OPEN ACCESS
IOP Publishing
Journal of Breath Research
J. Breath Res. **8** (2014) 027109 (10pp)
doi:10.1088/1752-7155/8/2/027109

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

U Kalluri, M Naiker and M A Myers

Biomedical Science, School of Health Sciences, Federation University Australia, Mt Helen, Victoria 3350, Australia

E-mail: u.kalluri@federation.edu.au

Received 5 December 2013, revised 17 March 2014
Accepted for publication 2 April 2014
Published 27 May 2014

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

(Some figures may appear in colour only in the online journal)

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

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]

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 ^{13}C-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

defences are insufficient ROS causes peroxidative damage to proteins, polyunsaturated fatty acids and DNA [25]. These peroxidative changes to DNA bases may be carcinogenic [26, 27]. Considerable evidence supports the hypothesis that oxidative stress appears to be increased in some cancers [28] including lung cancer [16].

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 and activation 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-butene 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.

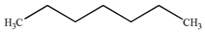
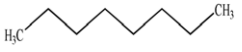
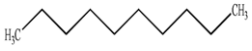
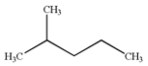
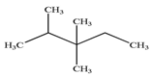
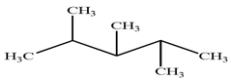
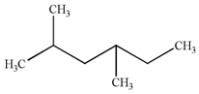
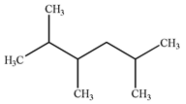
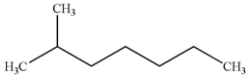
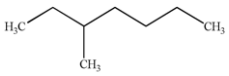
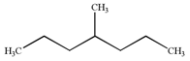
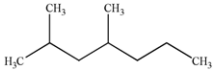
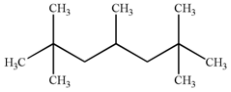
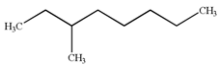
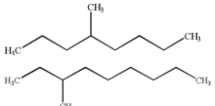
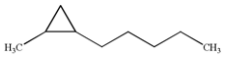
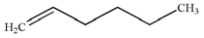
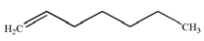
Class	Compound	Structure	Breath	*In vitro* (normal cells)	*In vitro* (cancer cells)	References
Hydrocarbons						
Alkanes (straight chain)	Pentane		+[a]	–	–	[54]
	Heptane		+	–	–	[54]
	Octane		+	↑ b(hFB)	↑ (A549)	[51, 54]
	Decane		+	–	↑	[16, 50, 54–56]
	Undecane		+	–	↑	[16, 43, 53]
Alkanes (branched)	2-methyl pentane		+	–	–	[49, 54]
	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		+			[16]
Alkenes	1-hexene		+	–	+	[16, 50]
	1-heptene		+	–	–	[16]

Table 3. (Continued).

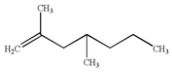
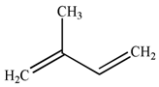
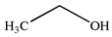
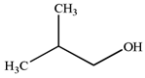
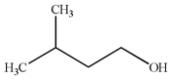
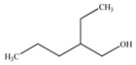
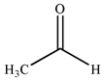
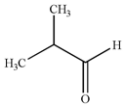
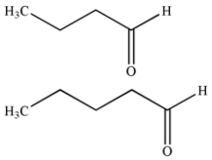
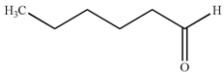
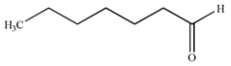
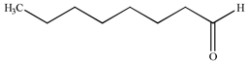
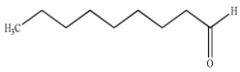
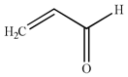
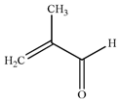
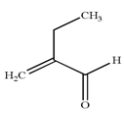
Class	Compound	Structure	Breath	*In vitro* (normal cells)	*In vitro* (cancer cells)	References
	2,4,dimethyl-1-heptene		–	↑	↑(A549)	[51]
	2-methyl -1,3-butadiene (Isoprene)		+	–	+	[16, 50, 54]
Alcohols	Ethanol		–	–	↑(A549)	[51]
	2-methyl-1-propanol		–	↑(hFB)	–	[51]
	2-methyl-2-propanol		–	↑(HBEpC)	–	[51]
	3-methyl-1-butanol		–	↑(hFB)	–	[51]
	2-ethyl-1-hexanol		–	↑(hFB)	↑(NCI−H2087)	[49]
Aldehydes	formaldehyde		+	–	–	[57]
	Acetaldehyde		+	↓	↓(NCI−H2087, CALU-1)	[49, 52, 53]
	2-methyl propanal		–	↓	↓(A549,NCI−H2087, Calu-1)	[49, 51, 53]
	Butanal		–	–	↓(A549)	[51]
	Pentanal		+	–	–	[58]
	Hexanal		+	↓	↓(NCI−H1666,Calu−1)	[16, 50, 58–60]
	Heptanal		+	–	+	[16,50,58–60]
	Octanal		+	↓	–	[58]
	Nonanal		+	–	–	[58]
	prop-2-enal		–	–	↓(Calu−1)	[53]
	2-methylprop-2-enal		–	↓(HBEpC)	↓(A549,NCI−H1666, Calu-1)	[51, 53, 59]
	2-ethylprop-2-enal		–	–	↓(A549,Calu−1)	[51, 53]

Table 3. (Continued).

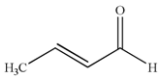
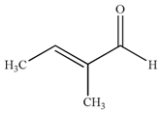
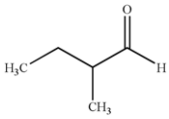
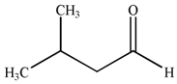
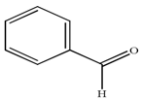
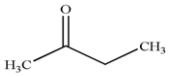
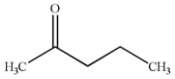
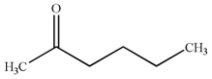
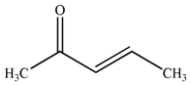
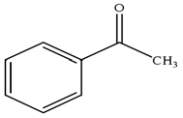
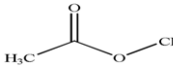
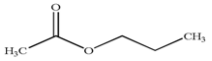
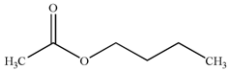
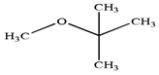
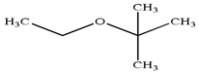
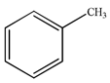
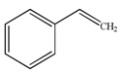
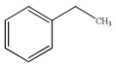
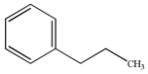
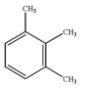
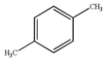
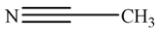
Class	Compound	Structure	Breath	*In vitro* (normal cells)	*In vitro* (cancer cells)	References
	2-butenal		–	↓	–	[51]
	2-methyl-2-butenal		–	–	↓ (A549,Calu−1)	[51, 53]
	2-methyl butenal		–	↓	↓ (NCI−H2087)	[49, 51]
	3-methyl butenal		–	↓	↓	[49, 51, 53, 59]
	Benzaldehyde		–	↓	↓ (Calu−1)	[51, 53]
Ketones	Acetone		–	↑	↑ (A549)	[51]
	2-butanone		–	–	↓ (Calu−1)	[53]
	2-pentanone		–	↑	↑	[51]
	2-hexanone		–	↑	–	[51]
	3-pentene-2-one		–	↓	–	[51]
	1-phenyl ethanone		+	–	–	[16]
Esters	Methyl acetate		–	↑	–	[51]
	n-propyl acetate		–	↑	–	[51]
	n-butyl acetate		–	↓	–	[51]
Ethers	Methyl-tert-butyl ether		–	↑ (HBEpC)	↑ (A549) ↓ (Calu−1)	[51], [53]
	Ethyl-tert-butyl ether		–	↑ (hFB)	↑ (A549) ↓ (Calu−1)	[51], [53]
Aromatics	Benzene		+	↑ (hFB)	+	[16,50, 51, 54–56, 61]
	Toluene		+	–	–	[54]
	Styrene		+	–	+	[16, 50, 51, 54–56, 61]

Table 3. (Continued).

Class	Compound	Structure	Breath	*In vitro* (normal cells)	*In vitro* (cancer cells)	References
	Ethyl benzene		+	–	–	[55]
	Propyl benzene		+	–	+	[50, 54, 56]
	Trimethyl benzene isomers		+	–	+	[16, 50, 54]
	Xylene isomers		+	–	–	[16, 62]
Heterocyclics	Tetrahydro Furan		–	–	↓ (Calu−1)	[53]
	pyrrole		–	–	↓ (A549)	[51]
Nitriles	Acetonitrile		–	–	↓ (Calu−1)	[53]

[a] All compounds in breath are reported as present or not present in lung cancer patients' breath.

[b] 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).

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 stabililization 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.

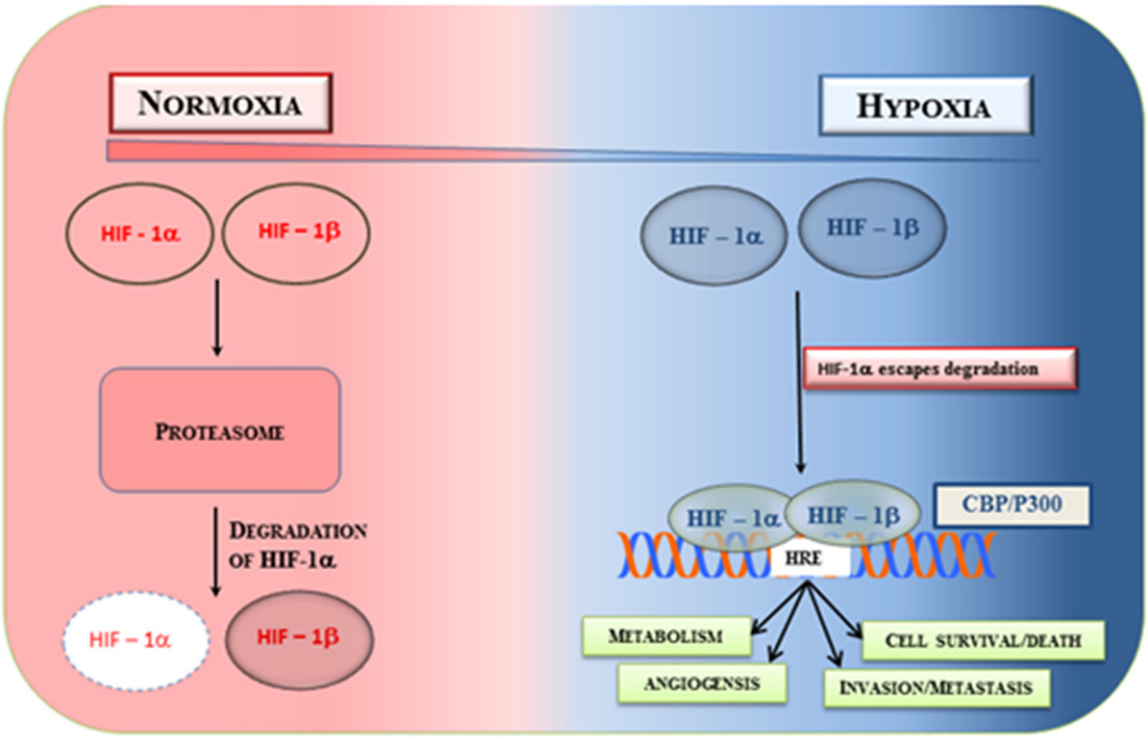


Figure 1. Activation of the hypoxia-inducible factor (HIF-1α) transcription factor in normoxia and hypoxia. Figure adapted from [71].

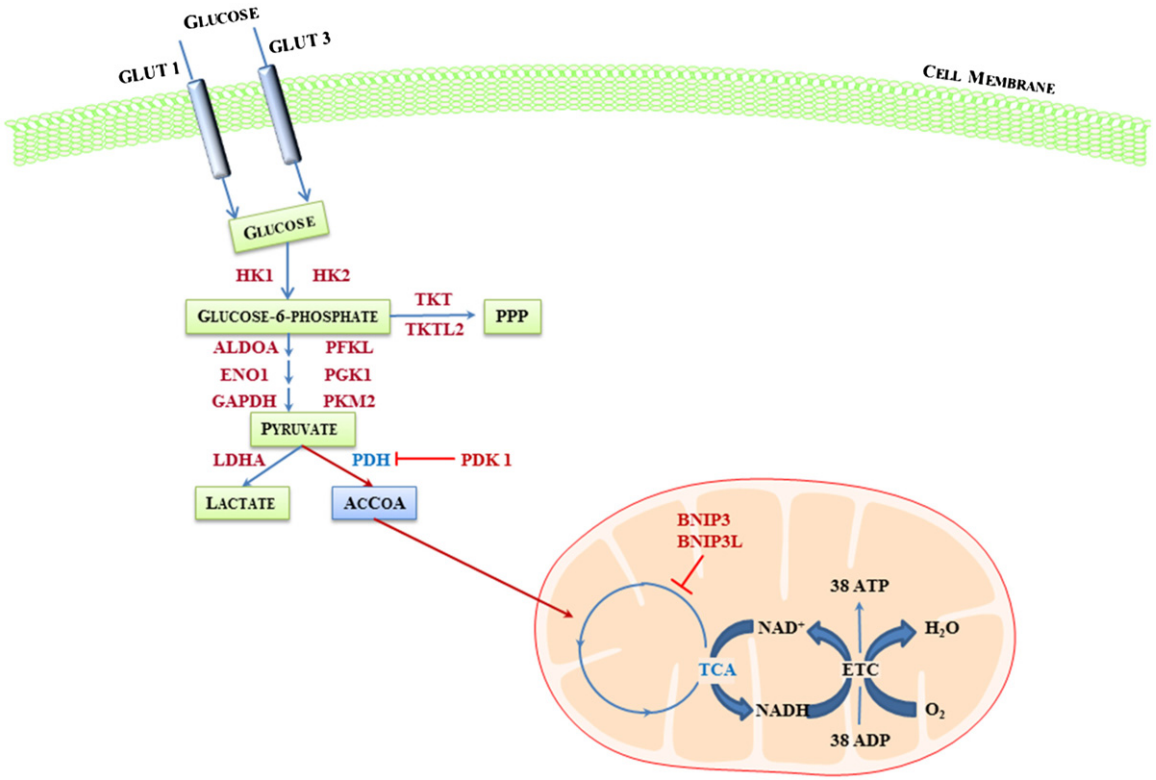


Figure 2. Metabolic reprogramming in hypoxia: activation of HIF1- α/β activates PDK1, an inhibitor of PDH which leads to the shunting of pyruvate away from the TCA cycle and instead it is converted to lactate. Figure adapted from [64].

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.

Acknowledgment

This work was funded by the Helen Macpherson Smith Trust, Project No. 6858.

References

[1] Ginsberg R J, Vokes E E and Rosenzweig K 2012 *Cancer: Principles and Practice of Oncology* vol 1 6th edn ed V T De Vita, S Hellman and S A Rosenberg (Baltimore, MD: Williams and Wilkins) p 667

[2] Mathers C D and Loncar D 2006 Projections of global mortality and burden of disease from 2002 to 2030 *PLOS Med.* **3** e442

[3] Jemal A, Center M M, DeSantis C and Ward E M 2010 Global patterns of cancer incidence and mortality rates and trends *Cancer Epidemiol. Biomar.* **19** 1893–907

[4] Phillips M, Cataneo R N, Cummin A R, Gagliardi A J, Gleeson K, Greenberg J, Maxfield R A and Rom W N 2003 Detection of lung cancer with volatile markers in the breath *Chest* **123** 2115–23

[5] Folkman J, Hahnfeldt P and Hlatky L 2000 Cancer: looking outside the genome *Nature Rev. Mol. Cell Bio.* **1** 76–79

[6] Vaupel P 2004 Tumor microenvironmental physiology and its implications for radiation oncology *Semin. Radiat. Oncol.* **14** 198–206

[7] Hakim M, Broza Y Y, Barash O, Peled N, Phillips M, Amann A and Haick H 2012 Volatile organic compounds of lung cancer and possible biochemical pathways *Chem. Rev.* **112** 5949–66

[8] Tisch U, Billan S, Ilouze M, Phillips M, Peled N and Haick H 2012 Volatile organic compounds in exhaled breath as biomarkers for the early detection and screening of lung cancer *CML Lung Cancer* **5** 107–17

[9] Miekisch W, Schubert J K and Noeldge-Schomburg G F 2004 Diagnostic potential of breath analysis—focus on volatile organic compounds *Clin. Chim. Acta* **347** 25

[10] Philips M 1992 Breath tests in medicine *Sci. Am.* **267** 74–79

[11] Amann A 2012 Breath analysis for clinical diagnosis and therapeutic monitoring *Siriraj Med. J.* **64** 18–19

[12] Risby T H and Solga S F 2006 Current status of clinical breath analysis *Appl. Phys.* B **85** 421–6

[13] Mitsubayashia K, Matsunagab H, Nishiob G, Todab S and Nakanishib Y 2005 Bioelectronic sniffers for ethanol and acetaldehyde in breath air after drinking *Biosensors Bioelectron.* **20** 1573–9

[14] Ochiaia N, Takinoa M, Daishimab S and Cardinc B D 2001 Analysis of volatile sulphur compounds in breath by gas chromatography-mass spectrometry using a three-stage cryogenic trapping preconcentration system *J. Chromatogr.* B **762** 67–75

[15] Pauling L, Robinson B A, Teranishi R and Cary P 1971 Quantitative analysis of urine vapor and breath by gas-liquid partition chromatography *Proc. Natl Acad. Sci. USA* **68** 2374–6

[16] Phillips M, Herrera J, Krishnan S, Zain M, Greenberg J and Cataneo R N 1999 Variation in volatile organic compounds in the breath of normal humans *J. Chromatogr.* B **729** 75–88

[17] Dallinga W J, Robroeks C M H T T, Van Berkel J J B N, Moonen E J C, Godshalk R W L, Jöbsis Q, Dompeling E, Wouters E F M and Van Schooten F J 2010 Volatile organic compounds in exhaled breath as a diagnostic tool for asthma in children *Clin. Exp. Allergy* **40** 68–76

[18] Van Berkel B N J J, Dallinga W J, Möller M G, Godschalk W L R, Moonen J E, Wouters F M E and Van Schooten J F 2010 A profile of volatile organic compounds in breath discriminates COPD patients from controls *Respir. Med.* **104** 557–63

[19] Hauschild C A, Baumbach I J and Baumbach J 2012 Integrated statistical learning of metabolic ion mobility spectrometry profiles for pulmonary disease identification *Genet. Mol. Res.* **11** 2733–44

[20] Baumbach J, Bunkowski A, Lange S, Oberwahrenbrock T, Kleinbölting N, Rahmann S and Baumbach J I 2007 IMS^2—an integrated medical software system for early lung cancer detection using ion mobility spectrometry data of human breath *J. Integr. Bioinform.* **4** 75

[21] Khalid Y T *et al* 2013 Breath volatile analysis from patients diagnosed with harmful drinking, cirrhosis and hepatic encephalopathy: a pilot study *Metabolomics* **9** 938–48

[22] Risby T H 2008 Critical issues for breath analysis *J. Breath Res.* **2** 030302

[23] Miekisch W and Schubert J 2006 From highly sophisticated analytical techniques to life-saving diagnostics: technical developments in breath analysis *Trend Anal. Chem.* **25** 665–73

[24] Kneepkens C M, Lepage G and Roy C C 1992 The hydrocarbon breath test in the study of lipid peroxidation: principles and practice *Clin. Invest. Med.* **15** 163–86

[25] Kneepkens C M, Lepage G and Roy C C 1994 The potential of the hydrocarbon breath test as a measure of lipid peroxidation *Free Radic. Biol. Med.* **17** 127–60

[26] Ray G, Batra S, Shukla N K, Deo S, Raina V, Ashok S and Hussain S A 2000 Lipid peroxidation, free radical production and antioxidant status in breast cancer *Breast Cancer Res. Treat.* **59** 163–70

[27] Loft S and Poulsen H E 1996 Cancer risk and oxidative DNA damage in man *J. Mol. Med.* **74** 297–312

[28] Hietanen E, Bartsch H, Bereziat J C, Camus A M, Mc Clinton S, Eremin O, Davidson L and Boyle P 1994 Diet and oxidative stress in breast, colon and prostate cancer patients: a case-control study *Eur. J. Clin. Nutr.* **48** 575–86

[29] Norberg Å, Jones W A, Hahn G R and Gabrielsson L J 2003 Role of variability in explaining ethanol pharmacokinetics *Clin. Pharmacokinet.* **42** 1–31

[30] Turner C, Španěl P and Smith D 2006 A longitudinal study of ethanol and acetaldehyde in the exhaled breath of healthy volunteers using selected-ion flow-tube mass spectrometry *Rapid Commun. Mass Spectrom.* **20** 61–68

[31] Risby T H 2005 Trace gas analysis in exhaled human breath for disease diagnosis *CLEO: Conf. on Lasers and Electro-Optics* vol 2 pp 898–9

[32] Ledochowski M, Widner B, Murr C, Sperner-Unterweger B and Fuchs D 2001 Fructose malabsorption is associated with decreased plasma tryptophan *Scand. J. Gastroenterol.* **36** 367–71

[33] Stone B G, Besse T J, Duane W C, Evans C D and DeMaster E G 1993 Effect of regulating cholesterol biosynthesis on breath isoprene excretion in men *Lipids* **28** 705–8

[34] Phillips M, Cataneo N R, Joel G, Gunawardena R and Naidu A 2000 Effect of age on the breath methylated alkane contour, a display of apparent new markers of oxidative stress *J. Lab. Clin. Med.* **136** 243–9

[35] Remmer H, Hintze T, Frank H and Müh-zange M 1984 Cytochrome P-450 oxidation of alkanes originating as scission products during lipid peroxidation *Xenobiotica* **14** 207–19

[36] Anderson M L, Beebe E L, Fox D S, Issaq J H and Kovatch M R 1991 Promotion of mouse lung tumors by bioaccumulated polychlorinated aromatic hydrocarbons *Exp. Lung Res.* **17** 455–71

[37] Benhamou S and Bonaïti-Pellié C 1995 Susceptibility to bronchial cancer: an example of genetic-environmental interaction *Ann. Biol. Clin.* **53** 507–13

[38] Hecht S S 1997 Approaches to cancer prevention based on an understanding of N-nitrosamine carcinogenesis *Exp. Biol. Med.* **216** 181–91

[39] Minro W 1998 Polymorphic CYP genes and disease predisposition—What have the studies shown so far? *Toxicol. Lett.* **167** 2–3

[40] Zevin S and Benowitz N L 1999 Drug interactions with tobacco smoking: an update *Clin. Pharmacokinet.* **36** 425–38

[41] Buszewski B, Kesy M, Ligor T and Amann A 2007 Human exhaled air analytics: biomarkers of diseases *Biomed. Chromatogr.* **21** 553–66

[42] Zolotov A Y 2005 Breath analysis *J Anal Chem.* **60** 497

[43] Jordan A, Hansel A, Holzinger R and Lindinger W 1995 Acetonitrile and benzene in the breath of smokers and non-smokers investigated by proton transfer reaction mass spectrometry (PTR-MS) *Int. J. Mass Spectrom.* **148** L1–3

[44] Kushch I *et al* 2008 Compounds enhanced in a mass spectrometric profile of smokers' exhaled breath versus non-smokers as determined in a pilot study using PTR-MS *J. Breath Res.* **2** 026002

[45] Nagrath D, Caneba C, Karedath T and Bellance N 2011 Metabolomics for mitochondrial and cancer studies *Biochim. Biophys. Acta* **1807** 650–63

[46] Kim Y S, Maruvada P and Milner J A 2008 Metabolomics in biomarker discovery: future uses for cancer prevention *Future Oncol.* **4** 93–102

[47] Nicholson J K, Lindon J C and Holmes E 1999 'Metabonomics': understanding the metabolic responses of living systems to pathophysiological stimuli via multivariate statistical analysis of biological NMR spectroscopic data *Xenobiotica* **29** 1181–9

[48] Nicholson J K, Connelly J, Lindon J C and Holmes E 2002 Metabonomics: a platform for studying drug toxicity and gene function *Nature Rev. Drug Discov.* **1** 153–62

[49] Sponring A, Filipiak W, Mikony T, Ager C, Schubert J, Miekisch W, Amann A and Troppmair J 2009 Release of volatile organic compounds from the lung cancer cell line NCI-H2087 *in vitro Anticancer Res.* **29** 419–26

[50] Chen X, Xu F, Wang Y, Pan Y, Lu D, Wang P and Zhang W 2007 A study of the volatile organic compounds exhaled by lung cancer cells *in vitro* for breath diagnosis *Cancer* **110** 835–44

[51] Filipiak W, Sponring A, Filipiak A, Ager C, Schubert J, Miekisch W and Tropmair J 2010 TD-GC-MS analysis of volatile metabolites of human lung cancer and normal cells *in vitro Cancer Epidemiol. Biomarkers* **19** 182–95

[52] Smith D, Wang T, Sulé-Suso J, Španěl P and Haj A E 2003 Quantification of acetaldehyde released by lung cancer cells *in vitro* using selected ion flow tube mass spectrometry *Rapid Commun. Mass Spectrom.* **17** 845–50

[53] Filipiak W, Sponring A, Mikoviny T, Ager C, Schubert J, Miekisch W, Amann A and Tropmair J 2008 Release of volatile organic compounds (VOCs) from the lung cancer cell line CALU-1 *in vitro Cancer Cell Int.* **8** 17

[54] Poli D, Carbognani P, Corradi M, Goldoni M, Acampa O, Balbi B and Mutti A 2005 Exhaled volatile organic compounds in patients with non-small cell lung cancer: cross sectional and nested short-term follow-up study *Respir. Res.* **6** 71

[55] Yu J, Wang D, Wang L, Wang P, Hu Y and Ying K 2009 Detection of lung cancer with volatile organic biomarkers in exhaled breath and lung cancer cells *AIP Conf. Proc.* **198** 1137

[56] Yu H, Xu L and Wang P 2005 Solid phase microextraction for analysis of alkanes and aromatic hydrocarbons in human breath *J. Chromatogr.* B **826** 69–74

[57] Wehinger A, Schmid A, Mechtcheriakov S, Ledochowski M, Grabmer C, Gastl G A and Amman A 2007 Lung cancer detection by proton transfer reaction mass-spectrometric analysis of human breath gas *Int. J. Mass Spectrom.* **265** 49–59

[58] Fuchs P, Loeseken C, Schubert J K and Miekisch W 2010 Breath gas aldehydes as biomarkers of lung cancer *Int. J. Cancer* **126** 2663–70

[59] Sponring A, Filipiak W, Ager C, Schubert J, Miekisch W, Amann A and Troppmair J 2010 Analysis of volatile organic compounds (VOCs) in the headspace of NCI-H1666 lung cancer cells *Cancer Biomark.* **7** 153–61

[60] Deng C, Zhang X and Li N 2004 Investigation of volatile biomarkers in lung cancer blood using solid-phase microextraction and capillary gas chromatography-mass spectrometry *J. Chromatogr.* B **808** 269

[61] Gordon M S, Szidon J, Krotoszynski B K, Gibbons R D and O'Neill H J 1985 Volatile organic compounds in exhaled air from patients with lung cancer *Clin. Chem.* **31** 1278–82

[62] Vaupel P, Mayer A and Höckel M 2004 Tumor hypoxia and malignant progression *Method Enzymol.* **381** 335–54

[63] Harris A L 2002 Hypoxia—a key regulatory factor in tumour growth *Nature Rev. Cancer* **2** 38–47

[64] Semenza G L 2003 Targeting HIF-1 for cancer therapy *Nature Rev. Cancer* **3** 721–32

[65] Simiantonaki N, Jayasinghe C, Michel-Schmidt R, Peters K, Hermanns I M and Kirkpatrick J C 2008 Hypoxia-induced epithelial VEGF-C/VEGFR-3 upregulation in carcinoma cell lines *Int. J. Oncol.* **32** 585–92

[66] Jain R K 2002 Tumor angiogenesis and accessibility: role of vascular endothelial growth factor *Semin. Oncol.* **29** 3–9

[67] Wilson G D 2007 Hypoxia and prognosis: the oxygen tension mounts *Front. Biosci.* **12** 3502–18

[68] Wenger R H 2002 Cellular adaptation to hypoxia: O_2-sensing protein hydroxylases, hypoxia-inducible transcription factors, and O_2-regulated gene expression *FASEB J* **16** 1151–62

[69] Papandreou I, Cairns R A, Fontana L, Lim A L and Denko N C 2006 HIF-1 mediates adaptation to hypoxia by actively downregulating mitochondrial oxygen consumption *Cell Metab.* **3** 187–97

[70] Kim W J, Tchernyshyov I, Semenza L G and Dang V C 2006 HIF-1-mediated expression of pyruvate dehydrogenase kinase: a metabolic switch required for cellular adaptation to hypoxia *Cell Metab.* **3** 177–85

[71] Brahimi-Horn M C and Pouyssegur J 2007 Oxygen, a source of life and stress *FEBS Lett* **581** 3582–91

[72] Vaupel P, Kallinowski F and Okunieff P 1989 Blood flow, oxygen and nutrient supply, and metabolic microenvironment of human tumors: a review *Cancer Res.* **49** 6449–65

[73] Frezza C, Zheng L, Tennant D A, Papkovsky D B, Hedley B A, Kalna G, Watson G D and Gottlieb E 2011 Metabolic profiling of hypoxic cells revealed a catabolic signature required for cell survival *PLOS One* **6** e24411