Cell culture metabolomics in the diagnosis of lung cancer—the influence of cell culture conditions

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Abstract
Lung cancer is the leading cause of cancer deaths. Unfortunately, lung cancer is often diagnosed only when it becomes symptomatic or at an advanced stage when few treatment options are available. Hence, a diagnostic test suitable for screening widespread populations is required to enable earlier diagnosis. Analysis of exhaled breath provides a non-invasive method for early detection of lung cancer. Analysis of volatile organic compounds (VOCs) by various mass spectral techniques has identified potential biomarkers of disease. Nevertheless, the metabolic origins and the disease specificity of VOCs need further elucidation. Cell culture metabolomics can be used as a bottom-up approach to identify biomarkers of pathological conditions and can also be used to study the metabolic pathways that produce such compounds. This paper summarizes the current knowledge of lung cancer biomarkers in exhaled breath and emphasizes the critical role of cell culture conditions in determining the VOCs produced in vitro. Hypoxic culture conditions more closely mimic the conditions of cancer cell growth in vivo. We propose that since hypoxia influences cell metabolism and so potentially the VOCs that the cancer cells produce, the cell culture metabolomics projects should consider culturing cancer cells in hypoxic conditions.

Keywords: volatile organic compounds (VOCs), cell culture, metabolomics, hypoxia, cancer

Introduction
Lung cancer is one of the five most commonly diagnosed cancers and is the leading cause of cancer-related deaths throughout the world [1–3]. The 5 year survival rate for lung cancer patients is poor, largely due to symptoms of lung cancer usually becoming apparent only once the disease has reached an advanced stage. Methods for the detection of lung cancer are generally invasive and not suited to widespread population screening; hence, there is a need for a non-invasive, accurate and rapid screening test for early detection.

The exhaled breath of lung cancer patients contains volatile organic compounds (VOCs), some of which may be useful biomarkers of the disease and, therefore, provide a non-invasive means to screen for lung cancer using gas chromatography-mass spectrometry (GC-MS). Previous results indicate that lung cancer can be diagnosed in this way with some accuracy [4]. The diagnostic VOCs identified in these studies were mostly alkanes that showed decrease in exhaled breath compared to room air, which is believed to occur due to metabolism by cytochrome P450 mixed function oxidases that are up-regulated in cancer. However, most lung cancer VOCs that have been reported are not disease specific and their metabolic origins remain unknown. Knowledge of all the VOCs produced by lung cancer cells should lead to a panel of diagnostic biochemical markers that can be measured...
in combination to increase the sensitivity and specificity of lung cancer diagnosis.

The use of cell culture metabolomics allows for both the discovery of novel biomarkers of pathological conditions and investigation of the metabolic pathways that produce them. However, previous studies have found poor correlations between the VOCs from cancer cells in culture and those found by breath analysis (see section 2 of this paper). We propose that one reason for this discrepancy is the use of hyperoxic in vitro culture conditions that have traditionally been used for growing cancer cell lines. In vivo cancer cells experience low oxygen or hypoxic conditions as a consequence of the diffusion limit within tissues, which has been measured to be around 150 μm [5, 6]. Consequently, once a tumour grows to greater than 300 μm diameter or approximately 15 to 20 cells across, the cells in the centre will be experiencing hypoxic conditions. VOCs however are generally hydrophobic and therefore lipid soluble and so should pass freely from the hypoxic regions of the tumour to enter the circulation to travel to the lungs for release by breath.

There have been several excellent recent reviews of the VOCs associated with lung cancer [7, 8]. In this paper, we summarize the current state of knowledge about biomarkers of lung cancer in exhaled breath but with an emphasis on the critical role of cell culture conditions in in vitro studies in determining the VOCs produced. Hypoxic culture conditions more closely mimic the conditions of cancer cell growth in vivo. Since hypoxia influences cell metabolism, then it will also influence the VOCs produced by the cancer cells. Consequently, cell culture metabolomics projects should consider culturing cancer cells in hypoxic conditions.

1. Breath analysis

1.1. Pros and cons of breath analysis

Breath analysis provides a non-invasive window to observe the biochemical processes of the body [9]. Ancient physicians knew that the smell of human breath can indicate a certain disease state which could be a useful diagnostic tool. For example, diabetes is associated with the sweet smell of acetone in breath, renal failure results in a urine-like smell and fishy odour in breath is linked to liver disease [10] (table 1).

VOCs are only a small fraction of the total chemical compounds present in human breath and occur at low concentrations in the nmol L−1–pmol L−1 range [9, 12].

The origin of these volatile substances may be endogenous (generated within the body) or exogenous (absorbed as contaminants from the environment). Targeting the volatile component of breath for analysis reduces many issues associated with analysis of total breath. Currently, clinically available breath tests include: breath-alcohol test which determines ethanol concentration [13], the nitric oxide (NO) test to detect asthma and diagnosis of Helicobacter pylori infection by 13C-urea or ammonia breath tests [14].

In 1971, Pauling et al detected the presence of large numbers of VOCs using microanalysis of breath by newly developed capillary gas chromatography (GC) [15]. Apparently, there are approximately 200 VOCs present in the exhaled breath in picomolar concentrations [16] and there have been studies which aim to correlate single substances or sets of exhaled markers and the clinical conditions of patients [17–21]. Analysis of exhaled breath has many advantages compared to other diagnostic techniques such as bronchoscopy or medical imaging. It is non-invasive and painless and exhaled air can be sampled as often as necessary without restriction; particularly important for the critically ill and for large scale screening in healthy populations for cancer, renal and liver diseases. The basic research in breath analysis relies on the advances of analytical technology to detect and identify the VOCs. The sample of exhaled breath is analysed using various high-performance equipment such as GC-MS, selected ion-flow tube mass spectrometry (SIFT-MS), ion-mobility spectrometry (IMS) and proton transfer mass spectrometry (PTR-MS).

These methods of diagnosis are potentially useful in clinical practice but they are not yet available as portable analytical devices. Also, standardization of protocols for collection and analysis of exhaled breath must occur in order to achieve consistency in VOC profile analysis [22, 23].

1.2. Biological mechanisms

The origins of many VOCs have now been explained through an improved understanding of the mechanisms and kinetics of VOC synthesis [4] (and for a review see [7]) (table 2). Alkanes and methylated alkanes in breath are markers of oxidative stress, which are the products of reaction of lipids with reactive oxygen species (ROS). ROS comprise oxygen free radicals and hydrogen peroxide and are constantly produced in the mitochondria from where they can leak into the cytoplasm [24]. Cellular anti-oxidant defences such as glutathione (in reduced form) usually protect cells from ROS, but when these

| Table 1. Volatile compounds associated with disease [11]. |
|--------------------------------------------------------|
| **Volatile compound(s)** | **Disorder(s)** |
| Ethane and pentane | Oxidative stress |
| Methylated hydrocarbons | Lung or breast cancer |
| Isoprene | Cholesterol metabolism |
| Acetone | Diabetes mellitus, ketonemia |
| Dimethylsulfide, methyl mercaptane, ethyl mercaptane | Liver damage |
| Ammonia, dimethylamine, trimethylamine | Uremia, renal damage |

| Table 2. VOCs—biological origin. |
|----------------------------------|
| **VOCs** | **Biological basis** |
| Acetaldehyde | Ethanol metabolism [29, 30] |
| Acetone | Decarboxylation of acetoacetate and acetyl-CoA |
| Ethane and pentane | Lipid peroxidation [9] |
| Ethylene | Lipid peroxidation [31] |
| Hydrogen and methane | Gut bacteria [32] |
| Isoprene | Cholesterol biosynthesis [33] |
| Methylamine | Protein metabolism [31] |
Breath methyl alkanes may be products of lipid peroxidation of polyunsaturated fatty acids in cell membranes, a process that also generates alkanes such as ethane and pentane that are found in exhaled breath [34]. Alkanes are metabolized to alkyl alcohols by cytochrome P450 (CYP)—mixed function oxidase enzymes [35] and a number of studies have demonstrated that these enzymes are activated in lung cancer [36–39]. For example, polyaromatic hydrocarbons in tobacco smoke induce CYP 1A1 and CYP 1A2 activity, resulting in the accelerated drug metabolism of some procarcinogens [40]. Consequently, the biotransformation of volatile alkanes and monomethylated alkanes that are produced by oxidative stress may be accelerated by CYP enzymes that have been activated in patients with lung cancer so producing aldehydes, alcohols and ketones in measurable quantities in breath [4].

1.3. VOCs identified in breath of cancer patients

A number of studies have detected chemical compounds in breath samples from patients with and without lung cancer [15, 16, 39–42]. Although the VOCs identified as markers of lung cancer differ between reports, the results have all shown significant variations between exhaled breath of lung cancer patients and healthy volunteers [41]. The source and physiological function of most lung cancer VOCs, however, are still unknown [42]. Some of them could be of exogenous origin and so be inhaled, absorbed from the lungs and metabolized in the body, and the metabolites excreted by expiration. Other VOCs that are of endogenous origin may be generated as products of internal metabolic processes or activity of intestinal bacteria [41].

VOCs found in the breath of lung cancer patients include a wide range of aldehydes, alkanes and methylated alkanes containing C2–C11 carbons. Some studies have reported alkenes and aromatic compounds such as benzene, ethyl benzene, xylene isomers, acetonitrile, 2-methyl furan, 2,5-dimethyl furan, furan, 1,3-cyclohexadiene, 1,3-cyclopentadiene, 2-methyl-1-buten and 1,4-pentadiene, which are all related to cigarette smoking [43, 44].

As analytical technology rapidly advances, so has the detection of compounds in breath. Many compounds have been detected whose biochemical origin is unknown and many VOC metabolites reported as biomarkers have been found not to be disease specific. Hence, validation of the biomarkers is a necessary step in developing a specific and sensitive test for the early detection of lung cancer. *In vitro* analysis of established cancer cell lines is an approach that should help identify endogenous VOCs and define the underlying mechanisms that lead to quantitative or qualitative changes in lung cancer.

2. Cell culture metabolomics

2.1. Validation of biomarkers

Metabolomics, a high-throughput global metabolite analysis, is a burgeoning field with an emerging role in cancer diagnosis, recurrence and prognosis through identifying novel cancer biomarkers [45].

The integrated analysis of metabolomics and other *omics* technologies may provide more sensitive ways to detect changes related to disease and discover novel biomarkers [46]. Subtle changes in metabolism can be detected by analyses of the products of cellular processes which in turn can lead to the development of prognostic models useful for early detection of cancer.

The metabolome is downstream of the transcriptome and proteome, and is considered to be complementary to genomics, transcriptomics and proteomics [47, 48]. Understanding the metabolome may also assist in identifying intermediate or surrogate cancer biomarkers for establishing preventive or therapeutic approaches for health [46].

VOCs in breath can derive from cancer cells, healthy cells, immune cells and microbes [49]. Several studies have investigated the release of VOCs from human cancer cells *in vitro* [49–53], for example, headspace on-line measurements by SIFT-MS were able to detect acetaldehyde release from the lung cancer cell lines SK-MES and CALU-1 [52].

If some breath markers of lung cancer do derive from the cancer cells themselves, then there should be an overlap between the set of VOCs produced by cancer cells in culture and the VOCs detected in the breath of lung cancer patients. Comparison of the VOC profiles of breath analysis and cell cultures (table 3) reveals that, of the 68 VOCs detected in either breath or cell culture, only 16 VOCs were detected in both cell culture and breath. There were an additional 17 VOCs detected only in breath and 22 found only in lung cancer cell cultures and 13 VOCs found only in controls (non-transformed cell lines). This poor relationship indicates that *in vitro* culture of lung cancer cells is not a good model for the production of VOCs in breath of lung cancer patients. A more detailed examination of the compounds identified shows that of the 16 compounds common to both cell culture headspace and lung cancer breath, five were straight chain alkanes and methylated alkanes, which is consistent with lipid breakdown associated with oxidative stress [13, 41]. Interaction of active oxygen species with polyunsaturated fatty acids such as linoleic acid and palmitic acid in the cell membrane results in a series of reactions called lipid peroxidation. During the process of peroxidation of polyunsaturated fatty acids volatile alkanes are formed that can be excreted in breath unchanged or distributed throughout the body, partly metabolized and then excreted in breath.

The remainder of the compounds common to breath and cell culture were alkenes, aldehydes and aromatic compounds, some of which are also associated with lipid peroxidation [9, 30]. Of the 22 compounds found only in cell culture most were alcohols, ketones, esters and ethers (table 3) suggesting that the VOCs produced by cancer cell culture are mostly oxidized breakdown products. Other analytical
Table 3. Comparison of VOCs found in breath and in vitro analysis of cells cultured under standard hyperoxic conditions.

| Class         | Compound                        | Structure | Breath (normal cells) | In vitro (cancer cells) | References |
|--------------|---------------------------------|-----------|------------------------|-------------------------|------------|
| Hydrocarbons |                                 |           |                        |                         |            |
| Alkanes      |                                 |           |                        |                         |            |
| (straight chain) | Pentane                         | +         | −                      | −                       | [54]       |
|               | Heptane                          | +         | −                      | −                       | [54]       |
|               | Octane                           | +         | ↑ (hFB)               | ↑ (A549)                | [54]       |
|               | Decane                           | +         | −                      | ↑                       | [16, 50, 54–56] |
|               | Undecane                         | +         | −                      | ↑                       | [16, 43, 53] |
| Alkanes      | 2-methyl pentane                 | +         | −                      | −                       | [49, 54]   |
| (branched)   | 2,3,3-trimethylpentane           | −         | ↑ (hFB&HBEpC)         | ↑ (NCI-H2087)           | [51, 53]   |
|               | 2,3,4-trimethyl pentane          | −         | ↑                      | −                       | [51]       |
|               | 2,4-dimethyl hexane              | −         | ↑                      | −                       | [51]       |
|               | 2,3,5-trimethyl hexane           | 1         | ↑                      | ↑ (Calu–1)             | [51, 53]   |
|               | 2-methyl heptane                 | +         | −                      | −                       | [16]       |
|               | 3-methyl heptane                 | −         | ↑                      | −                       | [51]       |
|               | 4-methyl heptane                 | −         | ↑                      | −                       | [51]       |
|               | 2,4-dimethyl heptane             | +         | −                      | ↑ (Calu–1)             | [16, 53]   |
|               | 2,2,4,6,6-pentamethyl heptane    | +         | −                      | −                       | [54]       |
|               | 3-methyl octane                  | +         | −                      | −                       | [16]       |
|               | 4-methyl octane                  | +         | −                      | ↑ (Calu–1)             | [53]       |
|               | 3-methyl nonane                  | +         | −                      | −                       | [16]       |
| Cycloalkanes | 1-methyl-2-pentyl cyclopropane   | +         | −                      | −                       | [16]       |
|               | Methyl cyclo pentane             | +         | −                      | +                       | [16, 50]   |
|               | Cyclo hexane                     | +         |                        |                         |            |
| Alkenes      | 1-hexene                        | +         | −                      | +                       | [16, 50]   |
|               | 1-heptene                        | +         | −                      | −                       | [16]       |
Table 3. (Continued).

| Class       | Compound                     | Structure                       | Breath (normal cells) | In vitro (cancer cells) | References |
|-------------|------------------------------|--------------------------------|------------------------|-------------------------|------------|
|             | 2,4-dimethyl-1-heptene       | ![Structure](image)             | −                      | ↑ (A549)                | [51]       |
| Alcohols    | Ethanol                      | ![Structure](image)            | −                      | −                       |            |
|             | 2-methyl-1-propanol          | ![Structure](image)           | −                      | ↑ (HBEpC)               | [51]       |
|             | 2-methyl-2-propanol          | ![Structure](image)           | −                      | −                       | [51]       |
|             | 3-methyl-1-butanol           | ![Structure](image)           | −                      | ↑ (HBEpC)               | [51]       |
|             | 2-ethyl-1-hexanol            | ![Structure](image)           | −                      | ↑ (NCI−H2087)           | [49]       |
| Aldehydes   | formaldehoy                  | ![Structure](image)           | +                      | −                       |            |
|             | Acetaldehyde                 | ![Structure](image)           | +                      | ↓ (NCI−H2087, CALU-1)   | [49, 52, 53]|
|             | 2-methyl propanal            | ![Structure](image)           | −                      | ↓ (NCI−H2087, Calu-1)   | [49, 51, 53]|
|             | Butanal                      | ![Structure](image)           | −                      | −                       | [51]       |
|             | Pentanal                     | ![Structure](image)           | +                      | −                       | [58]       |
|             | Hexanal                      | ![Structure](image)           | +                      | ↓ (NCI−H1666, Calu−1)   | [16, 50, 58–60]|
|             | Heptanal                     | ![Structure](image)           | +                      | −                       | [16, 50, 58–60]|
|             | Octanal                      | ![Structure](image)           | +                      | −                       | [58]       |
|             | Nonanal                      | ![Structure](image)           | +                      | −                       | [58]       |
|             | prop-2-enal                  | ![Structure](image)           | −                      | −                       |            |
|             | 2-methylprop-2-enal          | ![Structure](image)           | −                      | ↓ (HBEpC)               | [51, 53, 59]|
|             | 2-ethylprop-2-enal           | ![Structure](image)           | −                      | −                       | [51, 53]   |
Table 3. (Continued).

| Class   | Compound     | Structure | Breath (normal cells) | In vitro (cancer cells) | References |
|---------|--------------|-----------|-----------------------|-------------------------|------------|
|         |              |           |                       |                         |            |
|         | 2-butenal    | ![Structure](image1.png) | − | ↓ | − | [51] |
|         | 2-methyl-2-butenal | ![Structure](image2.png) | − | − | ↓ | [51, 53] |
|         | 2-methyl butenal | ![Structure](image3.png) | − | ↓ | ↓ | [49, 51] |
|         | 3-methyl butenal | ![Structure](image4.png) | − | ↓ | ↓ | [49, 51, 53, 59] |
|         | Benzaldehyde  | ![Structure](image5.png) | − | ↓ | ↓ | [51, 53] |
| Ketones | Acetone       | ![Structure](image6.png) | − | ↑ | ↑ | [51] |
|         | 2-butanone    | ![Structure](image7.png) | − | − | ↓ | [53] |
|         | 2-pentanone   | ![Structure](image8.png) | − | ↑ | ↑ | [51] |
|         | 2-hexanone    | ![Structure](image9.png) | − | ↑ | − | [51] |
|         | 3-pentene-2-one | ![Structure](image10.png) | − | ↓ | − | [51] |
|         | 1-phenyl ethanone | ![Structure](image11.png) | + | − | − | [16] |
| Esters  | Methyl acetate | ![Structure](image12.png) | − | ↑ | − | [51] |
|         | n-propyl acetate | ![Structure](image13.png) | − | ↑ | − | [51] |
|         | n-butyl acetate | ![Structure](image14.png) | − | ↓ | − | [51] |
| Ethers  | Methyl-tert-butyl ether | ![Structure](image15.png) | − | ↑ | (HBEpC) | (A549) | (Calu−1) | [51], [53] |
|         | Ethyl-tert-butyl ether | ![Structure](image16.png) | − | ↑ | (HFB) | (A549) | (Calu−1) | [51], [53] |
| Aromatics | Benzene       | ![Structure](image17.png) | + | ↑ | + | [16, 50, 51, 54–56, 61] |
|         | Toluene       | ![Structure](image18.png) | + | − | − | [54] |
|         | Styrene       | ![Structure](image19.png) | + | − | + | [16, 50, 51, 54–56, 61] |
methods such as PTR-MS and SIFT-MS have also identified alcohols and aldehydes including isopropanol, formaldehyde and acetaldehyde in the breath of lung cancer patients [52, 57].

The increased oxidation of alkanes to alcohols, esters and ketones in cell culture is perhaps, not unexpected when the environment in which cells are usually grown is considered. Most laboratories culture cells in air with 5% carbon dioxide, i.e. there is approximately 20% oxygen in the atmosphere surrounding the cells. This is in contrast to the in vivo environment.

3. Hypoxia

3.1. Hypoxia in cancer and hypoxia-inducible factor

Oxygen availability alters gene expression and metabolism in cells, hence raising the possibility that hypoxia will change the pattern of VOCs produced by the cancer cells. Tumours possess extensive regions of hypoxia relative to the corresponding normal tissue [62]. A number of adaptive responses are initiated during cellular hypoxic stress, including the activation of a group of transcription factors called hypoxia-inducible factors (HIFs). Hypoxia-inducible factor-1α (HIF-1α) has been extensively studied as an endogenous hypoxia marker and its mechanism of accumulation under hypoxia is well understood [63, 64]. HIF-1α regulates an increased production of VEGF [65]. VEGF induces neovascularization but in tumours this happens in an irregular fashion and at a slower pace when compared to the proliferation rate of the tumour [66, 67]. This could result in poor blood supply and so further hypoxia.

As described in figure 1, in normoxia, HIF-1α and HIF-1β subunits are constitutively expressed. While HIF-1α is rapidly degraded by the proteosomal system, the amount of HIF-1β remains constant. In hypoxia, HIF-1α escapes degradation, binds with its partner HIF-1β, and together they bind to a hypoxia-response element (HRE) in target genes in association with co-activators such as CBP/P300. This triggers the expression of multiple target genes that enable the tumour cells to adapt to and overcome the conditions of decreased oxygen by increasing oxygen transport, stimulating angiogenesis and regulating glucose uptake and metabolism [68].

Recent studies show that HIF1α stabilization under hypoxia leads to the expression of pyruvate dehydrogenase kinase 1(PDK1) [69, 70] that phosphorylates and inactivates pyruvate dehydrogenase (PDH), limiting the conversion of pyruvate to Acetyl-CoA in the mitochondria (see figure 2). Consequently, PDK1 induction decreases the tricarboxylic acid (TCA) cycle activity, so reducing oxygen consumption.

In 1920, Otto Warburg discovered that tumours show increased glucose consumption in converting glucose to pyruvate and then to lactic acid despite the availability of oxygen. Non-transformed cells also convert glucose to pyruvate but then metabolize pyruvate through the TCA cycle and mitochondrial oxidative phosphorylation (OXPHOS) [71]. The mitochondrial pathway requires oxygen and is much more efficient in ATP production than anaerobic metabolism, producing 38 versus 2 ATP molecules per molecule of glucose as shown in figure 2 [71]. However, in an expanding tumour mass, characterized by low levels of oxygen and a high glucose consumption rate, anaerobic glycolysis can become the predominant pathway of ATP generation [72]. In addition to glycolysis, a recent study has shown that under hypoxia, autophagy is present and is also required to support ATP production [73].

An anticipated outcome of this hypoxic environment would therefore be increased oxidative stress and a large proportion of metabolites being produced as a consequence of lipid metabolism leading to the production of alkanes and methylated alkanes, and reduced oxidative degradation.

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**Table 3. (Continued).**

| Class         | Compound               | Structure | Breath (normal cells) | Breath (cancer cells) | References |
|---------------|------------------------|-----------|-----------------------|-----------------------|------------|
|               | Ethyl benzene          | ![Structure of Ethyl Benzene](image) | +                     | −                     | [55]       |
|               | Propyl benzene         | ![Structure of Propyl Benzene](image) | +                     | −                     | [50, 54, 56] |
|               | Trimethyl benzene isomers | ![Structure of Trimethyl Benzene Isomers](image) | +                     | −                     | [16, 50, 54] |
|               | Xylene isomers         | ![Structure of Xylene Isomers](image) | +                     | −                     | [16, 62]   |
| Heterocyclics | Tetrahydro Furan      | ![Structure of Tetrahydro Furan](image) | −                     | −                     | (Calu−1) [53] |
|               | pyrrole                | ![Structure of Pyrrole](image) | −                     | −                     | (A549) [51]   |
| Nitriles      | Acetonitrile           | ![Structure of Acetonitrile](image) | −                     | −                     | (Calu−1) [53] |

*All compounds in breath are reported as present or not present in lung cancer patients’ breath.*

*Compounds in cell culture are either reported as increased or decreased arrows in quantity compared to medium controls or shown as ‘+’ where reported without quantitation (cell lines indicated as hFB—human fibroblasts, HBEpC—human bronchial epithelial cells).
Figure 1. Activation of the hypoxia-inducible factor (HIF-1α) transcription factor in normoxia and hypoxia. Figure adapted from [71].

Conclusion

Differences in the VOCs found in breath and in the headspace of cancer cell lines can be attributed to many causes such as different sampling methodology, mass spectral techniques and statistical approaches. Here, we propose that cell culture conditions also play a role, as it is known that hypoxia induces autophagy and increased lipid peroxidation. This could explain...
the presence of alkanes and methylated alkanes found in breath of the lung cancer patients. Little attention has so far been paid to the in vitro culture conditions used to grow the cancer cells. The hyperoxic culture conditions used to grow cells are likely to produce more alcohols and other oxidized products rather than the methylated alkanes and other products that are more abundant in breath. Hence, oxygen controlled culture conditions are the way forward for modelling the in vivo situation. This approach should help in validating breath VOC markers for diagnosis, clarify further how these compounds are produced and perhaps lead to the identification of novel VOC markers of cancer.

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