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Oxidative stress and volatile organic compounds: interplay in pulmonary, cardio-vascular, digestive tract systems and cancer

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Abstract: Oxidative stress (OS) can be defined as an imbalance between antioxidant systems and various pro-oxidants. This loss of balance is closely associated with initiation and development of a wide range of systemic or organ specific diseases.

Exhaled breath of healthy humans contains a large number of volatile organic compounds (VOCs) derived from cellular metabolism, released by microorganisms or taken up from the environment. Qualitative or quantitative changes in their composition are associated with diseases and various pathological conditions, also characterized by increased production of reactive oxygen species (ROS), such as superoxide radical, hydrogen peroxide, hydroxyl anion, peroxinitrite, etc. Several volatile organic compounds such as ethane and pentane are direct end-products of the reaction of ROS with various biological compounds (e.g., lipid peroxidation, DNA or protein damage). Being able to accurately identify ROS-generated VOCs could be of particular importance in devising sensitive tests that can diagnose and follow-up oxidative stress-related diseases.

This review describes current knowledge on the associations between oxidative stress and free radicals and the release of several marker volatile organic compounds in a number of diseases. A special focus will be placed on such VOCs in the cardiovascular pathologies, pulmonary diseases and gastro-intestinal tract affections.

Keywords: Oxidative Stress, Volatile Organic Compounds, Lipid Peroxidation, DNA Damage

1 Introduction

Of the total oxygen amount inspired by humans approximately 10% is used in different non-enzymatic chemical reactions while the majority (around 90%) is used by mitochondria for energy production in form of ATP. Normal oxygen metabolism releases a group of compounds collectively named reactive oxygen species (ROS). These free radicals may be formed under the influence of drug metabolism, environmental pollution, UV light or cigarette smoke. Most often they are produced in the mitochondria following either electron leakage during respiratory chain or chemical reactions of transition metals (usually iron or copper ions). ROS are reactive molecules due the presence of one or more unpaired electrons that occupy an orbital alone. Atomic oxygen [O] can be considered as a free radical but its reactivity is low due to a specific arrangement of the two unpaired electrons it contains. Most common ROS molecules include free radical species such as: hydroxyl radical or superoxide anion radical as well as non-radical species: hydrogen peroxide, ozone or hypochlorous acid. Some ROS, like hydroxyl radical, are extremely active, short-lived and act at close distance of their production. Others, more long-lived, like H_2O_2

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(which is freely diffusible across biomembranes) are suggested to be responsible for the signaling properties of ROS. It is also important to note the phenomenon of age-related increase of oxidative stress, while the defense mechanisms against ROS may decrease during aging. Under normal conditions, low ROS levels have beneficial biological effects being involved in cellular homeostasis and key molecular mechanisms such as: modulation of cellular metabolism or cellular redox state, cell signaling, inhibition or activation of different gene transcription factors, inhibition of bacterial growth, inactivation of viruses, and release of inflammatory cytokines. However as a result of intense environmental stress stimuli or infection, ROS levels can increase significantly. Oxidative stress (OS) can be defined as a redox imbalance between antioxidant systems and endo- or exogenous prooxidants in favor of prooxidants. Damage as a result of free radicals action can accumulate over time and can represent a major underlining cause for various OS-related human diseases. A solid body of research literature shows that this loss of redox balance is in many cases closely associated with the initiation and development of wide range of systemic or organ specific diseases including: cancer, diabetes, metabolic syndrome, cardiovascular pathology, pulmonary diseases or oral conditions. At cellular levels, increased concentrations of ROS are potent inducers of cellular damage targeting structures that include (but are not limited to) phospholipids as components of biological membranes, proteins and DNA. These changes further affect cellular components and microcompartments by causing cytoskeleton disorganization, proteins/enzymes dysfunction, and membrane function impairment with increased membrane permeability. Notably, increased OS can lead to cellular death either by necrosis or programmed cell death through intrinsic or extrinsic apoptotic pathways.

One important issue in free radical biological research is the need for accurate measurement, identification and quantification of OS-related biomarkers. There are few methods which can properly detect free radicals due to their very short lifetime span; one example is electron paramagnetic resonance (EPR) where the free radicals react with a molecule (spin-trap substance) that stabilizes the end product and thus allows identification and monitoring of ROS. Alternatively indirect methods have been devised where the tests measure the extent of OS damage of different cellular OS products: like malondialdehyde from lipid peroxidation; protein carbonyls from protein oxidation or 8-hydroxy-20-deoxyguanosine- 8OHdG as a marker for DNA oxidative damage. Interestingly, several end-products of lipid peroxidation (such as methylated alkanes) or protein oxidation (i.e3-methylbutanal and 2-methylbutanal following myofibrillar protein irradiation) represent volatile organic compounds which can be highly relevant for the breath analysis (Fig. 1). Some other, alternative methods for OS detecting can be suggested through quantitative analysis of VOCs released as a result of OS.

Exhaled breath contains a plethora of volatile organic compounds (VOCs) that can be grouped as follows: VOCs containing oxygen (such as acetone), sulfur (dimethyl sulfide, hydrogen sulfide, methyl-mercaptan), or nitrogen (dimethylamine, trimethylamine, ammonia); and also saturated and unsaturated hydrocarbons such as alkanes, alkenes, ketones or aldehydes. Several VOCs are end products of ROS interactions with underlying cellular components resulting in lipid peroxidation, protein oxidation or DNA damage. Importantly, different ROS may have different "affinities" for their cellular targets: thus DNA bases such as pyrimidine and purine as well as DNA deoxyribose can be damaged by the hydroxyl radical; lipid residues can be easily oxidized by metal induced formation of ROS, while protein damage is acquired following exposure to superoxide/hydroxyl radicals. The final products of these chemical interactions are usually hydrocarbons such as ethane, pentane or 2-hydroxy-nonenal. A better understanding of the interconnection between oxidative stress and the release of VOCs from target substrates would greatly help in precisely identifying the exact origin of VOCs compounds in various diseases.

The present work focuses on VOCs' release in several major human biological systems and their possible connection with OS.

1.1 Analytical techniques for VOCs detection

Analyzing exhaled breath and its components is still a relatively young field of research. The progress in the field is closely related with the advances in analytical equipment which ideally should allow for increased sensitivity, reliable compound identification coupled with fast measurement times and lower costs. An overview of the most common analytical methods used in the field of volatile organic compounds will be given bellow.

It is well established the gas chromatography coupled with mass spectrometry (GC-MS) represents the gold standard in VOCs detection. The method is usually associated with different pre-concentration techniques such as NTDs (needle trap devices), TD (thermo-desorption) or SPME (solid phase microextraction). Each preconcentration technique has its own characteristics:

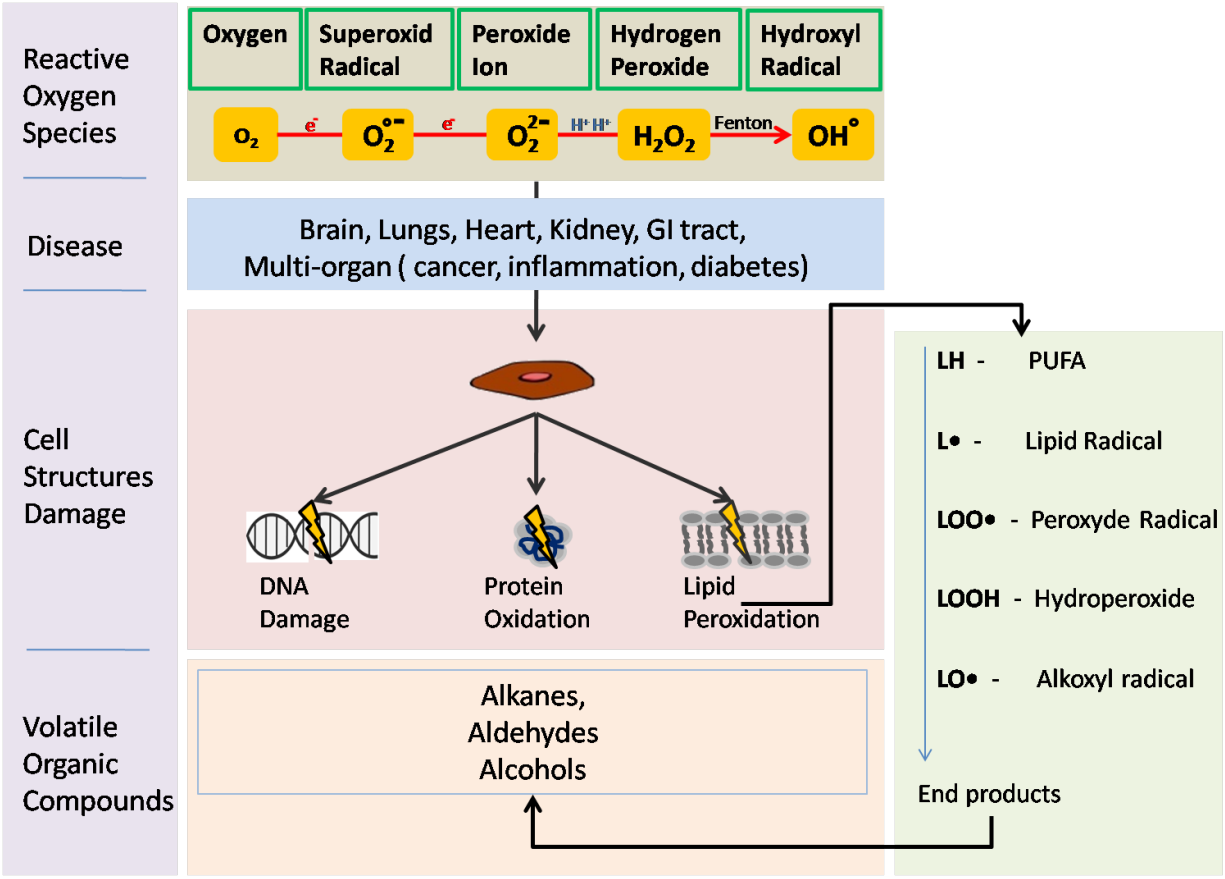


Figure 1: Interplay between VOCs – OS in local and systemic diseases.

SPME is easy to handle but has low sensitivity and is semi-quantitative; TD has a good sensitivity and reliable quantitative results; NTD is a versatile method but requires extra calibration steps. A critical point when analyzing chromatograms from CG-MS is the accurate identification of compound peak based on retention index together with spectral library identification. Correctly and reliably identifying the peaks is paramount in further elucidating the biochemical pathway connected to the volatile compound. A more recent variant GC-TOF-MS (gas chromatography-time of flight mass spectrometry), has the advantage of improved mass resolution with better peak identification together with faster measurements when compared to traditional GC-MS.

Another class of analytical techniques are direct mass spectrometric methods such as: secondary electrospray ionization mass spectrometry (SESI-MS, SESI-Q-TOF), proton-transfer-reaction time-of-flight mass spectrometry (PTR-TOF-MS), proton-transfer-reaction mass spectrometry (PTR-MS) or selected ion flow tube mass spectrometry (SIFT-MS). These methods do not separate the volatile compounds prior to analysis and can therefore analyze the samples much faster than GC-MS. In addition measurements can be performed in real-time which gives a clear advantage over blood tests allowing for assessing fast biological processes. The down side is that mass-spectrometry methods do not always clearly separate volatile compounds and may give less information than GC-MS.

Other instruments have the advantage of being easily transportable which makes them ideal for field experiments usage. Examples include ion mobility spectrometry (IMS) which aside their small size also offer high sensitivity and were produced with the main purpose of being used for military purposes. Also over the last decade a number of sensors for breath analysis applications have been developed for a wide array of applications.

1.2 OS and cellular damage – lipids, proteins, DNA

1.2.1 Lipid peroxidation

Lipids are a group of compounds with important biological functions in the human body such as being

key constituents of cell membranes, hormones or source of energy (ATP). Lipid peroxidation (LPO) represents the oxidative degradation of lipids usually containing double carbon bonds. Three main mechanisms can be involved in LPO: enzymatic oxidation, ROS independent non-enzymatic oxidation and ROS mediated oxidation. Each type is characterized by a specific end product and requires certain antioxidants to suppress the degree of OS. These end products play different roles depending on their concentration, localization in the cell and local conditions/ environment: pro- or anti-apoptotic effects, pro-and anti-inflammatory effects, cytotoxic or cytoprotective abilities. Several end products are also known to be toxic with a proven mutagenic and carcinogenic potential. To date it is well documented that lipid peroxidation is intimately connected with the development of a wide range of pathological and physiological processes: cancer, diabetes, cardio-vascular and neurological diseases, inflammation and aging.

Reactive oxygen species such as OH• are among the triggers that can initiate LPO. Cell membrane polyunsaturated fatty acids (PUFAs) contain reactive hydrogens bound to methylene groups and are important targets for LPO with ROS accumulation. The general mechanism develops along several steps: initiation step which leads to formation of (L•) by abstracting a hydrogen atom from a polyunsaturated fatty acid; further (L•) reacts with O_2 and forms (LOO•) peroxide radical in the propagation step; in a next step the peroxyl radical forms a hydroxiperoxide (LOOH) in a reinitiation reaction by abstracting hydrogen from a polyunsaturated fatty acid (LH); LOOH is then converted into an alkoxyl radical (LO•).

Two compounds can be nominated as major final products of lipid peroxidation: malondialdehyde and 4-hydroxy-2-nonenal. Notably, 4-hydroxy-2-nonenal has mutagenic potential and therefore represents an important toxic end product of lipid peroxidation. Other end products with high relevance for the exhaled breath diagnosis are formed during LPO of unsaturated fatty acids as a result of reactive oxygen species accumulation and include alkanes, such as ethane, alcohols - propanol, butanol or aldehydes such as hexanal, octanal or nonanal.

Pentane is a byproduct of LPO starting from linoleic acid that degrades to pentanyl radical (Fig. 2). Historically pentane was used as a marker of lipid peroxidation based on the assumption that it is produced but not metabolized. However recent studies show that the

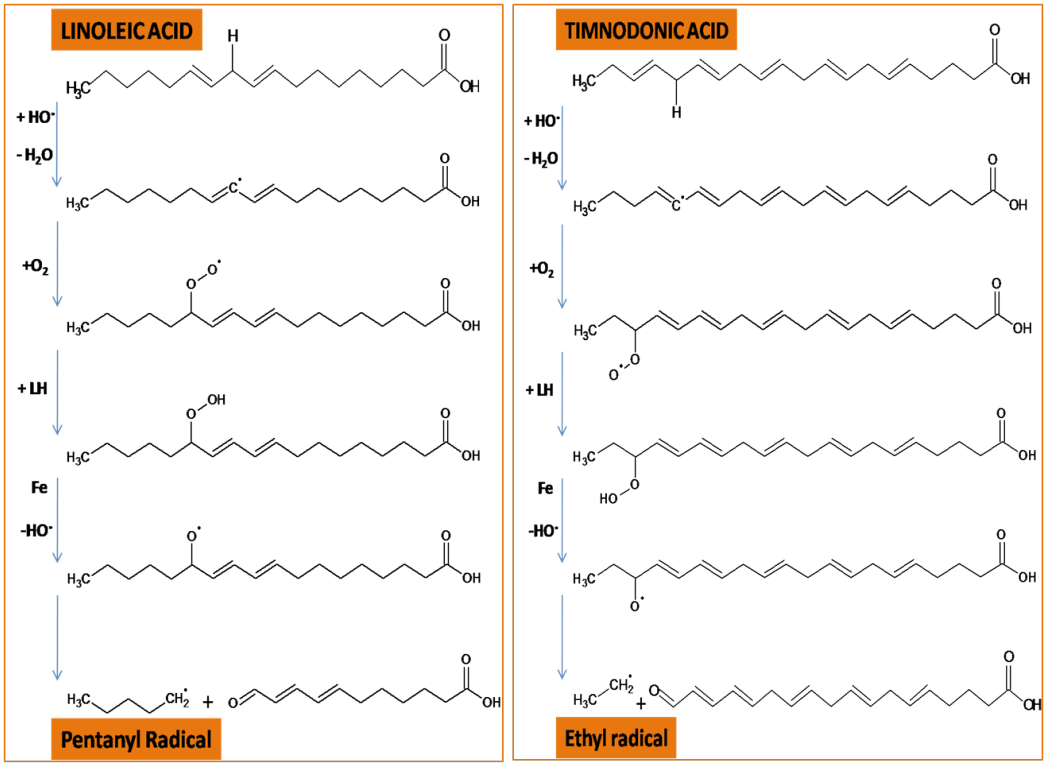
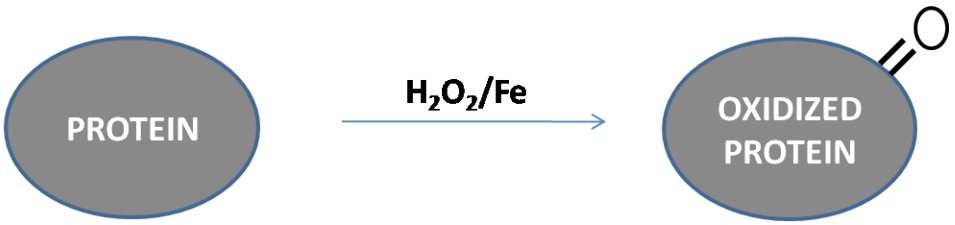


Figure 2: Lipid peroxidation triggered by reactive oxygen species. Production of pentane and ethane from polyunsaturated fatty acids – linoleic acid and timonodonic acid respectively.



Amino-acid side chain	Carbonyl groups
Lysine	Amino-adipicsemialdehyde
Threonine	Amino-ketobutyrate
Arginine	γ- glutamylsemialdehyde
Proline	γ- glutamylsemialdehyde

Figure 3: Protein oxidation and formation of carbonyl groups (aldehydes or ketones) from side chain amino-acids.

hydrocarbon can be metabolized to 2-pentanol in liver. Exhaled pentane concentration should therefore be taken into consideration in both the compound production and degradation through metabolism. The compound can be detected in exhaled breath from patients with different pathologies.

1.2.2 DNA damage and Protein Oxidation

Point or extended alterations of genetic material as a result of oxidative damage are among the first events involved in aging and the initiation and further development of cancer. Different ROS may have different "affinities" for their target substrates, thus DNA bases, such as pyrimidine and purine as well as DNA deoxyribose, can be damaged by the hydroxyl radical. Protein oxidation and its underlying mechanisms were studied using various models which involve proteins, peptides or amino-acids being exposed to ROS, such as superoxide/hydroxyl radicals formed as a result of ionizing radiation. ROS-mediated protein oxidation can potentially target any amino-acid in the peptide chain but it preferentially damages methionine and cysteine. Cysteine contains a thiol group that can bind to other molecules containing thiols and form reversible disulphide bonds. Protein oxidation can also be quantified by the analysis of carbonyl groups generated through oxidation process (Fig. 3). A number of tests based on these principles are currently in use for assessing oxidative damage extend. To the best of our knowledge, available literature on specific VOCs released as a result of protein or DNA damage is still lacking.

1.2.3 Pulmonary system

Lung diseases represent one direction where the research of specific volatile compounds and breath tests is rapidly expanding. The scope of this manuscript is not to present a thorough discussion on the relation between volatile compounds and pulmonary system diseases. This topic has been discussed in detail in Hakim et al., Amann et al. or Mazzone et al. Several volatile biomarkers have been proposed for lung cancer, chronic obstructive pulmonary disease (COPD), asthma, cystic fibrosis or interstitial lung disease. Asthma is one of the most prevalent chronic diseases worldwide. The condition is characterized by chronic inflammation of the airways associated with coughing, shortness of breath and wheezing. One of the most promising biomarkers of asthma in exhaled breath is nitric oxide (NO). This gaseous non ROS-related signaling molecule is frequently produced in response to cellular stress, may react with superoxide to form peroxynitrite, which serves as a marker of OS. Although increased levels of NO are not specific for asthma, the compound may be useful in differentiating asthma from other diseases associated with chronic cough. Oxidative stress has been shown to be one mechanism underlying the inflammatory processes that occur in lung disease. Therefore markers of lipid peroxidation such as the alkanes pentane or ethane can be significantly increased in patients with asthma. Levels of glutathione, aldehydes, isoprostanes together with pH changes may provide additional information regarding degree of oxidative stress in the airway. However, more longitudinal studies are needed that assess individual variations of the biomarkers

together with studies that can validate VOC patterns in a large population of asthmatics. Among all cancers, lung cancer is the leading cause of death. Approximately 85% of lung cancer patients die within the first 5 years from the diagnosis. A noninvasive, inexpensive, easy to use lung cancer test would be very important in the screening, diagnosis and management of lung cancer patients. An in-depth review on the use of volatile compounds as biomarkers in lung cancer has been recently published. These include ketones, aldehydes, alkanes and aromatic hydrocarbons. Aside from lung cancer, COPD is another major pulmonary disease associated with smoking. COPD is characterized by alterations in lung function, resulting from changes in lung parenchyma andlocal and systemic chronic inflammation. Patients with COPD may have higher levels of ethane and pentane in exhaled breath. Other markers of oxidative stress such as aldehydes have also been detected. However, using them in clinical settings may prove difficult due to lack of their specificity and chemical instability. One study, using electronic noses (e-nose) for VOC detection, which are based on an array of nano-sensors reacting with different fractions of volatile compounds, demonstrated the ability to discriminate between mild-to-severe asthma patients and COPD patients with accuracy of 96%. Another study also showed that an e-nose can discriminate between lung cancer and COPD patients with a cross validation value of 85%.

1.2.4 Cardiovascular system

First studies on a possible connection between certain volatile compounds in breath and heart diseases have been performed by Mendis et al. Based on the observation that lipid peroxidation generates pentane, this group analyzed exhaled breath of patients with stable and unstable angina. Their results showed no difference in pentane levels in coronary artery disease patients as compared to controls. In another study, the same group showed that pentane levels were the same in acute myocardial infarction patients, patients with stable angina and controls. However, isoprene levels were significantly higher in acute myocardial infarction patients than in stable angina patients or the control group and significantly decreased in chronic heart failure patients vs. controls. According to the authors, low isoprene levels may be result of both reduced sterol synthesis and peroxidation of a reduced pool of squalene by oxygen free radicals. Interestingly, the disease was associated with increased oxidative stress – thus ROS can negatively influence the contractile properties of cardiac cells or stimulate cardiac fibroblast proliferation leading to organ hypertrophy. This problem was further studied by Phillips et al. in patients with unstable angina. A VOC panel that could discriminate between healthy volunteers and unstable angina patients was selected by forward stepwise discriminate analysis as follows: 4-methyl-octane, 4-methyl-decane, hexane, 5-methyl-pentadecane, 7-methyl-hexadecane, 2-methyl-propane, pentane, 2-methyl-butane. The exhaled breath analysis was also employed for the assessment of heart transplant success. In 1994 Sobotka et al. measured breath pentane as a possible non-invasive candidate marker of acute cardiac allograft rejection. Pentane levels did not differ in patients undergoing transplantation without rejection and healthy control subjects. However, pentane concentrations in breath of patients with mild and moderate rejection were found to be higher than in patients with successful transplantation. The data were also confirmed by Holt et al. who found increased pentane concentrations during acute rejection of transplanted hearts. More recently Phillips et al. evaluated exhaled breath samples from 539 patients who received a heart transplant. The study showed that several hydrocarbons related to lipid peroxidation found in breath, can be used as biomarkers of heart transplant rejection. A recent study by Pabst et al. assessed the impact of heart surgery on breath biomarkers, with a special focus on compounds related to oxidative stress. Breath samples were taken after induction of anesthesia, after sternotomy and at specific time intervals after the end of surgery. The data show that acetone concentrations increased markedly at the end of the extracorporeal circulation procedure and were positively correlated with serum C-reactive protein and troponine-T. Also, pentane was significantly increased after sternotomy and dropped towards the end of surgery; moreover, pentane levels correlated well with serum creatinine. Isoprene concentrations rose following sternotomy and decreased to initial levels 30 min after surgery. The levels correlated with cardiac output, thus patients with low cardiac index had less isoprene in breath than patients with high cardiac index. Correlations between breath markers and clinical and biochemical parameters support the assertion that breath tests may be used in monitoring cardiovascular diseases.

1.2.5 Gastro-intestinal system

Crohn's disease or ulcerative colitis is characterized by acute intestinal inflammation of the mucosa and the intestinal lumen. Overproduction of ROS with subsequent lipid peroxidation has been proposed as one

of the possible mechanisms for these diseases. Pelli et al. showed that exhaled breath from patients with Crohn's disease and ulcerative colitis contains elevated levels of ethane, propane and pentane when compared to controls. At the same time, isoprene and butane had the same concentrations as in healthy volunteers. The results were only partially confirmed by Sedghi et al., who showed that ethane, but not pentane levels, are significantly increased in patients with ulcerative colitis and correlate with disease activity, symptom score and endoscopic score. Again, other reports support the observation that pentane is increased in exhaled breath as a results of intestinal inflammation in humans and in a rat model.

A distinct body of research demonstrates that volatile compounds, generating oral malodor, once released, can have toxic effects on the surrounding tissues at the starting parts of the gastro-intestinal tract. Interestingly, several studies show that physiological concentrations of H_2S can induce apoptosis either by intrinsic or extrinsic pathways in different cell types such as: keratinocyte stem cells, normal keratinocytes from human gingiva, oral keratinocyte stem cells, osteoblasts or dental pulp stem cells.

1.2.6 Cancer

As with other cancer types, in breast cancer reactive oxygen species can be produced in the cell as a result of progressive accumulation of genetic and epigenetic mutations. Oxidative stress leads to the accumulation of specific VOCs in the blood. Certain volatile biomarkers of breast cancer are also transported to the lung and can therefore be found in the exhaled breath.

One of the first volatile biomarker found to be significantly increased in patients with breast cancer was pentane, generated from lipid peroxidation of polyunsaturated acids in cell membranes. In a first study from 1994, Hietanen et al. found that exhaled breath of women with breast cancer had elevated concentrations of pentane in comparison to healthy subjects. Furthermore, Phillips et al. analyzed methylated alkane compounds in breath samples from three groups: women with breast cancer (confirmed by biopsy analysis), women with no histological evidence of breast cancer in the biopsy and healthy volunteers. For VOC detection the study employed gas chromatography and mass spectroscopy to determine the alveolar gradients of C4-C20 alkanes and mono-methylated alkanes. Based on their determinations, the VOCs biomarkers used to further identify breast cancer cases included: nonane, 5-methyl-tridecane, 3-methyl-undecane, 6-methyl-pentadecane, 2- methyl-propane, 3-methyl-nonadecane, 4-methyl-dodecane, 2-methyl-octane. The results were reported to have a sensitivity of 94.1% and a specificity of 73.8% for distinguishing between breast cancer patients and healthy individuals. In a continuation of this study the combination 2-propanol, 2,3-dihydro-1-phenyl-4(1H)-quinazolinone, 1-phenyl-ethanone, heptanal and isopropyl myristate was used. These markers were reported to identify breast cancer with 93.8% sensitivity and 84.6% specificity. The data from the group expanded the search for volatile biomarkers of breast cancer and proposed a new set of potential breast cancer volatile biomarkers.

1.2.7 Influence on breath sampling protocols and other cofactors

Exhaled air concentrations of different VOCs are highly dependent on one hand on various physiochemical properties such as water solubility, Henry constant, blood:breath and blood:fat ratios. These parameters help to estimate how different VOCs may be stored in different compartment of the body and thus, at which level they may appear in breath, blood, saliva or urine. In this context the difference in concentration levels can be several orders of magnitude. On the other hand, different physiological conditions present during exhaled air sampling have a huge influence on exhaled VOCs levels. One example is represented by exhalation kinetics during physical exercise which were investigated in several studies. In this respect the Fahri equation is very useful in modeling exhalation kinetics, reflecting the relation of breath concentrations and alveolar ventilation and cardiac output, as well.

From a methodological point of view, the gold standard in breath analysis is gas chromatography mass spectrometry, however the method gives information in only a single time point, without reflecting the possible changes in exhalation kinetics during sampling. The collection of exhaled air is often controlled by monitoring the CO_2-level focussing on the alveolar part. Besides the above, selecting the collection device (Tedlar bag, syringes) might have also an influence on the collected VOC concentrations caused mainly by adsorption of compounds on the inner wall, diffusion through the material of the container, or emission of contaminants. Exhalation kinetics can be examined using real-time breath analysis techniques such as proton-transfer reaction time of flight mass spectrometry resulting in a breath-to-breath resolution. Other factors such as expiratory flow, breath hold, inclusion of anatomic dead space, different

collection or transport materials, age, gender, diet, physical exercise, drugs, diet, smoking, sleep, pregnancy, environmental pollutions may all influence exhaled VOC levels. One of the long term goals of breath analysis that will ensure the successful implementation of the method in clinical settings, would be to clearly standardize breath sampling. Another possible method of increasing the specificity of a breath test would be to analyze multiple molecules together, as it is known that various disorders are associated with VOCs panels and thus have specific breath volatile fingerprints.

2 Conclusions and future considerations

The possibility to properly and accurately detect and analyze the "molecular breath print" generated by OS will provide important implications in designing breath tests that will screen, diagnose many OS related diseases and will allow suggestions of relevant follow-up treatment. However, it is important to note that most of studies that correlate VOCs with oxidative stress come from in vivo measurements of exhaled breath from patients with different affectations. The resulting compounds can be in some cases non-specific. In order to directly nominate specific VOCs as markers of oxidative stress, it would be most useful to analyze the release of VOCs also in 2D and 3D in vitro models. This approach has the advantage of investigating specific biological processes initiated by oxidative stress and elucidating the biochemistry of released VOCs in correlation with the expression of molecular biomarkers characteristic of the induced process.

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