Heated Tobacco Product Iqos Induces Unique Metabolic Signatures in Human Bronchial Epithelial Cells

Erin O. Curley

Omran Abu Aboud

Kenneth J. Chmiel

Ajay P. Nayak
Thomas Jefferson University

Oliver Fiehn

See next page for additional authors

Authors

Erin O. Curley, Omran Abu Aboud, Kenneth J. Chmiel, Ajay P. Nayak, Oliver Fiehn, Amir A. Zeki, and Pawan
Sharma

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To the Editor:

The tobacco epidemic is one of the most significant health threats, causing 8 million deaths annually [1]. Currently, major tobacco companies have shifted their strategies, effectively enticing the public including youth, through aggressive and misleading advertisements for new electronic nicotine and flavour delivery systems (ENDS), mainly e-cigarettes. More recently, a novel category of e-devices that heat tobacco in specialised cigarettes known as heated tobacco products (HTPs) has been introduced, which aims to replace the dwindling combustible cigarette (CC) market. Unlike traditional CCs, HTPs such as IQOS are a hybrid between a traditional CC and an e-cigarette. They work by heating a metal filament to heat a solid rolled tobacco sheet to 350°C to generate aerosol without combustion [2]. Differentiating biological effects of IQOS from e-cigarettes or CCs is a critical step to understanding mechanisms of disease, and to facilitate the development of a clear long-term strategy to prevent an e-cigarette or vaping use-associated lung injury (EVALI)-like epidemic in the future. Currently, there is a lack of published information regarding the metabolomics of disease pathology associated with IQOS in comparison to traditional CCs and e-cigarettes. The significance of this enquiry lies in the potential to unravel the biochemical and metabolic changes induced by such inhaled products. A deeper understanding of these alterations can provide critical insights into the underlying mechanisms of action leading to disease. Consequently, there is an urgent need to investigate the impact of IQOS and other emerging HTPs on airway structural cells, such as smooth muscle cells or epithelial cells. This research aims to identify unique and novel damaging biosignatures (metabolites) that can serve as indicators of lung pathology in individuals who consume these products. This, in turn, will guide and focus future research efforts.

IQOS induces greater cytotoxicity in bronchial epithelial cells compared to e-cigarettes [3], but it is equally toxic when compared to CCs [2]. In here, we aimed to investigate whether IQOS exposure can induce distinct metabolites that are either upregulated or downregulated in the human bronchial epithelial cells (HBE)-1 cell line (as described in [4, 5]) *versus* traditional CCs and e-cigarettes. We sought to identify novel metabolites and pathways associated with IQOS that may be different to CC smoke and/or e-cigarette vapour. e-Cigarette vapour was generated using e-liquid (18 mg·mL^{-1} nicotine, 1:1 propylene glycol (PG):vegetable glycerine (VG); Vapor Empire, CA, USA); IQOS aerosol was generated using IQOS HeatStick (Marlboro, USA); and CC smoke extract was generated using 1R6F Kentucky Research Cigarette (University of Kentucky, Lexington, KY, USA) using an automated exposure system (CH Technologies, USA). Confluent submerged HBE-1 cells were exposed to either room air (unexposed), CC smoke, e-cigarette vapour or IQOS aerosol for a total of 6 min. We chose a 6-min exposure time to align with the average duration it takes for an individual to smoke a combustible cigarette. It is noteworthy that IQOS allows only 14 puffs before extinguishing, a process also taking ~6 min. This exposure duration was selected to closely mimic or approximate the physiological real-life exposures observed in human subjects. Cells were then subsequently incubated (37°C, 5% carbon dioxide) for a total of 48 h with the exposed media, and then cell lysates were collected using liquid nitrogen with RIPA buffer (CST, USA) with protease and phosphatase inhibitors (Selleck Chemicals, USA). Cell lysates were stored at −80°C until they were ready to be processed and analysed at the UC Davis West Coast Metabolomics Center (Davis, CA, USA) for the abundance of primary metabolites, lipids and biogenic amines. MetaboAnalyst 5.0 (Canada) was used for the statistical analysis (one-factor). MetaboAnalyst combines the fold change (FC) analysis (FC threshold 2; false discovery rate 0.05) and t-test ($p < 0.05$) based on either biological or
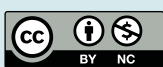
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Metabolic signatures are lacking for heated tobacco products, making it crucial to identify new biosignatures of lung damage. This will enable the establishment of product-specific guidelines and an understanding of associated toxicity. https://bit.ly/3TkhBox
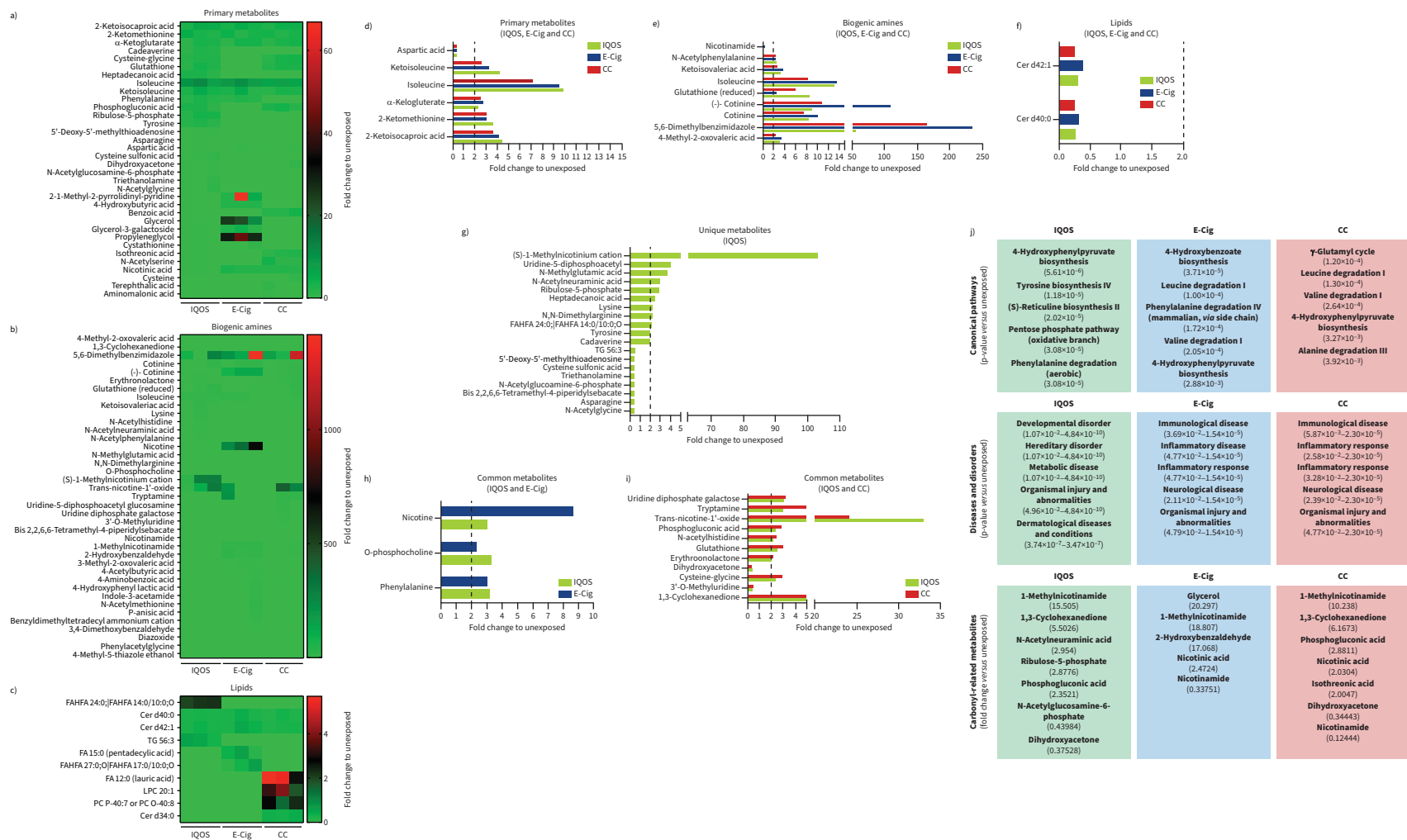
FIGURE 1 The effects of IQOS (heated tobacco product) on the airway epithelial cellular metabolome. Comparison of the effects of IQOS, e-cigarettes (E-Cig) and combustible cigarettes (CC) on human bronchial epithelial (HBE)-1 cells on the abundance of a) primary metabolites, b) biogenic amines and c) lipids. Heatmaps showing statistically significant upregulation or downregulation (p<0.1) for IQOS, E-Cig and CC were compared to unexposed cells using MetaboAnalyst 5.0. d) Primary metabolites, e) biogenic amines and f) lipids that are common between IQOS, E-Cig and CC when compared to unexposed cells. g) Unique metabolites that are seen only in IQOS when compared to unexposed cells. Common metabolites that are seen in h) IQOS and E-Cig and i) IQOS and CC when compared to unexposed cells. j) Canonical pathways and associated diseases and disorders and carbonyl-related metabolites for IQOS, E-Cig and CC when compared to unexposed cells. Data presented are the mean of three independent experiments carried out using HBE-1 cells while significance is shown for two-fold or greater change when compared to unexposed cells using MetaboAnalyst 5.0.

statistical significance to create the significant metabolites followed by Qiagen's Ingenuity Pathway Analysis (IPA) to rank the output pathways by relative abundance from the list and express their enrichment based on $p < 0.05$.

Using the MetaboAnalyst, we were able to determine the upregulated and downregulated metabolites after being exposed to IQOS, e-cigarette and CC. There were 34 primary metabolites, 39 biogenic amines and 10 lipids that were statistically different from unexposed cells *versus* IQOS-, e-cigarette- and CC-exposed cells (figure 1a–c). In addition, we found common primary metabolites, biogenic amines and lipids that were statistically significant in the IQOS-, e-cigarette- and CC-exposed groups *versus* unexposed cells (figure 1d–f). When IQOS-exposed cells were compared to unexposed cells, there were unique metabolites that were not common with e-cigarette- or CC-exposed cells (figure 1g). In addition, when compared to unexposed cells, there were common metabolites between IQOS and e-cigarette (figure 1h), as well as IQOS and CC (figure 1i). Even though IQOS has metabolites similar to e-cigarette- and CC-aerosol-exposed cells, our data suggest that IQOS has a unique biochemical fingerprint that is not observed with the other exposure devices.

e-Cigarettes and CCs are known to cause carbonyl exposure, and carbonyls are the most abundant toxic species in aerosols generated from smoking devices [6]. Each exposure group when compared to unexposed cells were enriched with a few metabolites that were linked to carbonyls (figure 1j). The common carbonyl-grouped metabolite in all three comparisons is 1-methylnicotinamide (a metabolite of nicotinamide, and product of nicotinamide N-methyltransferase) [7]. It was significantly increased by 10- to 18-fold in each group and recent studies have shown that nicotinamide N-methyltransferase is highly expressed in various cancers such as oral squamous cell carcinoma and gastric cancer [8, 9]. While there was no common carbonyl metabolite between all groups; 1,3-cyclohexanedione was abundant in both IQOS and CC *versus* unexposed. This finding is significant because the high levels of carbonyls in these products indicate that despite people using e-cigarettes and IQOS as a "safe alternative" to traditional CC smoking, these products may still be harmful.

Using the IPA, we were able to undertake a core analysis that identified canonical pathways and diseases and disorders associated with these metabolites. All exposures induced the canonical pathway of 4-hydroxyphenylpyruvate biosynthesis, which is synthesised by 4-hydroxyphenylpyruvate dioxygenase, which is found to be highly expressed in lung cancer [10]. IQOS exposure induced unique canonical pathways that were not observed with e-cigarette and CC exposure (figure 1j). Furthermore, the pathways induced by IQOS are associated with numerous human diseases and disorders, including developmental and hereditary disorders, metabolomic diseases, organismal injury and dermatological diseases. The most common pathology associated with IQOS exposure is organismal injury and abnormalities (figure 1j).

Smoking is still a leading cause of COPD, adversely impacts other airway diseases such as asthma, and negatively affects other organs in the body (*e.g.* aerodigestive tract, cardiovascular system, gut) [11]. Despite the declining use of traditional CC smoking, this trend could reverse with the introduction of emerging smoking products acting as a novel gateway to new generations of ENDS users. In our study, we utilised immortalised HBE-1 cells that are well established in the field [4, 12]. Airway epithelial cells serve as the initial defence barrier in the human lungs to environmental contaminants, particularly in the context of smoke exposure. Additionally, epithelial cells preserve the integrity of the mucosal lining, regulate local immune responses, mucus release, balance inflammatory responses and produce biologically active substances to maintain innate immunity function against infections and other noxious agents [13].

This study is the first to reveal the substantial increase in metabolites induced by IQOS, some of which are distinct compared to those induced by e-cigarettes and CCs. These metabolites activate pathways linked to various diseases including inflammation and cancer. Furthermore, the identification of unique metabolites associated with each type of exposure enables the creation of a distinct product-specific signature. Such signature profiles have the potential to aid in predicting the development of disease pathology in the future. In summary, our initial, yet significant, findings here will lay the groundwork for more extensive human and basic mechanistic studies focused on validating and further characterising the causal role of these metabolites.

Erin O. Curley[1], Omran Abu Aboud[2], Kenneth J. Chmiel[2], Ajay P. Nayak[1], Oliver Fiehn[3], Amir A. Zeki [2,4,5,6] and Pawan Sharma [1,6]

[1]Center for Translational Medicine, Division of Pulmonary, Allergy and Critical Care Medicine, Jane and Leonard Korman Respiratory Institute, Sidney Kimmel Medical College, Thomas Jefferson

University, Philadelphia, PA, USA. [2]UC Davis Lung Center, Davis, CA, USA. [3]West Coast Metabolomics Center, UC Davis Genome Center, University of California, Davis, CA, USA. [4]UC Davis Reversible Obstructive Airway Disease Center, Department of Internal Medicine, Division of Pulmonary, Critical Care, and Sleep Medicine, University of California, Davis, CA, USA. [5]Veterans Affairs Medical Center, Sacramento, CA, USA. [6]A.A. Zeki and P. Sharma contributed equally to this article as lead authors and supervised the work.

Corresponding author: Pawan Sharma (pawan.sharma@jefferson.edu)

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