LASER SPECTROSCOPY METHOD FOR NON-INVASIVE ANALYSIS OF BREATH IN PULMONARY DISEASE

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Abstract. Oxidative stress in the respiratory system after the normal metabolism of oxygen and production of reactive oxygen species (ROS) increase the production of mediators of pulmonary inflammation and initiate or promote mechanisms of carcinogenesis. The molecules contained in the exhaled breath can provide information about the physiological processes that occur in the body. In this study, we present a quantitative analysis of breath ethylene concentrations in non-small cell lung cancer before and immediately after the chemotherapy and in chronic obstructive pulmonary disease (COPD) to illustrate the importance of oxidative stress within these diseases, using CO_2 laser photoacoustic spectroscopy (CO_2LPAS) method.

Key words: oxidative stress, breath analysis, ethylene, lipid peroxidation, lung cancer, chornic obstructive pulmonary disease, laser photoacoustic spectroscopy.

1. INTRODUCTION

All aerobic cells require oxygen (O_2) for their energy production. The percentage of oxygen in the atmosphere is 21% in dry air [1] and this 21% of oxygen has harmful effects, most of them due to the formation of free oxygen radicals. Cells continuously produce free radicals as a part of their normal metabolic processes. Under normal metabolic conditions 2–5% of the O_2 consumed by mitochondria is converted to ROS [2] and the prevention of excess free radical formation is a vital step for cell survival [3]. ROS is a term used of highly reactive compounds which can also act as free radicals. ROS include *e.g.* hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), peroxynitrite ($ONOO^-$) and hypochlorous acid ($HOCl$) [4, 5]. Free radicals have very short half-life and are therefore difficult to measure. Also, a cell's internal environment is perturbed by infections, disease, toxins or nutritional imbalance leading to the formation of ROS [6]. Oxidative stress is defined in general as excess formation and/or insufficient removal of highly reactive molecules such as ROS. This "oxidative shielding" acts as a defense mechanism for either decreasing cellular uptake of toxic pathogens or

chemicals from the environment, or to kill the cell by apoptosis and thus avoid the spreading to neighboring cells [7]. Therefore, ROS formation is a physiological response to stress. Being highly reactive molecules, the pathological consequence of ROS excess is damage to proteins, lipids and DNA [7, 8]. Direct means of measuring free radicals include electron spin resonance and spin trapping methods [9]. Free radicals can also be labeled chemically and analyzed by spectroscopic methods. However, the most common approach is to measure the derivates or end-products of oxidation processes such as lipid peroxidation (LP) products [10]. ROS induce LP which is a marker of oxidative stress and is defined as the oxidative degradation of polyunsaturated fatty acids (PUFA) induced by free radicals [11]. The results of LP can lead to the loss of integrity of cellular membranes. Some of the stable end-products of LP such as ethane, ethylene, and 1-pentane are well suited for the estimation of cellular damage [12–14]. The fact that ethylene is highly volatile, not significantly metabolized by the body, and not soluble in body fat means that it diffuses rapidly into bloodstream after generation and it is transported to the lungs. The membranes separating the air in the lungs from the blood in the capillaries are very thin and are optimized for gas transport, so ethylene is easily emitted in exhaled breath and then collected [13, 14].

Breath analysis is a potentially powerful tool for diagnosis and study of medical diseases [15]. In breath analysis, biomarkers give information very quickly compared to blood or urine tests. Breath is a complex mixture of gases, vapors and aerosols. The bulk matrix of breath is a mixture of nitrogen (78.6%), oxygen (16%), carbon dioxide (0.9%), water vapor and gases: inorganic gases (*e.g.*, NO, CO_2, and CO), volatile organic compounds (VOCs) (*e.g.*, isoprene, ethane, pentane, and acetone), other typically non-volatile substances (isoprostanes, peroxynitrite, cytokines, and nitrogen). More than 3,500 different components have been identified in exhaled breath, among these more than 1,000 VOCs (35 breath biomarkers). Breath analysis is a method for non-invasive information on the clinical state of an individual by monitoring volatile organic compounds present in the exhaled breath and with great clinical potential [15–17]. Numerous in vivo studies have revealed that lipid peroxidation has a key role in carcinogenes [18–21]. LP markers can also be examined non-invasively. Ethylene from the human breath is an indicator of oxidant stress and can be directly correlated to physiological events in the patients (or biochemical events surrounding LP).

1.1. LUNG CANCER

Cancer is a multi-step genetic disease in which mutations is associated with growth and development a as tumor. Tumor cells have aberrant respiration caused and causes DNA damage and activates signaling pathways leading to uncontrolled growth, the inability to differentiate, and the malignant phenotype [22–99]. Lung cancer is the leading cause of cancer death worldwide, and is associated with over 1 million deaths annually [22]. There are two major types of lung cancer: i) non-small cell lung cancer (87%): squamous cell carcinoma (32%), adenocarcinoma

(26%), large cell carcinoma (29%); ii) small cell lung cancer (13%) (Grows more rapidly). Each grows and spreads in different ways and is treated differently.

Cigarette smoking is by far the greatest risk factor for lung cancer [22–29]. The longer a person uses tobacco and the more they use, the greater their risk. If a person quits before cancer develops, the damaged lung tissue gradually improves. Others at risk include nonsmokers who breathe in secondhand smoke and occupational or environmental exposure to radon, asbestos, certain metals, radiation or air pollution [24]. If people are exposed to the above carcinogens & also smoke, their risk is greatly increased. Symptoms of lung cancer may include a cough that does not go away or gets worse, chest pain that is often worse with deep breathing, coughing, or laughing, hoarseness, weight loss and loss of appetite, coughing up blood or rust-colored sputum (spit or phlegm), shortness of breath, feeling tired or weak. The detection of lung cancer may include: an X-ray image of the lungs that may reveal an abnormal mass or nodule; a CT scan can reveal small lesions in your lungs that might not be detected on X-ray; sputum cytology, that look at the sputum under the microscope, and can sometimes reveal the presence of lung cancer cells; tissue sample (biopsy), a sample of abnormal cells may be removed in a procedure called a biopsy. After the analysis results, the doctor may recommended the treatment options that includes: surgery to remove tumors, chemotherapy, and radiation in combination or alone are common treatments for lung cancer. Surgery represents the operation to remove cancer cells, radiation therapy uses high-energy rays to shrink or kill cancer cells, chemotherapy uses anticancer drugs that attack cancer cells and normal cells. These drugs are usually given by injection or by mouth. But, the treatment options depend on cancer type and stage of cancer.

Chemotherapy [30–33] is considered as first option for neoplasm in patients with advanced stage (III–IV), when the tumor can not be removed. Chemotherapy literally means drug treatment. In cancer treatment, the term chemotherapy means treatment with cell killing (cytotoxic) drugs using one chemotherapy drug or a combination of different chemotherapy drugs. There are more than 100 different drugs currently available and new ones are being developed all the time. Whether chemotherapy is a suitable treatment, and which drugs it is, depends on many things like: the type of cancer, where in the body the cancer started, what the cancer cells look like under the microscope (the grade), whether the cancer has spread, the general health of the patient. Current therapeutic options can reduce the size of the tumor, may relieve pain or other symptoms. Pain is one of the most important symptoms of patients with cancer, its characteristics are different from the pain that we feel normally. Therefore, and its treatment is particularly patients being administrated powerful painkillers.

1.2. CHRONIC OBSTRUCTIVE PULMONARY DISEASE

Chronic obstructive pulmonary disease (COPD) [34–43] is a progressive disease that makes it hard to breathe. "Progressive" means the disease gets worse over time. COPD can cause coughing that produces large amounts of mucus (a slimy substance), wheezing, shortness of breath, chest tightness, and other symptoms. Cigarette smoking is the leading cause of COPD. Most people who have COPD smoke or used to smoke [39]. Long-term exposure to other lung irritants such as air pollution, chemical fumes, or dust also may contribute to COPD. The term "COPD" includes two main conditions emphysema and chronic bronchitis. In emphysema, the walls between many of the air sacs are damaged. As a result, the air sacs lose their shape and become floppy. This damage also can destroy the walls of the air sacs, leading to fewer and larger air sacs instead of many tiny ones and the amount of gas exchange in the lungs is reduced. In chronic bronchitis, the lining of the airways is constantly irritated and inflamed which causes the lining to thicken. Lots of thick mucus forms in the airways, making it hard to breathe. Most people who have COPD have both emphysema and chronic bronchitis. COPD develops slowly, the symptoms often worsen over time and can limit the ability to do routine activities. Most of the time, COPD is diagnosed in middle-aged or older adults. COPD has no cure yet, and doctors don't know how to reverse the damage to the airways and lungs.

2. METHOD AND MATERIALS

2.1. EXPERIMENTAL SET-UP

Laser Photoacoustic Spectroscopy (LPAS) method is a very powerful investigation technique, which is capable of measuring trace gas concentration at sub parts per billion (ppb) level. To produce the photocoustic effect, the gas sample is confined into a resonant chamber, where the modulated (chopped) radiation enters via an IR-transparent window and is locally absorbed by IR-active molecular species, leading to a periodic expansion and contraction of the gas volume synchronous with the modulation frequency of the radiation. This generates a pressure wave that can be detected by sensors [44].

The experimental system of the photoacoustic detection system is presented in Fig. 1 and describe previously [44–49]. LPAS experimental setup used in the present work consists of a radiation source, a photoacoustic (PA) cell where the gas sample is enclosed and analyzed, a vacuum/gas handling system, and a detection unit. As a radiation source is used a CO_2 laser home-built, line-tunable between 9.2 and 10.8 μm on 73 different vibrational-rotational lines and frequency-stabilized, that emits continuous wave radiation with an output power of 2–5 W. The requirement for gases to be detected is that they should possess high absorption

strength and a characteristic absorption pattern in the wavelength range of the CO_2 laser. Inside the PA cell, traces of gas can absorb the laser radiation and the absorbed energy is released into heat, which creates an increase in pressure inside a closed volume. The laser beam is amplitude modulated by an optical chopper, focused by a ZnSe lens and introduced in the PA cell. The laser power used to excite the sample gas inside the PA cell is measured by a two channel powermeter. The acoustic waves produced in the PA cell are detected with four miniature microphones connected in series. The PA signal, proportional to the trace gas concentration is applied to a lock-in amplifier which detects and measures very small single frequency AC signals. The output signals of the lock-in amplifier and of the powermeter are then converted into digital signals and processed by a computer. A software program (TestPoint) for graphic and instrumentation permits to obtain and process the experimental results. The absolute trace gas concentrations are processed by the computer and the results are displayed on the screen. The gas handling system is an important part of the experimental set-up for the gas level concentration measurements (upper part of the general scheme of the photoacoustic detection system from Fig. 1), ensuring gas purity in the PA cell. The handling system can be used to introduce the sample gas in the PA cell at a controlled flow rate, to pump out the gas sample from the cell, and to monitor the total and partial pressures of gas mixtures.
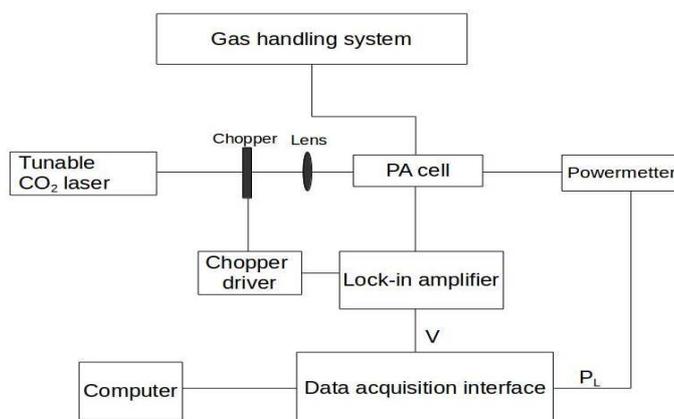

Fig. 1 – CO_2 LPAS scheme.

2.2. PROTOCOL ANALYSIS

We have analyzed the ethylene concentration from the exhaled breath of subjects with: i) squamous non-small lung cell ($n = 5$) in stage III and IV and the breath samples were collected from the 3rd to the 6th session of chemotherapy, with

age between 61 and 72 years, ii) subjects with COPD ($n = 5$) ($FEV_1 < 60\%$) with age between 58 and 79 years. All participants with COPD underwent flow volume tests, including measurements of FEV_1. All samples were given in the laboratory between 09:00 and 11:00 in the morning and analyzed after 2 to 6 hours, and at the subjects under chemotherapy treatment, after the treatment session the breath analysis were collected in the afternoon from 14:00 to 16:00 and analyzed immediately after. Informed consent was obtained from all individuals. Subjects breath samples were collected inside aluminized bags (0.75-liter aluminum-coated bags) using a disposable mouthpiece connected to the sample bags via mouthpiece adaptor. After the collection, the sample gas is transferred to the PA cell to be measured. During the transfer of the exhaled air from the collecting bag to the PA cell, the sample gas was passed through a glass cell filled with potassium hydroxide (KOH) pellets to remove the CO_2 from the exhaled breath [50]. All measurements were made at 10P(10) CO_2 laser line (10.53 μm), where the ethylene absorption coefficient has the maximum value of 30.4 cm^{-1} atm^{-1}. To ensure the quality of each measurement, an intensive cycle of N_2 washing was performed between samples, in order to have a maximum increase of 10% for the background PA signal. The exhaled air sample was transferred to the PA cell at a controlled flow rate of 600 sccm (standard cubic centimeters per minute), and the total pressure of the gas in the PA cell was measured.

3. RESULTS AND DISCUSSIONS

The oxidative damage to membrane lipids can be evaluated by a quantitative analysis of ethylene, a sub-product for lipid peroxidation. The objective of this study was to evaluate the oxidative stress response of subjects with squamous lung cancer (stage III and IV) before and after chemotherapy treatment and subjects with COPD, by measuring the ethylene concentration from the exhaled breath samples. Table 1 summarizes exhaled ethylene levels in patients with squamous lung cancer before and after chemotherapy session and Table 2 summarizes the exhaled ethylene level in patients with COPD.

Lung cancer represents a major health concern in the world. A growing body of evidence has indicated that lung cancer, among other cancers, is associated with increased production of ROS. Also, many chemotherapeutic agents seem to be associated with the generation of free radicals and increased lipid peroxidation. Despite accumulating data on ROS in cancer, there are few studies examining antioxidants, cytokines and markers of oxidative stress during cancer treatments

[51]. To address the problem of oxidative damage in normal tissues exposed to chemotherapeutics, we have studied the exhaled breath of five subjects diagnosed with squamous lung cancer in the III and IV disease stage and under the chemotherapy treatment. This patients did not have surgery for removal surgery of the tumor or radiotherapy sessions. Quantitative measurements of oxidative damage in subjects with lung cancer before and after chemotherapy was realized by measuring ethylene concentrations in the exhaled breath. The induced oxidative stress in non-targeted tissues lead to normal tissue injury. The chemotherapy cycle is of 21 days and the duration of a session is of 2 days, when the cancer patient cancer receive by perfusion the drug or drugs and vitamins. Before every chemotherapy session the oncologist determines treatment after results of blood tests. In a healthy person the breath ethylene concentration found with our CO_2LPAS system is in the range of 10 ppb to 25 ppb, but the concentration depend also the food or beverage ingested and if the subject brush their teeth. We observe in increase level of ethylene as biomarker of LP in patient with lung cancer before chemotherapy treatment and a high ethylene concentration immediately after the chemotherapy. The findings here would indicate that lung cancer is associated with increased oxidative stress.

The sources of the increased oxidative stress in the respiratory compartment of COPD patients derive from the increased burden of oxidants from environmental exposures, such as cigarette smoke and air pollutants, and from the increased amounts of ROS [40, 52]. These ROS are capable of causing oxidative damage to DNA, lipids, carbohydrates and proteins, and thereby mediate an array of downstream processes that contribute to the development and progression of COPD. They also activate resident cells in the lung, particularly epithelial cells and alveolar macrophages, to generate chemotactic molecules that recruit additional inflammatory cells (neutrophils, monocytes and lymphocytes) into the lung [38-40], which in turn perpetuates oxidative stress in the lung. Collectively, these events lead to a vicious cycle of persistent inflammation, accompanied by chronic oxidative stress, which lead to disturbances in the protease-antiprotease balance, defects in tissue repair mechanisms, accelerated apoptosis and enhanced autophagy in lung cells, which have all been linked to the severity and progression of COPD [41–43]. From the results represented in Table 2 we found an increased level of breath ethylene concentration in patients with COPD. The highest level of breath ethylene was found at the COPD patients that continued to smoker and smoke for a very long time 40 and 50 years. Before the measurements for the patients with COPD were measured FEV_1 and FEC parameters (where FEV_1 is forced expiratory volume in 1 second and FVC is forced vital capacity).

Table 1

Ethylene concentration at the subjects with squamous non-small lung cancer
before and immediately after chemotherapy

Subject	Gender	Age	Ethylene [ppbb] before chemotherapy	Ethylene [ppb] after chemotherapy
C1	M	61	46.4	92
C2	M	72	108	480
C3	M	63	72.6	180
C4 (type 2 diabetes)	M	65	223	373
C5	M	68	52	103

Table 2

Ethylene concentration at the subjects with COPD

Subject	Gender	Age [years]	Ethlene [ppb]	Smoker/ non-smoker	Observations
S1	M	68	108	Ex smoker from 6 years	Mixed ventilation dysfunction, diagnosed 10 years ago. $FEV_1 = 59.9\%$, FEC = 47.6%
S2	M	58	225	Smoker from 40 years	Mixed ventilation dysfunction, diagnosed 1 year ago. $FEV_1 = 44.7\%$, FEC = 33.2%
S3	M	79	97.8	Ex smoker	Mixed ventilation dysfunction, diagnosed 3 years ago. $FEV_1 = 43.8\%$, FEC = 32.6%
S4	M	71	480	Smoker from 50 years	Mixed ventilation dysfunction, diagnosed 5 years ago. $FEV_1 = 28\%$, FEC = 26.4%
S5	M	63	70.7	Ex smoker from 4 years	Mixed ventilation dysfunction, diagnosed 4 years ago. $FEV_1 = 33.4\%$, FEC = 28.6%

The subjects with squamous lung cancer present a low level of breath ethylene comparing with the subjects with COPD, but after the chemotherapy session they present a very high concentration of ethylene in the breath. These differences in breath ethylene concentrations can be observed in Fig. 2. Form the

patients group with lung cancer, one has also type 2 diabetes, disease that present a high level of oxidative stress due to hyperglycemia and insulin deprivation [53, 54].

Ethylene concentration in healthy subject, COPD and lung cancer before and after chemotherapy
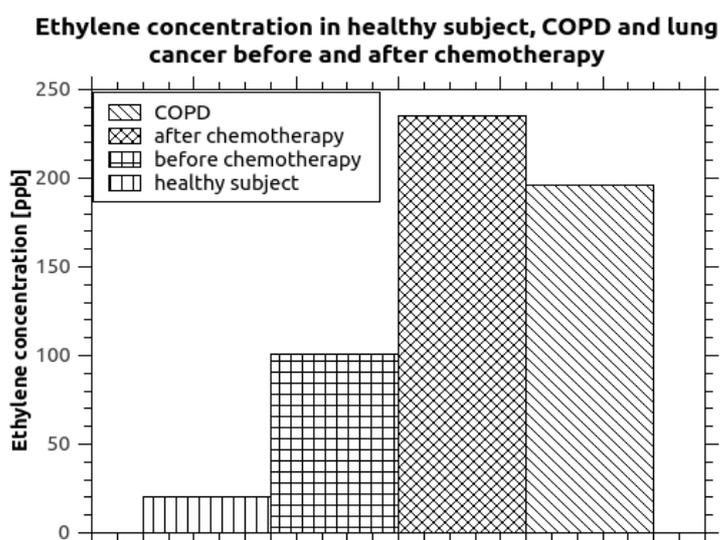

Fig. 2 – Mean breath ethylene concentration in subjects with squamous lung cancer before and after chemotherapy treatment, from 3rd to 6th session and at subjects with COPD.

There has been increasing interest in non-invasive monitoring of respiratory tract inflammation and oxidative stress. Exhaled air analysis has many advantages: measurements are non-invasive, simple and repeatable. Analysis may also be performed on patients with severe airflow obstruction or other lung diseases where more invasive techniques are not possible [55]. Exhaled ethylene represents an indirect indicator of LP, and measurement of it is considered highly sensitive [56].

4. CONCLUSIONS

In this study we present a real-time monitoring of breath ethylene concentration in patients with COPS and squamous lung-cancer (stages III and IV) under chemotherapy treatment (from 3rd to 6th chemotherapy session) and patients with COPD. This quantification is superior to the direct measurement of free radical because can be estimated the cellular damage and the adverse effects.

Chemotherapy induced ROS species and their oxidative damage to lipids and cellular membranes and chemotherapeutic treatment is suspected for the toxic side effects. The changes of biochemical pathways proceeded inside cells might be

observed in expired air. In the experiment, breath analysis was carried out before and after anticancer therapy. The data showed that cytostatic drugs increase the concentration of ethylene in the breath collected after chemotherapy. Although, chemotherapy improves the survival rates of cancer patients, but oxidative stress in normal tissues increase and decreases the quality of life of patients.

Patients with COPD present a high level of oxidative stress. The sources of the increased oxidative stress in the respiratory compartment of COPD patients derive from the increased burden of oxidants from environmental exposures, such as cigarette smoke in this case, and from the increased amounts of ROS. Oxidative stress lead to defects in tissue repair mechanisms, accelerated apoptosis and enhanced autophagy in lung cells, which have all been linked to the severity and progression of COPD. The results suggest that patients with COPD are characterized by increased systemic and pulmonary oxidative stress breath marker.

Therefore, novel real-time monitoring of endogenous LP in patients with pulmonary injuries provides important details of endogenous production of ethylene. Further research is still needed on the topic.

Compliance with Ethical Standards.

Conflict of interest. Nothing to disclose.

Human and Animal Rights. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. This article does not contain any studies with animals performed by any of the author.

Informed consent. Informed consent was obtained from all individual participants included in this study.

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