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# Molecular Diagnostics of Pulmonary Diseases Based on Analysis of Exhaled Breath Condensate

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Tereza Kačerová, Petr Novotný, Ján Boroň and  
Petr Kačer

Additional information is available at the end of the chapter

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## Abstract

Measurements of biomarkers in exhaled breath condensate (EBC) extend a novel route for monitoring lung physiology and provide a beneficial insight into the pathophysiology of a specific disease. From the medicinal point of view, biomarkers present in EBC depict rather the processes occurring in lungs than those in the entire system. Therefore, particular profiles of exhaled biomarkers (e.g. cys-LTs, LTB<sub>4</sub>, 8-isoprostane, etc.) apparently reveal information exclusively applicable to differential lung disease diagnoses. This chapter describes the developed analytical method being applied to a clinical study for differential diagnostics of various phenotypes of asthma, chronic obstructive pulmonary disease, lung cancer, etc. In particular, having determined cys-LTs and LXs by the described method, and having applied them as biomarkers of bronchial asthma, their distinctive potential was demonstrated to differentially diagnose the specific disease, clearly suggesting this method to be reckoned as a beneficial alternative to existing diagnostic methods. Consecutively, the developed method was expanded to other asthma markers as aldehydes, nitrotyrosine, 8-isoprostane, PGE<sub>2</sub>, adenosine and finally, a supplementary study was carried out, engaging in detecting serotonin. The multi-marker screening and importance in the diagnostics of pulmonary diseases are referenced in the text as well.

**Keywords:** exhaled breath condensate, pulmonary diseases, leukotrienes, lipoxins

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## 1. Introduction

From the very beginnings of civilizations, with tracks from Mesopotamia, Egypt, and ancient Greece, medical practitioners examined the potential of exhaled breath (EB) parameters as health-related signs usable for identifying various ailments and essentially mapping different

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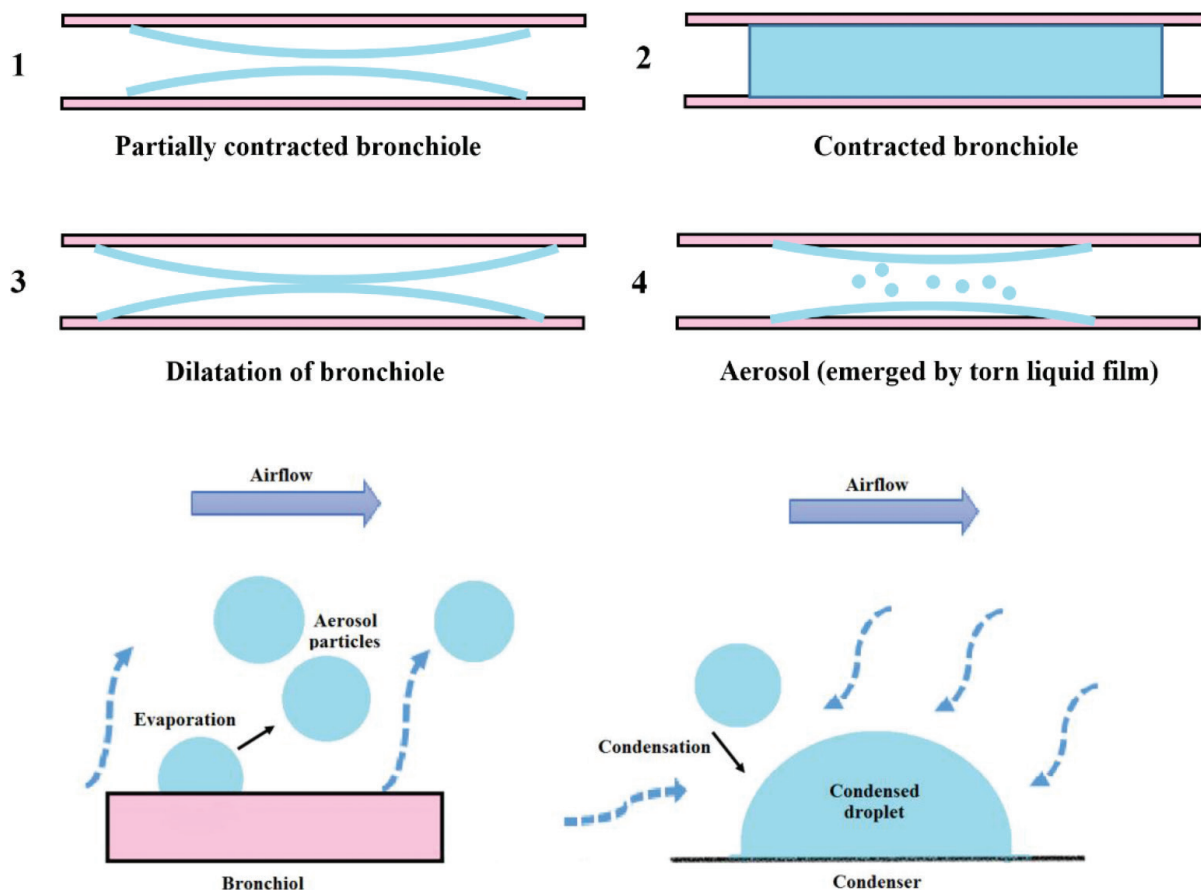
physiological states. Via different odors, sounds, and breath dynamics often attributed to supernatural powers and superstitious beliefs, various lung diseases could be relatively well diagnosed and further progression could be predicted. For instance, the odors in EB as, for example, fruity traces of acetone aided to identify diabetes; a rather pungent characteristic odor was associated with a lung inflammation, while volatile vapors from urine revealed a kidney disease [1]. Modern investigations enlisted approx. 250 frequently detected volatile organic compounds (VOC's) in EB [2]. Early analyses did not incorporate sample pre-treatments as sample concentration and exclusively depended on relatively modest gas chromatography (GC) methods. The progress of technology, however, over the decades has permitted much more precise and sophisticated analyses of EB, some of which have been implemented to the clinical practice, as, for example, ethanol levels in blood or typical inflammations caused by common pathogens as *Helicobacter pylori* using  $^{13}/^{14}\text{C}$ -urea [3]. As mentioned above, the prime advantage of EB analysis is the patient's comfort, especially eliminating the stressful intrusions to human organisms, yet there are challenges ahead. For instance, a breakthrough task is to find common internal standard reliably standardizing diagnoses for each pathological status. Furthermore, an opposite selection of multi-marker panel is to be conspicuously correlated to different health phenomena, providing the knowledge of characteristic concentrations. Moreover, it is often unclear which metabolic pathways in relation to different measured biomarkers are involved and some are probably yet to be discovered or decoded. Last but not the least, technological and procedural challenges include also the standardization in terms of the sample collection and treatment, and conceivably, endeavors to automatization of the complete process in the clinical practice.

## 2. Exhaled breath condensate

Compared to the currently widespread invasive and semi-invasive diagnostic methods, the analysis of exhaled breath condensate (EBC) is relatively new and has the first-rate potential to become a preferred and completely noninvasive alternative. EBC is a biological matrix reflecting the composition of the bronchoalveolar extra-cellular lung fluid. The main advantage of EBC as of a matrix is its specificity for the respiratory tract (the liquid is not influenced by process occurring in other parts of human organism). Many important biomolecules are present in exhaled breath in the form of an aerosol [4, 5] (**Figure 1**) which is condensed by cooling during the collection, forming the EBC matrix.

The collection of EBC is performed while using the condenser, which is currently available at a specialized clinical facility. During the collection, the exhaled air is led through the condenser into the cooling box that is pre-cooled to the temperature  $-20^{\circ}\text{C}$ . In the cooling box, the aerosol particles are obtained and the gaseous phase is liquidized.

In the obtained liquid, typically known as EBC, more than 2000 compounds [6] have been identified so far and many of them are considered to represent sensitive biomarkers of lung diseases [7, 8]. The determination of the concentration of these molecules in EBC allows



**Figure 1.** Formation of exhaled breath condensate.

assessing the type and severity of ongoing pathological process or even the efficiency of a therapeutic procedure, etc. In case of numerous pulmonary diseases,  $H_2O_2$ , cysteinyl leukotrienes (cys-LTs), lipoxins (LXs), malverines, resolvins, isoprostanes, prostaglandines, glutathione, adenosine, thiobarbituric acid, aldehydes, nitrotyrosine, cytokines represent a specific group of biomarkers and their concentration levels are elevated (eventually lowered) in airways and lungs as a result of an ongoing allergic reaction, inflammation, oxidative stress, and other processes [9–12].

The most significant advantage of EBC compared to other biological matrices (as are, for example, urine and blood) is the fact that EBC is a highly specific fluid for the respiratory system, so any other biochemical processes in human organism do not influence it.

### 3. The collection of EBC

During the collection of EBC, the exhaled air is led through the condenser, where some components are condensed. The patients should breath calmly and regularly during the whole

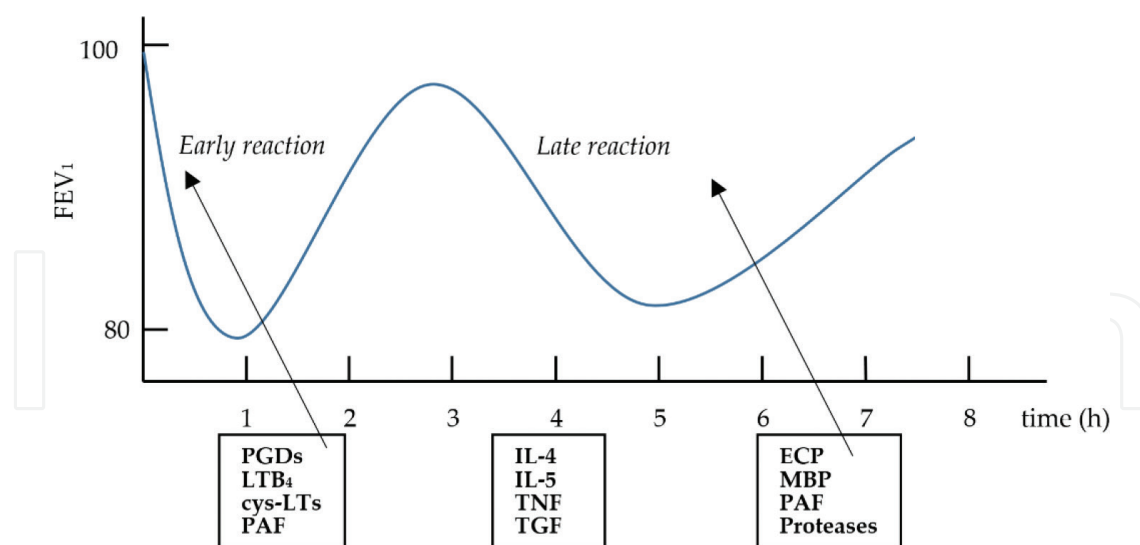
process. The exhaled air flows through the mouthpiece and the one-way valve into the cooling cuff that is pre-cooled at the temperature of  $-20^{\circ}\text{C}$ . In the cooling cuff, the aerosol particles and the obtained gaseous phase are condensed. This liquid is then gathered in the sample collection vial (the temperature remains the same) [13]. The whole process lasts approximately 7–12 min. It is necessary to obtain 120 l of EB in total, which corresponds to 1–2 ml of the condensate. The obtained condensate is then conserved in a micro-test-tube. In order to monitor the degrading process, the samples were labeled by deuterium-labeled internal standards. The prepared samples are then subsequently frozen and stored for a period not exceeding 6 months ( $-80^{\circ}\text{C}$ ).

As the collection of EBC is a noninvasive diagnostic method that does not burden the patient, it can be used in several different clinical studies. A regular collection of EBC enables, for example, monitoring of the impact of climate conditions on the patients. Globally, collection of EBC is a method that is suitable for clinical studies that are trying to understand the process in the organism which corresponds to some external impulses (physical activity, air quality, allergens, etc.)

#### 4. Bronchial asthma

Bronchial asthma is a relatively common pulmonary disease, which is usually characterized by dyspnoea combined with intervals of a normal breathing [14–19]. Typical symptoms of asthma include constricted bronchial tubes and an increased secretion of sputum, which is abnormally dense and viscous [16]. Various sources agree that on the global scale, the asthma incidence accounts for around 300 million people, while the prognoses that are negative in the sense of the future number will keep rising. On the other hand, wide ranges of relatively efficient anti-asthmatic therapies are available (e.g., glucocorticoid therapy,  $\beta_2$ -receptors agonists, etc.) [17] enabling the majority of patients to live normal lives. However, there is still a small group of patients, who do not respond to any kind of current therapy. These patients are usually diagnosed as severe refractory asthmatics (SRA) [6], whose common feature is a lack of any response to any contemporarily available pharmacotherapy. SRA accounts for approximately 5% of all asthmatics, which represents 10 million of people [6].

**Figure 2** describes the immunopathogenesis of asthma [20]. The asthma attack starts by exposure to an allergen, which causes synthesis of immunoglobulin E (IgE). IgE then binds to the surface of mast cells. As there occurs a re-exposure to the same allergen, the interaction between allergen and antibody triggers the release of mediators as are prostaglandins (PGDs), cys-LTs,  $\text{LTB}_4$  and platelet-activating factor (PAF). These mediators cause bronchoconstriction that is connected to an immediate drop in FEV1 (= forced expiratory volume in 1 s; the FEV1 is the volume exhaled during the first second of a forced expiratory maneuver started from the level of total lung capacity). The allergen-antibody interaction also causes production of a wide range of cytokines (e.g., interleukin 4 and 5 (IL-4 and IL-5), tumor necrosis factor (TNF) and tissue growth factor (TGF)). These cytokines then activate neutrophils and eosinophils. Neutrophils produce proteases and PAF, and at the same time, eosinophils produce eosinophil cationic protein (ECP) and major basic protein (MBP). These



**Figure 2.** Immunopathogenesis of asthma [20].

products, eosinophils and neutrophils, cause mucus hypersecretion, edema, and constriction of smooth muscles. This is usually associated with the late asthma phase and it causes the second drop in FEV<sub>1</sub>.

#### 4.1. The diagnostics of asthma

There are several options for the diagnostics of asthma; however, only an early and correct diagnosis of this life-threatening disease permits the physician to timely initiate an effective therapy and minimize the harm to the patient [18]. Several noninvasive methods are already in clinical use (e.g., spirometry, bronchomotoric tests, etc.). In some cases, invasive and *semi*-invasive methods appear to be an inevitable option to gain the correct diagnosis (e.g., open-lung biopsy and bronchoalveolar lavage) [21], yet it is to an unambiguous expense of the patient and often the health cost as well as a demanding laboratory examination.

Currently, a significant part of the relevant research centers focuses on methods of the so-called personalized diagnostics (or methods of personalized medicine), with the aim to stratify patients to characteristic groups (e.g., phenotypes) and thus achieve a more efficient therapy reflecting an individual phenotypic disposition (inclusive of genomic, proteomic and metabolomic profiles) [22, 23]. One of the examples of these endeavors (particularly for diagnostics of pulmonary diseases) is the measurement of a fractional exhaled nitric oxide (FeNO) [24–26] in EBC, helping to distinguish asthma from other pathogenetic processes diagnosed as chronic cough, gastroesophageal reflux disease (GERD), vocal cord dysfunction, bronchitis, chronic obstructive pulmonary disease (COPD), etc.

#### 4.2. Asthma phenotypes

As asthma is a disease affecting millions of people of all ages worldwide, many criteria can be used for its classification. Nevertheless, the predominantly used criterion is the severity of the disease, as is presented in **Figure 3**, followed by the age of the first exacerbation [9, 26].



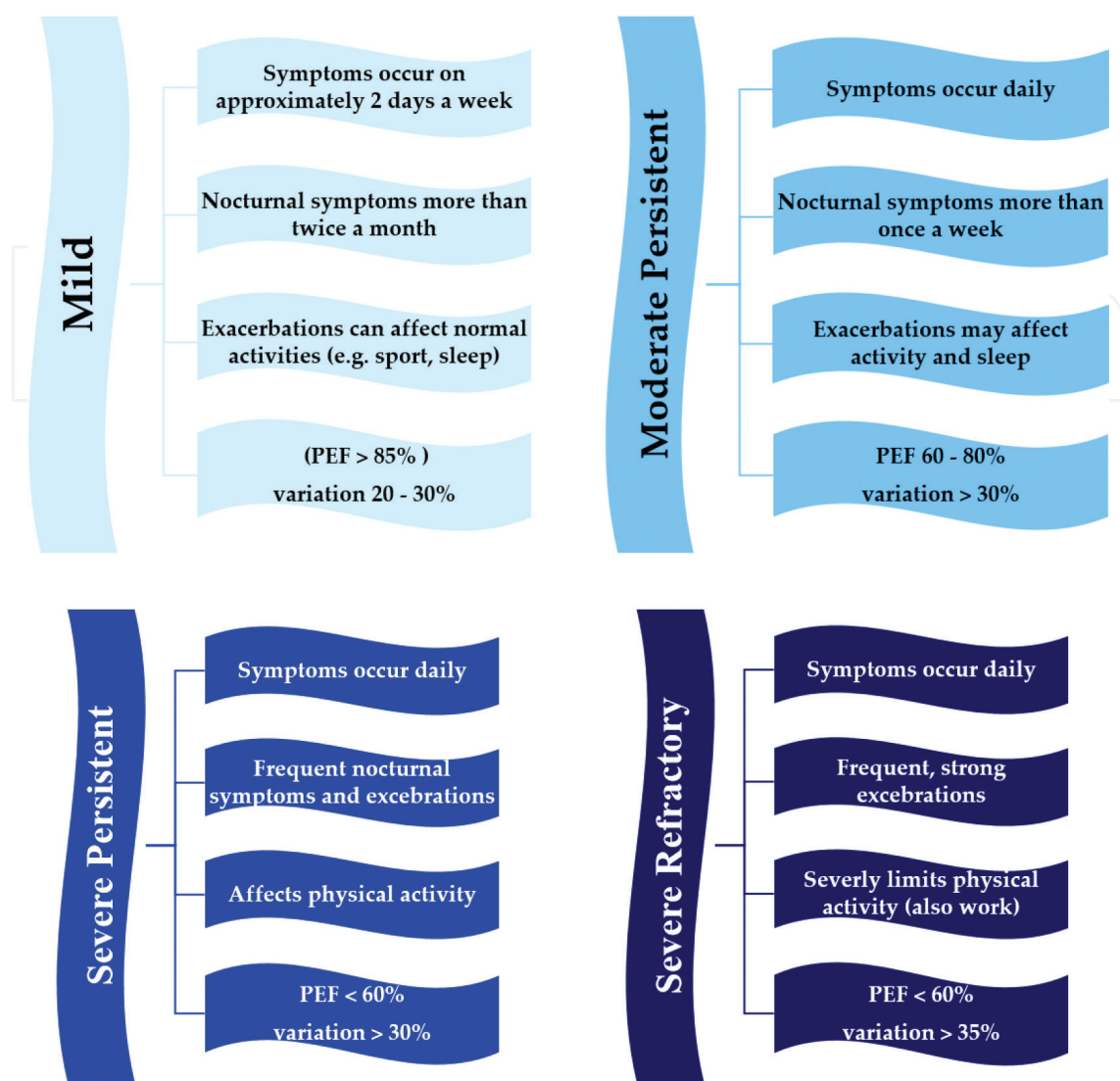


Figure 3. Asthma phenotypes.

### 5. Chronical obstructive pulmonary disease (COPD)

Chronical obstructive pulmonary disease (COPD) is a chronical inflammatory pulmonary disease [27–29]. The development of COPD usually lasts many years. During these years, bronchial tubes of COPD patients are getting more and more narrowed. COPD is also characterized by attacks of dyspnoea and persistent dry cough. The cough is often accompanied by expectorated mucus. In a late stage, it can cause obstructive, effortful, and painful breathing. These complications can be a hindrance also during simple physical activity. COPD patients are also prone to pneumonia. The main cause of COPD is often smoking. Other contributing factors include the genetic inheritance, a long exposition to dust particles, or a regular and frequent lung infection.

COPD is often divided into two main groups (phenotypes): chronic bronchitis and emphysema.

## 5.1. Chronic bronchitis

In chronic bronchitis [28], a typical symptom is a permanent constriction of bronchial tubes. Furthermore, an inhalation of harmful substances cause impairment of the respiratory mucous membrane, while a repeated damage to the membrane makes it thicker and lowers the tissue transparency. As a result, the affected cells increase the production of mucus, leading to the characteristic cough.

## 5.2. Emphysema

Emphysema is characterized by a loss of the pulmonary tissue, while the respiratory ways are abnormally widened distantly from terminal bronchioles [28].

The main cause of emphysema is smoking. The substances that are inhaled during smoking are led through the respiratory ways to bronchioles. In bronchioles, the substances provoke a local immune reaction, which is linked with the production of aggressive compounds via leucocytes (mainly free radicals responsible for oxidative stress). This reaction thus initiates a degradation of bronchioles. The afflicted bronchioles merge into huge lung sacs. These sacs have a smaller surface of the pulmonary tissue and thus the gas exchange between lungs and blood is limited.

The second cause of this disease can be disequilibrium between proteases and their inhibitors—anti-proteases. Some COPD patients suffer from the lack of alfa-1-trypsin (an anti-protease), which is the reason for a higher number of proteases in the respiratory ways, which damage the pulmonary tissue [29].

## 5.3. Asthma and COPD

Similar to asthma, COPD is a pulmonary disease and shares many similar symptoms (e.g., pulmonary obstruction, over-production of mucus, attacks of cough and dyspnoea, etc.).

| Parameter                                | Asthma                  | COPD                    |
|------------------------------------------|-------------------------|-------------------------|
| Age (origin of the disease)              | Childhood, anytime      | 40+                     |
| Development of the disease               | Abrupt attack           | Slower                  |
| Dyspnoea                                 | Rather abrupt, variable | Often, rather permanent |
| Pulmonary obstruction                    | Mainly reversible       | Often irreversible      |
| Smoking                                  | Not very common         | 80% of cases            |
| Allergy                                  | Often (or parents)      | Rarely                  |
| Inflammation (can differ)                | Rather eosinophil       | Rather neutrophil       |
| Bronchial hyperreactivity                | Distinct                | Less common             |
| Glucocorticoid therapy                   | Mainly efficient        | Rather inefficient      |
| Mortality (inhabitants per year (world)) | 300 million (decreases) | 600 million (increases) |

**Table 1.** Asthma and COPD comparison.



Especially, these common characteristics cause that asthma and COPD are sometimes misdiagnosed [30–32]. This can cause an incorrect pharmacotherapy administration, followed by their health state not (or just slightly) improving.

However, several factors can be used to distinguish asthma from COPD (**Table 1**).

## 6. Biomarkers of pulmonary diseases present in EBC

The term biomarker herein refers to a measurable biomolecular factor applicable for the measurement of a disease progression or treatment-related biomolecular changes in the human organism. On a molecular scale, biomarker refers to “a subset of markers that might be discovered using metabolomics, proteomics, genomics and other -omics technologies or imaging technologies.” Biomarkers play a major role in medicinal biology. Biomarkers may be foreseen as a promising tool in the near future due to their unique potential for early diagnoses, which obviously permit disease prevention, a drug target identification, a drug response monitoring, etc. The collection and analyses of substances present in EBC provide a simple, noninvasive, real-time, point-of-care clinical and research tool for the evaluation of lung pathophysiology.

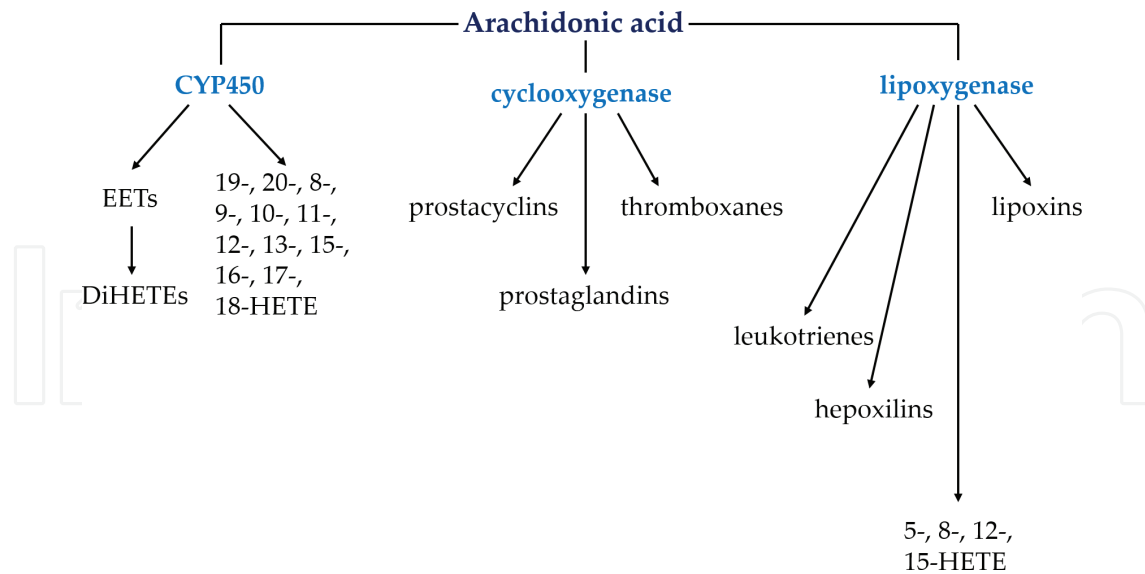
Very significant role is played by some biomarkers that are produced from the arachidonic acid (some of them were already mentioned above). Arachidonic acid ((5Z,8Z,11Z,14Z)Eicosa-5,8,11,14-tetraenoic acid) is a polyunsaturated omega-6 fatty acid present in phospholipid cell membranes [11, 12]. The products of the metabolism of arachidonic acid are called eicosanoids. These molecules are characterized by the 20C chain. The production of eicosanoids is enabled by different enzymes (**Figure 4**), the only exception are isoprostanes which emerge through oxidation of arachidonic acid (non-enzymatic pathway).

### 6.1. Arachidonic acid metabolites

Arachidonic acid is a polyunsaturated fatty acid present in phospholipid bilayer. In human organism, arachidonic acid acts as a vasodilator or regulates inflammation as a key intermediate. There are several pathways which allow transformation of the arachidonic acid in a number of different metabolites (**Figure 4**). Among the most significant products of its metabolism can be classified leukotrienes, lipoxins, isoprostanes, and prostanoids [6, 33].

#### 6.1.1. Leukotrienes

Leukotrienes (LTs) [6, 33] represent a group of biologically active molecules. LTs are produced by various tissue cells (e.g., leukocytes, macrophages, mastocytoma cells) as a response to both immunological and non-immunological stimuli. LTs are potent pro-inflammatory [33] mediators and their release is usually triggered by the organism coming in contact with an allergen. The interaction between LTs and their receptors can lead to a wide range of biological effects: leukocytes activation, bronchial smooth muscles contraction, vascular permeability stimulation and increased mucus production, etc. All of the described symptoms are typically connected not only to pathophysiology of bronchial obstruction, especially to asthma, but also to other lung inflammatory disorders.



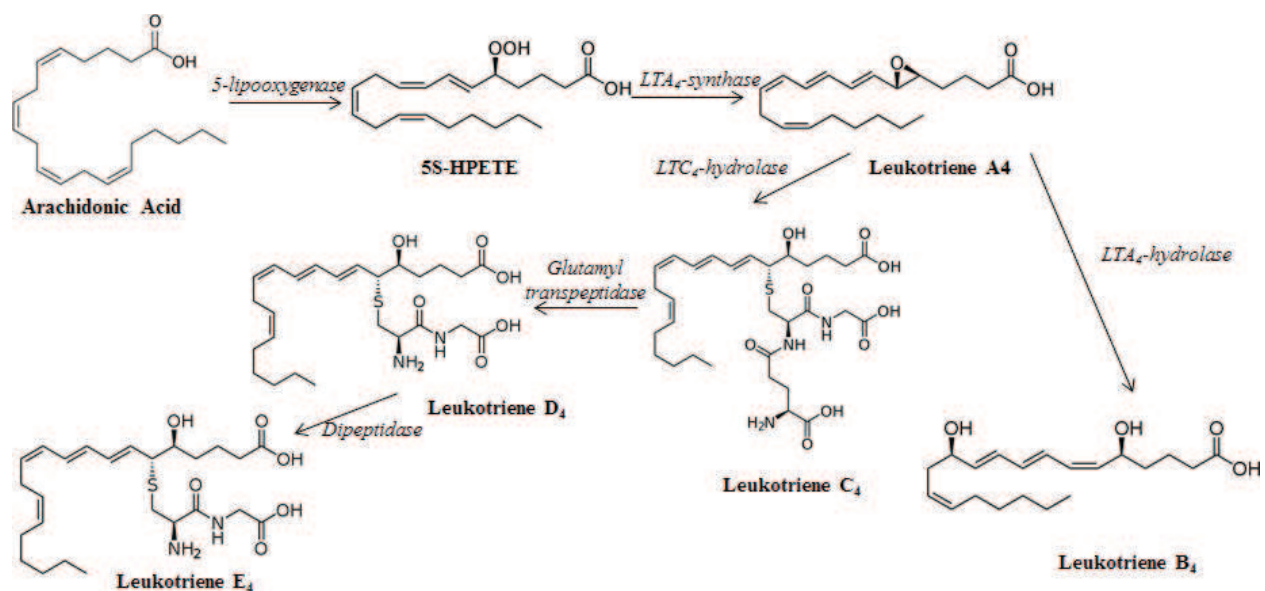
**Figure 4.** Biomarkers generated from arachidonic acid.

LTs are derivatives of arachidonic acid that are synthesized *via* the 5-lipoxygenase pathway (**Figure 5**). The major problem in the determination of LTs in body matrices is their low stability due to their sensitivity toward oxidation. This explains challenging analytical determination of the used assays and to a relatively high variability of the published data.

### 6.1.2. Lipoxins

Lipoxins (LXs) function in our organism as “natural antiasthmatics” as they are the anti-inflammatory mediators. Binding of LXs to their receptors also support the reconstructive process that is initiated in lungs immediately after the asthma attack.

LXs and LTs are derivatives of arachidonic acid and they are generated in three different metabolic pathways [34]. The first one is enabled by acetylsalicylic acid (ASA, in aspirin induced



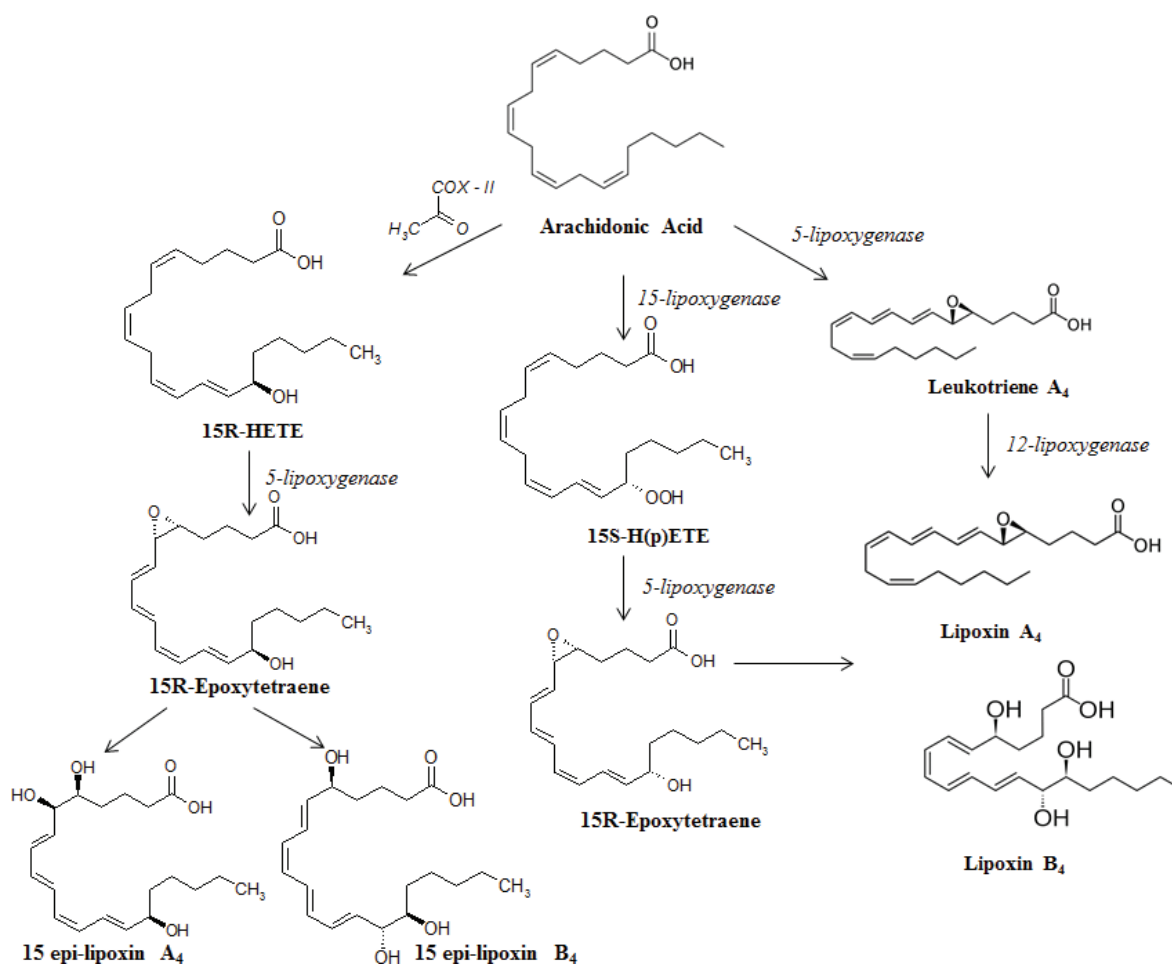
**Figure 5.** Biosynthesis of leukotrienes.

asthma), the second one by the enzyme 15-lipoxygenase, and the last one by 5-lipoxygenase which transforms arachidonic acid into  $\text{LTA}_4$  and then into  $\text{LXA}_4$  eventually into  $\text{LXB}_4$  (**Figure 6**). On the other hand, the levels of LXs are usually lowered during inflammation.

### 6.1.3. Prostanoids

Prostanoids represent another group of biomarkers that are generated from the arachidonic acid. The synthesis is enabled by the enzyme cyclooxygenases ( $\text{COX}_1$ ,  $\text{COX}_2$ ) [6, 33]. Three major groups of biomarkers belong to the prostanoid family: prostacyclins, prostaglandins ( $\text{PGD}_2$ ,  $\text{PGE}_2$ , and  $\text{PGF}_2$ ), and thromboxanes ( $\text{TXA}_2$ ,  $\text{TXB}_2$ ). All of them represent significant participants in the inflammatory response. Thromboxanes are mainly responsible for vasoconstriction, while prostaglandins play an important role in the inflammatory and anaphylactic reactions. Another important function of thromboxanes and prostaglandins is their ability to adapt the inflammatory response and affect symptoms, such as fever, pain, or swelling.

The effect of prostanoids can be both pro- and anti-inflammatory with regard to the type of the inflammatory stimulus. Increased levels of some prostanoids with bronchoconstrictive effects ( $\text{PGE}_2$ ,  $\text{PGD}_2$ ,  $\text{PGF}_2$ , and  $\text{TBX}_2$ ) have been detected in EBC; however, the significance of their presence has not been sufficiently explained yet.



**Figure 6.** Biosynthesis of lipoxins.

## 6.2. Resolvins and protectins

Although the resolution of inflammation may have been regarded as a passive process, it has been proved that it can be actually described also as an active process in which numerous chemical mediators are involved. An example of these molecules may be resolvins and protectins. Both of them are synthesized from  $\omega$ -3-PUFA precursors. Based on the model systems, it has been proved that resolvins and protectins participate in the anti-inflammatory response. In connection, the disproportion in their molecular levels can lead to diseases that are characterized by prolonged inflammation [33, 35]. At the same time, resolvin receptors may represent interesting targets for the future pharmacotherapies.

## 6.3. Oxidative stress biomarkers

### 6.3.1. Biomarkers of lipid peroxidation

#### 6.3.1.1. Isoprostanes; 8-iso-prostaglandin F<sub>2</sub> $\alpha$ (8-iso-PGF<sub>2</sub> $\alpha$ or 8-isoprostane)

Isoprostanes are prostaglandin-like compounds formed *in vivo* from the free radical-catalyzed peroxidation of essential fatty acids (primarily arachidonic acid) without the direct action of cyclooxygenase (COX) enzymes [6, 8, 9, 33]. These non-classical eicosanoids possess potent biological activity as inflammatory mediators that augment the perception of pain. These compounds are accurate markers of lipid peroxidation in both animal and human models of oxidative stress.

8-iso-prostaglandin F<sub>2</sub> $\alpha$  (also known as 8-epi-PGF<sub>2</sub> $\alpha$  or 8-isoprostane) is a biomarker that has been shown to be useful for the assessment of oxidative stress *in vivo*. It is produced in the phospholipid membranes from the non-cyclooxygenase peroxidation pathways derived from arachidonic acid. It is present in EBC in physiological concentration levels which grows in the course of lifetime as a consequence of aging. Pathological levels in EBC are reasonably increased as a result of several lung diseases and disorders that are induced by oxidative stress (asbestosis, silicosis, lung cancer, COPD, etc.).

### 6.3.2. Biomarkers of nucleic acids damage

#### 6.3.2.1. 8-hydroxy-2'-deoxyguanosine, 8-hydroxyguanosine, and 5-hydroxymethyl uracil

The steady-state levels of nucleic acids damage biomarkers represent the balance between formation and repair. As reviewed by Valavanidis et al. [36], increased levels of 8-hydroxy-2'-deoxyguanosine (8-OHdG), the principal product of DNA oxidation, represent a valuable biomarker of DNA damage by oxidative stress.

8-Hydroxyguanosine (8-OHG) is a nucleoside that is an oxidative derivative of guanosine. Measurement of the levels of 8-OHG is used as a biomarker of RNA damage by oxidative stress.

In a rat model, 8-OHdG was found to have anti-inflammatory effect. Rats treated with lipopolysaccharide (LPS) exhibited inflammatory lung injury dependent on neutrophils with an increase in pro-inflammatory cytokines such as interleukins 6 and 18 (IL-6, IL-18) and

tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ). Rats pre-treated with 8-OHdG prior to LPS treatment showed inhibited LPS-induced inflammatory responses. 8-OHdG anti-inflammatory action was found to be higher than for aspirin and other nucleosides (8-OHG, deoxyguanosine, guanosine, adenosine). 8-OHG and adenosine also exhibited anti-inflammatory activity, but it was much lower than for 8-OHdG. Deoxyguanosine was found to be almost ineffective. Compared to aspirin, which acts through cyclooxygenase (COX) inhibition, 8-OHdG seems to be more versatile and, therefore, more effective as it was found that 8-OHdG suppresses ROS formation in human neutrophils. However, in human organism, 8-OHdG is excreted in much lower concentrations than in rats and, therefore, only exogenously administered 8-OHdG could have a therapeutic potential as an anti-inflammatory agent. 8-OHdG is also considered to be a potential biomarker of cancers related to smoking (e.g., lung cancer).

5-Hydroxymethyl uracil (5-OHMeU) is an example of oxidized-pyrimidines. Low levels of these molecules have been detected as a consequence of DNA oxidation initialized by oxidative stress. Oxidized-pyrimidines are more likely to be repaired than other relative molecules, which may represent an explanation of their low detected pathological concentration levels. As the excision rate from DNA is different for various bases, participation of specific excision-repair enzymes might occur.

### 6.3.3. Biomarkers of peptides damage

#### 6.3.3.1. *o*-Tyrosine, 3-chlorotyrosine and 3-nitrotyrosine

*o*-Tyrosine (*o*-Tyr), 3-chlorotyrosine (3-ClTyr), and 3-nitrotyrosine (3-NO<sub>2</sub>Tyr) are among the most prominent biomarkers of oxidative protein damage and are present in the body fluids of patients with diseases related to oxidative stress [6].

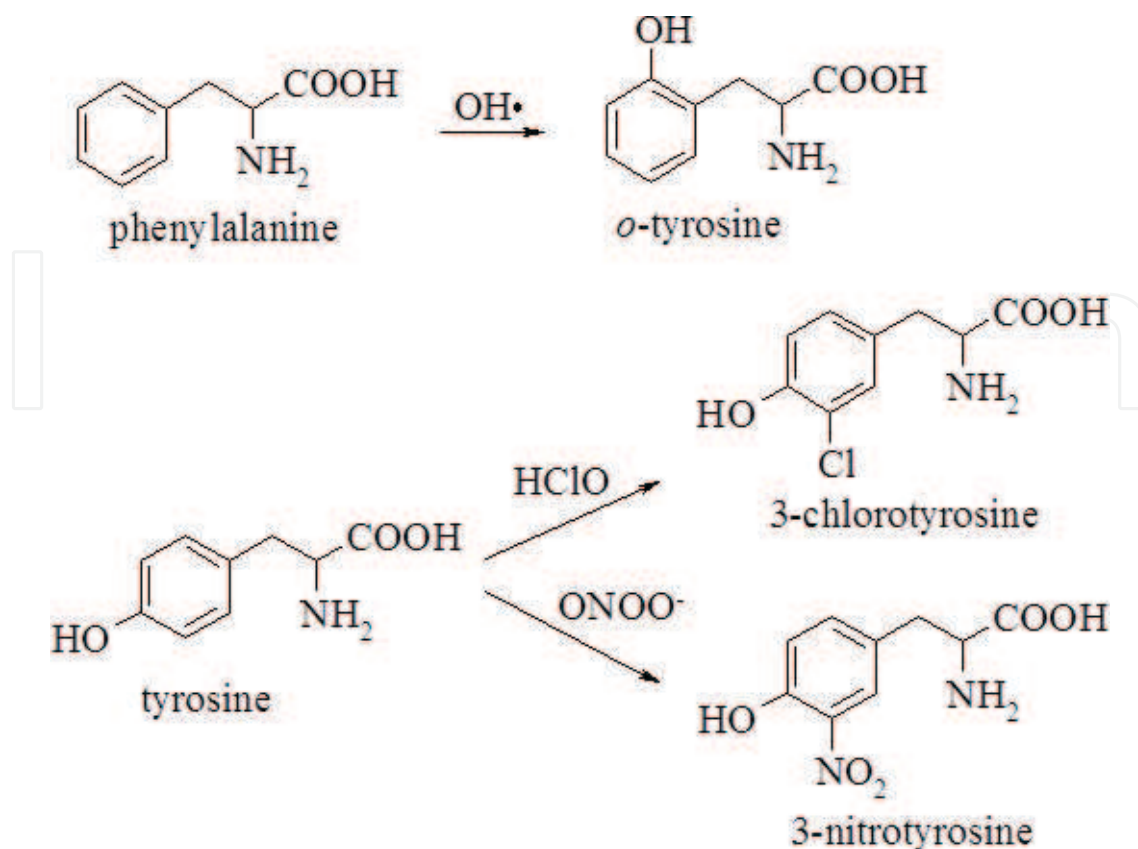
Free radicals cause alterations in cellular protein structure and function. Oxidized, nitrated, and chlorinated modifications of aromatic amino acids including phenylalanine and tyrosine are reliable biomarkers of oxidative stress and inflammation in clinical conditions. In human organism, tyrosine is formed from phenylalanine. Physiological *p*-tyrosine (*p*-Tyr) occurs by enzymatic oxidation of phenylalanine by phenylalanine hydroxylase. Important derivatives of tyrosine are catecholamines (dopamine, adrenaline, and noradrenaline) or thyroid hormones. *o*-Tyr and *m*-tyrosine (*m*-Tyr) are formed by the attack of ROS on phenylalanine. Unlike *p*-Tyr, *o*-Tyr and *m*-Tyr are not natural amino acids and are considered to be oxidative stress biomarkers. The biomarkers that are formed during protein oxidative damage are amino acids *o*-Tyr, 3-ClTyr, and 3-NO<sub>2</sub>Tyr (**Figure 7**).

## 6.4. The other biomarkers

### 6.4.1. Cytokines

Cytokines are proteins secreted by immune cells (e.g., B lymphocytes, T lymphocytes, macrophages, and mast cells) or fibroblasts and endothelial cells. Cytokines are fundamental regulators of the immune system and they play various roles in human organism (not only in immune system), as they influence: regeneration of the tissue, embryonal development, carcinogenesis, angiogenesis, etc. The function of numerous cytokines can be triggered by oxidative stress. In





**Figure 7.** Formation of *o*-tyrosine, 3-chlorotyrosine, and 3-nitrotyrosine.

human organism, they can act as both inflammatory and anti-inflammatory molecules, however in the respiratory tract they are mainly considered to represent biomarkers of chronic inflammation.

A wide range of cytokines has been detected in EBC so far. An example of such cytokine can be tumor necrosis factor (TNF) or interferon (IFN). Low concentration levels [37–40] of both of these biomarkers have been detected in EBC. Specifically, TNF represents a biomarker of oncological diseases as its increased levels have been mainly described among lung cancer patients [41, 42]. Other cytokines that are detectable in EBC are various members of the interleukin family (IL-1, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10 and IL-13).

#### 6.4.2. Glutathione

Glutathione (GSH) is a tripeptide that functions in organism as an endogenous antioxidant. The main task of GSH is to prevent the cells to be damaged by free radicals and reactive oxygen species and thus protect the organism from oxidative stress. An important part of this process is the oxidation of GSH to glutathione disulfide (GSSG). This process occurs, for example, in the airway cells, where it is essential to protect the lungs and airways tissue which are exposed to the effect of external oxidants. Simultaneously, GSH is one of the regulators of the NO cycle. Decreased levels of GSH and proportionally increased levels of GSSG, which are mainly connected to the disproportion in the redox balance, represent a reliable biomarker of oxidative stress, usually coupled with inflammation [43]. Decreased levels of GSH in EBC have been mainly monitored



in case of patients suffering from bronchial asthma. The results of another conducted study showed that significantly increased levels of GSSG occur in EBC of alcoholics [44].

## 6.5. Other molecules determined in EBC

### 6.5.1. Proteins and metabolites

The majority of pulmonary diseases is also characterized by alternations in the protein profile of the patients. Many of these changes are measurable in EBC and can be used for monitoring of pathological process occurring (mainly) in the respiratory tract. The changes in the structure and concentration levels of various proteins have recently become a popular and reliable tool for monitoring of the process and molecular alterations in lungs and airways. Based on the proteomic analysis of EBC, 44 unique proteins [45, 46] have been detected so far. Many of these proteins might become steady biomarkers of inflammation or oxidative stress, when scanning of the differences between the proteome profiles of healthy control subjects and subjects with various pulmonary diseases may represent a significant shift toward detecting new prognostic and/or diagnostic biomarkers.

### 6.5.2. Serotonin

Serotonin (5-hydroxytryptamin (5-HT)) is a neurotransmitter that is predominantly located in central nervous system and gastrointestinal tract (GIT). In GIT, 5-HT regulates bowel movements. In CNS, it is responsible for the regulation of mood, sleep, muscle contraction, and some cognitive functions (involving memory and learning abilities). It is also present in thrombocytes, where it is involved in the regulation of homeostasis and coagulation [47].

5-HT plays a significant role in many pathological and neuropsychiatric diseases [47, 48]. The serotonergic substances are also important in pharmacology. The genes that code various components of 5-HT system are the subject of the study as they could be factors of depression, schizophrenia, obsessive-compulsive disorder, aggression, alcoholism, migraine, and autism [49].

## 7. Experimental part

### 7.1. Analytical method for multi-marker screening

The following analytical methods combined with various pre-treatment methods are currently referenced in the literature for the determination of biomarkers present in EBC: HPLC-MS, GC-MS and EIA (ELISA). Based on validation parameters (e.g., accuracy, precision, limit of quantification (LOQ), limit of detection (LOD), linearity, selectivity, etc.) the methods described above can be compared.

LC-MS method in a highly selective and accurate SRM mode affords both quantitative and qualitative information about the monitored biomarkers and today seems to be method of the first choice. Liquid chromatography can be used in UHPLC, which is characterized by the fact that the separation of substances occurs at higher flow rate of the mobile phase (1 mL/min) on LC columns with smaller average particle size of the stationary phase (diameter of particles

<2 µm) and by shortening the time of LC-MS analysis. When using the so-called “stable-isotope-dilution assay,” the accuracy and precision of the LC-MS method can be increased by suitable deuterated internal standards. However, the main disadvantage of the LC-MS analysis is the inclusion of the pre-treatment step (SPE, immunoaffinity extraction, etc.), when the EBC sample is recommended to exclude a contact to room temperature, ideally temperature above 0°C. This problem can be prevented by using the 2D technology for liquid chromatography. In the first dimension, an on-line SPE is carried out and the subsequent dimension uses the UHPLC. For detection of selected biomarkers, 2D UHPLC-MS method was developed and because of the sensitivity of biomarkers mentioned above, it is highly recommended.

Analysis of substances were realized on the LC-MS system consisting of quaternary pump and mass spectrometer operating on the principle of triple quadrupole equipped with electrospray ionization (ESI). To implement multimarker screening, it was necessary to carry out two types of analyses. The first one were determined substances containing amino group in its structure. The second one serves to determine substances with aldehyde and carboxylic groups. These two analyses were necessary because of the different conditions of derivatization reactions (acid vs. alkaline environment) and the resulting liquid chromatography at different conditions (different composition of the mobile phase used on different chromatographic columns).

#### 7.1.1. Determination of the amino compounds

For the derivatization of compounds containing an amino group in its structure (*o*-tyrosine (*o*-Tyr), 3-nitrotyrosine (3-NO<sub>2</sub>-Tyr), 3-chlorotyrosine (3-Cl-Tyr), hydroxyguanosin (8-OHG) and 8-hydroxy-2'-deoxyguanosine (8-OHdG) were used as derivatization reagent 3-aminopyridyl-N-hydroxysukcinimidyl carbamate (= APDS). To 500 µl of the EBC sample-containing deuterium labeled analyte analogues was added to 450 µl of borate buffer (pH 8.5) and 50 µl of APDS derivatization agent (concentration of 1 mg/ml of acetonitrile). Derivatization reactions were carried out for 10 min at 4°C. Thus prepared sample was subjected to LC-ESI-MS/MS analysis on chromatographic column XTerra MS (C18 50 × 1 mm × 3.5 µm) (Waters, Republic of Ireland). The substances were subjected to analysis where isocratic elution method with a mobile phase composed of acetonitrile: water (60:40—v/v) (water = 0.1% formic acid) was used. The column was tempered to 25°C. Mobile phase flow rate was 150 µl/min. The volume of the analyzed samples was 10 µl. Mass spectrometer parameters were optimized to the following values: capillary voltage –2500 V, the inlet capillary temperature 300°C, the temperature of the evaporator HESI 300°C, sheath gas (nitrogen) pressure 45 psi, auxiliary gas (nitrogen) 10 ArbU. Measurement parameters were optimized for use in neutral loss mode in the interval 250–500 Da (Q1) → 130–380 Da (Q3) with CID energy 15 eV in the negative electrospray ionization (ESI-).

#### 7.1.2. Determination of aldehydes and carboxylic acids

Derivatization of aldehydes (n-aliphatic aldehydes (C6–C12), malondialdehyde (MDA), 4-hydroxynonenal (4-HNE), 4-hydroxyhexenal (4-HHE) and substances with a carboxyl group in its structure 8-isoprostane (8-iso-PGF2α), cys-LTs, LTB<sub>4</sub> was carried out using derivatization with Girard's reagent T (GirT) in the presence of N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) and N-hydroxysuccinimide. To the sample containing 100 µl of EBC with deuterium-labeled internal standards were added 10 µl of

each derivatization reagent GirT ( $c = 100 \mu\text{l/ml}$ ), reagent EDC together with  $10 \mu\text{l}$  of sulfo-N-hydroxysuccinimide,  $10 \mu\text{l}$  of 1% hydrochloric acid and  $270 \mu\text{l}$  of propan-2-ol. Derivatization proceeded for 30 minutes and in such way prepared sample was immediately analyzed by LC-ESI-MS/MS. Chromatographic column used was a Thermo Hypercarb ( $100 \times 21 \text{ mm} \times 5 \mu\text{m}$ ) with pre-column Hypercarb (Thermo Electron Corporation, USA). For separation of substances, was used the isocratic elution method with a mobile phase composed of methanol: water (40:60—v/v) (pH adjusted with ammonium hydroxide to 9). Flow rate of mobile phase was  $150 \mu\text{l/min}$ . Chromatographic column temperature was  $30^\circ\text{C}$  and the sample volume was  $10 \mu\text{l}$ . Mass spectrometer parameters were optimized to the following values: capillary voltage 3000 V, capillary inlet temperature  $300^\circ\text{C}$ ; HESI evaporator temperature  $300^\circ\text{C}$ , sheath gas (nitrogen) pressure 45 psi and auxiliary gas (nitrogen) 10 ArbU. Measurement parameters were optimized for use in neutral loss mode in the interval 150–750 Da (Q1)  $\rightarrow$  91–691 Da (Q3) with CID energy – 16.5 eV in the positive electrospray ionization (ESI+).

## 8. Case studies

### 8.1. Asthma phenotyping

The first aim of the study was to determine levels of the pro-inflammatory cys-LTs and levels of the anti-inflammatory LXs in EBC of patients suffering from different asthma phenotypes (including also SRA), compare the obtained data between the groups of asthmatics, and make the comparisons with healthy control subjects.

As is presented in **Figure 8**, the study showed that both levels of cys-LTs and LXs were changing among different asthma phenotypes. According to the results, EBC of SRA patients contained the highest levels of the pro-inflammatory cys-LTs but at the same time the lowest levels of the anti-inflammatory LXs. The results of the analysis of EBC of healthy control subjects were inverse to these (i.e., EBC of health controls contained the highest levels of LXs but on the contrary the lowest levels of cys-LTs).

The remaining groups have spread in the interval from healthy control subjects to SRA. The order of these groups was based on the raising severity of the asthma phenotype (mild asthma  $\rightarrow$  moderate persistent asthma  $\rightarrow$  difficult asthma) (**Figure 8**).

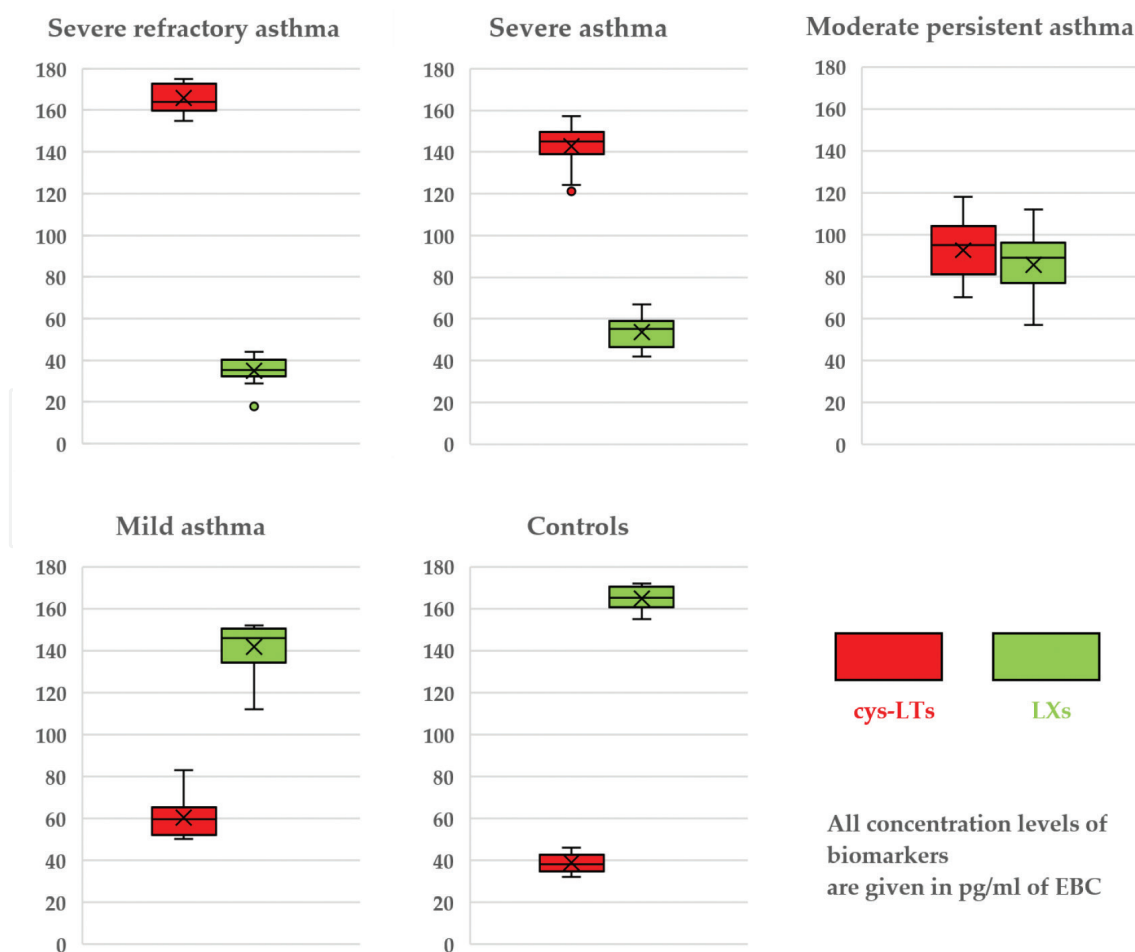
According to the results (**Figure 15**), it is possible to use cys-LTs and LXs for the differential diagnostics of asthma and identify various asthma phenotypes. The diagnosis can be assessed on the phenomenon that the concentration levels of LXs and cys-LTs are complementary and connected by dynamic equilibrium (i.e., increasing levels of the inflammatory LTs lead to a corresponding decrease in the levels of the anti-inflammatory LXs). This occurs due to the fact that biochemical synthesis (both cys-LTs and LXs are generated from  $\text{LTA}_4$ ) enhancing the production of LXs simultaneously lower the generation of LTs. Combining cys-LTs with LXs offers an interesting alternative to the currently used methods of molecular diagnostics of bronchial asthma. **Figure 8** describes the principle of equilibrium between the pro-inflammatory LTs and the anti-inflammatory LXs. The developed method represents a potential tool for asthma phenotyping accuracy improvement, which was proved in a clinical study, which enabled the separation of patients into five groups:

- a. Severe refractory asthma.
- b. Severe asthma.
- c. Moderate persistent asthma.
- d. Mild asthma.
- e. Non-asthmatics—healthy control subjects.

## 8.2. Monitoring of efficacy of the used pharmacotherapy

The developed method was used in a parallel study. The study was conducted to prove whether the method could be applied for monitoring of efficacy of the used pharmacotherapy. In this case, *per oral* and inhaled glucocorticoid treatments have been compared. Results of the study are present in **Figure 9**. In the clinical study of 35 patients with *per oral* glucocorticoid therapy, 35 patients with inhaled glucocorticoid therapy and 32 people from the healthy control group were involved.

From the results, it is obvious that the PCA analysis divided the subjects into three groups. The first group contained only healthy control subjects; however, the two remaining have



**Figure 8.** Statistically evaluated clinical results: levels of cys-LTs and LXs in different asthma phenotypes.

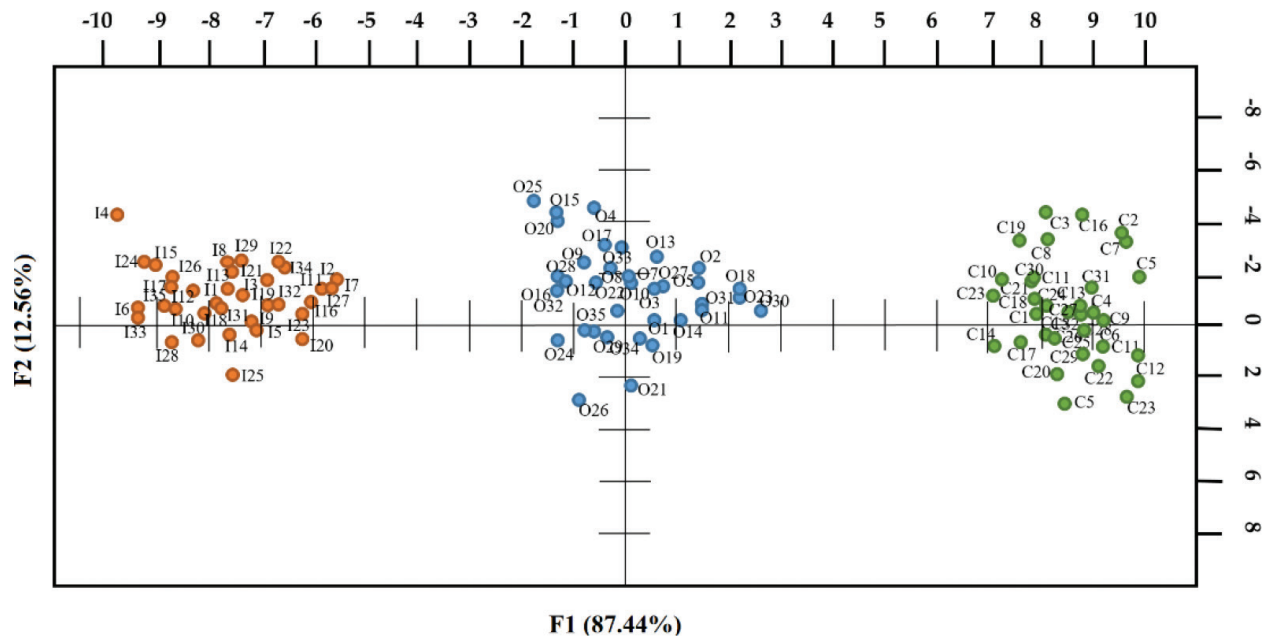


Figure 9. Monitoring of efficacy of the used pharmacotherapy.

been separated according to the type of glucocorticoid application. The results also show that on these terms more efficient was the *per oral* glucocorticoid therapy, as the cluster representing patients with *per oral* treatment is in the spectrum closer to the controls.

The study has also confirmed that the developed method can be used for such monitoring, which could in the future make the asthma pharmacotherapy more accurate. Furthermore, the method could also enable controlling of dosing and comparing of the efficacy of different anti-asthmatic drugs, which would globally improve asthma treatment.

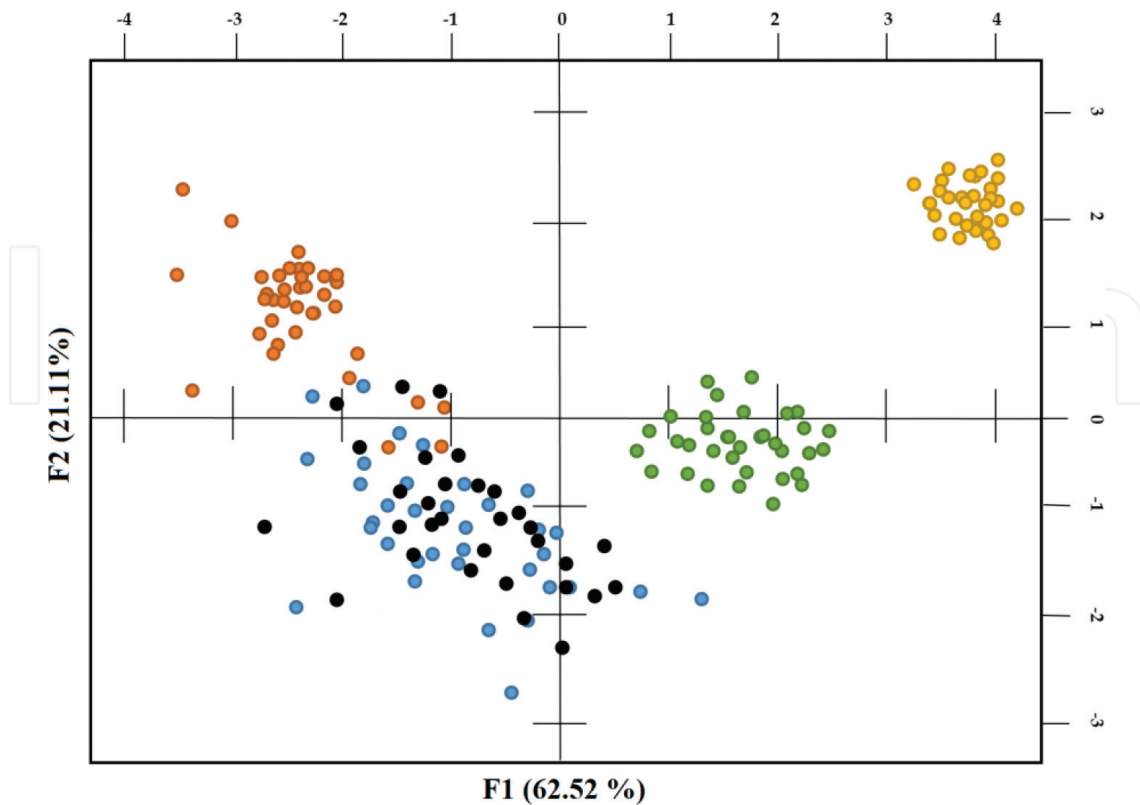
8.3. Asthma and COPD separation

Apart from cys-LTs and LXs, EBC contains a wide range of other different biomarkers. The research has shown that biomarkers of oxidative stress play a significant role in the development of some pulmonary diseases. Examples of such biomarkers can be 8-isoprostan, MDA, HHE, HNE and other aldehydes and biomarkers connected to damage of proteins (*o*-Tyr, 3-ClTyr and 3-NOTyr) or nucleic acids (8-OHdG, 8-OHG and 5-OHMeU).

These biomarkers allowed extension of the developed method, which was originally based on the detection of levels of cys-LTs and LXs. An example of such extensions can be separation of asthma and COPD on molecular level.

The metabolic fingerprinting of EBC of patients suffering from COPD showed a significant increase of biomarkers of neutrophil inflammation—LTB<sub>4</sub> and also biomarkers of oxidative stress (mainly *o*-Tyr and 8-isoprostane). The developed method was used in a clinical study that was aimed at detection and description of differences between COPD patients and SRA (SRA were chosen because their profile is quite similar to the profile of COPD patients and thus their diagnosis is often altered). The obtained results were compared to the analysis of EBC of healthy control subjects (two control groups were chosen—one for COPD patients and one for SRA).





**Figure 10.** Results of clinical study: separation of COPD patients (light blue—bronchitis, dark blue—emphysema) and SRA patients (orange); control groups: green—COPD controls; yellow—SRA controls.

According to the results (**Figure 10**), the PCA analysis has divided the patients into four groups based on their biomarker profiles. The results show that profiles of SRA and COPD patients were different, which allows an accurate separation of these two diseases. The figure also shows that the control groups were separated. Further, the results show that it is not possible with this method to separate (on the molecular level) the two phenotypes of COPD—chronical bronchitis and emphysema.

#### 8.4. Biomarker panel for monitoring of pathogenesis of pulmonary diseases

As a significant part of the study, a panel of biomarkers that can be used for differentiation of various pulmonary diseases was designed. The analyzed biomarkers are divided into two main groups. The first group contained biomarkers of eosinophil inflammation—cys-LTs ( $\Sigma \text{LTC}_4$ ,  $\text{LTD}_4$ ,  $\text{LTE}_4$ ), the anti-inflammatory eicosanoids—LXs ( $\Sigma \text{LXA}_4$ ,  $\text{LXB}_4$ ) and anti-inflammatory resolvins (RvD1). The second group contained biomarker of neutrophil inflammation— $\text{LTB}_4$ , 8-isoprostane which is biomarker of oxidative stress connected to damage of phospholipid membrane, biomarkers of damage of proteins ( $\Sigma$  *o*-tyrosin, NO-tyrosin and Cl-tyrosin) and biomarkers of damage of nucleic acids ( $\Sigma$  5-OHMeU, 8-OHG and 8-OHdG).

The first two graphs show results of the analysis of EBC of patients suffering from SRA and moderate persistent asthma. The results are compared with the analysis of EBC of healthy control subjects.

According to the graph (Figure 11), it is obvious that EBC of patients who suffer from asthma contained increased levels of cys-LTs (the highest levels—SRA, this confirms the study mentioned above). On the contrary, EBC of asthmatics contained lowered levels of the anti-inflammatory LXs and resolvins. Considering the asthma-phenotyping-study, it can be also said that the results of analysis of EBC of SRA and controls were inverse.

Figure 12 shows the results of the monitoring of  $LTB_4$ , 8-isoprostane, biomarkers of damage of proteins and nucleic acids. Levels of  $LTB_4$  showed the same trend as cys-LTs, for example, the highest levels were detected in EBC of SRA and the lowest in EBC of healthy controls. At the same time, levels of 8-isoprostane were slightly elevated among the group of patients with moderate persistent asthma and even more among SRA. The differences in levels of biomarkers responsible for damage of proteins and nucleic acids were slightly higher in EBC of asthmatics, but the differences were not so significant, which means that these biomarkers are not so specific and influential in case of bronchial asthma.

Figures 13 and 14 show same biomarkers as the previous Figures 11 and 12, but in EBC of patients who suffer from COPD, asbestosis and lung cancer.

From Figure 13, it is quite obvious that biomarkers cys-LTs, LXs and resolvins do not play a significant role in pathogenesis of these diseases, as their levels are comparable to those detected among healthy control subjects (the levels are just slightly elevated and only COPD patients show some more noticeable deviations).

Figure 14 shows that illnesses characterized by damage of the pulmonary tissue are usually connected to increased levels of biomarkers of oxidative stress. One of these significant

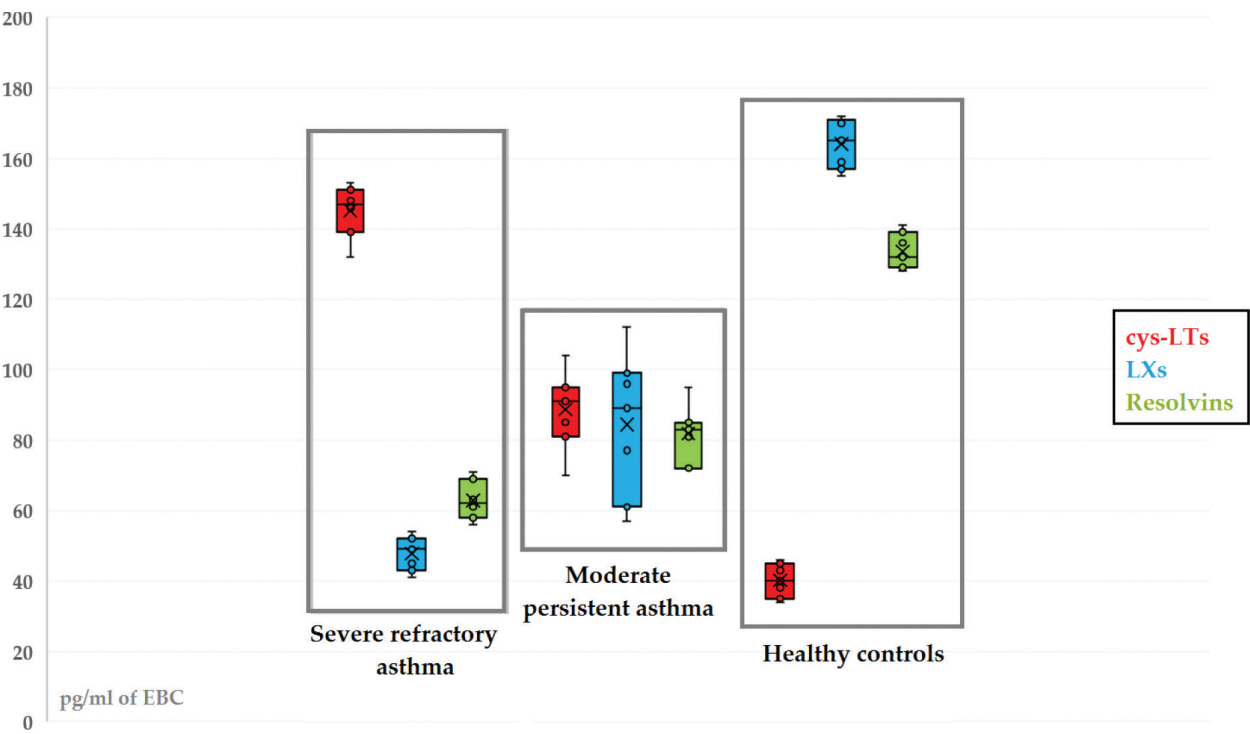
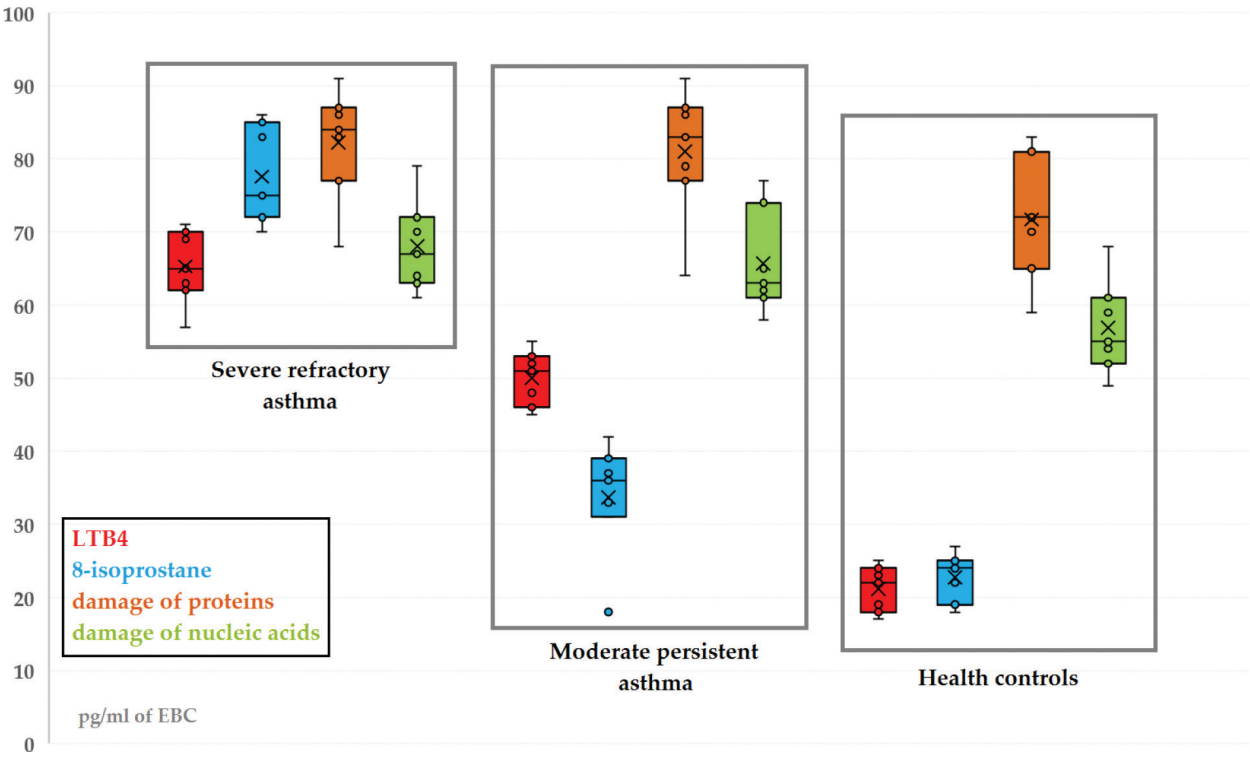
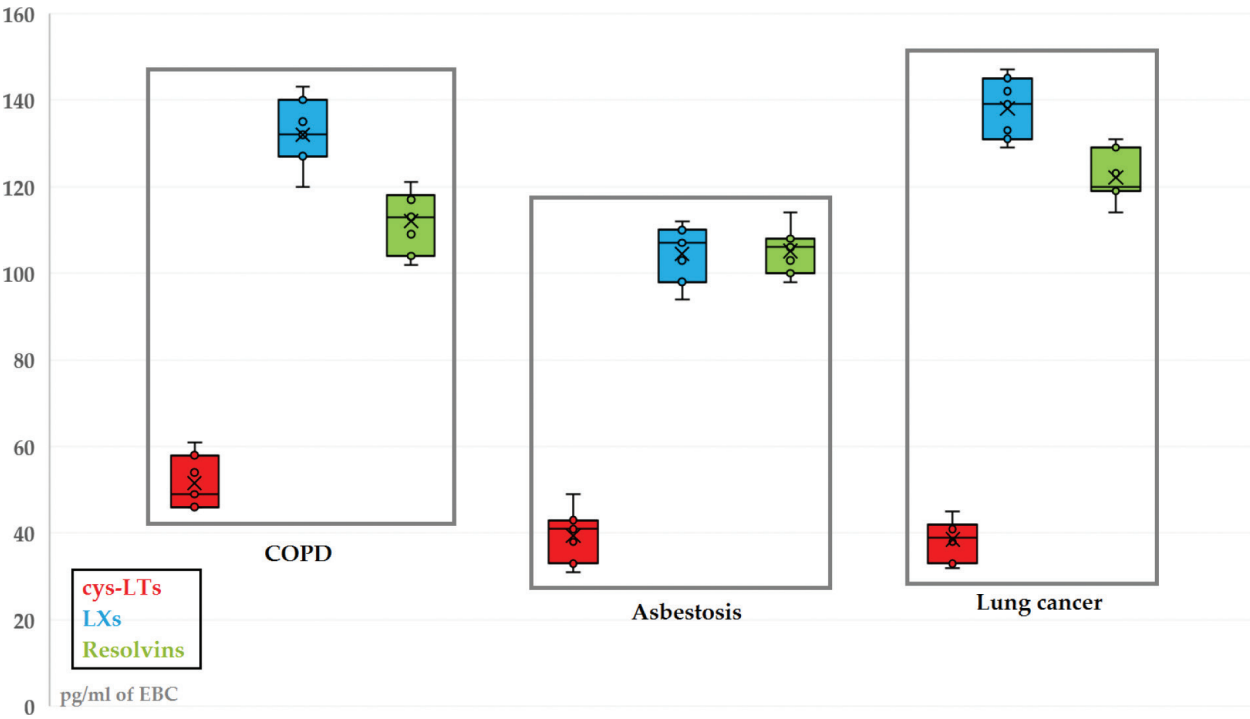


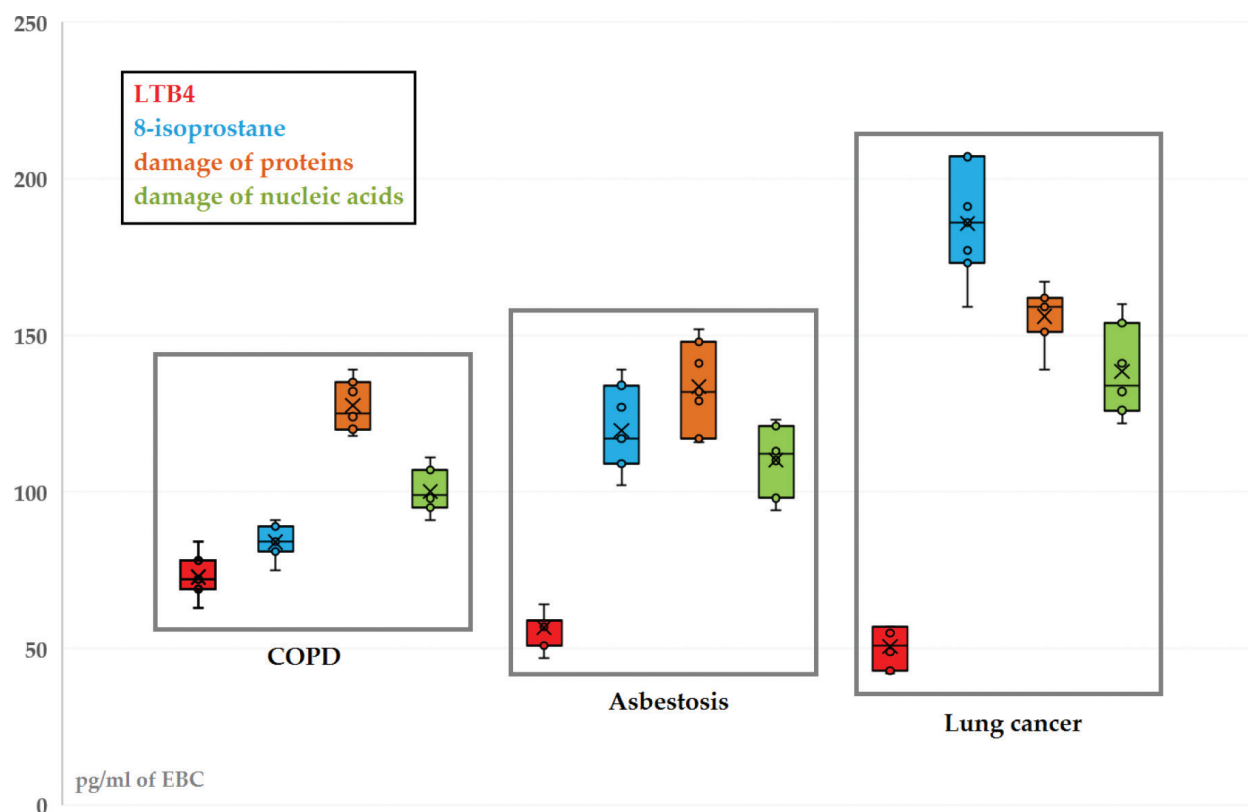
Figure 11. Evaluated clinical results: levels of cys-LTs, LXs and resolvins in EBC of SRA, moderate persistent asthma and healthy controls.



**Figure 12.** Evaluated clinical results: levels of LTB<sub>4</sub>, 8-isoprostane, biomarkers of proteins and nucleic acids damage in EBC of SRA, moderate persistent asthma and healthy controls.



**Figure 13.** Evaluated clinical results: levels of cys-LTs, LXs, and resolvins in EBC of COPD, asbestosis, and lung cancer patients.



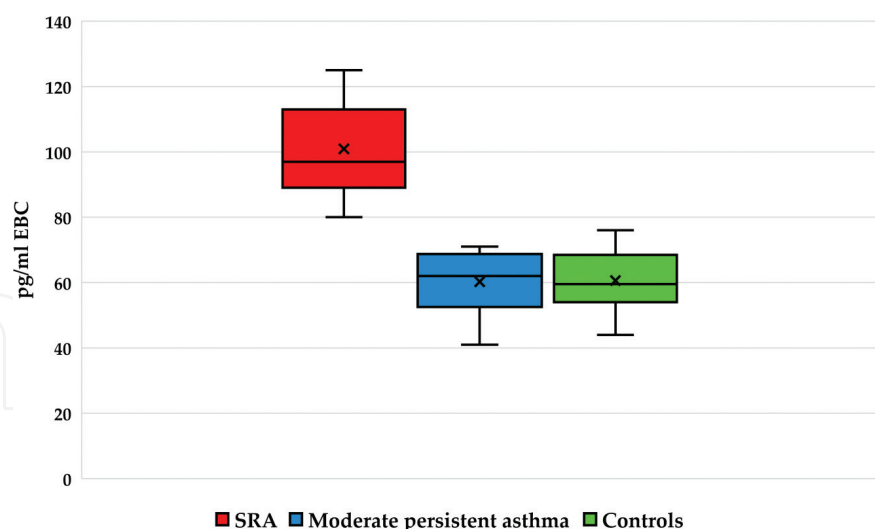
**Figure 14.** Evaluated clinical results: levels of  $LTB_4$ , 8-isoprostane, biomarkers of proteins and nucleic acids damage in EBC of COPD, asbestosis, and lung cancer patients.

indicators of ongoing tissue necrosis processes is 8-isoprostane. The analysis of EBC showed that the levels of this biomarker are increased among COPD and asbestosis patients and even more among people suffering from lung cancer. Similar information is provided by the biomarkers of proteins damage (tyrosines) and nucleic acids damage (5-OHMeU, 8-OHG, and 8-OHdG). The levels of these molecules were elevated in EBC of patients with COPD and asbestosis and it can be said that the highest levels are specific for lung cancer (average concentration of tyrosines is approximately 75 pg/ml of EBC for healthy controls and 160 pg/ml of EBC for patients with lung cancer).

### 8.5. Serotonin in EBC of SRA

Based on the clinical experience, it is proved that SRA patients positively respond to SSRI (selective serotonin reuptake inhibitors) antidepressants therapy. SSRI antidepressants usually improve physical state of patients, which may seem as a quite logical coincidence. However, much more surprising is the fact that when SRA patients are prescribed SSRI antidepressants, their breath functions improve significantly. This phenomenon prompted to performed research aimed at the detection of serotonin in EBC of SRA. The obtained results were compared with serotonin levels in EBC of other asthma phenotypes and healthy control subjects.

According to the results (**Figure 15**), it is obvious that the levels of serotonin in EBC of SRA are different as compared to other asthma phenotypes and healthy control subjects. However,



**Figure 15.** Evaluated clinical results: levels of 5-HT in EBC of SRA, moderate persistent asthma and healthy controls.

surprisingly, the levels were significantly elevated (in case of SRA patients) which is against all expectations (it was expected to detect lower levels of serotonin, which would provide a possible explanation of positive SRA's responsiveness to SSRI antidepressants therapy). Probably, even more interesting is the fact the levels of serotonin of other asthma phenotypes and health controls were the same, which indicates that the deviation appears only among SRA.

The interpretation of these results is quite complicated. One of the possible hypotheses is that SRA could be a different disease that would only demonstrate itself as asthma (i.e., patients have similar symptoms as asthmatics, but the cause of the disease could be different). However, this theory will require further research in the future. One of the possible extensions could be monitoring of levels of serotonin in cerebrospinal fluid, which would provide information about the process behind the blood-brain barrier. On the other hand, the study proved that there many significant physiological differences between SRA and other asthmatics, which could be used in the future for the development of a possible drug against SRA.

## 9. Conclusions

Measurements of biomarkers in EBC offer a novel way of monitoring lung inflammation, damage by oxidation stress with an insight into the pathophysiology of different diseases. The described diagnostic method was based on the detection and quantification of biomarkers in a matrix specific for the respiratory tract—EBC. As the collection of EBC is completely noninvasive, the method offers a broad spectrum of application. The method is applicable to children as well as to senior people and it is appropriate also in case of longitudinal studies that are trying to precisely understand the processes occurring on the molecular level in the respiratory tract. The method can be easily repeated which proves its suitability for regular monitoring of the pharmacotherapy efficiency or the impact of various allergens. The results obtained from the EBC analysis represent reliable characterization of the exhaled biomarkers



profile (LXs, cys-LTs, LTB<sub>4</sub>, 8-isoprostane, tyrosines, etc.), which is relevant for diagnostics, separation, and phenotyping of different respiratory diseases. Nevertheless, EBC analysis requires standardization and validation including sample collection and sample pre-analysis treatment (e.g., internal standardization, storing, pre-treatment method application, etc.).

Model clinical studies were carried out as a part of the work, which applied a methodology based on the molecular diagnostics of EBC. The method allowed an asthma phenotyping, which was founded on the fact that the concentration levels of cys-LTs and LXs are not only complementary but also intra-related by a dynamic equilibrium. This phenomenon, however, affords not only asthma phenotyping but also other diagnostics as, for example, monitoring of efficacy of the used pharmacotherapy. The analysis of EBC also showed that the detected biomarkers can be used for the differentiation of various pulmonary diseases (more specifically (apart from asthma) COPD, asbestosis, and lung cancer). Increased (or decreased) levels of some biomarkers are specific only for some diseases and thus these can be selectively differentiated as much as, for example, asthma from COPD.

Additionally, an experiment was conducted and focused on determining serotonin in EBC. The aim of this study was to assess the positive effects of the SSRI (selective serotonin re-uptake inhibitors) antidepressants on SRA. High levels of serotonin were detected in EBC of SRA patients, which was in contradiction to the initial assumption. Simultaneously, a hypothesis was formulated stating that SRA probably functions on different molecular principles. This could have probably been the reason for SRA inefficiency with the commonly used drugs.

For the future research, one can only recommend focusing on large longitudinal studies to ascertain whether sequential measurements in individual patients reflect asthma severity and the degree of a lung inflammation, and on studies engaged to the relationships between the concentrations of asthma biomarkers and its symptoms. In order to implement the EBC analysis to the clinical practice as well as reliably guiding the pharmacological treatment of asthma and the effect of drugs on asthma markers present in EBC, further controlled studies are required to be conducted. In particular, studies are recommended determining the expediency of the EBC analysis for predicting a treatment response, and assessing new therapies. Obviously, this outlines a great deal of work to be done. The fact that EBC analyses are currently used in various clinical trials and studies corroborates the above arguments. On the other hand, it is important to proclaim that the fact whether and when EBC analyses will become applicable to the clinical settings is still difficult to predict.

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## Author details

Tereza Kačerová<sup>1,2</sup>, Petr Novotný<sup>3</sup>, Ján Boroň<sup>3</sup> and Petr Kačer<sup>1, 4\*</sup>

\*Address all correspondence to: [petr.kacer@1lf.cuni.cz](mailto:petr.kacer@1lf.cuni.cz)

1 BIOCEV, 1st Medicinal Faculty Charles University, Vestec, Czech Republic

2 Grammar School Nad Štolou 1, Prague 7, Czech Republic

3 ESSENCE LINE, s.r.o., Prague 5, Czech Republic

4 National Institute of Mental Health, Klecany, Czech Republic

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