

Volatile Organic Compounds of Lung Cancer and Possible Biochemical Pathways

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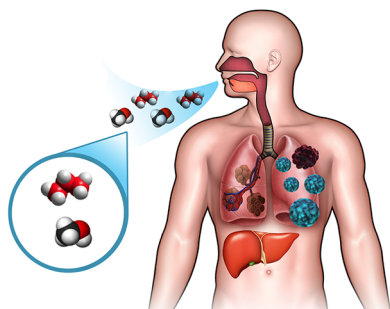
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1. INTRODUCTION

Lung cancer (LC) is the leading cause of cancer mortality, with more than 1 million deaths worldwide every year.^{1,2} There are two major forms of LC disease: nonsmall cell LC (NSCLC; *ca.* 85% of all LCs) and small-cell LC (SCLC; *ca.* 15% of all LCs). NSCLC is divided into three major histologic subtypes: squamous-cell carcinoma, adenocarcinoma, and large-cell carcinoma. SCLC and squamous-cell carcinoma are most

strongly linked with smoking, and adenocarcinoma is the most common type of LC in patients who have never smoked.^{1,2}

The evaluation of a patient's prognosis of LC requires (i) confirming the presence of the disease and (ii) staging the disease. Different methods for diagnosis and staging are used in clinical practice, including blood tests, chest X-ray,³ computed tomography (CT),⁴ magnetic resonance imaging (MRI),⁵ and positron emission tomography (PET).⁶ In most cases, removal of cells or tissues is required in order to determine the presence and the extent of the LC.⁷ Tissue samples are usually examined under a microscope to determine the shape and/or concentration of the LC cells. Based on the tissue analysis, one can specify and tailor therapy according to the histology type, mutation analysis, specific staining, and other specifications. Tissue specimens can be obtained, for example, by fiberoptic bronchoscopy guided biopsy⁸ or by CT scan guided needle biopsy.⁹

Gene profiling^{10–19} and/or protein profiling^{20–24} are new promising techniques for determining cancer risk, prognosis, and targeted (or personalized) therapy, thus improving the clinical outcome for LC patients. Despite the recent progress in the identification of biomarkers, gene mutations, and genomic signatures, some formidable obstacles must still be overcome on the way for developing effective biomarkers, including the following: tumor heterogeneity; highly complex tumor–host interplay; and complexity, multiplicity, and redundancy of tumor-cell signaling networks involving genetic, epigenetic, and microenvironmental effects. Additionally, the technologies associated with these approaches are often expensive, time-consuming, and unavailable in many medical facilities and require relatively large amounts of tissue for analysis. Comprehensive reviews dealing with these approaches have been published elsewhere.^{25–31}

An emerging approach for diagnosing LC relies on volatile organic compounds (VOCs), *viz.* organic compounds with relatively high vapor pressure or volatility, that can be detected in the headspace of cancer cells or blood samples, and/or in the exhaled breath.^{32–67} Impressive empirical data have confirmed the potential of these compounds to serve as a basis for a noninvasive, simple, inexpensive, and easy-to-use diagnostic tool. In fact, monitoring VOCs in the breath may soon become an

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interesting supplement (or even an alternative) to conventional medical diagnostics, thanks to the rapid advances in the techniques for breath collection and gas-analysis. This novel approach could revolutionize LC care and management by allowing noninvasive *in vivo* differential diagnosis, *in vitro* prediction of the metastatic potential of the cancer cells, tailoring of individual treatment, and real-time monitoring of the therapeutic success.^{68,69} Nevertheless, the pathophysiology underlying the alteration of the VOC levels and compositions in LC patients has been vague to a large extent. In this review, we present a comprehensive, comparative examination of the LC VOCs collected from different body fluids (including headspace of cells, blood, breath, saliva, and urine), that have been reported by different research groups over the past three decades. The results of this examination are evaluated and discussed with respect to one or to a combination of different mechanisms, including oxidative stress, cytochrome p450, carbohydrate metabolism (such as the glycolysis/gluconeogenesis pathways), and lipid metabolism. Based on the obtained understanding, the review highlights the advantages and challenges of VOC-based diagnostic methods for LC and shows possible routes toward clinical practice.

2. VOLATILE ORGANIC COMPOUNDS OF LUNG CANCER

VOCs of LC can be detected (i) directly from the headspace of the cancer cells (i.e., the mixture of VOCs trapped above the cancer cells in a sealed vessel),^{32–41} (ii) *via* the blood,⁴² and/or (iii) *via* the exhaled breath.^{43–57} The principle behind the latter is that cancer-related changes in the blood chemistry are reflected in measurable changes in the breath through exchange *via* the lungs.⁷⁰ It was found that some gases exchange in the airways, rather than the alveoli, depending on the blood/air partition coefficient, $\lambda_{b/a}$. Theoretical and experimental studies have shown that gases with low solubility in blood, mainly nonpolar VOCs ($\lambda_{b/a} < 10$), exchange almost solely in the alveoli, while highly blood-soluble gases, mainly polar VOCs ($\lambda_{b/a} > 100$), tend to exchange in the airways.⁷¹ Further studies predicting the location of the pulmonary gas exchange have shown that VOCs with $10 < \lambda_{b/a} < 100$ interact significantly both with the airways and with the alveoli.⁷¹ An important conclusion of these studies is that the airways play a larger role in pulmonary gas exchange than previously assumed.^{72,73} Hence, the implications of pulmonary tests and breath tests might have to be re-evaluated.⁷¹ The VOC profile is also influenced by the retention of VOCs in the lungs, viz. the fraction of the molecules that remains in the respiratory tract at any time, after inhalation and exhalation, because of the blood/air partition coefficient.⁷⁴ Thus, the final partition and exhalation of the VOCs depends on their physical and chemical properties and on their interaction with the different alveolar clearance processes.^{74,75}

Hundreds of spectrometric studies, mainly using gas chromatography–mass spectrometry (GC-MS) and proton transfer reaction mass spectrometry (PTR-MS), have been carried out in order to identify the LC VOCs. More than 1000 trace VOCs have been found in human breath at concentrations ranging from parts per million by volume (ppmv) to parts per trillion by volume (pptv).^{44–46,49,51–56,58–67} Typical examples are isoprene, methanol, acetone, and 2-propanol (appearing in all human breath samples), acetonitrile, furan, 2-methyl furan (primarily found in smokers), limonene (perhaps of exogenous origin), hydrogen (from bacteria in the gut of persons suffering

from fructose mal-absorption), methane (from bacteria in the gut), or ethane and pentane (as products of lipid peroxidation).

In spite of the promising advances, the lack of normalization and standardization has led to significant variations in the VOC profiles and/or concentrations between the different studies reported in the literature. These inconsistencies can be attributed to the following:

- (a) Variances in the control groups used in the clinical studies. For example, control groups might consist of healthy nonsmokers, healthy smokers, age-matched groups, chronic obstructive pulmonary disease (COPD) control groups, hospital personnel, relatives and spouses of the patients, *etc.*
- (b) Lack of standardization in the medium used for growing cell cultures, types of LC cell lines, period of sample collection, and algorithms for analyzing the data in the headspace. Moreover, there is no clearly defined control for the LC cell lines (medium or healthy cells).
- (c) Variance in the equipment (e.g., GC-MS,^{53,63} PTR-MS,^{38,57} *etc.*). Note that the identification of the VOCs by GC-MS or PTR-MS is not 100% certain, even if the identification by spectral library match and retention time for GC-MS is quite reliable.^{34,35,37}
- (d) Variances between the sampling procedures used in various studies, such as, but not limited to mixed expiratory breath collection,⁵⁷ end-tidal breath collection with CO₂-controlled sampling,⁶³ sampling with Tedlar or Mylar bags,^{57,76} use of a portable breath collection apparatus (BCA), which was developed and used by Phillips et al.,⁵³ *etc.*
- (e) Variance in the preconcentration procedures used in the various studies, such as solid phase microextraction (SPME) fibers,^{45,59} solid-phase extraction with subsequent thermal desorption, cryo-focusing,⁵⁵ *etc.*
- (f) Variances in the normalization procedures. While part of the studies normalized the data according to the concentration of a specific VOC in the exhaled breath,^{57,59} other studies have normalized the data according to the difference between the concentrations in the exhaled and the inhaled air.^{53–55} Non-normalized data were reported as well.
- (g) Variance in the data analysis. For instance, the analysis of the GC-MS raw data includes:
 - (i) Identification, separation, and integration of the peaks in measured chromatograms for each sample. This might involve the use of Gaussian and non-Gaussian peak-fitting software, algorithms for the numerical calculation of the peak area, and algorithms for background compensation. Using internal standards might improve the reliability of the results, allowing a compensation of spectral shifts prior to the peak integration.
 - (ii) Quantification of the area under each peak in order to allow quantitative comparisons between different chromatograms. This is not straightforward and requires the use of well-selected internal and/or external standards. Objective quantification in terms of VOC concentration requires elaboration and time-consuming calibration procedures using exact, high-purity calibration standards.
 - (iii) Quantitative comparisons of the chromatogram peak areas or VOC concentrations between differ-

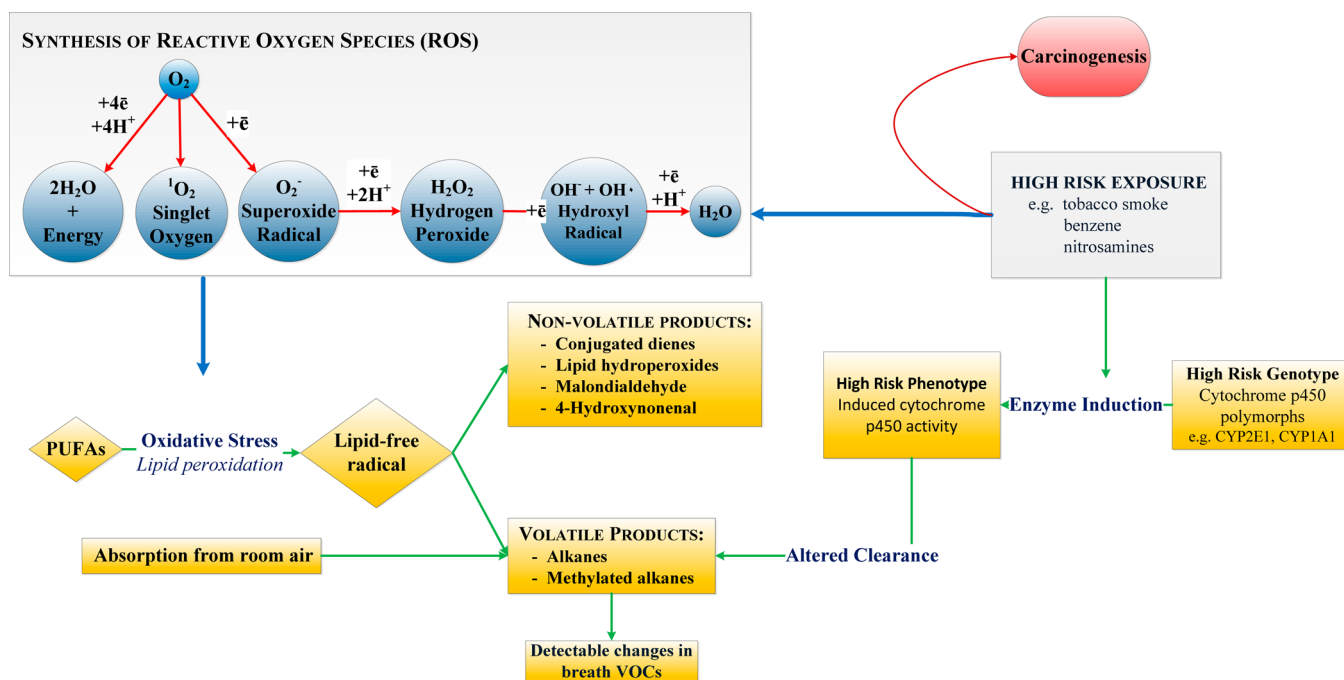


Figure 1. Hypothetical basis of the breath test for LC: LC may result from the interaction of hereditary and environmental factors. Several cytochrome p450 mixed oxidases are activated by exposure to environmental toxins such as tobacco smoke. The induced phenotype may increase the risk of LC by increased conversion of precursors to carcinogens. An altered pattern of cytochrome p450 mixed oxidase activity could potentially modulate catabolism of endogenous VOC products of oxidative stress and generate an altered pattern of breath VOCs.

ent study groups. The available statistical tests differ in the assumptions concerning the tested groups or populations: Gaussian and non-Gaussian populations, paired and unpaired groups, comparison between two or more groups, etc. For example, if the null hypothesis for the normal population distribution is fulfilled, the student's *t*-test may be used to detect significant similarities or differences between the examined groups.⁷⁷ Otherwise, the use of the nonparametric statistical test may be indicated, such as Wilcoxon/Kruskal–Wallis one-way analysis of variance.^{57,78}

- (iv) Classification, regression, and cluster analysis of VOC patterns that are derived directly from the (unidentified) chromatogram peaks. Patterns distinguishing the study groups may be obtained from the collective GC-MS results through a variety of supervised or unsupervised statistical pattern recognition algorithms. For example, forward stepwise multilinear regression — a supervised method — was used by Phillips et al.^{46,52} in order to establish LC patterns based on (unidentified) chromatogram peaks.⁷⁷ VOC patterns of LC and histologically different types of LC were studied using unsupervised methods such as principal component analysis.

Several accounts focusing on the technical aspects of the analysis of LC VOCs in the exhaled breath, such as sample collection, analyte preconcentration, vapor desorption, and measurement techniques, have been published elsewhere.^{58,62,79}

Excellent reviews on VOCs in the exhaled breath for the diagnosis of lung cancer can be found in refs 43–45, 68, and 80.

3. BIOCHEMICAL PATHWAYS OF VOLATILE ORGANIC COMPOUNDS OF LUNG CANCER

Despite the lack of standardization, possible sources of LC VOCs have been reported and discussed. It has been proposed that pathological processes, such as metabolic disorders, can produce new VOCs or change the ratio between the VOCs that are produced normally by the body. This idea is based on the principles of cell biology, such as the effect of the liver enzymes on the cell membranes, which primarily consist of amphipathic phospholipids, carbohydrates, and many integral membrane proteins.^{81,82}

A pathophysiologic model that could account for these observations is illustrated in Figure 1.⁵⁴ In normally occurring oxidative stress (see Box 1), reactive oxygen species leak from the

Box 1

Oxidative stress: The overall balance between formation and scavenging of reactive oxygen species (ROS) and free radicals in the body. ROS are molecules or ions with an unpaired electron in the outer shell, which are constantly produced in the mitochondria as part of the cellular respiration process. In addition, they can be caused by fungal and viral infections, or they can stem from exogenous sources, such as cigarette smoke, pollution, and radiation.

Cytochrome p450: A large and diverse group of mixed oxidase enzymes which catalyze the oxidation of organic substances. These enzymes are overactivated in LC.

mitochondria or from the peroxidate polyunsaturated fatty acids in the cell membranes and generate volatile alkanes and methylated alkanes that are excreted in the breath.⁸³ It is possible that the induction of cytochrome p450 (CYP1B1, CYP2E1, and CYP1A1, cf. Box 1) genotypes by polycyclic

aromatic hydrocarbons in tobacco smoke⁸⁴ increases the risk of LC, while accelerating the catabolism of the oxidative stress products of certain VOCs, thereby modifying their abundance in breath. This hypothesis is consistent with the chemical composition of several LC VOCs and their production in nonendobronchial tumors.

In a complementary pathophysiologic model, it was proposed that part of the normal cells proliferating at rapid rates during the early stages of cancer development reach the oxygen diffusion limit and become hypoxic (less than 0.1% oxygen in the gaseous phase).⁸⁵ As a result, some cancer cells demonstrate impaired mitochondria respiration and high glycolysis, namely the Warburg effect,^{86–89} in order to survive in the hypoxic microenvironment.^{90,91} As a consequence of the excessive lactate production (which helps to select motile cells) and of the high glycolysis rates, the tissue becomes acidic. This acidic environment permits breakeage of the basement membrane and accessibility to the blood vessels.⁹² At the same time, this acidic environment protects the tumor from the immune system.⁹² Moreover, the metastatic potential of the cancer cells is strongly related to the tumor's local environment and the tumor–host interaction.⁶⁹ The process of metastasis consists of selection and sequential steps that include angiogenesis, detachment, motility, invasion of the extracellular matrix, intravasation, circulation, adhesion, extravasation into the organ parenchyma, and growth.^{93,94} Since tumor growth is accompanied by gene changes and/or protein changes,^{95,96} the expression of individual alleles also creates a specific VOC signature which is secreted in body fluids.⁹⁷ Davis and co-workers have studied the production of VOCs in human cell lines with single-gene differences, where the variability of the VOC production is expected to arise solely from the presence of a specific human leukocyte antigen (HLA) allele, and its impact on downstream signal transduction and metabolite pathways.⁸³ It was found that tumorous and nontumorous cells, immune cells, and infectious agents may all contribute to the production of VOCs. Still, further isolation of the human cell components (e.g., cancer cell lines, immune cells, microbes, etc.) is needed to gain comprehensive understanding on the exact role of each component on the release of the VOCs.

The previously proposed pathophysiologic models were related to VOCs which are endogenous, viz. compounds which are produced by the tissues of a living organism. From a complementary point of view, exogenous VOCs detected in breath reflect the history of a specific person in terms of cigarette smoking and air pollution, and as a result, they are of great interest, even though they did not get enough focus in the field of breath analysis so far. These exogenous VOCs can leak into the cytoplasm and attach to organs or organelles in the body, causing peroxidative damage to proteins, polyunsaturated fatty acids, and DNA.⁹⁸ In particular, very lipophilic molecular species are stored in the fat compartments of the human body and subsequently released during weeks or even months after the exposure. In this review, we will give an overview over the exogenous compounds that should be taken into consideration when discussing the LC VOCs.

The optimal way to validate or to determine the pathophysiologic pathways of LC VOCs is to compare VOC profiles from different sources (organs or clinical samples) in the same LC patient and/or the same animal model. Within this approach, the simplest starting point would be a comparison between VOC profiles collected from the headspace of LC tumor, the (headspace of) blood samples, and the breath samples. However, many technical challenges have been hindering this approach.

With these challenges in mind, the current review presents a comparison between LC VOCs reported by different research groups over the last three decades from different clinical sources: cancer cells, blood samples and breath samples, and, in few cases, urine samples and saliva. In each clinical sample, we have compared the presence/absence of each experimentally validated VOC in the LC states, relative to the control states. Our initial “mapping” has revealed 112 VOCs in the breath of LC patients and 88 VOCs found in lung derived cell lines. Out of this list, we have considered only those VOCs that were reported by at least two independent studies, to focus on the most reproducible and validated LC VOCs. This action narrowed the full list to 36 VOCs in total, which were divided into seven compound families, as presented below:

Table 1: hydrocarbons: alkanes, branched-chain alkanes, and branched-chain alkenes

Table 2: primary and secondary alcohols

Table 3: aldehydes and branched aldehydes

Table 4: ketones

Table 5: esters

Table 6: nitriles

Table 7: aromatic compounds

Following the categorization of the LC VOCs, we have expressed each individual VOC in terms of its concentration trend, where “positive trend” means higher concentration in LC states, relative to healthy states, and *vice versa* for “negative trends”. We then compared between the trends that were obtained for the different clinical sources and/or body organs (see Figure 2). Match or mismatch between the trends in the different sources is evaluated and discussed by one or a combination of different mechanisms: oxidative stress and cytochrome p450, liver enzymes, carbohydrates metabolism (glycolysis/gluconeogenesis pathways), and lipid metabolism, resulting in either increased or decreased concentrations of the

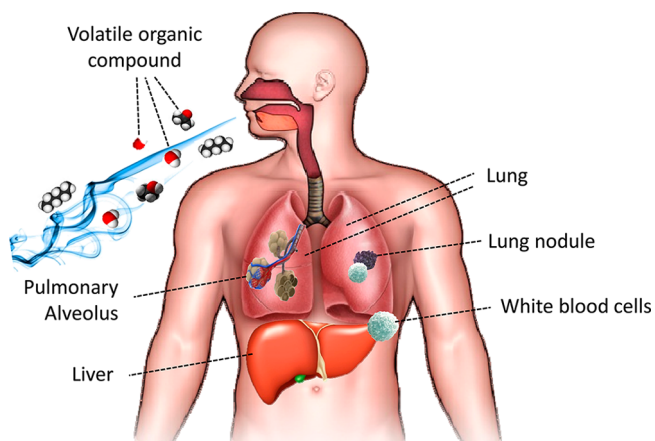













Figure 2. Scheme of a human body representing the different factors that are believed to affect the concentration and composition of LC VOCs: (i) The VOCs emitted from the cancer cells are different than those emitted from the surrounding microenvironment, leading to high glycolysis rates (Warburg effect) and low influence from the immune system. (ii) Liver enzymes, which accelerate the catabolism of VOC products of oxidative stress, are induced by aromatic hydrocarbons in tobacco smoke, leading to an increased risk for cancer. (iii) Cancer-related changes in the blood chemistry could be reflected in the breath, through instantaneous equilibrium between their concentration in the pulmonary blood and the air in the alveoli of the lungs by concentration gradient.

Table 1. Hydrocarbons (Alkanes), Branched-Chain Alkanes, and Branched Chain Alkenes That Appear in Exhaled Breath and *in-Vitro* Cells of LC

Compound	Structure	Breath		<i>In-vitro</i> (Normal cells)		<i>In-vitro</i> (Cancer cells)	
		Increased ^a	Decreased ^b	Increased	Decreased	Increased	Decreased
Hydrocarbons (Alkanes)							
Pentane						↑ ^{c,37}	
Heptane		COPD > Controls ⁵²	Smokers > NSCLC ⁵²				
Octane				↑ ³⁴ (Dermis ^d)		↑ ³⁴	
Decane		NSCLC > Controls ⁵² COPD > Controls ⁵²					
Branched-Chain Alkanes							
2-Methylpentane		NSCLC > Controls ⁵²				↑ ³⁷	
2,3,3-Trimethylpentane				↑ ³⁴ (Dermis, Bronchial ^e)		↑ ³⁵ ↑ ³⁷ (n.s ^f)	
2,3,5-Trimethyl hexane				↑ ³⁴ (Dermis)		↑ ³⁵	
2,4-Dimethyl-1-heptane						↑ ³⁵ ↑ ³⁷ (n.s)	
4-Methyloctane						↑ ³⁵ ↑ ³⁷ (n.s)	
Branched-Chain Alkenes							
2,4-Dimethyl-1-heptene				↑ ³⁴ (Dermis, Bronchial)		↑ ³⁷ (n.s) ↑ ^{34*}	
2-Methyl-1,3-butadiene (isoprene)		NSCLC > COPD ⁵²	Controls > LC ⁵⁹ Smokers > COPD ⁵²				

^aIn breath analysis: a higher concentration of a VOC appears in the breath of subjects with LC, compared to control subjects. In headspace analysis: a higher concentration of a VOC appears in LC or normal cells, compared to medium. ^bIn breath analysis: a lower concentration of a VOC appears in the breath of subjects with LC, compared to control subjects. In headspace analysis: a lower concentration of a VOC appears in LC or normal cells, compared to medium. ^cVOC increased in normal or cancer cells compared to medium. ^dHuman fibroblast (hFB) derived from the dermis. ^ePrimary human bronchial epithelial cells (HBEPc). ^fNonsignificant. (*) For VOC measurement different numbers of cells were used. “*” means that a significant increase or decrease in the VOC concentration was found only in part of the experiments and not in all.

VOCs.⁹⁹ It is important to emphasize in this context that the reported comparisons might not provide precise or definite answers to the puzzles encountering the biochemical pathways of LC VOCs. However, these comparisons will help, first and foremost, stimulating constructive discussions of potential pathophysiologic scenarios that shall retrieve more attention in the upcoming research and development in the field of LC VOCs. Additionally, the above-mentioned discussion would enable pointing out the LC VOC(s) that could serve as potential biomarkers for further R&D and, probably, for real-world clinical applications.

3.1. Hydrocarbons

Table 1 presents a list of the hydrocarbons found in the breath of LC patients and/or in the headspace of LC cells. The hydrocarbons contain three families: (i) straight alkanes (pentane, heptane, octane, and decane); (ii) branched-chain alkanes (2-methylpentane; 2,3,3-trimethylpentane; 2,3,5-trime-

thylhexane; 2,4-dimethyl-1-heptane; and 4-methyloctane); and (iii) branched-chain alkenes (2,4-dimethyl-1-heptene and 2-methyl-1,3-butadiene).

2,4-Dimethyl-1-heptane and 4-methyloctane were found only in the headspace of the *in vitro* LC cells, but neither in the breath nor in the blood samples. The concentrations of these two VOCs in the headspace of the LC cells were higher than their constituent growth medium.^{35,37} Similar findings were recorded also for pentane,³⁷ which was considered by stepwise discriminant analysis as one of the best nine LC VOCs.⁵³ Four VOCs (octane; 2,3,3-trimethylpentane; 2,3,5-trimethylhexane; and 2,4-dimethyl-1-heptene) were found in the headspace of LC cells as well as in the headspace of normal cells.^{34,35,37} The concentrations of these four VOCs in the headspace of cancer cells were higher in the constituent (growth) mediums. Regarding the VOCs detected in breath, heptane was found in decreased levels in NSCLC patients, in comparison to healthy

smokers, and in increased levels in COPD patients, in comparison to nonsmoking healthy volunteers.⁵² This fact shows that smoking has a direct effect on the mechanism producing heptane. Decane was found in higher concentrations in both NSCLC and COPD patients, in comparison to healthy volunteers, implying its involvement in lung malfunction.⁵² 2-Methylpentane was detected both *in vivo* and *in vitro*.^{37,52} The concentration of 2-methylpentane was higher in NSCLC than in controls and higher in LC cells than in the medium.

The main mechanism which affects the emission of hydrocarbons in the body is oxidative stress (see Box 1). Alkanes are mainly produced by peroxidation of polyunsaturated fatty acids (PUFA), known as lipid peroxidation by ROS (see Box 2). PUFA

Box 2

Lipid peroxidation: Auto oxidation of lipids exposed to oxygen is responsible for *in vivo* tissue damage. It may be a cause of cancer, inflammatory diseases, atherosclerosis, and aging. It is oxidative degradation of lipids, *viz.* a process in which free radicals “steal” electrons from the lipids in cell membranes, resulting in cell damage. This process proceeds by a free radical chain reaction mechanism. It most often affects polyunsaturated fatty acids, because they contain multiple double bonds in between which lie methylene (CH₂) groups that possess especially reactive hydrogens. The fatty acid radical is not a very stable molecule, so it reacts readily with molecular oxygen, thereby creating a peroxy-fatty acid radical. This too is an unstable species that reacts with another free fatty acid, producing a different fatty acid radical and a lipid peroxide, or a cyclic peroxide if it had reacted with itself. This cycle continues, as the new fatty acid radical reacts in the same way. The radical reaction stops when two radicals react and produce non-radical species. As a matter of fact, the human body tries to control and reduce lipid peroxidation by the use of antioxidants.

are found mainly in cellular and subcellular membranes in the body. They tend to undergo lipid peroxidation since they contain multiple double bonds with methylene –CH₂– groups in between that possess especially reactive hydrogen (see Box 2).⁹⁷ The products of this reaction are saturated hydrocarbons such as ethane and pentane, which are derived from ω3 and ω6 PUFA, respectively. Expired pentane or ethane has been widely used as a sensitive and noninvasive indicator of lipid peroxidation *in vivo*.¹⁰⁰ While the presence of other saturated hydrocarbons (e.g., C₃–C₁₁) can be attributed to the lipid peroxidation process, the

same mechanism seems irrelevant for branched hydrocarbons. Indeed, Kneepkens et al. claimed that branched hydrocarbons cannot result from lipid peroxidation, for the simple reason that there are no branched polyunsaturated fatty acids in the body.⁹⁷

2-Methyl-1,3-butadiene, known as isoprene, is the most abundant VOC in human breath. Isoprene is formed along the mevalonic pathway of cholesterol synthesis in the cytosolic fraction and is known to be related to age and gender.⁶⁵ The concentration of isoprene was varied between different studies, perhaps because isoprene concentration changes quickly during even minor physical effort.^{101,102} For example, Poli et al.⁵² found an increased amount of isoprene in NSCLC in comparison to COPD patients, whereas Wehinger et al.⁵⁷ and Bajtarevic et al.⁵⁹ detected a decrease of isoprene in the breath of LC patients, in comparison to healthy controls. Isoprene increases in concentration even during the slightest effort,^{101–104} such as leg or arm contractions or getting up from a sitting to a standing position. The end-expired values of isoprene are sensitive to changes not only in pulmonary ventilation and perfusion but also perfusion of storage sites during exercise. Hence, differences in sampling protocol may lead to quite different concentrations of isoprene in exhaled breath.^{52,57,59,65,101–104}

Several studies have shown that some of the investigated alkanes were present in lower concentrations in the breath of patients with LC than in samples obtained from controls.^{55,56,66,70} The proposed explanation for these findings was that, during carcinogenesis, cytochrome p450 (CYP) enzymes are induced, resulting in the hydroxylation of several VOCs:¹⁰⁵


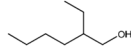


The hydrocarbons that do not metabolize are excreted into the breath within minutes due to their low solubility in the blood.^{106,107}

3.2. Alcohols

Alcohols mostly originate from food and alcohol beverages and are absorbed from all parts of the gastrointestinal tract, largely by simple diffusion into the blood. Alcohols are also derived from the metabolism of hydrocarbons. Since alcohol has high affinity to water, it is found in body tissues and fluids and is absorbed rapidly in the blood. This may explain the small amount of alcohols in the breath of people who have not had alcoholic beverages close to the time of the breath testing (Table 2). A major factor which needs to be taken into consideration is the effect of confounding factors on the alcohol metabolism in the

Table 2. Primary and Secondary Alcohols Appear in Exhaled Breath and *in-Vitro* Cells of LC

Compound	Structure	Breath		<i>In-vitro</i> (Normal cells)		<i>In-vitro</i> (Cancer cells)	
		Increased ^d	Decreased ^b	Increased	Decreased	Increased	Decreased
Primary and Secondary Alcohols							
1-Propanol		LC > Controls ^{59,63}				↑ ^{c,37} (<i>n.s.</i> ^f)	
2-Ethyl-1-hexanol		LC > Controls ¹⁰⁸		↑ ³⁴ (Dermis ^d)		↑ ^{32,37}	↓ ^{g,32}

^aIn breath analysis: a higher concentration of a VOC appears in the breath of subjects with LC, compared to control subjects. In headspace analysis: a higher concentration of a VOC appears in LC or normal cells, compared to medium. ^bIn breath analysis: a lower concentration of a VOC appears in the breath of subjects with LC, compared to control subjects. In headspace analysis: a lower concentration of a VOC appears in LC or normal cells, compared to medium. ^cVOC increased in normal or cancer cells compared to medium. ^dHuman fibroblast (hFB) derived from the dermis. ^fNonsignificant. ^gVOC decreased in normal or cancer cells compared to medium.

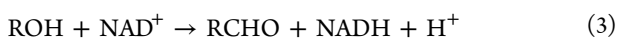
body, mostly the water and fat content that vary between different people and genders.

Table 2 presents two alcohols: 1-propanol and 2-ethyl-1-hexanol. Only 1-propanol was found in the breath of humans. In this case, 1-propanol was found at increased concentrations in the case of LC, as compared to healthy subjects.^{59,63} This is probably because of the cytochrome p450 enzymes, which hydroxylate the lipid peroxidation biomarkers to produce alcohols. The concentration of 1-propanol also slightly increased in the headspace of cancer cells in comparison to the concentration level in the medium.³⁷ On the other hand, analysis of the headspace of dermis and cancerous cells yielded another alcohol, i.e., 2-ethyl-1-hexanol, at an increased concentration level compared to the medium in the LC cell line, NCI-H2087.^{34,37} In a recent study, 2-Ethyl-1-hexanol was exclusively found in the *saliva* samples corresponding to the patients suffering from LC and was not identified in samples from patients with other types of cancer.¹⁰⁸ In another work, 2-ethyl-1-hexanol was found to be increased in NSCLC cell lines but decreased in SCLC cell lines, as compared to the medium.³² These results indicate that the origin of the alcohol is probably from the metabolism of the medium and of alkanes, which may be altered in different histologies of LC. Arguably, these alcohols are metabolized in the body by various enzymes, such as alcohol

Box 3

Alcohol dehydrogenase (ADH): A member of a general class of enzymes called oxidoreductases which form aldehydes or ketones from an alcohol through the reduction of nicotinamide adenine dinucleotide (NAD⁺ to NADH) mainly in the liver. ADH can catalyze the oxidation of many different alcohols in humans, including primary, secondary, cyclic secondary, or hemiacetal.

dehydrogenase (ADH) (see Box 3) and cytochrome p450 (CYP2E1), which mostly operates in the liver, into aldehyde:

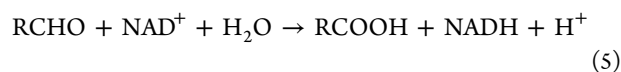


The remaining alcoholic LC VOCs are eliminated through the excretion of alcohol in breath, urine, sweat, feces, breast milk, and saliva.

3.3. Aldehydes

Aldehydes are produced during frequent physiological processes and biotransformation events. Part of the aldehydes is necessary to certain functional processes. The other part of the aldehydes is believed to be cytotoxic intermediates with various functions, such as signal transduction, gene regulation, and cellular proliferation.^{109,110}

There are several sources of aldehydes in the body. The first source relates to metabolized alcohol in the body (see section 3.2). For example, ethanol is degraded by alcohol dehydrogenase to produce acetaldehyde, and methanol is degraded to formaldehyde. Aldehydes that are formed in the body are oxidized by aldehyde dehydrogenase (ALDH) (see Box 4) to yield carboxylic acids:



Box 4

Aldehyde dehydrogenase (ALDH): An enzyme family which is responsible for the oxidation of aldehydes into carboxylic acids. These enzymes are classified into more than 20 putatively functional genes and function mainly in the liver. ALDH activity could be a functional marker for some types of cancer, including LC.^{111–113} Cells with high activity of ALDH are likely to be “cancer stem cells” (CSC), i.e., have distinct phenotypic and functional characteristics compatible with cancer initiating stem cells or early progenitor cells. During the growth of tumors, *in vivo*, a change in ALDH activity (ALDH1A1 and ALDH3A1) is detected.¹¹²

The second source for the aldehydes in the body relates to the reduction of hydroperoxide by cytochrome p450 as a secondary product of lipid peroxidation (see Box 2). The hydroperoxy bond undergoes a stepwise one-electron reduction in which the first reductive step yields an alkoxy radical. This radical undergoes the well-known β -scission reaction to yield a ketone or an aldehyde and a derived radical, R'• (see Figure 3).¹¹⁴

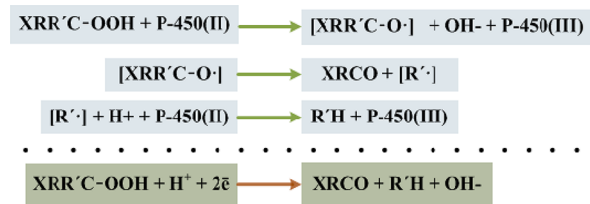


Figure 3. One of the pathways for aldehyde formation: reduction of hydroperoxide by cytochrome p450 as a secondary product of lipid peroxidation. The hydroperoxy bond undergoes a stepwise one-electron reduction in which the first reductive step yields an alkoxy radical. This radical undergoes the well-known β -scission reaction to yield a ketone or an aldehyde and a derived radical.

The third source for the aldehydes in the body relates to cigarettes. Aldehydes in tobacco smoke are both saturated compounds, such as formaldehyde, acetaldehyde, propionaldehyde, and butyraldehyde, and unsaturated compounds, such as acrolein and crotonaldehyde.¹¹⁵ In particular, it is thought that sugars are a major source of formaldehyde in cigarette smoke. Cellulose has been suggested to be the major precursor of mainstream smoke acetaldehyde.

The fourth source for the aldehydes in the body is a byproduct of tobacco metabolism in the body by cytochrome p450 as part of the detoxification process. The detoxification process catalyzes an additional oxygen atom to the foreign compound and thus turns it into a more water-soluble substance. In other words, the process converts the hydrophobic carcinogens into a form that is easier to excrete from the body.^{116,117} An example for such a process is the metabolism of a strong carcinogen, known in cigarettes, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK). NNK is metabolized by cytochrome p450 enzymes to α -hydroxy-NNKs. The hydroxy-NNKs spontaneously decompose to diazonium ions and aldehydes. The diazonium ions react with DNA to form adducts (see Figure 4).

Table 3 presents the aldehydes and branched aldehydes that are related to LC. Straight aldehydes include all C1–C9

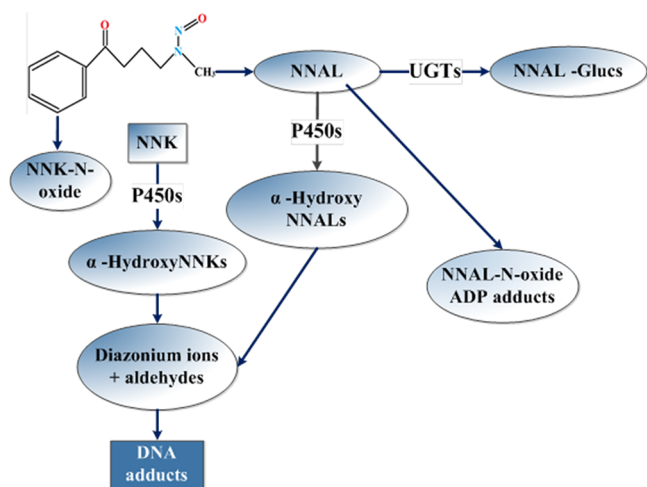


Figure 4. Mechanism of NNK damage to the DNA. NNK is metabolized by p450 enzymes to α -hydroxyNNKs. The hydroxyl NNKs spontaneously decompose to diazonium ions and aldehydes. The diazonium ions react with DNA to form adducts.

aldehydes: methanal (i.e., formaldehyde), ethanal (i.e., acetaldehyde), propanal, butanal, pentanal, hexanal, heptanal, octanal, and nonanal. Branched aldehydes include the following: 2-methylpropanal, methacrolein (i.e., methacrylaldehyde), 2-methylbutanal, 3-methylbutanal, and 2-methyl-2-butenal. All of the straight aldehydes listed above (C1–C9), apart from acetaldehyde, were found in higher concentrations in the breath of LC patients, in comparison to controls.^{51,57,63,78} On the other hand, aldehydes in the headspace of LC cells showed an opposite trend relative to the breath studies, where a decrease in the concentration of a specific aldehyde was quantified in comparison to the medium. This trend was observed for butanal,³⁸ pentanal,³⁸ hexanal,^{35–38} and heptanal.³⁸ The decrease in the concentration of aldehydes in the headspace of LC cell lines may be due to the impairment of the oxidation phosphorylation process in the cancer cells (Warburg effect). A decrease in aldehydes was also found in normal cells, in comparison to the medium. Haick and co-workers reported a decrease in concentration of nonanal in the case of SCLC cell lines, as compared to the growth medium. No significant change was observed for the same compound in immortal bronchial epithelium (IBE) cells, as compared to their medium. Moreover, a significant decrease in the concentration of nonanal was found for squamous cell carcinoma, but no significant change was observed for adenocarcinoma cell lines.³² Still, more studies are needed in order to quantify the difference in the concentration of aldehydes in the headspace of LC versus normal cells, and between histologically different LC cells.

The concentration of acetaldehyde in the breath of LC patients was found to be similar to that of healthy controls. The concentration of acetaldehyde in *in vitro* studies have not shown a clear trend but rather varied between the different studies. This could be connected to the finding that acetaldehyde concentration depends on the tissue histology. For example, the concentration of acetaldehyde was found to be decreased in dermis,³⁴ bronchial,³⁴ and fibroblast³⁹ cells, as compared to the medium, but was increased in epithelial cells.³⁹ Generation of acetaldehyde was documented in SK-MES cell lines (derived from SCLC patients) and in CALU-1 cell lines (derived from NSCLC patients) too.⁴¹ This observation supports the hypothesis that the VOC production/elimination profile is







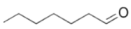
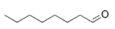






modified in LC cells, as compared to the medium.⁴¹ Moreover, the acetaldehyde levels increased proportionally to the number of cells in the cultures. This clearly demonstrates that LC cells generate and release acetaldehyde into the medium and, hence, into the headspace. The rate of acetaldehyde production was found to be on the order of one million molecules per cell per minute at a temperature of about 37 °C.

Formaldehyde is detected in the breath of LC patients in higher concentration than in controls.⁵⁷ It is suspected that formaldehyde is released from immune and LC cells⁵⁷ during the degradation of tryptophan,¹¹⁸ which could impair the function of the immune system during cancerogenesis.^{119,120} Fuchs et al. found elevated levels of formaldehyde in the breath of LC and healthy nonsmokers, as compared to healthy smokers.⁷⁸ Formaldehyde is an exogenic compound originating from many industrial processes and is considered to cause different types of cancers, among them LC. Formaldehyde is a highly water-soluble gas and is almost entirely absorbed in the respiratory system when inhaled. In the respiratory tissue, formaldehyde is oxidized by formaldehyde dehydrogenase (FDH), a member of the ALDH enzyme family (see Box 4), after binding to glutathione (GSH), to form the adduct S-hydroxymethylglutathione. This process plays an important role in the way our body fights against the formaldehyde toxicity and can explain why we see higher levels of formaldehyde in LC patients than in healthy smokers. The same researchers observed higher concentrations of formaldehyde in healthy nonsmokers, as compared to healthy smokers.⁷⁸ Formaldehyde may be a significant biomarker for LC, but further work needs to be carried out.

Hexanal and heptanal were detected in blood, breath, and urine samples. Deng et al.⁴² analyzed the headspace of blood for ten normal subjects and ten NSCLC patients with stage-I disease. High levels of hexanal and heptanal were found only in blood samples of the LC patients, in concentrations higher than 1.8 μM . In contrast, low concentrations of hexanal and heptanal were detected in 70% of the normal blood samples (concentrations higher than 0.2 μM). Similar results were reported by Lili et al.,¹²¹ even though the detection of the aldehydes was performed differently, *viz.* by a dispersive liquid-liquid microextraction combined with high-performance liquid chromatography (HPLC).¹²¹ Hexanal and heptanal were found in elevated levels in breath samples of LC patients and in urine samples of pediatric patients with various forms of cancers, as compared to healthy controls.¹²² Hexanal was consumed by CALU-1 cancer cells.³⁵ Both hexanal and heptanal were consumed by lung epithelium tumor cells A-549.³⁸ The presence of hexanal and heptanal in the various clinical samples collected from LC patients indicates that these VOCs originate from the LC cells *per se*, from where they are transferred to the blood and are furthered, by diffusion, to the pulmonary airways and to the exhaled breath.

In the following, we will raise a few scenarios that might explain the elevated levels of aldehydes in the breath of LC patients. The first hypothesis relates to an increase in ALDH activity (ALDH1A1 and ALDH3A1) in the LC cell *per se*.^{40,112} Krupenko et al.¹²³ have shown that ALDH1L1 (also known as 10-formyltetrahydrofolate dehydrogenase) is down-regulated in human liver, lung, prostate, pancreas, and ovary cancers.¹²³ Manzer and co-workers¹²⁴ found that rabbit ALDH1A1 is catalytically active and efficiently oxidizes some of the major products of lipid peroxidation: hexanal, 4-hydroxynonenal, and malondialdehyde. They also found similar results in the human recombinant ALDH1A1 protein. Arguably, the enhanced tumor

Table 3. Aldehydes and Branched Aldehydes Appear in Exhaled Breath and *in-Vitro* Cells of LC




Compound	Structure	Breath		<i>In-vitro</i> (Normal cells)		<i>In-vitro</i> (Cancer cells)	
		Increased ^a	Decreased ^b	Increased	Decreased	Increased	Decreased
Aldehydes							
Methanal (Formaldehyde)		LC > Controls ⁵⁷ (Nonsmokers + LC) > Smokers ⁷⁸					
Ethanal (Acetaldehyde)				↑ ^{c,39} (epithelial ^h)	↓ ^{g,34} (Dermis ^d , Bronchial ^e) ↓ ³⁹ (fibroblast ^f)	↑ ^{39,41}	↓ ^{35,37,38}
Propanal		LC > Controls ⁶³ NSCLC > Controls ⁵¹					
Butanal		LC > Smokers ⁶³ NSCLC > Controls ⁵¹	Nonsmokers > smokers ⁶³				↓ ³⁸
Pentanal		NSCLC > Controls ⁵¹ LC > Controls ⁷⁸			↓ ³⁸ (Retinal ^l , Immortal ^k)		↓ ³⁸
Hexanal		LC > Smokers ⁶³ NSCLC > Controls ^{42,51} LC > Controls ^{78,121,122} SCLC > NSCLC ⁷⁸	Nonsmokers > smokers ⁶³		↓ ³⁴ (Bronchial) ↓ ³⁸ (Retinal, Immortal)		↓ ^{35,38} ↓ ³⁵⁻³⁸ (<i>n.s</i>)
Heptanal		NSCLC > Controls ^{42,51} LC > Controls ¹²²			↓ ³⁸ (Retinal)		↓ ³⁸
Octanal		NSCLC > Controls ⁵¹ LC > Controls ^{78,121}			↓ ³⁴ (Bronchial)		
Nonanal		NSCLC > Controls ⁵¹ LC > Controls ⁷⁸					↓ ³²
Branched Aldehydes							
2-Methylpropanal					↓ ³⁴ (Dermis, Bronchial)		↓ ^{34,35,37}
Methacrolein (or methacrylaldehyde)					↓ ³⁴ (Bronchial)		↓ ³⁴⁻³⁶
2-Methylbutanal					↓ ³⁴ (Dermis)		↓ ³⁷
3-Methylbutanal					↓ ³⁴ (Dermis, Bronchial)		↓ ³⁴⁻³⁷
2-Methyl-2-butenal							↓ ³⁵ (<i>n.s</i>) ↓ ³⁴

^aIn breath analysis: a higher concentration of a VOC appears in the breath of subjects with LC, compared to control subjects. In headspace analysis: a higher concentration of a VOC appears in LC or normal cells, compared to medium. ^bIn breath analysis: a lower concentration of a VOC appears in the breath of subjects with LC, compared to control subjects. In headspace analysis: a lower concentration of a VOC appears in LC or normal cells, compared to medium. ^cVOC increased in normal or cancer cells compared to medium. ^dHuman fibroblast (hFB) derived from the dermis. ^eVOC decreased in normal or cancer cells compared to medium. ^fNormal lung epithelial cells (NL20). ^gTelomerase positive lung fibroblast cells (35FL121 Tel+). ^hRetinal pigment epithelium cells (hTERTRPE1). ⁱImmortalized human bronchial epithelial cells (BEAS2B). ^jFor VOC measurement, different numbers of cells were used. * means that a significant increase or decrease in the VOC concentration was found only in part of the experiments and not in all.

proliferation associated with the ALDH1A1-related activities may, indirectly, be (one of) the reasons for the increased aldehyde levels in the breath. Another hypothesis for the elevated aldehydes in breath is related to the ALDH2 enzyme. Several ALDH2 mutant alleles are known to cause catalytic inactivation, which increases the risk of cancer.¹⁰⁹ This enzyme encodes a

mitochondrial matrix enzyme that is constitutively expressed in lung tissues, with the acetaldehyde being the major, but not only, compound. It is likely that some of these VOCs (hexanal, acetaldehyde, propanal; see Table 3) emitted in breath are a result, or a byproduct, of signal transduction during the cancerous pathological process. More specifically, it is likely

Table 4. Ketones That Appear in Exhaled Breath and *in-Vitro* Cells of LC

Compound	Structure	Breath		<i>In-vitro</i> (Normal cells)		<i>In-vitro</i> (Cancer cells)	
		Increased ^a	Decreased ^b	Increased	Decreased	Increased	Decreased
Ketones							
Acetone		LC > Smokers ⁶³	Controls > LC ⁵⁹ non-smokers > smokers ⁶³	↑ ^{c,34} (Bronchial ^e)		↑ ³⁹ ↑ ^{34*}	
2-Butanone (methyl ethyl ketone)		LC > Controls ⁵⁹				↑ ³⁷	↓ ^{g,35}
2-Pentanone				↑ ³⁴ (Dermis ^d , Bronchial)		↑ ³⁴	

^aIn breath analysis: a higher concentration of a VOC appears in the breath of subjects with LC, compared to control subjects. In headspace analysis: a higher concentration of a VOC appears in LC or normal cells, compared to medium. ^bIn breath analysis: a lower concentration of a VOC appears in the breath of subjects with LC, compared to control subjects. In headspace analysis: a lower concentration of a VOC appears in LC or normal cells, compared to medium. ^cVOC increased in normal or cancer cells compared to medium. ^dHuman fibroblast (hFB) derived from the dermis. ^ePrimary human bronchial epithelial cells (HBEpC). ^fNonsignificant. ^gVOC decreased in normal or cancer cells compared to medium. ^hNormal lung epithelial cells (NL20). ⁱTelomerase positive lung fibroblast cells (3SFL121 Tel+). ^jRetinal pigment epithelium cells (hTERT/PE1). ^kImmortalized human bronchial epithelial cells (BEAS2B). ^lFor VOC measurement, different numbers of cells were used. * means that a significant increase or decrease in the VOC concentration was found only in part of the experiments and not in all.

that these VOCs result from signaling by the reactive species,^{125,126} which is one of the physiological processes producing aldehydes during lipid peroxidation. Signal transduction is based on an extracellular signaling molecule which triggers a cell membrane receptor, causing a second messenger to start a physiological response in the cell.¹²⁷ While there is no direct experimental evidence to support these arguments, several studies have shown that reactive aldehydes, such as 4-hydroxy-2-nonenal (HNE), can be classified as second messengers in signal transduction events. Throughout exposure to different pathologies that involve the production of ROS, lipid peroxidation resulting in HNE production increases, and the local concentration of HNE in tissues can increase to 10 μM or more. Most HNE would partition into membranes and, therefore, occur at much larger concentration near the membrane.¹²⁸ 4-HNE is a potent alkylating agent that reacts with DNA and proteins capable of inducing specific cellular stress responses such as cell signaling and apoptosis.¹¹⁰ It has been stressed that reactive species are not just harmful for cellular activity but of normal cellular physiology.¹²⁶ Moreover, dysregulated ROS signaling may contribute to a host of human diseases.¹²⁹ With these findings in mind, it is reasonable to hypothesize that some of the VOC changes in the body's VOC levels and, hence, in the breath, can be connected to signaling processes altering normal cell activity.

3.4. Ketones

Table 4 presents three ketones: acetone, 2-butanone (a.k.a. methyl ethyl ketone), and 2-pentanone. The concentration levels of acetone, a secondary product of lipid peroxidation, varied strongly from study to study. For example, Kischkel et al.⁶³ observed an increase of the acetone concentration in the breath of LC subjects, relative to healthy controls, but Bajtarevic et al.⁵⁹ reported the opposite trend. High levels of acetone were obtained in the headspace of LC and normal cells, as compared to the growth medium of the examined cells.^{34,39}

Acetone is usually a good predictor of ketosis.⁷⁶ Ketone bodies including acetone are formed under metabolic conditions associated with a high oxidation rate of fatty acids in LC as well as with a weight loss (one of the symptoms of cancer).¹³⁰

The liver produces considerable quantities of acetoacetate and β -hydroxybutyrate. Acetoacetate continually undergoes spontaneous decarboxylation to yield acetone. *In-vivo*, the liver appears to be the only organ in nonruminants to add significant quantities of ketone to the blood. Acetone is produced in smaller amounts than other ketone bodies and is exhaled via the breath because of its high vapor pressure. Ketone bodies are also derived from the amino acid metabolism. Under normal conditions, the rate of tissue protein catabolism is more or less constant throughout the day. However, protein metabolism and, thus, the amount of ketone bodies is increased in cachexia, which is associated with advanced cancer and other diseases.¹³⁰ The formation of ketone bodies in general and of acetone in particular occurs mostly in the final stages of the disease, when cachexia usually occurs. This may explain the inconsistencies between different studies.^{59,63} In any case, the strong effect of exercising, fasting, and/or food consumption on the acetone level in the breath^{131,132} excludes the candidacy of acetone as a biomarker for LC.

Elevated concentrations of 2-butanone were found in the breath of LC patients.⁶³ The analysis of cell-line headspace samples showed a decrease³⁵ in one study and an increase in another study.³⁷ 2-Butanone is absorbed to the body from exogenous sources: it occurs naturally in the environment, and it is used in the chemical industry as a precursor for the synthesis of paints, glues, and other coatings. Although 2-butanone has not been proven to increase cancer risk, it is likely to assume that breathing 2-butanone along with other hazardous or carcinogenic chemicals increases the damage to the person's health.¹³³ We recommend the use of animal models for studying the role of 2-butanone in LC.

2-Pentanone appears in the exhaled breath of a large part of the population.^{104,134} In addition, 2-pentanone was found in increased concentration levels in LC cells and normal cells, as compared to the growth medium.³⁴ Different ketones were also detected in *urine* samples from mice with lung tumors, such as 2-heptanone and 6-hydroxy-6-methyl-3-heptanone.¹³⁵ The observed variation of ketones as a function of tumor growth suggests that ketogenic pathways may be involved in LC. Using animal models allows controlling many of the variables which can

affect the body odor volatiles, such as genetic and dietary differences.¹³⁵

3.5. Esters

Esters could be found in large quantities in natural sources such as fats and fatty oils, waxes, and fruit ethers/essential oils. Esters can be split into alcohol and acid via hydrolysis (see Figure 5).¹³⁶

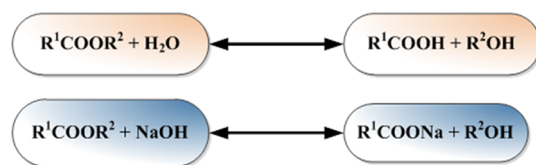


Figure 5. Hydrolysis of an ester under acidic conditions (red) and alkaline conditions (blue).¹³⁶

In humans, specific enzymes, or esterase, hydrolyze esters at temperatures below 40 °C. A wide variety of such enzymes take part in the body's metabolism, such as lipase, which catalyzes lipid hydrolysis.

Butyl acetate is the only ester that could be linked to LC (see Table 5). Butyl acetate was found in decreased concentration in the headspace of LC and healthy cells, as compared to the growth medium, irrespective of the cell type.^{34,37} Hence, there is apparently no LC-specific esterase cellular activity.¹³⁷ Accordingly, no change in butyl acetate was found in breath samples. This could be attributed to the observation that butyl acetate emitted from the LC cell is hydrolyzed in the body into butanol, which, in turn, converts to butanal and to butyric acid in blood and tissue. Since the hydrolysis rate is extremely fast, no (or negligible) amount of butyl acetate is excreted into the breath.

3.6. Nitriles

Table 6 lists the details of one nitrile (acetonitrile—a component of tobacco smoke¹³⁸) that was found in both breath and cell culture samples. Bajtarevic et al. observed a higher concentration of acetonitrile in the breath of LC smokers, as compared to LC ex-smokers.⁵⁹ Kischkel et al. found acetonitrile in higher concentration in LC ex-smokers as compared to healthy smokers.⁶³ Similar results were published for a higher concentration of acetonitrile in smokers in comparison to nonsmokers,^{57,63} which is consistent with previous observations in blood and urine samples.¹³⁹ The proposed pathway is the biotransformation of acetonitrile by cytochrome p450 mono-oxygenase to cyanohydrine, which further decomposes spontaneously into hydrogen cyanide and formaldehyde. The

acetonitrile metabolism in the body is rather slow. Therefore, considerable acetonitrile amounts are excreted as-is via exhaled breath and via urine.¹³⁸ Although acetonitrile is an exogenous compound, it could be of interest for the follow-up of LC patients.

In cancer cell lines, the levels of acetonitrile, which is widely used as a solvent in the biological cell culture treatment and handling,^{140,141} were found to be decreased relative to the growth medium.³⁵ This decrease is probably due to consumption of the acetonitrile in the growth medium through the LC cell metabolism. The lack of additional data regarding normal cell lines makes it difficult to fully understand if there is a real difference between cancer cells and normal cells with regard to this compound.

3.7. Aromatic Compounds

Table 7 presents four aromatic compounds including the following: benzene, toluene, styrene, and 2,5-dimethyl furan. Benzene, toluene, and 2,5-dimethylfuran were increased in the breath of smokers versus nonsmokers and LC patients.^{52,59,63} Styrene, however, was found in elevated concentrations in the breath of COPD patients, as compared to nonsmoking controls and LC patients. Styrene was also found in decreased levels in LC cell lines as compared to their medium. These compounds are considered exogenous pollutants. Exogenous sources include exposure to cigarette smoke, alcohol, pollution, and radiation. Since these molecules are highly reactive, they leak into the cytoplasm, attacking organs or organelles in the body and causing peroxidative damage to proteins, PUFA, and DNA. This damage accumulates during life and is assumed to lead to age-dependent diseases such as cancer.⁹⁸ It is reasonable to assume that LC patients that have been exposed to excessive smoking and/or have experienced continuous occupational exposure to such exogenous compounds might uptake the compounds in the fatty tissues of the body. These absorbed compounds might be then released slowly and constantly into the breath. In addition, mechanical, cellular, and enzymatic defense mechanisms act to eliminate hazardous compounds and xenobiotics. In the enzymatic defense reaction, the compound is first functionalized by phase I enzymes, usually by the cytochrome p450 enzyme system, and then conjugated to a more soluble and excretable form by other enzyme systems, such as glutathione S-transferases, sulfotransferases, and N-acetyltransferases.¹⁴²


Tobacco-related carcinogen biomarkers can potentially be used to predict which smoker or smokeless-tobacco user will get cancer, by identifying those individuals who activate tobacco

Table 5. Esters That Appear in Exhaled Breath and *in-Vitro* Cells of LC

Compound	Structure	Breath		<i>In-vitro</i> (Normal cells)		<i>In-vitro</i> (Cancer cells)	
		Increased ^a	Decreased ^b	Increased	Decreased	Increased	Decreased
Esters							
Butyl acetate					↓ ^{34,37} (Dermis ^d , Bronchial ^e)		↓ ^{34,37} ↓ ^{36*}





^aIn breath analysis: a higher concentration of a VOC appears in the breath of subjects with LC, compared to control subjects. In headspace analysis: a higher concentration of a VOC appears in LC or normal cells, compared to medium. ^bIn breath analysis: a lower concentration of a VOC appears in the breath of subjects with LC, compared to control subjects. In headspace analysis: a lower concentration of a VOC appears in LC or normal cells, compared to medium. ^dHuman fibroblast (hFB) derived from the dermis. ^ePrimary human bronchial epithelial cells (HBEpC). ^gVOC decreased in normal or cancer cells compared to medium. ^{*}For VOC measurement, different numbers of cells were used. ^{*} means that a significant increase or decrease in the VOC concentration was found only in part of the experiments and not in all.

Table 6. Nitriles That Appear in Exhaled Breath and *in-Vitro* Cells of LC

Compound	Structure	Breath		<i>In-vitro</i> (Normal cells)		<i>In-vitro</i> (Cancer cells)	
		Increased ^a	Decreased ^b	Increased	Decreased	Increased	Decreased
Nitriles							
Acetonitrile (m/z=42)			Smokers > Nonsmokers ^{57,59,63,139} Smokers > LC ⁶³				↓ ^{g,35}

^aIn breath analysis: a higher concentration of a VOC appears in the breath of subjects with LC, compared to control subjects. In headspace analysis: a higher concentration of a VOC appears in LC or normal cells, compared to medium. ^bIn breath analysis: a lower concentration of a VOC appears in the breath of subjects with LC, compared to control subjects. In headspace analysis: a lower concentration of a VOC appears in LC or normal cells, compared to medium. ^gVOC decreased in normal or cancer cells compared to medium.

Table 7. Aromatic Compounds That Appear in Exhaled Breath and *in-Vitro* Cells of LC

Compound	Structure	Breath		<i>In-vitro</i> (Normal cells)		<i>In-vitro</i> (Cancer cells)	
		Increased ^a	Decreased ^b	Increased	Decreased	Increased	Decreased
Aromatic Compounds							
Benzene			Smokers > Nonsmokers ^{59,63} Smokers > LC Smokers > NSCLC > Controls ⁵²	↑ ^{c,34} (Dermis ^d)			
Toluene		Smokers > Nonsmokers ⁵⁹ Smokers > NSCLC > Controls ⁵²					
Styrene		COPD > Controls ⁵²	COPD > NSCLC ⁵²				↓ ^{g,32}
2,5-Dimethyl-Furan			Smokers > Nonsmokers ^{59,63} Smokers > LC ⁶³				

^aIn breath analysis: a higher concentration of a VOC appears in the breath of subjects with LC, compared to control subjects. In headspace analysis: a higher concentration of a VOC appears in LC or normal cells, compared to medium. ^bIn breath analysis: a lower concentration of a VOC appears in the breath of subjects with LC, compared to control subjects. In headspace analysis: a lower concentration of a VOC appears in LC or normal cells, compared to medium. ^cVOC increased in normal or cancer cells compared to medium. ^dHuman fibroblast (hFB) derived from the dermis. ^gVOC decreased in normal or cancer cells compared to medium.

carcinogens more efficiently and/or detoxify them less efficiently.¹⁴³ In addition, these VOCs could be used for following-up of LC progression.

4. CONCLUSIONS AND OUTLOOK

Empirical data on VOCs associated with LC have been accumulated for over three decades. Nevertheless, the pathophysiology underlying the alteration of the LC VOCs has been vague to a large extent. So far, it has been hypothesized that the abnormal LC VOCs are produced by tumor cells that are excreted into the endobronchial cavity,⁵⁴ from where they are exchanged in alveoli and excreted via the breath. Arguably, an optimal approach to check this hypothesis would be to compare VOC profiles from different sources (organs or clinical samples) in the same LC patient and/or the same animal model. Within this approach, the simplest starting point would be a comparison between the VOC profiles in the headspace of LC tumor tissue or LC cells, in (headspace of) blood samples, and in breath samples. However, many technical challenges have been hindering the implementation of such an approach.

With these challenges in mind, the current review presents a comparison between LC VOCs collected from different sources (cancer cells, blood, breath, and, where available, saliva and urine

samples), as reported by different research groups over the past three decades. This comparison might not provide precise or definite answers to the puzzling pathophysiological pathways of LC VOCs. However, it will help stimulating constructive discussions and new ideas.

The outcome of the presented comparative study could be summarized as follows (cf. Figure 2). Each of the validated LC VOCs has its own particular biochemical or chemical background. Both endogenous and exogenous compounds could be interesting candidates for LC biomarkers. Hydrocarbons, aldehydes, and some ketones are produced in the body as a result of oxidative stress, which occurs when increased quantities of reactive oxygen species (ROS), originating mainly from exogenous factors such as pollution, radiation, and cigarette smoke, are produced in the mitochondria. The body reacts to this by activating the detoxification process, in which the liver enzyme cytochrome p450 catalyzes the addition of an oxygen atom to the foreign compound, thus turning it to a more soluble substance in water—an alcohol and, as byproducts, aldehydes are formed. Other liver enzymes may also alter the concentration of the VOCs, such as ADH, which turns alcohols to aldehydes, and ALDH, which oxidizes aldehydes into carboxylic acids. Some aldehydes are emitted via the breath as a result of signal

transduction during the cancerous pathological process. Another mechanism is associated with the high glycolysis rates, which is characteristic for cancer cells. In this process, the acidic microenvironment of the cells protects them from the immune system cells and helps them to become motile and to reach blood vessels. Exogenous sources of VOCs include cigarette smoking, alcohol consumption, air pollution, and radiation. The reactive nature of these exogenous molecules can cause peroxidative damage to biological organelles, stimulating cancer, and hence, they are of great interest.

Although the biological mechanisms discussed above affect the concentration of the VOCs in both blood and breath, we presume that there is an enormous advantage for breath sampling in comparison to blood sampling. First, the blood and breath concentrations are related through the respective Henry constant of each compound, where the blood concentration can be higher or lower than the breath concentration. For example, the blood/breath Henry constant of isoprene is ~ 0.9 .¹⁴⁴ Hence, the concentration of isoprene in the blood is $\sim 90\%$ of the concentration in the breath. On the other hand, the Henry constant of butane is ~ 0.183 . Hence, the concentration of butane in the blood is only about 18.3% of the concentration in exhaled breath.¹⁴⁵ Another aspect considers the reliability of the sampling technique. In the common process of blood sampling, VOCs are quickly released into the surrounding air. Hence, the sampling of VOCs from blood needs very careful preparation and processing of the sample to avoid evaporation (and therefore the loss) of the compounds in interest. This is one of the reasons that the measured isoprene concentrations from blood are relatively unreliable. A third aspect relates to the analytical techniques. Measuring VOCs in gaseous samples is well developed and comparatively simple, because all the other (nonvolatile) compounds do not interfere. However, measuring VOCs in blood samples (where they are surrounded by a much more complicated matrix) is rather complicated. The last aspect concerns medical applications. Breath sampling is noninvasive, and breath can be sampled as often as is desirable. Exhaled breath can even be sampled continuously during an ergometer challenge or during sleep,^{146,147} as opposed to blood, which cannot be sampled continuously.

Open questions to be addressed are the delineation of the metabolic pathways leading to the generation of potential biomarkers, as well as the impact of potential confounding parameters such as high inspiratory concentrations. With this in mind, we raise in the following important issues in relation with LC VOCs. We present ideas to investigate these issues with the aim to gain a better understanding of the mechanisms of VOC production/consumption in the body. *First issue:* Many metabolic pathways, such as glycolysis, apoptosis, loss of tumor suppressor genes, and angiogenesis, are activated or over-activated in the case of cancer.¹⁴⁸ These pathways may alter the production of VOCs in the body. In order to identify the exact change in the VOC pattern, we propose blocking such metabolic processes in various cell lines, each in a separate assay. This could be achieved by deactivating the specific enzyme (e.g., hexokinase, pyruvate kinase dehydrogenase, or matrix metalloproteases) that initiates or is crucial to the process, in order to compare between the measured VOC profiles before and after the blocking. According to the specific blocking, the LC VOCs can be associated with the different mechanisms occurring in the same cancer cell. *Second issue:* The hypothesis that certain VOCs are associated with the cell metabolism *per se*, rather than with the microenvironment of the cancer or other indirect metabolic

pathways in the human's or animal's body, needs to be confirmed through direct observation. This issue could be resolved by using cell lines from well-documented sources, so that they can be directly correlated to metabolic pathways without any confounding factors. In this context, using a variety of different cell lines, rather than replicas of the same cell line, could be helpful to stimulate the natural diversity of LC while eliminating potential confounding effects that are associated with clinical samples. *Third issue:* Many LC VOCs are related to environmental and tobacco compounds. Following inhalation, these molecules might affect the respiratory system, and later on the blood too. Therefore, it is important to examine the effect of inhaling these compounds on the blood, as any change in the composition of blood can affect the body's metabolism and, hence, the breath VOC profile. Using an animal model, such compounds could be introduced either *via* inhalation, or they could be directly introduced into the bloodstream, in order to monitor the resulting breath VOC profile of the treated animals. In addition, oxidative stress could be determined through measuring the amount of glucose and the activity of G-6 PD. Comparing between the animal model and the introduction of the same molecules *in vitro* to cancer cells would allow obtainment of a detailed understanding of how these VOCs affect the body both on a cellular level and as a whole. *Fourth issue:* It is hypothesized that cancer is a "free organ", having its own cancer stem cells (CSC). These cells are a chemotherapy-resistant population capable of self-renewal. Stem cells were found to have high levels of ALDH activity. However, there is a variance in the ALDH activity between the different cells. A focused study on CSC both *in vitro* and *in vivo* might, therefore, reveal variances in the VOC patterns that are released as a response to different ALDH activity. This could serve as a launching-platform for developing a CSC (and/or ALDH activity) biomarker, namely a single VOC or a VOC pattern that could be indicative for recurring tumor initiation, metastasis initiation, thus aiding the prediction of a patient's prognosis and the tailoring of personalized treatments.

Ultimately, these discoveries would contribute to the improvement of breath testing systems that could be used in clinical practice for personalized screening, diagnosis, and treatment monitoring of LC states. The results could be translated into management advances that could also serve as a model for development of breath tests for other diseases.

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NOMENCLATURE

$\lambda_{b/a}$	partition coefficient of blood/air
ADH	alcohol dehydrogenase
ALDH	aldehyde dehydrogenase
AUC	area under curve
COPD	chronic obstructive pulmonary disease
CSC	cancer stem cells
CT	computed tomography
CYP	cytochrome p450
DNA	deoxyribonucleic acid
FDH	formaldehyde dehydrogenase
GC-MS	gas chromatography–mass spectrometry
GSH	glutathione
HLA	human leukocyte antigen
HNE	4-hydroxy-2-nonenal
HPLC	high-performance liquid chromatography
HS-MS	headspace sampler–mass spectrometry
IBE	immortal bronchial epithelium
LC	lung cancer
MRI	magnetic resonance imaging
NNK	4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone
NSCLC	nonsmall cell lung cancer
PCA	principal component analysis
PET	positron emission tomography
ppmv	parts per million by volume
PTR-MS	proton transfer reaction–mass spectrometry
pptv	parts per trillion by volume
PUFA	polyunsaturated fatty acid
ROS	reactive oxygen species
SCLC	small cell lung cancer
SPME	solid phase microextraction
VOC	volatile organic compound

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