way hierarchical cluster analysis of metabolites associated with cotinine, separation of individuals with very low cotinine was evident (Fig. 2A). The low cotinine group included more infants ($n = 11$) than children ($n = 5$), posing a potential limitation in interpretation if the metabolic differences of infants and children were large relative to the metabolic differences associated with second-hand exposures. To test whether the association with cotinine was dependent upon the number of infants with very low cotinine values, we eliminated six of the 11 infants with lowest values and repeated analyses. Elimination of more than half of the lowest values did not change the pattern, thereby supporting the interpretation that the metabolic separations were associated with cotinine and not the number of infants (data not shown). Similar analyses to determine whether associations were with CF instead of cotinine were not needed because infants and children with CF were distributed in all clusters. Thus, in the absence of evidence that metabolic associations with cotinine were compromised by numbers of infants or individuals with CF, we continued analyses with all infants and children.

Of the 5093 metabolic features, 2320 had positive (red dots) and 2773 had negative (blue dots) associations with cotinine (Fig. 2B). Pathway enrichment analysis was used to determine whether the metabolic features associated with cotinine were enriched in specific

metabolic pathways. The results showed that 18 metabolic pathways varied with cotinine (Fig. 2C), including amino acid and urea pathways previously found to be associated with cotinine [47]. Most amino acid pathways were included, *i.e.*, arginine, proline, tryptophan, tyrosine, asparagine, aspartate, lysine, alanine, histidine, cysteine, glycine, serine, and threonine. These pathway associations included metabolites with confirmed identities (Schymanski Level 1) and are consistent with previous literature [28]. Newly identified pathways included fatty acid (e.g., linoleate, fatty acid β-oxidation), carbohydrate (hexose phosphorylation, glycolysis), and xenobiotic detoxication pathways; metabolite confirmations and annotations are provided in Supplemental Table 2. Overall, these results show that major metabolic effects are associated with plasma cotinine in infants and children with second-hand exposures to use of nicotine-containing products.

Generation of flavorant metabolites and mwas of menthol glucuronide

To test for potential redox effects of flavorant exposures from second hand exposures, we selected menthol because it is commonly used in both cigarettes and electronic cigarettes, and menthol glucuronide was annotated as a match in associations with cotinine (Supplemental Table 3). Menthol has previously been reported to have both reductive and oxidative activities, depending on cell type and route of exposure [11,48]. We used an enzyme-based biotransformation system to generate metabolites from mint oil and found time-dependent generation of an accurate mass match to menthol glucuronide. Level one identification of menthol-glucuronide shows that menthol glucuronide peak was dependent both upon the enzyme system and the availability of the co-substrate, UDP-glucuronic acid and had the same *m/z* and chromatographic retention time. Additionally, this peak also positively associated with three other metabolites annotated as menthol metabolites, neomenthol glucuronide, menthone-glycerol-ketal, and menthofuran providing orthogonal evidence that the peak was menthol glucuronide. Menthol glucuronide correlated with cotinine (Fig. 3A), and MWAS of menthol-glucuronide showed that 4328 (FDR<0.2) features were associated with menthol glucuronide. As seen with cotinine, hierarchical cluster analysis showed that subclusters with low menthol glucuronide were present (Fig. 3B). Also similar to cotinine, more negative associations (2553; blue, Fig. 3C) were observed than positive associations (1775; red, Fig. 3C). Pathway enrichment analysis revealed associations of diverse metabolic pathways (Fig. 3D), including many of the same pathways as cotinine, i.e., multiple amino acid pathways (tryptophan, tyrosine, asparagine, aspartate, alanine, histidine, lysine, arginine, proline, glutamate) and the urea cycle. Importantly, many additional pathways were also associated with menthol glucuronide, including complex lipids (glycosphingolipids, glycerophospholipids), mitochondria and energy-related [TCA cycle, vitamin B_3 (niacin), ubiquinone], antioxidant (vitamin C, vitamin E), vitamin B_6 and amino sugars and N-glycan metabolism (Fig. 3D). Confirmed metabolites and annotations within each pathway are provided in Supplemental Table 4.

We used xMSannotator [49] to test for possible oxidative stress markers associated with menthol glucuronide. Relevant metabolites positively associated with menthol glucuronide included 4-hydroxynonenal (**4-HNE**; Fig. 4A) and 4-oxo-2-nonenal (**4-ONE**; Fig. 4B), well established products of lipid peroxidation of arachidonic and linoleic acids [50–54]. Additionally, other metabolites positively associated with menthol glucuronide included the

medium chain fatty acid (MCFA) lipid peroxidation product decenedioc acid (Fig. 4C), and the MCFA 2-hydroxydecanedioc acid (Fig. 4D).

Flavorants and flavorant metabolites associated with cotinine

To test for other flavorants associated with second-hand nicotine product exposure, we examined the other 40 flavorant and flavorant metabolites which were positively associated with cotinine (Supplemental Table 3). Most consistent among these were several cinnamon-related metabolites including cinnamic acid (Fig. 5A), hydrocinnamic acid (Fig. 5B) and methyl cinnamate (Fig. 5C). Cinnamic acid had a relatively strong correlation to cotinine ($r = 0.43$, $p = 1.2 \times 10^{-4}$). Additional flavoring metabolites associated with cotinine included vanillin butylene glycol acetal (Fig. 5D), and norfuraneol (Fig. 5E). Additional analyses showed that many of these flavorant metabolites also correlated with menthol glucuronide. Collectively, the results show that second-hand exposure of infants and children to nicotine products is associated with major metabolic responses and that co-exposure to flavorants exacerbate these responses and/or cause additional metabolic responses.

Determination of flavorants-associated metabolic perturbation using network analyses

To gain insight into possible network responses to flavorants, we used the differential network analysis tool, xMWAS [40] to identify communities associated with the flavorant metabolites (Fig. 6). The flavorant metabolites tended to have correlation coefficient with cotinine $r < 0.6$; to minimize effects of cotinine associations on the metabolic associations with flavorant metabolites, we set the correlation cutoff for xMWAS at 0.6. Analyses were performed separately for the two chromatographic analyses, HILIC and C18, and both showed that metabolic communities differed for the flavorants. With the HILIC data, results showed three metabolic communities (Fig. 6A), with the spicy flavorant cinnamyl phenylacetate associated with multiple amino acids, nucleotides, and vitamin B_3 metabolism, and other flavorant metabolites, including vanillic acid sulfate and hydrocinnamic acid, associated with vitamin E, fatty acids, and carnitine shuttle (Figure 6B; See Supplemental Table 5 for annotated metabolites associated with communities). Although one must note that these analyses do not show whether community associations are due to common exposures to the different flavorants or due to common metabolic responses to the flavorants, the results show that some flavorant metabolites have widespread associations with metabolic pathways while others do not.

The C18- data showed 2 distinct metabolic communities (Fig. 6C) with several butyrate species, as well as menthyl derivatives which were associated with fatty acid pathways (Figure 6D; Supplemental Table 6) for annotated metabolites associated with communities). As with the HILIC+ data, many of the metabolites showed no apparent associations. Collectively, the results show that flavorants found in second-hand exposures to nicotine-containing products, along with endogenously produced metabolites of the flavorants, are associated with metabolic effects in infants and children beyond those which can be attributed to cotinine or its precursor, nicotine.

Discussion

The present research extends previous research on second-hand exposure to electronic cigarettes, as well as studies focusing on the flavorant, vanillin, in plasma of infants and children with second-hand exposures to traditional or electronic cigarette exposures [20,55–57]. The results from this study show that we need to more broadly consider possible redox effects of flavorants that could add to or independently impact health outcomes related to second-hand exposures to cigarette smoke and electronic cigarette vapors. The results show that second-hand exposures to the redox-active flavorant, menthol, measured as menthol glucuronide in plasma, is associated with plasma 4-HNE and 4-ONE and has multiple metabolic effects which were distinguished from those associated with the nicotine product, cotinine. Although the study population was small and additional studies will be needed to determine applicability to other populations and age groups, the key findings show that targeted research is needed to evaluate health impacts of specific metabolic products that are generated endogenously in humans from environmental exposures to flavorants present in electronic cigarettes and many other products.

According to recent data, prevalence of smoking and electronic cigarette use remains high [2]. Health effects from second-hand exposures in adults are linked to poorer outcomes in chronic lung diseases such as asthma [58], and health effects in infants and children can be severe and sometimes contribute to death [1,2]. Although information is available indicating potential adverse effects of flavorants associated with use of nicotine-containing products [6–8,20,59], little information is available for infants and children concerning effects of flavorants from second-hand exposure to nicotine product use. The present study therefore provides important evidence for the need to further evaluate risks of second-hand exposures to flavorants in nicotine products.

The results also indicate that untargeted HRM data include many uncharacterized environmental and redox-related metabolites in addition to identified metabolites. While tools are available for annotation of many of these, one should note that annotation scores are not identification scores. Generally, annotations can be considered identification confidence Level 5 (based upon accurate mass *m/z*), Level 4 (having accurate mass match and additional evidence supporting correct molecular formula), or Level 3 (tentative candidates based upon *m/z* and other information) [27]. Annotation of metabolic features included menthol glucuronide, a metabolic product of menthol previously used as biomarker of menthol exposure [60,61]. Confidence in identification in the present study used biology-based criteria based upon the demonstration that co-occurrence of multiple products from the same xenobiotic chemical can support identification [23] and that correlation of enzymatic precursors and products can also support identification of metabolic features matching environmental agents can be obtained by subsequent detective study [62]. Thus, the database of flavorant metabolites generated using human liver S9 to generate metabolites followed by LC-HRMS characterization (Supplemental Table 3), enables identification based upon three criteria, accurate mass match to predicted *m/z*, co-occurrence with related metabolites in the same samples, and significant correlation of metabolites with common precursor or product. Hydroxyl-maltol, vanillic acid sulfate and cinnamic acid and neomenthol glucuronide are examples in addition menthofuran and others specifically

described (Fig. 5 and Table 3). The chemical structures for these flavorants can be found in Supplemental Figure 1.

The parent compound of menthol glucuronide, menthol, is used in both traditional smoking as well as electronic cigarettes, and recent legislation has suggested to ban menthol in tobacco products [63]; however, current legislation has exempted the menthol from prohibition in the flavoring of traditional or e-liquid commercial products [64]. In the current analyses, the positive correlation of menthol metabolites with cotinine supports the interpretation that the source of menthol was second-hand nicotine use rather than dietary or another source. However, this correlation was relatively weak ($r = 0.2$), which could indicate other sources of exposure or differences in distribution into plasma or biologic half-life of cotinine and menthol glucuronide. The inability to verify the source of exposure is a weakness that may be unavoidable when using archival samples or data for surveillance purposes.

The availability of metabolomics data in accessible data repositories, such as Metabolomics Workbench, facilitates the translation of mechanistic studies in model systems to human populations. Laboratory studies show that cigarette smoke and electronic cigarette vapors cause oxidative stress and produce toxicants such as acrolein [6–8]. The present research translates these findings to second-hand human exposures by showing that menthol glucuronide was positively associated with 4-HNE and 4-ONE levels, known lipid peroxidation products and inducers of oxidative stress (Fig. 5A, B). Importantly, in addition to menthol flavorant metabolites, which were also positively associated with these measures of oxidative stress, cinnamic acid, a cinnamon-related metabolite, may also be of special interest because of association with both pyrimidine and purine metabolic pathways, indicating that cinnamon metabolism may have more substantial effects on nucleotide metabolism than other flavorants (Fig. 6A, B and Supplemental Table 4). Other flavorants, like vanillin, have known antioxidant effects, and it is therefore noteworthy that many signals annotated as flavorant metabolites (Supplemental Table 7) were negatively associated with cotinine. This negative associations could indicate that flavorants or their metabolites could inhibit conversion of nicotine to cotinine or vice versa., and this is supported by previous studies which have shown that menthol species have slowed the metabolism of nicotine to cotinine, [65,66], with other flavorants having similar effects [67,68]. An alternative interpretation is that exposure to second-hand smoke or electronic cigarette vapors causes enhanced elimination of flavorant metabolites through activation of other elimination systems.

Because of this complexity in responses to flavorants and knowledge that many commercial e-liquids contain different flavorants [69], the data-driven differential network analysis tool, xMWAS [40] provides a useful way to visualize relationships between multiple exposures and biologic responses. The integration of flavorant metabolites with the untargeted metabolome datasets (Fig. 6) showed central metabolic pathway associations, *i.e.*, sphingolipid, energy metabolism (citric acid cycle, hexose), and amino acid metabolism (arginine, glutamate, aspartate), which were previously associated with idiopathic pulmonary fibrosis [24] Prior research also shows that sphingolipid, energy (citric acid cycle, hexose, pentose, butanoate), amino acid (tryptophan, branched chain

amino acids, arginine), and micronutrient (pyridoxine, biopterin) metabolism are associated with asthma [25]. Studies of bronchoalveolar lavage fluid showed that lipid (phospholipid, arachidonate, linoleate), energy (citric acid cycle, hexose, fatty acid), amino acid (tyrosine, methionine, glutamate, branched chain amino acids), and pyrimidine metabolism are associated with acute respiratory distress syndrome [26]. These overlapping pathways suggest biologic importance, and appearance of major clusters of metabolic pathway responses to different flavorant metabolites indicates that *1)* flavorants have effects beyond those associated with cotinine, *2)* individual flavorants have different metabolic effects and *3)* that flavorants are likely to have interactive effects. Thus, in addition to the exposure surveillance opportunities for second-hand exposures created by the availability of untargeted human metabolomics data in data repositories, the present findings indicate a need for more extensive mechanistic and dose-response studies of flavorants used in electronic cigarettes and other products. One limitation of this study is the availability of clinical data for the children enrolled and clearly defining what type of cigarette exposure each subject was exposed to, limiting translatability and adjustment for covariates; however, these pilot results can still inform mechanistic studies in pre-clinical models.

In conclusion, exposure to second-hand traditional cigarette smoke or electronic cigarette vapors are recognized to pose health risks for lung disease, especially among children. The present results using untargeted metabolomics data from a public repository support the interpretation that flavorants and their elimination products from second-hand exposure are associated with metabolic perturbations and those found with cotinine, a marker of nicotine exposure. Of particular concern, some of these pathways are associated with lung disease. Detailed studies of individual flavorants and their metabolites are needed to identify and eliminate those of greatest concern to minimize both acute and chronic lung disease from second-hand exposures in infants and children.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Data availability

Data will be made available on request.

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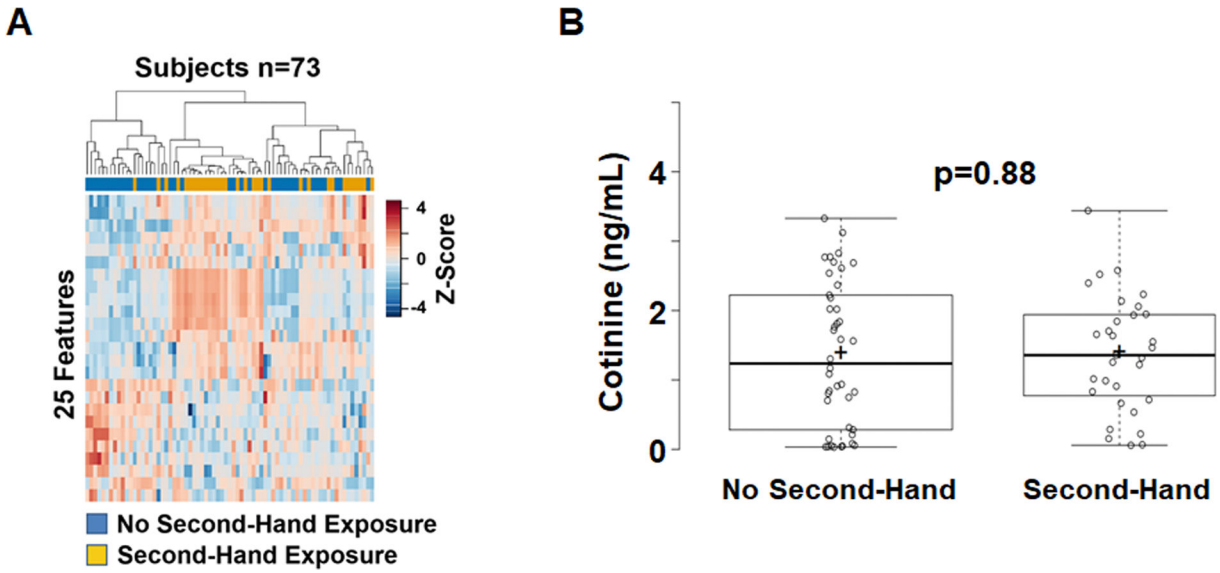

Fig. 1.

Dendrogram of exposure to second-hand smoke in human plasma. A) Unsupervised hierarchal clustering heatmap using the HILIC column coupled with positive ionization indicates mixed separation and that 25 features are changed between children who were reported to have been exposed to second-hand smoke. B) Box plots showing quantification of cotinine concentrations for both non-exposed and exposed in ng/mL. $n = 41$ for reported no smoke exposure and $n = 32$ for reported second-hand smoke exposure.

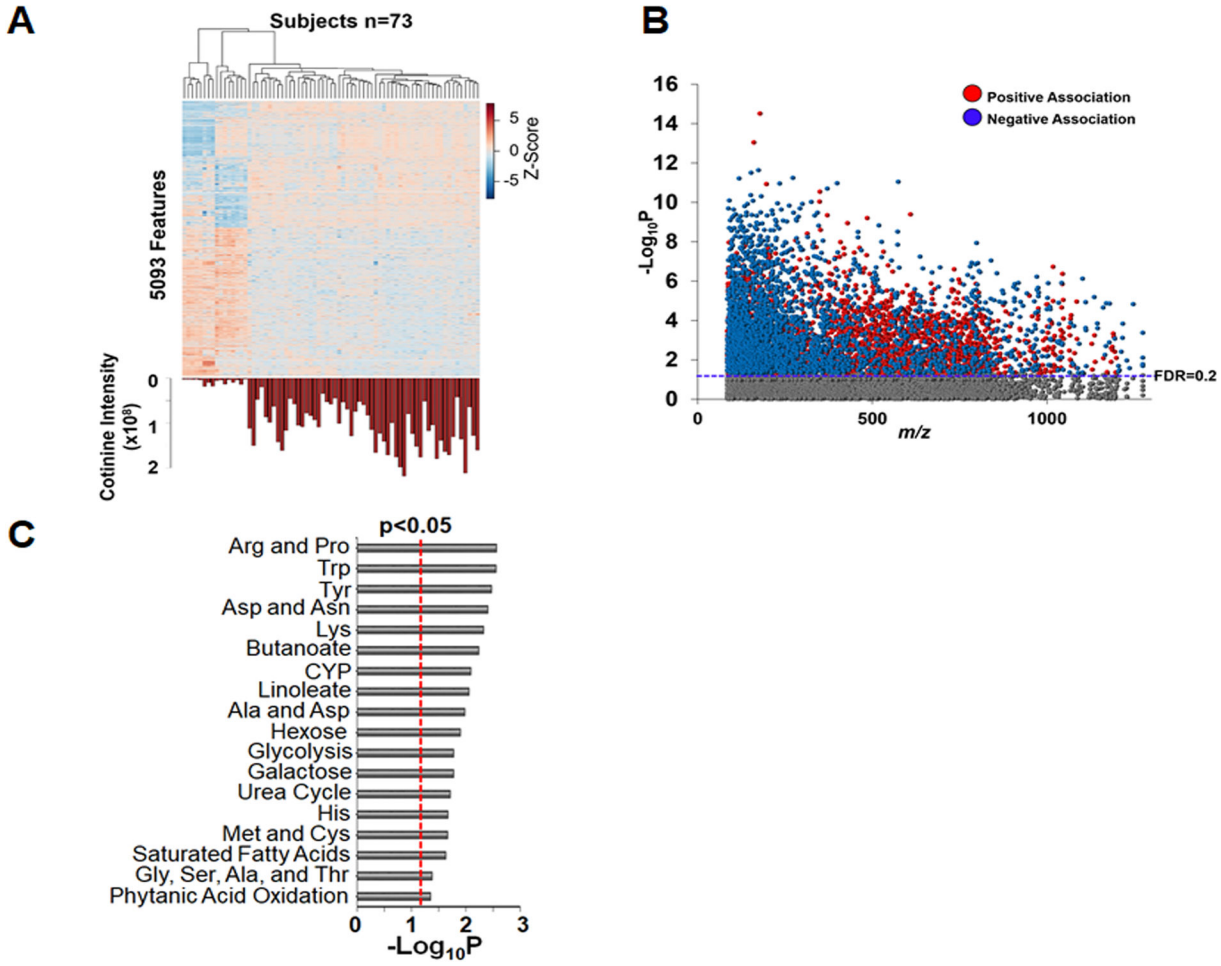

Fig. 2.

Metabolome-wide association study of cotinine exposure in human plasma. A) Unsupervised hierarchal clustering heatmap using the HILIC column coupled with positive ionization (HILIC+) indicates that 5093 metabolic features are associated with cotinine. B) Type I Manhattan plot using m/z plotted against $-log_{10} P$ reveals that 2773 metabolic features are negatively associated (blue) and 2320 metabolic features are positively associated (red) with cotinine. FDR cutoff indicated by the dashed dark blue line, $p < 0.05$ cutoff indicated by dashed grey line. C) Pathway enrichment analysis of cotinine-associated metabolites using HILIC+ data. A total of 18 enriched pathways were determined to be associated with cotinine. Filled gray bars indicate significance ($p < 0.05$) is indicated by the dotted line. $n = 73$ human plasma.

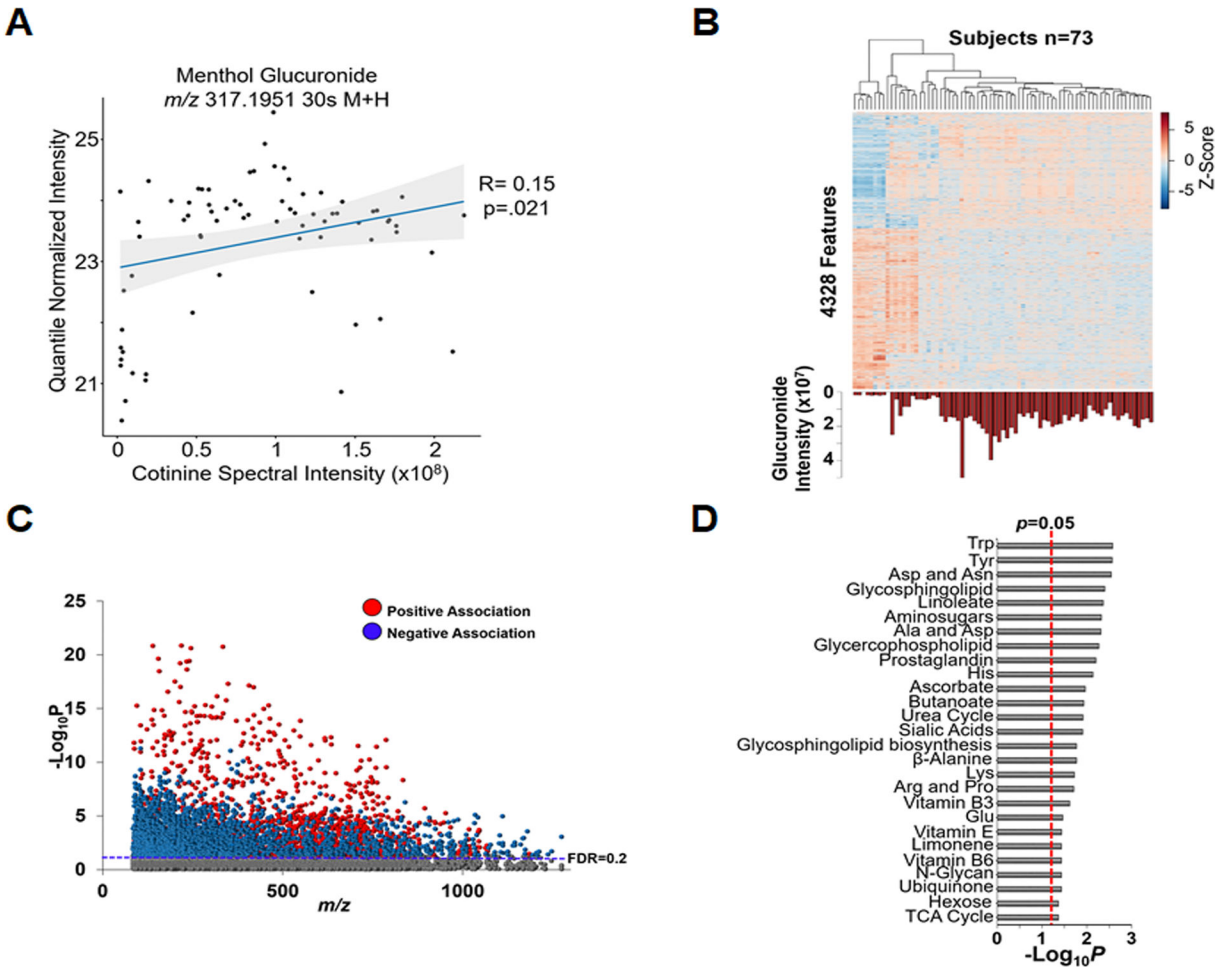

Fig. 3.

Metabolome-wide association study of menthol glucuronide exposure. A) Regression of menthol glucuronide (*m/z* 317.1951, 30 s, *M* + *H*) against cotinine. Data is represented as quantile normalized abundance, with the 95 % confidence interval indicated in the grey shaded area, and the regression curve indicated by the blue line. B) Unsupervised hierarchal clustering heatmap using the HILIC column coupled with positive ionization (HILIC+) indicates that 4328 features are associated with the metabolite. C) Type I Manhattan plot reveals that 1775 features are positively associated (red) with menthol glucuronide and 2553 features are negatively associated (blue) with glucuronide. FDR cutoff indicated by the dashed dark blue line. D) Pathway enrichment analysis of menthol glucuronide associated metabolites using the HILIC+. A total of 27 enriched pathways were determined to be associated with menthol glucuronide. (Filled gray bars indicate significance and the cutoff ($p < 0.05$) is indicated by the dotted line). $p < 0.05$ cutoff indicated by dashed red line. $n = 73$ human plasma.

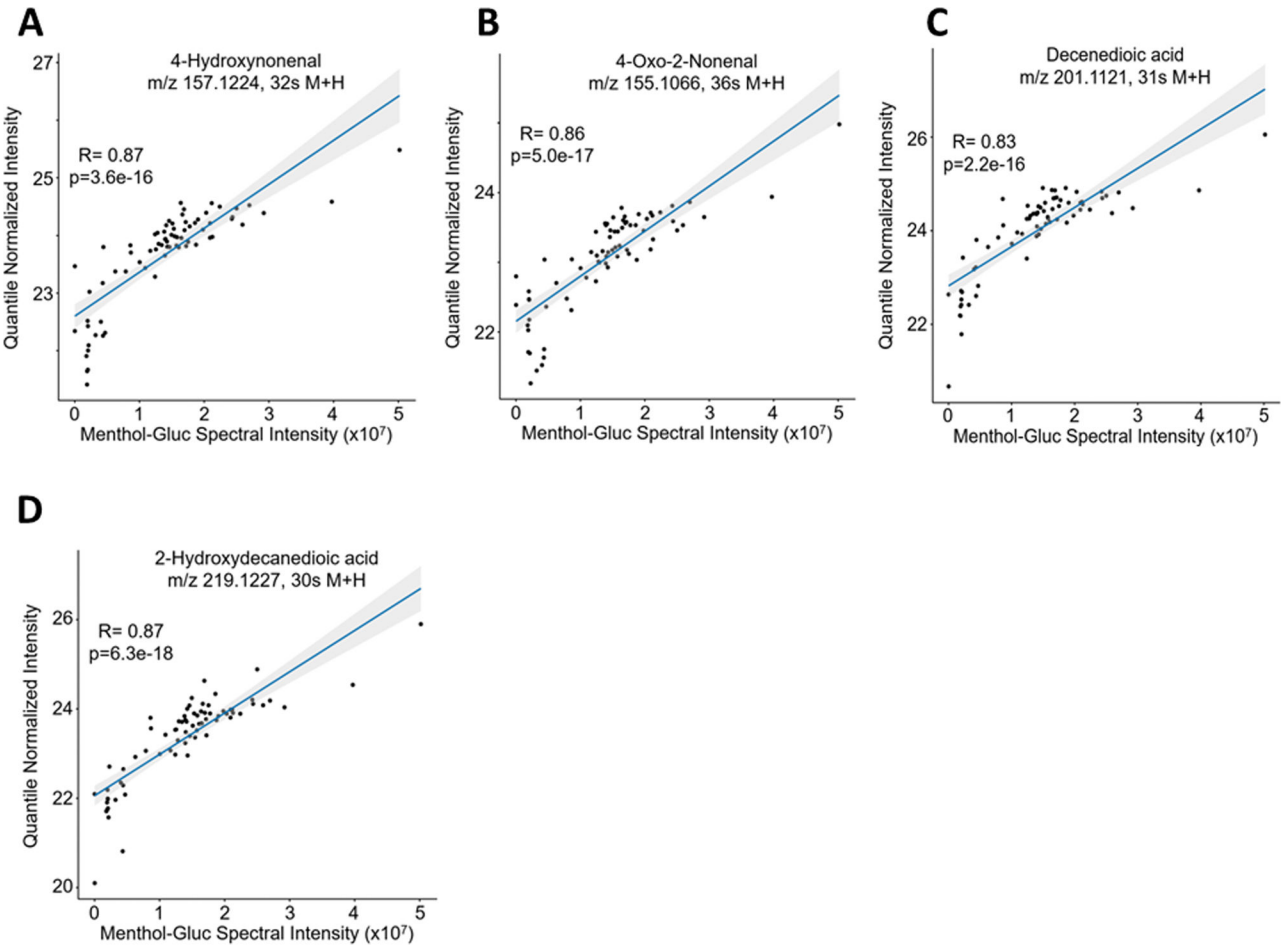

Fig. 4.

Association of lipid peroxides, fatty acids with menthol glucuronide. Association of lipid peroxides and fatty acids (A-D) with menthol glucuronide are shown by regression curve. A) 4-Hydroxynonenal (*m/z* 157.1224, 32 s, *M* + *H*), B) 4-Oxo-2-Nonenal (*m/z* 155.1066, 36 s *M* + *H*), C) Decenedioc Acid (*m/z* 201.1121, 31 s; *M* + *H*), D) 2-Hydroxydecanedioc Acid (*m/z* 219.1227, 30 s, *M* + *H*). Data is represented as quantile normalized abundance, with the 95 % confidence interval indicated in the grey shaded area, and the regression curve indicated by the blue line. *n* = 73 human plasma.

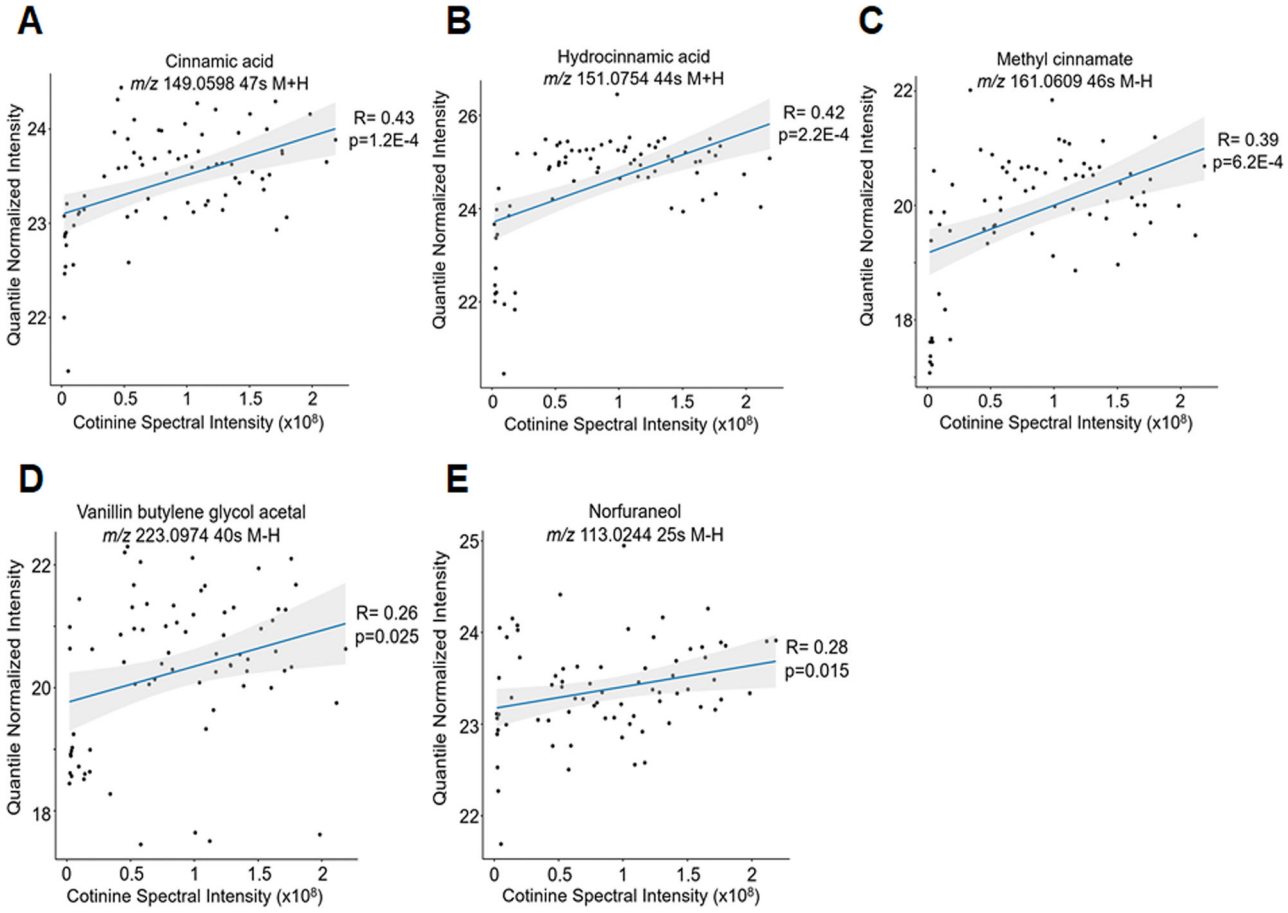

Fig. 5.

Flavoring agents positively associated with cotinine exposure. Correlation of cinnamon-related metabolites, vanillin butylene glycol acetal (D) and norfuraneol (E) with cotinine are shown by the regression curve. A) Cinnamic Acid (*m/z* 149.0598, 47 s, $r = 0.43$), B), Hydrocinnamic Acid (*m/z* 151.0754, 44 s, $r = 0.42$), C) Methyl cinnamate (*m/z* 161.0609, 46 s, $r = 0.39$), D) Vanillin butylene glycol acetal (*m/z* 223.0974, 40 s, $r = 0.26$), and E) Norfuraneol (*m/z* 113.0244, 25 s, $r = 0.28$). Data is represented as quantile normalized abundance, with the 95 % confidence interval indicated in the grey shaded area, and the regression curve indicated by the blue line. $n = 73$ human plasma.

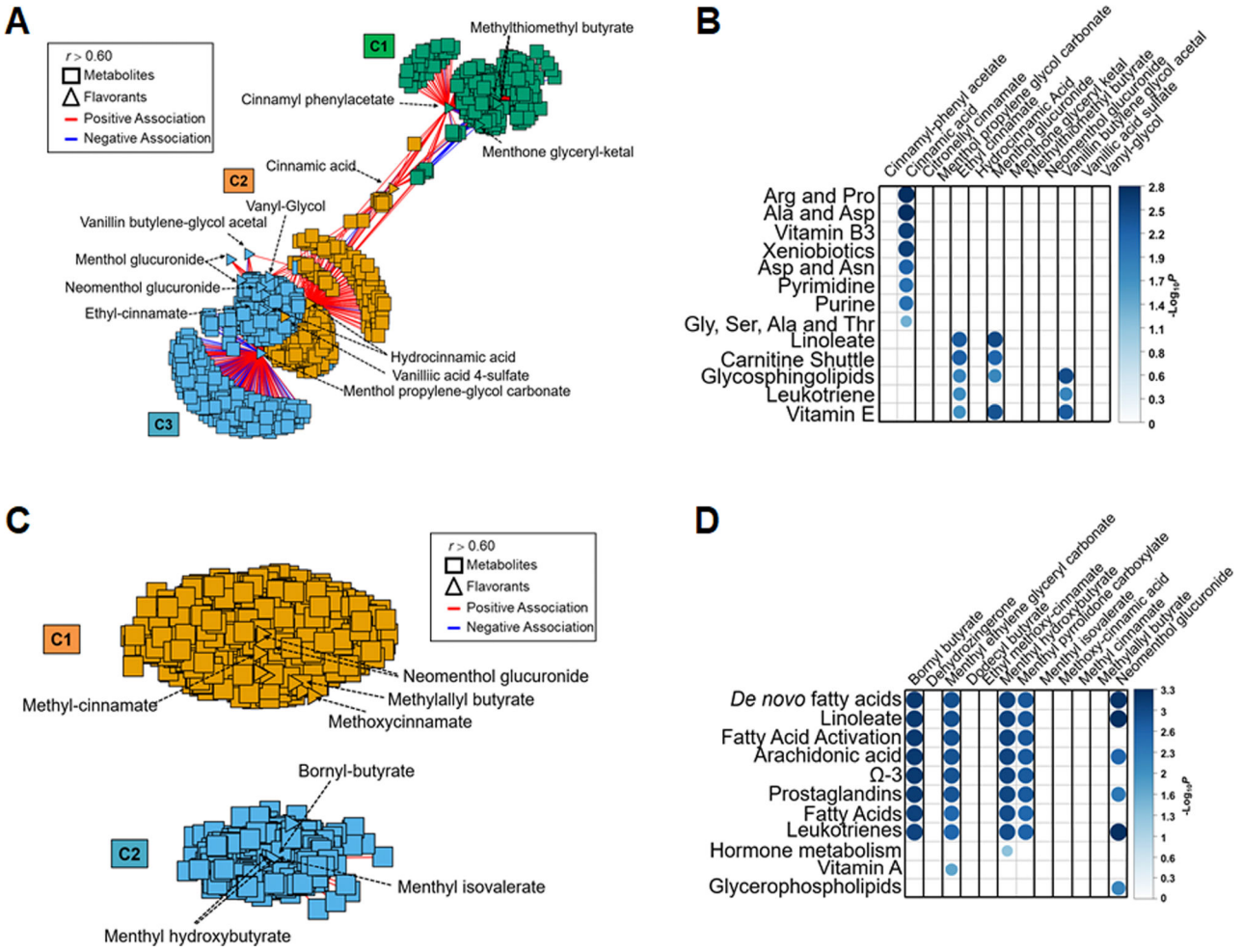

Fig. 6.

Association of flavorants and flavorant metabolites with the metabolome A) xMWAS network of plasma reveals 3 metabolic communities in HILIC+ dataset. Community 1 (green) has 236 metabolic features (squares) which are associated with the flavorants (triangle) cinnamyl-phenyl acetate, methylthiomethyl butyrate (multiple adducts), and menthone glyceryl-ketal. Community 2 (orange) has 226 metabolic features associated with vanillic acid sulfate, cinnamic acid, and hydrocinnamic acid. Lastly, community 3 (blue) has 436 features which are associated with vanillin butylene glycol acetal, menthol glucuronide (multiple adducts), neomenthol glucuronide, vanyl-glycol, ethyl cinnamate, citronellyl cinnamate, hydrocinnamic acid, and menthol propylene glycol carbonate. B) Bubble plot showing metabolic pathways associated with flavorants using the HILIC+ network data. C) xMWAS network of plasma reveals 2 metabolic communities in C18- dataset. Community 1 (orange) has 765 metabolic features (squares) which are associated with the flavorants (triangle) neomenthol glucuronide (multiple adducts), methyl cinnamate, dehydrozingerone, ethyl methoxy−cinnmate, methoxy−cinnamic acid, methylallyl butyrate, and menthyl pyrrolidone carboxylate. Community 2 (blue) has 334 metabolic features associated with bornyl butyrate (multiple adducts), dodecyl butyrate, menthyl hydroxybutyrate (multiple adducts), menthyl isovalerate, and menthyl ethylene glycol carbonate. D) Bubble plot

showing metabolic pathways associated with flavorants in the C18- network data. Metabolic pathway analysis was performed using metabolic features associated with flavorants and metabolites at |r|>0.60 and $p < 0.05$ using mummichog. Red lines indicate positive associations, and blue lines indicate negative associations in network data. For bubble plots, the size and color of the bubbles represents the pathway significance level based on $-\log_{10}$P.

Table 1

Demographic information of exposed cohort.

Demographic Information	Infant	Children	Female	Male	Reported Pathology	Normal	Projected Smoke Exposure	Total Samples
Number	31	42	36	37	57	16	56 %	73

Table 2

Cotinine levels in the cohort.

Cotinine	Median	Mean	Max	Minimum	1st Quartile	2nd Quartile	3rd Quartile	4th Quartile
ng/mL	1.32	1.37	3.44	0.03	0.66	1.32	2.06	3.44

Table 3

Flavorants and flavorant metabolites associated with menthol glucuronide ($P < 0.05$).

Flavorant	Metabolites	Pearson correlation, τ
Menthol	Neomenthol glucuronide	0.68
	Menthone-glycerol-ketal	0.23
	Menthofuran	0.14
Vanillin	Vanyl-glycol	0.71
	Vanillic acid sulfate	0.38
	Homovanillic acid sulfate	0.34
	Homovanillin	0.16
	Vanillin butylene glycol acetal	0.15
Cinnamon	Hydrocinnamic acid	0.79
	Isocinnamyl propionate	0.76
	Cinnamyl alchohol	0.68
	Cyclohexyl cinnamate	0.45
	Cinnamic acid	0.37
	Cinnamyl propionate	0.35
	Cinnamyl phenylacetate	0.2
Other	5-hydroxymaltol	0.46