

Analyses of lung cancer-derived volatiles in exhaled breath and *in vitro* models

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Impact Statement

Lung cancer is a debilitating disease with an average 5-year survival rate of about 10–20%. As a result of its rising global incidence, lung cancer has become the most common cause of cancer deaths. The development of novel diagnostic methods and tools for early disease detection is critical to increase the rate of successful treatment. Recently, the detection of cancer-derived volatile organic compounds (VOCs) in human breath has been proposed to provide insight into the endogenous metabolic processes of cancer. Due to the non-invasive nature of sample collection, monitoring VOCs and exhaled breath volatiles as cancer biomarkers has gained traction for the potential to be used for disease diagnostics. Advancements in analytical platforms have allowed the screening of hundreds of volatile compounds; it is thus critical to evaluate the biological origins of these compounds in the context of lung cancer to pave the way for early diagnosis, disease monitoring, and therapy outcomes.

Abstract

Lung cancer is one of the leading causes of cancer incidence and cancer-related deaths in the world. Early diagnosis of pulmonary tumors results in improved survival compared to diagnosis with more advanced disease, yet early disease is not reliably indicated by symptoms. Despite of the improved testing and monitoring techniques for lung cancer in the past decades, most diagnostic tests, such as sputum cytology or tissue biopsies, are invasive and risky, rendering them unfeasible for large population screening. The non-invasive analysis of exhaled breath has gained attentions as an innovative screening method to measure chemical alterations within the human volatilome profile as a result of oncogenesis. More importantly, volatile organic compounds (VOCs) have been correlated to the pathophysiology of disease since the source of volatile compounds relies mostly on endogenous metabolic processes that are altered as a result of disease onset. Therefore, studying VOCs emitted from human breath may assist lung cancer diagnosis, treatment monitoring, and other surveillance of this devastating disease. In this mini review, we evaluated recent human studies that have attempted to identify lung cancer-derived volatiles in exhaled breath of patients. We also examined reported volatiles in cell cultures of lung cancer to better understand the origins of cancer-associated VOCs. We highlight the metabolic processes of lung cancer that could be responsible for the endogenous synthesis of these VOCs and pinpoint the protein-encoding genes involved in these pathways. Finally, we highlight the potential value of a breath test in lung cancer and propose prominent areas for future research required for the incorporation of VOCs-based testing into clinical settings.

Keywords: volatile organic compounds, lung cancer, breathomics, metabolic deregulation

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Introduction

Lung cancer is a debilitating disease with an average 5-year survival rate ranging between 10% and 20%.¹ As a result of its rising global incidence, lung cancer has become the second most frequently diagnosed cancer and responsible for 18% of the total cancer deaths.² Lung cancer patients are generally diagnosed at the age of 65 years or above with late-stage disease,³ where the histotypes of these tumors are commonly divided into small cell lung carcinoma (SCLC) and non-small cell carcinoma (NSCLC) with specific DNA mutations permitting further molecular stratification.⁴ Smoking is still considered the primary culprit responsible for most

respiratory cancers, including esophageal adenocarcinoma and esophagogastric junctional adenocarcinoma.^{5,6} Early diagnosis of lung cancer results in improved survival compared to diagnosis with more advanced disease, as increased accessibility to positron emission tomography with computed tomography (PET-CT) scanning and endobronchial ultrasound for the sampling of mediastinal lymph node have improved the accuracy of staging for lung cancers.⁷ Lower stages of lung cancer (I–III) are treated with surgical resection since the tumors have yet to metastasize. Surgery affords a 5-year survival rate of 70–90% for localized tumors.⁸ Patients with early-stage disease exhibit general symptoms, such as fatigue and chest pains,⁹ thus the cancer is not reliably

indicated by symptoms. Therefore, up to 75% of patients present advanced disease at the time of diagnosis.¹⁰ Most of the currently used diagnostics for lung cancers, such as tissue biopsy and sputum cytology, have yet to meet the standards of screening, as they are insufficiently accurate, invasive, and risky,¹¹ rendering them impracticable for large population screening. Meanwhile, advancements in high-throughput analytical technologies have greatly enhanced our understanding of cancer itself. Substantial efforts have been focused toward uncovering the relationship between the volatile signatures of the human body and the presence of cancer.^{12,13} VOCs emitted through the endogenous metabolic processes of cells have been hypothesized to be released into the blood where they subsequently diffuse across the pulmonary alveolar membrane and are exhaled through breath.¹⁴ Non-invasive breath analysis has been proposed as an innovative means to study some of the hundreds of VOCs exhaled by humans, as exhaled breath contains many reported VOCs that can reflect biochemical processes are deregulated as a result of biological activities, such as oncogenesis, oxidative stress, or inflammation.¹⁴ Since the source of volatile compounds relies mostly on endogenous metabolic processes primarily related to oxidative stress,¹⁵ which are tightly coupled with oncogenesis,¹⁶ VOCs analyses have been considered to provide unique markers for the detection of cancer.

Case-control studies have attempted to analyze specific VOCs in exhaled breath of cancer patients and healthy individuals, with promising results identifying cancer-specific VOCs in liver and breast cancers.^{17,18} In addition, pioneering work from Nakhleh et al.¹⁹ achieved 813 independent diagnoses of 1 of 17 different diseases, including colon, prostate, and esophageal cancers, using exhaled breath samples from 1404 subjects and 591 control subjects. Evidence from several published studies in lung cancer patients highlights abnormal VOCs in exhaled breath.^{13,20,21} This has been confirmed with many analytical methods, such as gas chromatography-mass spectrometry (GC-MS).^{4,22,23} In addition, exhaled breath analysis revealed altered VOC profiles in lung cancer patients after tumor resection.²⁴ Thus, it can be postulated that in addition to disease diagnosis; volatilomics can also offer a non-invasive method to detect drug treatment response in patients. Promising evidence for this method has emerged where drug metabolites have been reportedly detected in exhaled breath condensates.²⁵ Taken together, breath volatiles appear to bolster a potential utility as part of the screening protocols for the evaluation of lung cancer onset in patients.

Over the past decade, researchers have reported numerous candidate compounds as diverse as toluene, acetaldehyde, benzene derivatives, and alcohols to serve as potential biomarkers of lung cancer.²² It has now become the time to place a greater emphasis on using this gained knowledge of lung cancer-associated volatiles to enhance our understanding of their potential biological origins and involvement in cancer pathogenesis. The goal of this mini review is to discuss some highlights of the recent analytical methods and research progress made in the analysis of lung cancer-derived VOCs in human and cell studies. Since the potential endogenous

origins have yet to be elucidated, we probed publicly available databases to identify endogenous metabolic pathways in which the identified VOCs can be generated based on several clinical studies; we also conducted a secondary analysis of the available data to highlight the biochemical pathways *in vivo*.

Analytical requirements and emerging technologies for human breath volatile analysis

Detection of breath volatiles requires robust analytical techniques that allow chemical identification of low abundance volatiles, which can range in concentrations between parts-per-million (ppmv) and parts-per-trillion by volume (pptv).²⁶ Among several analytical approaches that have been implemented for the assessment of exhaled VOCs, the two mainstream ones are as follows: (1) semi-selective analytical sensors aimed at pattern recognition to uncover disease fingerprints^{19,27} and (2) high-throughput mass spectrometry practices that seek to detect and quantify the emitted VOCs in complex biological matrices.^{28,29} Utilizing analytical sensors provides an advantage since analyses are conducted on a preselected subset of compounds. These sensors can include colorimetric or electronic chemical sensor arrays, which are akin to targeted analyses in mass spectrometry platforms, where only a subset of analytes is considered in the investigation. Upon detecting the desired analytes, the electronic chemical sensors will determine the signal as a shift in electrical current.³⁰ Conversely, colorimetric sensors entail chemically reactive colorants printed on a disposable cartridge which change color when interacting with the desired analytes.³¹ While this analytic method is convenient and portable, it does include some exhaled breath from nasal airways. Thus, it is important to standardize sampling protocols to fully capture the VOCs excreted by the lungs.

In the case of mass spectrometry-based analyses, several analytical platforms have been used for VOC identification, including selective-ion flow-tube mass spectrometry (SIFT-MS)³² and proton-transfer reaction mass spectrometry (PTR-MS),³³ while GC-MS²⁰ has been deemed the gold standard. In addition to increased sensitivity of volatile analyte detection, mass spectrometry-based analyses offer a unique advantage in the ability to detect and identify new analytes that may not have been previously reported³⁴ as they are able to detect a wider array of VOCs reported by untargeted analyses to unravel prominent VOCs as biomarkers. Untargeted analyses have exhibited better discriminatory ability in cancer patients,³⁵ which is attributed to the broader range of chemicals detected. In addition, the semi-quantitative nature of mass spectrometry analyses also affords a unique advantage in the form of comparative analyses to monitor changes in lung cancer-derived volatiles in response to drug-based therapies. Emerging methods for volatile analyses in exhaled breath have been reviewed elsewhere.³⁶⁻³⁸ While the constantly evolving technologies for volatile analyses empower great research discoveries, this review seeks to highlight the

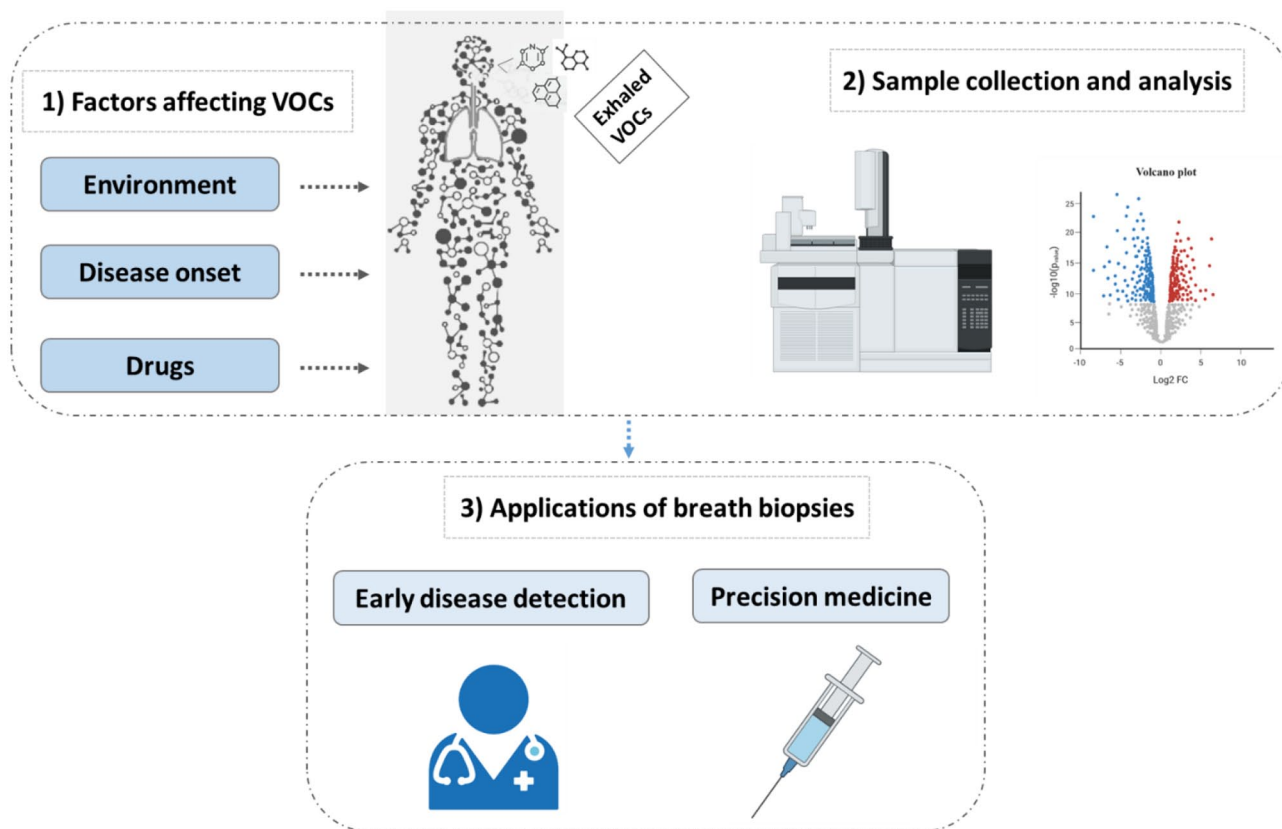


Figure 1. General schematic of breathomics workflow and clinical applications. (A color version of this figure is available in the online journal.)

detected volatiles in lung cancer studies and their biological and clinical relevance.

The composition of lung cancer volatilome

Exhaled breath contains thousands of trace volatiles that are generated endogenously within the body or absorbed from the environment (Figure 1). Exhaled breath is proposed to consist of three phases:³⁹ Phase I, II, and III. Phase I, deemed dead space, consists of exogenous volatiles from the environment and offers little clinical insight. This is followed by Phase II which represents the transition phase as it is a mixture of exogenous and endogenous volatiles from the upper respiratory tract. These VOCs may provide clinical relevance, yet analyses must be taken with background influence in mind. Phase III, termed alveolar phase, consists of the biologically relevant volatiles that originate from endogenous metabolic processes. Collectively, VOC patterns in breath are considered to mirror a person's health condition since the volatiles can be products of many metabolic pathways.⁴⁰ To better understand the chemical composition of the lung cancer volatilome, we sought to first summarize the volatiles reportedly deregulated in breath of lung cancer patients. We probed the literature for studies using mass spectrometry-based methods to analyze volatiles in either lung cancer patients or cell lines *in vitro* ranging from 2015 to 2021. Herein, it was evident that GC-MS-based methods were the most frequently used (Table 1). In lung cancer

patients, it was apparent that 145 unique VOCs encompassing 20 unique compounds classes were reportedly deregulated in exhaled breath of lung cancer patients with respect to healthy controls (Figure 2(A)).

Detection of acetonitrile, toluene, and some benzene derivatives in exhaled breath, for example, is proposed to be mainly associated with smoking,⁴⁶ yet these volatiles were also reported in non-smokers, suggesting they can also arise from other environmental exposure to gas pollutants or secondhand smoking.⁴⁷ Nonetheless, 13 VOCs were reported in at least three independent studies which screened different population of patients (Figure 2(B)). Acetone, benzene, and ethylbenzene were the most frequently reported VOCs as they were shown to be elevated in lung cancer patients in five distinct studies (Table 1). Four studies reported 1-propanol, 2-propanol, cyclohexane, cyclohexanone, and nonanal. Benzene and its derivatives are most commonly reported in breath of smokers but have also been proposed as lung cancer markers in breath.⁴⁸ Detection of ketones, such as acetone, is also common since it is a by-product of acetyl-CoA decarboxylation, a process catalyzed by the enzyme acetyl-CoA carboxylase. Acetyl-CoA carboxylase is involved in fatty acid synthesis mediated by the expression of the ACC gene, which is upregulated in lung cancer cells.⁴⁹ Propanol has been proposed to be formed endogenously in humans when ethanol is present.⁵⁰ Ethanol is also reported in breath of lung cancer patients and is a downstream product of glycolysis.⁵¹ Increased glycolytic activity in pulmonary tumors could explain this finding. Collectively, multiple

Table 1. Summary of the study populations and analytical methods in the selected relevant literature.

Study	Chen et al.	Huang et al.	Koureas et al.	Sakumura et al.	Schallschmidt et al.	Song et al.	Sun et al.	Tsou et al.
Sample size (age)	122 (49.58 ± 7.71) 160 (61.19 ± 9.91)	30 (59) 20 (70)	53 (66.8 ± 13.2) 51 (70.9 ± 8.1)	29 (n/a) 107 (n/a)	23 (61.4) 37 (65.9)	43 (47.9 ± 6.5) 41 (58.1 ± 8.1)	30 (34.27 ± 9.38) 30 (52.35 ± 15.5 and 59.40 ± 8.97)	168 (31.4 ± 10.4) 148 (64.5 ± 11)
Smoking status	171 131	N/A N/A	86 18	70 66	25 35	21 63	24 36	57 259
Disease stage	i: 56, II: 18, III: 22, IV: 58, N/A: 6	N/A	N/A	i: 55, II: 15, III: 28, IV: 9	N/A	i: 13, II: 7, III: 6, IV: 17	N/A	i: 1, II: 4, III: 35, IV: 105
Analytical tools	GC-MS	SPME GC-MS	SPME GC-MS	GC-MS	SPME GC-MS	SPME GC-MS	PTR-MS	SIFT-MS
Reference	Choueiry and Zhu ⁴¹	Phillips et al. ⁴²	Graff et al. ⁴³	de Vries et al. ⁴⁴	Dillon et al. ⁴⁵	Chen et al. ²⁵	Sun et al. ⁷⁸	Bianchi et al. ²⁸

GC-MS: gas chromatography–mass spectrometry; PTR-MS: proton-transfer reaction mass spectrometry; SIFT-MS: selective-ion flow-tube mass spectrometry; SPME: solid-phase microextraction.

reports of the same VOCs across different studies increase the confidence that these volatiles are truly associated with lung cancer incidence. A full list of the reported VOCs is summarized in Table 2 and encompasses all the reported classes, including alcohols, aldehydes, and benzene derivatives, among others.

To better understand which volatile results strictly from cancer metabolism compared to other metabolic processes, we next screened the literature for *in vitro* volatiles of cultured lung cancer cells in efforts of excluding the contribution of systemic individual metabolism on the chemical composition of the volatilome. It was apparent that 84 unique VOCs were detected in lung cancer cell cultures and pertain to 11 distinct chemical classes (Figure 2(C)). Alkanes, benzene derivatives, and alcohols were among the most notable compound classes identified. A summary of the reported volatiles emitted from *in vitro* cell cultures is outlined in Table 2. Among these volatiles, 2-ethyl-1-hexanol, benzaldehyde, decanal, and nonanal were the most prevalently identified volatiles in culture headspace (Figure 2(D)). These findings are not surprising since alkanes were the most frequently reported compound classes in both the *in vitro* and *in vivo* literature.

As of yet, there exists no unanimous approach to probe VOCs, as evident by the various analytical techniques and sample matrices explored. This results in convoluted lists of identified VOCs, with no mutually agreed on volatiles as putative markers. Thus, it may be beneficial to compare VOCs in the headspace of cell cultures and exhaled breath of lung cancer patients to identify overlapping volatiles as potential markers for disease. We highlighted 16 reported VOCs in both breath and the headspace of cell cultures (Figure 3). Namely, these compounds are nonanal, propylbenzene, toluene, decanal, 1,2,4-trimethylbenzene, ethylbenzene, hexanal, 2-ethyl-1-hexanol, limonene, acetone, styrene, benzaldehyde, cyclohexanol, propanal, dodecane, and o-Xylene. Detection of benzene derivatives from the headspace of cell cultures was a little surprising since metabolic pathways responsible for their endogenous synthesis have not been described. It is thus highly probable that some of the reported aromatic derivatives (e.g. trimethylbenzene and 2,6-butylated hydroxytoluene), halogenated volatiles (e.g. dichloromethane and chlorobenzene), and silylated compounds (bis(trimethylsilyl)ester and hexamethylcyclotrisiloxane) are a result of background contamination during sample collection or the reflection of recent human exposures to airborne, food, or water contaminants. In addition, these compounds are generally associated with smoking and environmental exposure.⁵² Thus, one must be cautious before suggesting benzene derivatives solely as lung cancer markers. Alcohols and aldehydes, such as 2-ethyl-1-hexanol and benzaldehyde, were also among the most reported compound classes. Since lung cancers heavily rely on glycolysis for sustained proliferation,⁵³ it is suspected that some of these VOCs are downstream products of endogenous energy metabolism. Additional validation experiments, such as metabolic flux analysis, could be further performed. Therefore, it is imperative to continue building knowledge of metabolic alterations in lung cancers to uncover the biochemical origin of exhaled

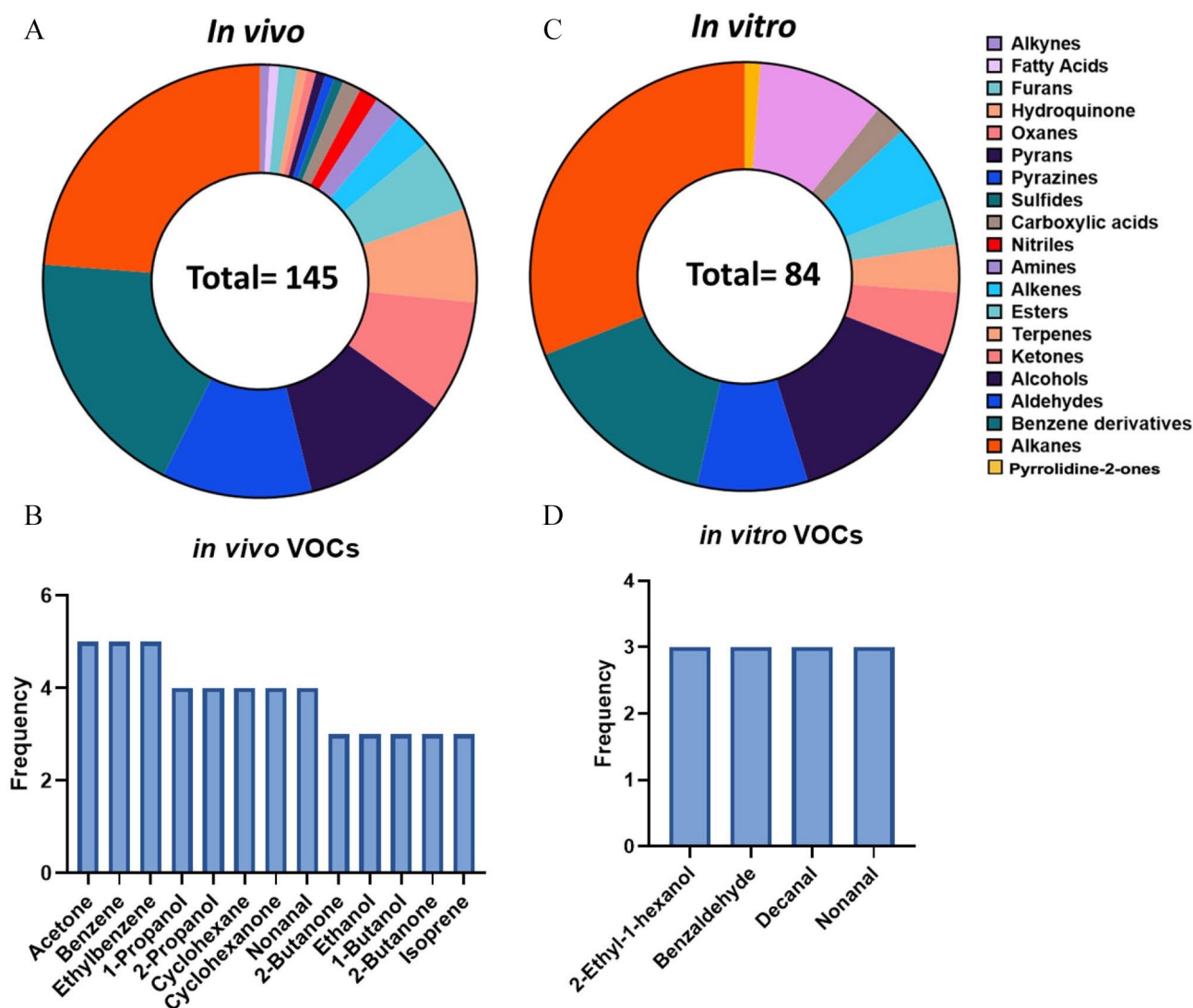


Figure 2. Classes and frequency of lung cancer-derived VOCs reported in *in vivo* and *in vitro* studies. A total of 20 unique compound classes were reported in the literature, where 84 distinct VOCs were identified in (A) *in vitro* cell lines compared to (B) 143 reported *in vivo* from exhaled breath. (C) In total, 4 VOCs were reported in three or more cell line studies compared to (D) 13 VOCs in three or more breath studies. (A color version of this figure is available in the online journal.)

volatiles in efforts of cementing VOCs as critical markers for lung cancer diagnosis.

Endogenous origins of lung cancer VOCs

The source of the VOCs and semi-volatile VOCs in breath is largely under-investigated and hard to trace.⁵⁴ While endogenous volatiles are assumed to be related to changes to oxidative stress and metabolic activities, exogenous VOCs can be attributed to smoking habits or air pollution.⁴⁶ As reported before, the vast majority of VOCs exhaled by lung cancer patients pertain to classes of alkanes, alcohols, aldehydes, ketones, nitriles, and aromatic compounds.⁵⁵ This was also commensurate with our literature summary for this mini review. To better understand the metabolic origins of VOCs in humans, we explored the Kyoto Encyclopedia of Genes and Genomes (KEGG) for the identified compounds in metabolic pathway databases and attempted to link the

volatiles to biochemical alterations that occur in lung cancer (Table 3). In total, 11 metabolic pathways were identified that assisted our interpretation of biological origins of seven lung cancer-associated volatiles. Those metabolic processes include glycolysis/gluconeogenesis, pyruvate metabolism, glycerophospholipid metabolism, propanoate metabolism, glutathione metabolism, glyoxylate and dicarboxylate metabolism, biosynthesis of unsaturated fatty acids, fatty acid elongation, fatty acid degradation, fatty acid biosynthesis, and steroid hormone biosynthesis. It was also apparent that hexadecanoate and acetaldehyde were the most involved compounds, as they were reported in four and three pathways, respectively. Hexadecanoate is a fatty acid anion and the conjugate base of palmitic acid, the primary product of the fatty acid synthase, and an integral part of cell membrane structure.⁵⁶ It is not surprising that it was frequently identified as an intermediate of fatty acid biosynthesis, elongation, and degradation. Studies have demonstrated that unbalanced redox environment in which cancer cells grow

Table 2. VOCs and their respected classes reported in the selected literature.

Class	VOC
Alcohols	Dimethylbenzyl alcohol* ⁺ , 1-butanol*, 1-pentanol*, 1-propanol*, 2-ethyl-1-hexanol* ⁺ , 2-methylpropanol*, 2-propanol*, 3-methyl-1-butanol*, cedrol*, cyclohexanol* ⁺ , dodecanol*, ethanol* [†] , methanol*, n-butanol*, n-nonanol*, n-octanol alcohol*, trans-2-hexenol*, 1-decanol*, 1-nonanol*, 1-undecanol*, 2,4-decadien-1-ol [†] , 2-butanol*, 2-ethyl-1-hexanol*
Aldehydes	(E)-2-heptenal*, 2-ethylhexanal*, 2-methyl-2-propenal*, acrolein*, benzaldehyde* ⁺ , ethanal* [†] , ethanedial*, hexanal* ⁺ , n-butanal*, n-decanal* ⁺ , n-hexanal* [†] , n-octanal*, nonanal* ⁺ , n-pentanal*, octanal*, propanalaldehyde*, 1-dodecanal ⁺ , undecanal ⁺ , 4-hydroxynonenal ⁺ , propanal ⁺
Alkanes	1,5-diaminopentane*, 2,2,4-trimethylpentane*, 2,2-dimethylpentane*, 2,2-dimethylbutane*, 2,3-dimethylpentane*, 2,4-dimethylheptane*, 2-methylhexane*, 2-methylpentane* [†] , 3-methylhexane*, 3-methylpentane* [†] , 4-methyloctane*, 4-methylheptane*, butane*, chloroform*, cyclohexane*, dichloromethane* [†] , dodecane*, ethylcyclohexane*, heptane*, 3-ethyl-3-methylheptane*, hexamethylcyclotrisiloxane*, hexane*, methylcyclopentane* [†] , methylcyclohexane*, methylpentane*, n-heptane* [†] , n-hexane* [†] , n-nonane nonyl hydride*, n-octane*, n-pentane*, octane*, pentanal*, propylcyclohexane*, trichloromethane* [†] , undecane*, pentadecane*, hexadecane*, heptadecane*, octadecane*, tridecane*, 2-methylpentane*, 2,4-dimethyldodecane*, 2-bromododecane*, 2-methyl-5-ethylheptane*, 4,6-dimethyldodecane*, n-dodecyl chloride [†] , heneicosane*, tetradecane*, trimethyl[4(trimethylsilyl)butoxy]silane ⁺
Alkenes	1-butene*, 1-heptene*, 1-hexene*, 2,4-dimethyl-1-heptene*, 2,4-dimethyl-2-pentene, 1-decene*, 1-dodecene*, heptamethylnonene*
Alkynes	1-butyne*
Amines	1-methylpyrrolidine*, isopropylamine*, trimethylamine*
Benzene derivatives	1,2,3,4-tetrahydronaphthalene*, 1-methylindan*, 2,6-butylated hydroxytoluene* [†] , 4,7-methano-1H-indene*, octahydro-, benzene* [†] , benzene*, 4-ethyl-1,2-dimethyl, phenol*, 1,2,3-trimethylbenzene*, 1,2,4-trimethylbenzene* ⁺ , 1-methyl-3-propylbenzene*, 2-ethyl-p-xylene*, 4-ethylbenzamide*, benzoic acid*, chlorobenzene*, cymene*, dichlorobenzene*, ethylbenzene* [†] , n-propylbenzene*, n-propylbenzene*, o-Xylene*, p-Cymene*, propylbenzene* ⁺ , pyridine*, styrene* ⁺ , tetramethylbenzene* [†] , toluene* [†] , xylene* [†] , phenol*, benzene*, 1,3-bis(1,1-dimethylethyl)-, 2-ethyl-1,3-dimethyl-benzene*
Benzofurans	3,7-dimethyl, hexahydro-benzofuranone*
Carboxylic acids	Formic acid*, propanoic acid*
Esters	2,2,4-trimethyl-3-carboxyisopropyl pentanoic acid isobutyl ester* [†] , butylacetate*, DL-sec-butyl acetate*, ethylacetate*, ethylbutyrate*, methylacetate*, methylundecanoate*, methylmyristate*, propylacetate*, 2-ethylhexyl benzoate*, methyl salicylate*
Fatty acids	Palmitate*, propionate*, 3-methyl pentanoate*, caproate*, heptanoate*, acetate*, formate*, isobutyrate*, butyrate*, valerate*, methyl acetate [†] , ethyl acetate [†]
Furans	2,5-dimethylfuran*, tetrahydrofuran*
Hydroquinones	2,6-bis(1,1-dimethylethyl)-1,4-benzenediol*
Ketones	2,3-butanedione*, 2-butanone*, 2-pentanone* [†] , 3-hydroxy-2-butanone*, 4-heptanone*, acetoin*, acetone* [†] , acetophenone* ⁺ , camphor*, cyclohexanone* [†] , cyclopentane*, 2-dodecanone*, 2-pentadecanone*
Nitriles	Acetonitrile*, acrylonitrile*
Oxanes	2-hydroxy cineole*
Pyrans	2,3-dehydro-1,8-cineole*
Pyrazines	2-methylpyrazine*
Pyrrolidine-2-ones	1-methyl-2-pyrrolidinone
Sulfides	Dimethylsulfide*
Terpenes	3-methylbutanal*, 4-isopropyl toluene*, 5-isopropenyl-2-methyl-7-oxabicyclo heptan-2-ol*, alpha-pinene*, alpha-terpinene*, beta-caryophyllene*, eucalyptol*, isoprene*, limonene* ⁺ , pinene*, 3-carene*

VOC: volatile organic compounds.

**in vivo*, + *in vitro*, † *Huang et al.*

induces oxidative stress.¹⁸ Peroxidation of polyunsaturated fatty acids (PUFA) comprising membranes of organelles can form alkanes, leading to the degradation of phospholipids.⁵⁷ Elevated lipid contents of the cellular membranes in lung cancer cells coupled with increased oxidative stress render this hypothesis plausible.

Furthermore, peroxidation of lipids in cancer can lead to the endogenous production of volatile ketones, alkanes, aldehydes, and furans in the body.^{24,58} Ketones and alcohols can also be generated when the glycolytic pathway takes place in hypoxic environments of proliferating tumors.⁵⁹ Among the reported ketones, acetone was found both in breath and cell culture headspace. In humans, hepatocytes can produce considerable quantities of acetoacetate, the simplest keto acid and prominent cellular fuel supply.⁶⁰ Decarboxylation of acetoacetate, which is achieved in both fatty acid degradation and the metabolism of ketogenic amino acids, yields endogenous acetone.⁶¹ In addition, increased oxidative stress could impair glutathione metabolism since glutathione is an

active antioxidant in the cell. However, 1,5-diaminopentane was reportedly involved in this glutathione metabolism, and it serves a role in amino acid export.⁶²

Degradation of membrane lipids has been postulated in several reports to be the endogenous origin of alkanes and other hydrocarbons in lung cancer patients.^{15,55} Cancers of various histological origins are responsible for the increased levels of circulating alkanes.⁴² It is hypothesized that CYP450 may be a contributing factor for this phenomenon and responsible for the increased levels of methylalkanes.⁶³ Metabolism of hydrocarbons can also generate alcohols. Circulating levels of alcohols differ as a result of intake. In addition, endogenous acetaldehyde can serve as a precursor for ethanol in anaerobic conditions. CYP450 isoenzymes contribute to alcohol metabolism as well, by the hydroxylation of lipid peroxidation biomarkers. In particular, CYP2E1 can convert alcohol into acetaldehyde and produce more reactive oxygen species to increase oxidative stress in the tumor environment.⁶⁴ Cancers have also been thought to

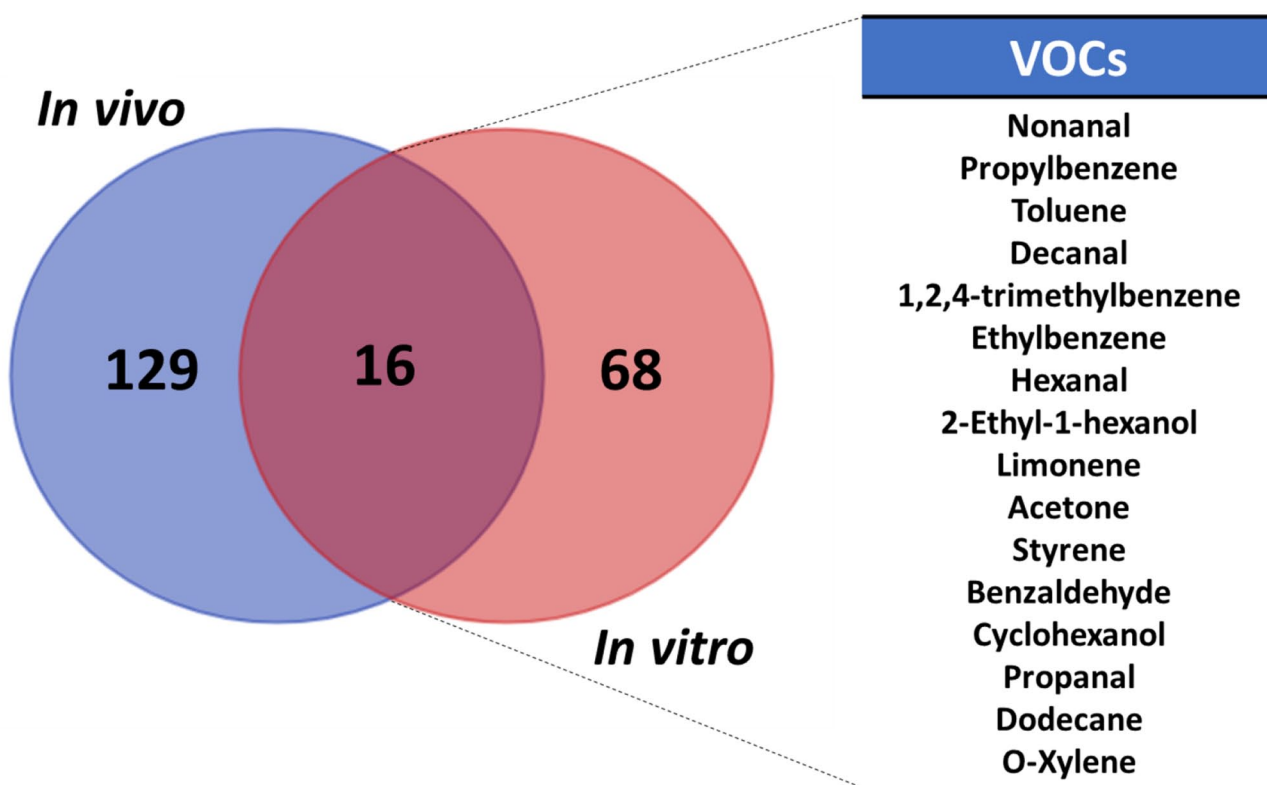


Figure 3. Overlap of VOCs detected both *in vivo* from exhaled breath of lung cancer patients and *in vitro* in headspace of lung cancer cell cultures. (A color version of this figure is available in the online journal.)

Table 3. KEGG pathways that contained one of the identified breath VOCs.

Compound	KEGG entry ID	Metabolic pathway	KEGG pathway ID
Acetaldehyde	C00084	Glycolysis/gluconeogenesis	hsa00010
		Pyruvate metabolism	hsa00620
		Glycerophospholipid metabolism	hsa00564
Ethanol	C00469	Glycolysis/gluconeogenesis	
		Propanoate metabolism	hsa00640
Propanoate	C00163	Propanoate metabolism	hsa00480
1,5-Diaminopentane	C01672	Glutathione metabolism	hsa00630
Formate	C00058	Glyoxylate and dicarboxylate metabolism	hsa01040
Hexadecanoate	C00249	Biosynthesis of unsaturated fatty acids	hsa00062
		Fatty acid elongation	hsa00071
		Fatty acid degradation	hsa00061
		Fatty acid biosynthesis	hsa00140
4-Methylpentanal	C02373	Steroid hormone biosynthesis	

KEGG: Kyoto Encyclopedia of Genes and Genomes.

metabolize alcohols *in vitro* since 2-ethyl-1-hexanol has been reported both in human breath and headspace of lung cancer cell cultures.⁶⁵

Digging deeper into the origins of VOCs from humans, we conducted a secondary analysis on the reported VOCs by Huang *et al.*⁶⁶ In that report, pleural effusions were collected from 50 elderly individuals divided into two groups: malignant population consisting of 20 diagnosed cancer patients and benign inflammatory population encompassing 30 patients with tuberculous pleurisy or other diseases (Table 1). The study reported a total of 76 identified volatiles from the effusions, with a subset of 24 deemed to be associated with cancer (Table 2). While this dataset does not represent the

whole lung cancer volatilome, it provides vital information to determine the metabolic origins of these VOCs. Global pathway analysis using MetaboAnalyst's established pathway module was able to attribute the origins of three cancer-associated VOCs to four metabolic pathways. Acetaldehyde was determined to be involved in glycolysis/gluconeogenesis, glycerophospholipid metabolism, and pyruvate metabolism. In addition, pathway analysis confirmed glycolytic origins of endogenous ethanol. Steroid hormone biosynthesis was also responsible for the formation of 4-methylpentanal in cancer cells. However, 4-methylpentanal is a by-product of cholesterol metabolism to pregnenolone which is catalyzed by the enzyme cholesterol side-chain cleavage enzyme in the

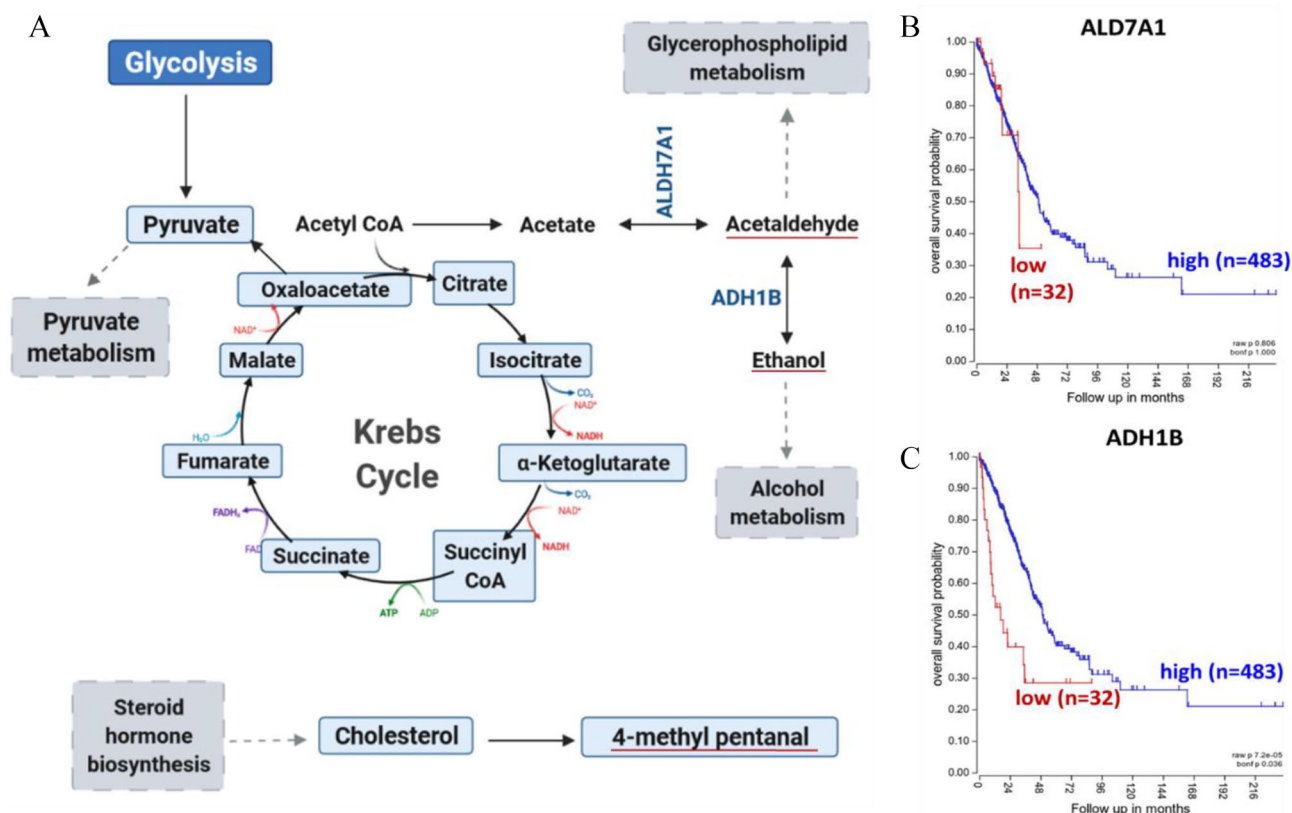


Figure 4. Secondary analysis of reported VOCs in Huang *et al.* with clinical relevance. (A) Endogenous synthesis of acetaldehyde and ethanol in humans, downstream of glycolysis, as outlined in KEGG (B and C) Kaplan–Meier in lung adenocarcinoma patients revealing lower survival of patients with elevated expression of genes involved in endogenous VOC synthesis. (A color version of this figure is available in the online journal.)

mitochondria. It can also serve as a substrate for aldehyde formation in cells.

In humans, alcohol metabolism is primarily catalyzed by alcohol dehydrogenase (ADH).⁶⁷ For instance, endogenous ethanol can be derived from metabolism of hydrocarbons, another group of cancer-associated VOCs, by ADHs.⁶⁷ In addition, alcohol can serve as a precursor for acetaldehyde. In the mitochondria, acetaldehyde is rapidly oxidized to acetate by aldehyde dehydrogenase (ALDH) to free up carbon sources for carbon dioxide or fatty acid synthesis.⁶⁸ This downstream metabolic process of glucose metabolism is visualized in Figure 4(A). Endogenous acetate can be synthesized in the body through acetyl-CoA hydrolysis. Acetate can then be converted by acetaldehyde through ALDH7A1 among other ALDHs.⁶⁹ Aldehydes can subsequently be reduced to their corresponding alcohols through ADHs.⁷⁰ Elevated expression of both ALDH7A1 (Figure 4(B)) and ADH1B (Figure 4(C)) was associated with worse survival outcomes in lung cancer patients.⁷¹ Increased expression of these genes would catalyze the reactions of ethanol and acetaldehyde, which could lead to their accumulation in the tumor. Upregulation of a certain gene would reflect in increased synthesis of the product metabolite since increasing levels of the enzyme catalyzing the reaction would be present.⁷² This would provide greater abundance for these compounds to transfer to exhaled breath. Thus, the detection of these compounds in breath could be used to reflect on the genetic anomalies in lung cancers and assist in identifying

disease severity.⁷³ Collectively, reports of these VOCs are of particular interest since ¹³C-glucose labeling studies in lung cancer patients have highlighted glycolytic dependence of pulmonary tumors.⁷⁴ ¹³C-enrichment of lactate and succinate in malignant tissues corroborates reliance of lung cancers on glycolysis and pyruvate carboxylation for sustained energy.⁷⁵ As these metabolic processes funnel through to acetyl-coA and acetaldehyde metabolism, the detection of these VOCs in breath could indicate disease onset. Taken together, these findings suggest the importance of understanding the biochemical origins of breath VOCs so we can improve early diagnosis of pulmonary carcinomas in efforts of mitigating cancer progression using breath analysis.

Novel advancements for VOC-based research in clinical settings

Since VOCs are tightly linked to lung cancer's metabolic deregulations, their abundance in breath varies accordingly to the disease status.⁷³ Thus, exhaled volatiles can serve as prospective markers where changes in the detected levels could be used to determine disease stage or patient's response to treatment.⁷³ Here, we explore the diverse applications of VOC analyses in the clinical settings. For instance, the inferred correlation between lung cancer progression and endogenously produced VOCs in humans suggests that volatilomics in breath has the potential to detect tumor formation at the very early stages of the disease. Meanwhile,

reports have suggested that the concentration of some exhaled VOCs, such as cyclohexane and xylene, increases in breath as lung cancer progresses to later stages.⁷⁶ This could reflect the increased metabolic rate of tumors to sustain growth, which in turn produces more volatile by-products that could be detected in breath. In addition, abundance of VOCs was shown to be varied between patients exhibiting different histological types of lung cancer.⁷⁶

In addition to assisting lung cancer diagnosis, exhaled VOCs analysis also has the great potential to be utilized for the monitor and evaluation of lung cancer treatment efficacy. As chemotherapy can disrupt tumor growth, it is expected that the underlying metabolic mechanisms responsible for VOC production will be inherently interrupted. Accordingly, after identifying prominent volatiles for lung cancer diagnosis, it would be of particular interest to monitor the changes in their abundance throughout treatment regimens. Thus, the development of a novel screening method to monitor patient responses to treatment is a vital clinical concern. Reports have shown the detection of 29 altered volatile compounds in lung cancer patients after tumor resection,⁷⁷ of which 12 were statistically significant. The significantly deregulated VOCs included butyl glycol and cyclohexanone where the levels were found to be decreased with a fold change of -0.85 and -1.75 , respectively. The abundance of cyclooctanemethanol was the most noticeably increased, with a reported fold change of 10.7. In addition, significant changes to VOC levels were revealed after subjecting patients to systemic therapy cycles.²³ The levels of α -phellandrene and 4-methyl-dodecane were decreased in exhaled breath of lung cancer patients compared to baseline controls. After treatment, the exhaled levels of these volatiles were restored to the levels similar to that of the baseline. Conversely, styrene exhibited an inverse trend where baseline levels were higher than progressive lung cancer patients and levels were decreased after treatment. Drug efficacy was assessed using computerized tomography scan and corroborated the results of the exhaled breath analysis. Treatment monitoring using headspace volatiles was also possible in cell cultures. Using online sampling, creatinine, methyl-3-hydroxybutyric acid, 2-phenylacetamide, L-histidine, and decyl acetate were identified in the headspace of lung cancer cell cultures.⁴¹ After cisplatin treatment, the levels of all but creatinine were increased in headspace gas. Decreased levels of histidine were reported in cultured lung cancer cells and consistent with the reports of occult metastases in patients.⁴³ Increased levels of histidine in the headspace of *in vitro* cell cultures after treatment suggest that VOCs can also provide valuable information for treatment responses in exhaled patient breath.

Another exciting finding highlighting the diverse utility of VOCs in the clinical setting was the prediction of non-small cell lung cancer patients' response to immunotherapy by the analysis of exhaled breath in treatment-naïve patients.⁴⁴ Lung cancer patients were subjected to routine standard clinical care, including computed tomography (CT) scans and blood tests prior to immunotherapy regimens. Patient response to anti-PD-1 therapy was monitored throughout the course of the study along with the collections of exhaled breath. After training a linear discriminant analysis-based model using 92 patients, the results were validated in an independent

cohort of 51 lung cancer patients. After validating the findings, the exhaled breath profiles of anti-PD-L1 responders were unique from the breath of non-responders with an area under the curve (AUC) of 0.85 (95% CI: 0.7–0.96). Strikingly, 12 out of the 51 patients in the validation set (24%) were predicted and subsequently proven to be non-responders to immunotherapy. These promising findings corroborate the unique exhaled molecular fingerprint of lung cancers and highlight the potential of VOC-based diagnostics.

Current clinical applications of these breathomics screenings are hindered by the availability of expensive instruments, such as mass spectrometers. Yet, they are the primary cost to run breath analyses as sample cost is considerably low. While PET-CT scans are currently effective, the elevated cost per screening may be a financial burden for patients. Besides, if a panel of known lung cancer VOC biomarkers is established, the technology can be afforded for the masses by implementing this biomarker panel into cheaper and portable breathalyzers. Exhaled breath analyses of volatiles have been demonstrated to (1) identify unique exhaled breath VOC patterns associated with lung cancer, (2) monitor changes in VOC abundance to monitor patients' response to treatment, and (3) predict patients' response to treatment prior to therapy. If done correctly with proper exclusion of environmental volatiles, exhaled breath prints of lung cancer patients could possibly be utilized in conjunction with the current clinical standards of care, such as CT scans or blood tests, for disease diagnosis and monitoring in efforts of affording patients rapid and individualized treatments.

Conclusion and future directions

Analyses of VOCs in exhaled breath and cell cultures have been considered as promising and non-invasive tools for evaluating the disease status and treatment outcomes of lung cancer in patients. Endogenously derived volatiles can mirror the metabolic process that is deregulated by oncogenesis. The literature is abundant with studies highlighting unique lung cancer-associated volatiles in breath of patients that could reflect early disease onset. While the endogenous origins of VOCs remain elusive, the curated knowledge of metabolic mechanisms and gene functions could facilitate our understanding of the effect of cancer on exhaled VOCs. In addition, our review has highlighted the potential use of volatiles to pinpoint genes used for patient survival prediction. Advancements in breath analyses could also offer an alternative to the current screening methods, which are incapable of detecting lung cancer in the early stages. However, it is acknowledged that VOC analysis is not mature enough to be broadly applied in clinical settings. Standardization of exhaled breath collection and analytical analyses is currently lacking and could explain the variation in proposed lung cancer biomarkers. Another limitation arises due to the ambiguity surrounding the metabolic processing responsible for many volatiles. Putative validation of the deregulated metabolic processes responsible for VOCs would provide invaluable information for scientists and clinicians investigating cancer-related VOCs. Nonetheless, research highlighted in this mini review suggests that exhaled volatiles can play a prominent role in future clinics for lung

cancer-related applications. The rise of more accurate and sensitive methods, such as secondary electrospray ionization mass spectrometry,⁴⁵ for VOC detections could validate the utility of these volatile biomarkers in reflecting the efficacy of treatment in lung cancer patients. Moving forward, we believe that the advancement of exhaled breath analysis will yield great results that will lay the foundation for the use of VOCs as markers for cancer diseases and for monitoring disease progression. The non-invasive collection of this limitless sample is likely to facilitate better patient compliance and increase willingness to get tested. We hope this mini review will stimulate new quantitative experimental and theoretical studies of cancer-related VOCs to provide most personalized treatment options for lung cancer patients.

AUTHORS' CONTRIBUTIONS

JZ contributed in conceptualization, resources, writing – review and editing, project administration, and funding acquisition; JZ and FC participated in methodology and investigation; FC performed the formal analysis and contributed in writing – original draft preparation; FC and AB participated in data curation. All authors have read and agreed to the submitted version of the article.

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