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Determination of Biomarkers in Exhaled Breath Condensate: A Perspective Way in Bronchial Asthma Diagnostics

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1. Introduction

The nowadays commonly used term asthma comes from Greek language and means "panting". Currently, asthma is defined as a chronic inflammatory disorder of the airways where many cells and cellular elements play significant roles. In susceptible individuals, this inflammation causes recurrent episodes of wheezing, breathlessness and chest tightness accompanied by coughing, occurring very often during the night or early morning. These episodes are widely associated with a variable airflow obstruction that is either spontaneously reversible, or controllable by a suitable treatment. Asthma can be controlled by recognizing its alarming signs and by avoiding stimuli triggering the attack. During an asthma episode, the airways become extremely narrow due to a muscle constriction, swelling of the inner lining and a mucus production. Fig. 1 compares the airway bronchi of a healthy subject and a patient with an ongoing asthmatic bronchoconstriction. These repetitive episodes can cause a very limited airflow and may lead to unexpected fatalities. Factors, playing role in bronchial asthma can be divided into three groups. Among the first, internal factors are such as genetic predisposition and the state of immune system. The second factors are classified as external triggers and include for example allergens like pollen, mold spores, dust mites or animal dander. However, indoor and outdoor pollutants and irritants such as smoke, perfumes, cleaning agents, etc., can also belong to this group. The third group consists of physical factors, especially exercise and cold air, and physiological factors like stress, gastroesophageal reflux disease (GERD) or viral and bacterial upper respiratory infection. Severity of asthma is traditionally classified as mild, moderate and severe depending on its symptoms, rescue inhaler use and function parameters of lungs. While on controller therapy, each of these groups is further classified as well controlled, not well controlled or poorly controlled, based on the presence and frequency of symptoms, and lung function (GINA 2010).

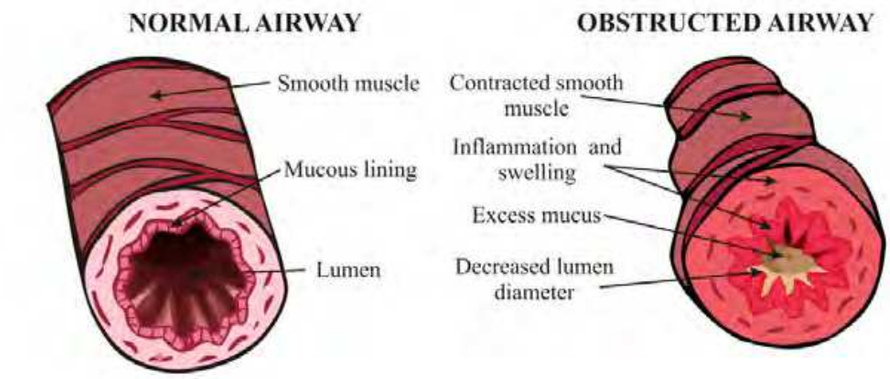

Fig. 1. Airways of healthy (left) and asthma (right) subjects

Bronchial asthma is one of the top public health problems affecting both children and adults globally. According to WHO, asthma is currently underdiagnosed and it is more than obvious it often unnecessarily reduces the quality of life, restricting individuals' activities for a lifetime. It is the most common chronic illness in children, while currently, 300 million people are suffering from it and 250,000 annual deaths are attributed to this disease. It is therefore a significant burden to the public health (GINA 2010). Workplace conditions, such as exposure to fumes, gases or organic and/or inorganic dust, are responsible for 11 % of asthma cases worldwide (occupational bronchial asthma), while about 70 % of asthmatics also suffer from various allergies. The prevalence of asthma increased by 95 % from 1995 to 2010 and the asthma rates in children under the age of five increased by more than 190 % from 1995 to 2010. It is estimated that the number of people with asthma will grow by more than 100 million by 2025. Without a proper management, asthma exacerbations often require frequent treatment and monitoring at emergency departments (ED), hospitalizations, and may lead to premature deaths. In the United States, almost 2.2 million people visited an ED in 2010 because of asthma and almost half a million of those were hospitalized. It generally affects people of all races and ages but some populations are burdened by the disease disproportionately more than others. Although asthma most often starts in the childhood, affecting more boys than girls, it affects more women than men in the adulthood (WHO, 2011).

However, asthma diagnosis can be rather difficult. Currently, there is no precise physiological, immunological, or histological test for diagnosing asthma. Early diagnosis of this potentially life-threatening disease is essential to allow a physician initiating an effective therapy and minimize the harm to the patient. The diagnosis is usually made based on the pattern of symptoms (airways obstruction and hyper-responsiveness) and/or response to the therapy (partial or complete reversibility) over a period of time. Signs and symptoms can range from mild to severe and are often similar to those of other conditions, including chronic obstructive pulmonary disease (COPD), early congestive heart failure or vocal cord problems. Children often develop temporary breathing conditions that have symptoms similar to asthma. For example, it can be hard to distinguish asthma from wheezy bronchitis, pneumonia or reactive airway disease. In order to rule out other possible conditions, lung function is generally tested first in an asthma diagnosis process. Tests to measure the lung functions include: (1) Spirometry Pulmonary Function Tests (PFT), which measures the narrowing of bronchial tubes by measuring how much air is exhaled after a deep breath and how fast it is breathed out. (2) Peak

Expiratory Flow Rate method (PEFR) measures the degree of obstruction in the airways. PEFR is a simple method that measures the level by which the peak flow readings are lower than usual. This is a sign that the airways may have deteriorated their functioning and that asthma could be exacerbating. Lung function tests are often done before and after taking a bronchodilator such as salbutamol to open the airways. If the lung function improves with the use of a bronchodilator, it is likely to be asthma. (3) Methacholine or histamine challenge tests consist in provoking bronchoconstriction or narrowing of the airways by a methacholine or histamine stimulus. Histamine causes nasal and bronchial mucus secretion and bronchoconstriction *via* the H1 receptor, whereas methacholine utilizes the M3 receptor for bronchoconstriction. The degree of narrowing can then be quantified by spirometry. Subjects with pre-existing airway hyperreactivity, such as asthmatics, will react to lower doses of bronchoconstriction drug. This test may be used if the initial lung function test is normal. (4) Fractional exhaled nitric oxide (FeNO) test appears useful to diagnose and monitor asthma, where the amount of nitric oxide present in patient breath is measured. If airways are inflamed – a sign of asthma – the nitric oxide levels are higher than normal. However, this test is not widely available.

At present, quantification of inflammation in the lungs is based on invasive (open lung biopsy (Chuang et al., 1987; Jarjour et al. 1998; Jeffery et al., 2000), bronchoalveolar lavage (Jarjour et al., 1998; Reynolds, 2000)) or *semi*-invasive (for example, induced sputum (Dworski et al., 2004; Green et al., 2002; Holz et al., 2000)) methods and the measurement of inflammatory markers in plasma and urine, which are likely to reflect systemic rather than lung inflammation. The analysis of exhaled breath condensate (EBC) is a relatively novel method with a good potential to become the preferred and completely non-invasive alternative to the currently practiced invasive and *semi*-invasive diagnostic methods for bronchial asthma. New approaches are based on attempting to identify robust biomarkers which could be utilized in establishing the diagnosis of asthma. The former studies investigated the predictive value of EBC pH for asthma, the latter chose to research hydrogen peroxide, nitrogen oxides, arachidonic acid derivatives, cytokines and others. Besides arachidonic acid derivatives, especially cysteinyl leukotrienes (cys LTs) have shown the most consistent results for the diagnosis of asthma (Hatipoglu & Rubinstein, 2004).

Thus, in the current clinical practice, spirometry and symptom scores are used to assist in the diagnosis of the disease severity and control in individual patients. EBC analysis and determination of concentration levels of the bronchial asthma biomarkers is an exciting new approach to monitoring lung inflammation. Many studies have attempted to associate changes in the EBC biomarkers – pH, hydrogen peroxide, arachidonic acid derivatives, especially cys LTs with diagnostic parameters, stratification of asthma severity, therapy effectiveness, etc. Because the technique is relatively inexpensive, it might be useful in large clinical studies and in clinical practice. In the near future, it might be possible to detect multiple asthma biomarkers in EBC (multimarker screening) to aid diagnosis, to predict the most effective therapy, and monitor the response to a treatment. The detection of elevated inflammatory mediators in EBC of subjects with relatively asymptomatic asthma and normal pulmonary function tests could offer a novel way monitoring the lung inflammation and perhaps initiating treatment in an earlier stage. They could also be helpful for the diagnosis of occupational asthma (Klusáčková et al., 2008) and monitoring work-related asthma control at the condition of either elimination from the workplace or reducing exposure, as the clinical benefit from workplace interventions is not sufficiently proven.

2. Exhaled Breath Condensate – A matrix for diagnostics

Every person breathes out 15 to 25 m^3 of air *per* day. In addition to gas exchange, lungs are involved in many metabolic processes (defence against pathogens, airway clearance, arachidonic acid metabolism etc.). They contain different cell types responsible for various functions (respiratory regulation, defence reactions and surfactant production). The surface of lungs and airways is abundant with a number of substances (e.g. enzymes, tumour markers, antibodies, proteins, metabolites etc.) whose presence and concentration level reflects the physiological/pathological conditions of an organism. Metabolites generated in the lungs can be examined by invasive or semi-invasive methods - bronchoalveolar lavage (BAL), methods of induced sputum and open lung biopsy. These diagnostic methods impose a considerable strain on the patients and cannot be repeated as often as the efficient health monitoring would require. By contrast, the measurements of metabolite products in the EBC are non-invasive and conspicuously reflect the composition of the extracellular lung fluid (Piotrowski et al., 2007).

During the collection of an EBC, it is often assumed that the monitored biomolecules are merely contained in the gas phase of the exhaled air. This assumption neglects the fact that the exhaled air also contains a liquid fraction, i.e. aerosol which inevitably carries important biochemical information as well (Fig. 2). In addition to gases as nitrogen, oxygen, carbon dioxide or carbon monoxide contained in the gaseous phase, there are substances with a sufficient vapour pressure at the body temperature and the atmospheric pressure such as water, hydrogen peroxide, hydrocarbons and other volatile organic compounds. In parallel, there are substances insoluble in water which form binary systems with water in the epithels of lungs and airways. In this case, the vapour pressure of water and hardly volatile biomolecules add up, greatly facilitating the evaporation of biomolecules. As a consequence, these can be present in the vapour phase of the exhaled air. Eicosanoids (leukotrienes and prostaglandins) are the example of substances entering the exhaled air by this described mechanism. Molecules of water-soluble substances, for example vasoactive peptides, enzymes, DNA and proteins flow within the exhaled air as aerosol particles. They are released from the mucous surface due to a turbulent airflow throughout bronchi and bronchioles (Effros et al., 2004). EBC is a water-based matrix. The collection of an EBC sample is a simple, non-invasive procedure that can be beneficially applied especially to children (older than 3 years), seniors as well as patients with different disease-impaired health conditions.

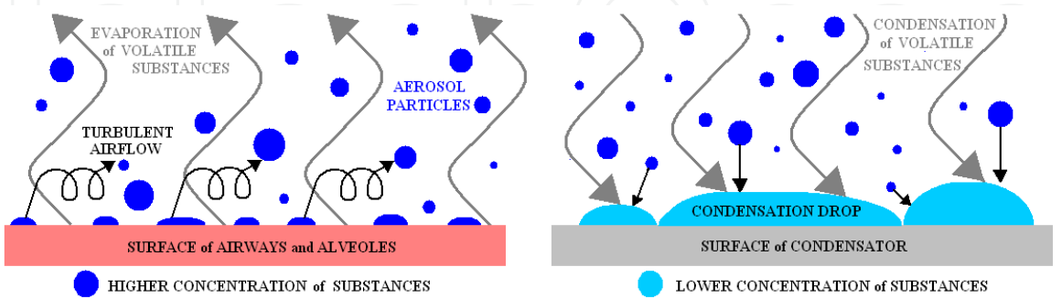

Fig. 2. Generation of exhaled breath condensate. Occurring on the airways surface and in alveoli: (1) evaporation of volatile substances and substances insoluble in water forming a binary system with water on the airways surface and (2) aerosol particles carried away by the turbulent airflow. Aerosol droplets are collected in the exhaled air condenser, where condensation of the water vapour and other volatile substances occurs.

Furthermore, its repeated usage in short time intervals is another advantage compared to invasive methods, enabling monitoring of an ongoing disease as well as an effective pharmacotherapy. The equipment for the collection of EBC generally consists of a mouthpiece with a one-way valve connected to a collecting system (Fig. 3).

The subjects wear a noseclip and breathe tidally *via* the mouthpiece and the one-way valve, in which expiratory and inspiratory air is separated. The valve block is connected to the collecting system which is composed of a lamellar condenser and a polypropylene tube (a sample collection container) that is inserted into a cooling cuff maintained at a stable cold temperature by a refrigerator.

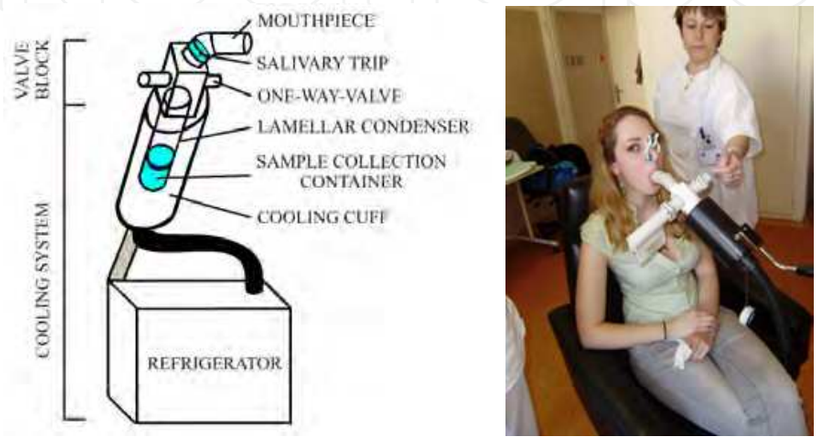

Fig. 3. Scheme of an exhaled breath condenser (left) and clinical EBC collection (right)

During the collection of EBC, the following parameters must be monitored: (1) the condenser temperature, (2) the nasal contamination, (3) the contamination by saliva, (4) humidity and (5) the time of EBC collection. The condenser temperature plays a crucial role in monitoring biomarkers contained in the gas phase (e.g. aldehydes, eicosanoids) and the amount of the condensed water (affecting the dilution rate of the EBC sample). In condensers utilizing cooling water at the temperature of 10 °C, about 81 % of water vapour condenses from exhaled air, whereas using ice, 89 % is captured on the condenser surface. The condensers using a counter flow cooling system reach temperatures of -10 °C achieving 93 % of the water condensed (Horváth et al., 2005). This low temperature is important for the preservation of heat-labile substances, i.e. a number of biomarkers including leukotrienes, aldehydes, etc. Inflammatory mediators such as leukotrienes and prostaglandins are also formed in the nose and paranasal sinuses (Howarth, 2000). Using simple experiments, the nasal contamination (alternatively the determination of whether the monitored biomarkers occur in the mucosa of the nose and nasopharynx and influence the quantity of substances detected in EBC) can be determined. In particular, the level of monitored substances in EBC is determined with/without using a nasal clip during the collection of EBC. Since leukotrienes are contained in high concentrations in saliva (McKinney et al., 2000; Tufvesson et al., 2010; Zakrzewski et al., 1987), it is important to exclude the contamination of EBC by saliva. This can be achieved by measuring amylase concentrations in the samples (the limit of detection for amylase activity in saliva is 0.078 $U.mL^{-1}$, it is approximetly the contamination of 0.2 μL of saliva in 1 mL of EBC (Gaber et al., 2006)). When collecting EBC using devices equipped with a saliva trap, the

contamination by saliva has not been evidenced (Montuschi et al., 2000; Syslová et al., 2011). The often neglected factor is the humidity, affected either by the environment (air humidity) in which the collection is carried out or in which the individual was present just prior to the collection and the amount of water that entered into the lungs from the body (dependent on the hydration/dehydration of the organism). Experiments carried out with the constant exhaled air volume of 120 L and the equal humidity demonstrated that a significant difference in monitored biomarkers could be determined in one subject owing to different bodily hydration (the first situation - hydration: drinking about 1 L of fluid before the collection of EBC *versus* the second situation – dehydration: drinking absence for 12 hours before the collection). With the fluid intake, the concentration of biomarkers was reduced by 33.8 ± 5.22 % for LTC_4, 36.8 ± 4.27% for LTD_4 and 36.3 ± 6.99 % for LTE_4. The absence of the fluid intake caused a concentrating of the monitored analytes to 162.7 ± 15.63 % for LTC_4, 161.3 ± 7.23 % for LTD_4 and 155.7 ± 14.50 % for LTE_4. Further to the humidity factor, the timing of the collection of EBC can affect the final detected concentration. The level of substances can be increased by 15 % during 18-20 hours without sleeping (valid for the sum of cys LTs) (Syslová et al., 2011).

EBC has been determined to have its pH in the range of 7.8 – 8.1 immediately after the collection; however it rapidly decreased (pH 6.0 – 6.5) due to the absorption of CO_2 from the ambient air, which could have been one of the causes for the consecutive changes occurring in this matrix after its collection (Effros et al., 2004). It was demonstrated that storing EBC under an inert atmosphere (argon) did not cause any change in pH; however this did not appear to be the main parameter with regards to the determination of some biomarkers concentration levels (e.g. leukotrienes, prostaglandins). The storage and the manipulation with the samples after the collection of EBC were the parameters capable of affecting the levels of the substances detected in EBC. The main risk factor was primarily the thermal and light instability of certain substances, in particular leukotrienes, prostaglandins, aldehydes and protein substances. For this reason, samples are required to be stored frozen at the temperature of -80 °C (or at -20 °C for up to one month). At this temperature, it was proven that the decomposition had been preserved and possible *in vitro* changes in the composition of the most important EBC asthmatic biomarkers had been prevented. Otherwise, the deteriorating changes started already at room temperature and took place in a short time interval (see section 4) (Syslová et al., 2008, 2011). A repeated freezing/thawing of the sample (freeze-thaw cycle was performed between -80 °C and laboratory temperature) had a negative impact on the stability of substances. Apparently, the critical point for the stability of the substances was the transformation of the sample from their solid to their liquid state (temperature of 0 °C). With regards to the lability of the biomarkers to a number of factors (described above), it is critical to respect them and prevent the circumstances that would otherwise had a negative effect on their precise determination. It is essential that an isotope-labelled internal standard (known amounts of deuterium labelled analytes to 1 mL of EBC) be added to the sample immediately after the collection. Performing the so-called "stable-isotope-dilution assay" allows a highly precise quantification as well as monitoring of changes in the sample composition, occurring during its processing.

3. Biomarkers of bronchial asthma present in exhaled breath condensate

Asthma is a chronic inflammatory disease of the airways characterized by reversible airways obstruction. Clinically, the severity of asthma is determined by evaluation of the expiratory flow rate and the symptoms. Smouldering inflammation is present even in patients with mild

to moderate asthma. Long-term presence of inflammation and repeated episodes of acute inflammation result in structural changes in the airways (airways remodelling), which might cause chronic airflow obstruction. It is apparent that molecular diagnostics based on the detection of inflammatory biomarkers facilitate the diagnosis earlier than clinical symptoms develop, and in parallel, the start of a therapy decreasing a potential harm to the patient. It also allows optimal monitoring of the inflammation and the disease activity. All the facts head to an improved asthma management beneficial to the patient as well as to the health system owing a predictable enhanced cost-effectiveness of the medical treatment.

In medicine, a biomarker is a term often used to refer to a substance (small/large size molecule), gene, cell, etc. measured in a biological matrix whose concentration reflects the severity or even presence of a certain disease state. More generally, a biomarker is anything that can be used as an indicator of a particular disease state or another physiological state of an organism. They may indicate either normal or diseased processes in the body. Although the term biomarker is relatively new, biomarkers have been used in pre-clinical research and clinical diagnosis for a considerable period of time (e.g. acetone present in breath as a sign of ketoacidosis, which may occur in diabetes, cholesterol as a biomarker and a risk indicator for coronary and vascular diseases, C-reactive protein (CRP) as a marker of inflammation, etc.). A biomarker is a parameter that can be used to measure the progress of a disease or the effects of its treatment. In molecular terms, biomarker is "the subset of markers that might be discovered using metabolomics, proteomics, genomics and other "-omics" or imaging technologies. Biomarkers also play a major role in medicinal biology. Biomarkers probably represent the future paradigmatic approach allowing us an early diagnosis, a more efficient disease prevention and monitoring of a drug response, a faster drug target identification etc.

Compound/Factor	**pH**
	H_2O_2
	Eicosanoids
	1) LTs: a) cys LTs = (LTC_4, LTD_4, LTE_4), b) LTB_4
	2) 8-isoprostane
	Nitrate/Nitrite, Nitrotyrosine
	Aldehydes (Malondialdehyde)
	Thiobarbituric acid reactive products (TBAR)
	Glutathione
	Adenosine
	Cytokines (IL-4, IL-6, TFN-α)
	Interferon-γ (IFN-γ)

Table 1. Inflammatory mediators detected in EBC in bronchial asthma

Collection and analysis of substances present in EBC procures a simple, non-invasive, real-time, point-of-care clinical and research tool for evaluating lung pathophysiology based on a molecular diagnostics approach. In the case of asthma, a chronic inflammatory disease of the airways, a row of biomarkers/factors present in EBC have been referenced (Table 1). Various inflammatory markers present in EBC have been investigated as possible asthma biomarkers.

The pH value of the airway lining fluid reflects the underlying homeostatic balance between acid and the base production of inflammatory cells and the buffering capacity of resident airways cells. EBC pH has been proved to be one of the robust biomarkers of asthma associated inflammation and resulting acid stress. In general, EBC pH values are lower in

asthmatics and well correlate with sputum eosinophililia, which is the hallmark of asthmatic airway inflammation, and resulting oxidative stress (Murugan et al., 2009). EBC pH is unstable at room temperature because CO_2 diffuses in and out of solution readily. Determination of EBC pH obtained after de-aeration with an inert gas (usually argon or nitrogen) has provided the most reproducible pH values to date (Kullmann et al., 2007). EBC pH measurement and its application to non-invasive assessment of asthmatic airway inflammation had first been received with optimism, but there have later been published several negative studies as well (Zhao et al., 2008; Ojoo et al., 2005). This fact may be explained by the proposition that EBC pH reflects asthma control more than it reflects asthma severity, and hence is unable to distinguish healthy subjects from asymptomatic quiescent asthmatics. In spite of these drawbacks, pH values of EBC posses established reference values in healthy subjects (normal median pH is 8.0 with interquartile 25 – 75 % range of 7.8 – 8.1) (Paget-Brown et al., 2006) and also shows a good reproducibility in asthmatics (the intra-class correlation ICC = 0.97) over a one-year period with minimal seasonal variation (Accordino et al., 2008). Asthma subjects demonstrate values with the median 7.4. Endogenous airway acidification has been assessed in asthma in many studies (Rozy et al., 2006; Ojoo et al., 2005; Hunt et al., 2000; Niimi et al., 2004). Regarding EBC pH values in non-deaerated samples, the mean values ranged from 6.0 to 7.4 (Brooks et al., 2006; Gessner et al., 2003). Nevertheless, EBC pH value after deaeration has been extensively validated and it has been found to represent a simple, robust and reproducible biomarker. Continuous EBC pH measurement may provide an option for monitoring of airways inflammation.

Hydrogen peroxide (H_2O_2) is one of the best asthma biomarkers ever studied. The activation of inflammatory cells in the airways leads to the production of superoxide anion (O_2^-), which undergoes spontaneous or enzyme-catalyzed dismutation to the less reactive H_2O_2. It has strong oxidizing properties which lead to further cellular injury. Thus, elevated values of exhaled H_2O_2 indicate airway oxidative damage. As hydrogen peroxide is unstable, it must be manipulated carefully. It has been determined spectrophotometrically (colorimetric assay) (Dekhuijzen et al., 1996), spectrofluorimetrically (fluorimetric assay) (Hyslop & Sklar, 1984; Ruch et al., 1983; Nowak et al., 2001), by a flow injection analysis with flurescence detection (Svensson et al., 2004), by a chemiluminescent method (Zappacosta et al., 2001) and with an amperometric biosensor (Ecocheck, Jaeger, Germany) (Gerritsen et al., 2005; Thanachasai et al., 2002). Besides pH, EBC H_2O_2 is the only other biomarker with established reference values in healthy subjects. The mean normal value is reported to be 0.13 μM, with a 2.5 – 97.5 % reference range of < 0.01 – 0.48 μM. In asthma subjects, they have been found to be elevated when compared to the healthy controls (Koutsokera et al., 2008). Elevated exhaled H_2O_2 levels enable distinction of asthmatics from controls and correlate with the disease severity and the lung function deterioration (Emelyanov et al., 2001). In parallel to H_2O_2 concentration, a reduction has been found in patients treated with inhaled corticosteroids (monitoring of pharmacotherapy). A series of studies have also shown correlations between airway senzibilization and H_2O_2 concentration levels. These findings suggested that exhaled H_2O_2 might be more useful than the FeNO test in monitoring asthmatic patients. However, poor reproducibility due to the high variability in values (including variability due to changes in flow) and poorer correlations in corticosteroid-naive asthma patients (Horváth et al., 1998) have been reported and thus the optimism with use of this marker has been moderated.

As mentioned, exhaled nitric oxide (NO) is an established marker of airway inflammation and is increased in patients with asthma. NO is a free radical due to its unpaired electron

and it may react with oxygen to yield nitrogen oxides (NO_x) or with superoxide anion to yield peroxynitrite, a highly reactive substance that may lead to the production of NO-derived products (Ricciardolo et al., 2006). NO-related products (nitrite, nitrate, nitrotyrosine and nitrosothiols) form some other groups of substances elevated in asthma patients compared to healthy controls (Kharitonov & Barnes, 2001). NO synthesis and release in the respiratory system has been determined indirectly by quantifying nitrite/nitrate, nitrotyrosine and *S*-nitrothiols in EBC. Nitrite/nitrate detection in EBC has been performed by the following methods: colorimetric assay (Griess reaction), fluorimetric assay (DAN reaction) (Marzinzig et al., 1997), chemiluminescence (Nguyen et al., 2005) and ion chromatography/conductivity detection (Tate et al., 2002). The normal values of nitrite/nitrate exhibited a significant discrepancy in the literature (Koutsokera et al., 2008) which is attributed to different assays used, efficiency of the method utilized for the reduction of nitrite to nitrate and NO_x present in the environment as a pollutant. Nitrite and/or nitrate elevated levels in asthma patients were determined (68 μM) and compared to healthy controls (9.6 μM) (Koutsokera et al., 2008). In contrast, a subsequent report showed no difference between concentration levels of nitrite/nitrate in asthmatic and healthy subjects (Kazani & Israel, 2010).

Several independent studies (Csoma et al., 2002; Montuschi & Barnes, 2002a; Koutsokera et al., 2008; Kazani & Israel, 2010) have indicated the presence of elevated levels of arachidonic acid derivatives (Cys LTs, leukotriene B_4 (LTB_4), 8-isoprostane) in EBC of asthma patients (Fig. 4). Therefore, measurements of these mediators are considered as potentially effective in the establishment of the diagnosis of asthma in a large cohort.

Inflammatory molecules called leukotrienes belong to the group of several substances released by mast cells during an asthma attack, and it is leukotrienes which are primarily responsible for bronchoconstriction. In chronic, more severe cases of asthma, general bronchial hyperreactivity (or smooth muscle twitchiness) is largely caused by eosinophils, which are attracted into the bronchioles by leukotrienes (and other chemoattractants) and which themselves also produce leukotrienes. Thus leukotrienes seem to be critical both in triggering acute asthma attacks and in causing a longer term hypersensitivity of the airways in chronic asthma. Leukotrienes are derived from arachidonic acid, the precursor of prostaglandins. There are two families of leukotrienes (LTB_4 and cys LTs – LTC_4, LTD_4 and LTE_4). The first group acts primarily in conditions in which the inflammation is dependent on neutrophils, such as cystic fibrosis, inflammatory bowel disease, and psoriasis. The second group (cys LTs) is concerned primarily with eosinophil and mast cell induced bronchoconstriction in asthma. They bind to highly selective receptors on bronchial smooth muscle and other airway tissue. Drugs have now been designed which can interfere with the activity of leukotrienes. Both leukotriene synthesis inhibitors and cysteinyl-leukotriene receptor antagonists have recently been shown to protect asthmatic patients against asthma attacks, but they are not useful as "rescue remedies" once an attack has already started. They act by preventing the relevant leukotriene release from mast cells and eosinophils or by blocking the specific leukotriene receptors on bronchial tissues, thus preventing bronchoconstriction, mucus secretion, and oedema. These drugs also reduce the influx of eosinophils and this way limit the inflammatory damage in the airway. For patients with moderate to severe asthma symptoms despite corticosteroid treatment, concomitant treatment with LT modifiers significantly improves asthma control.

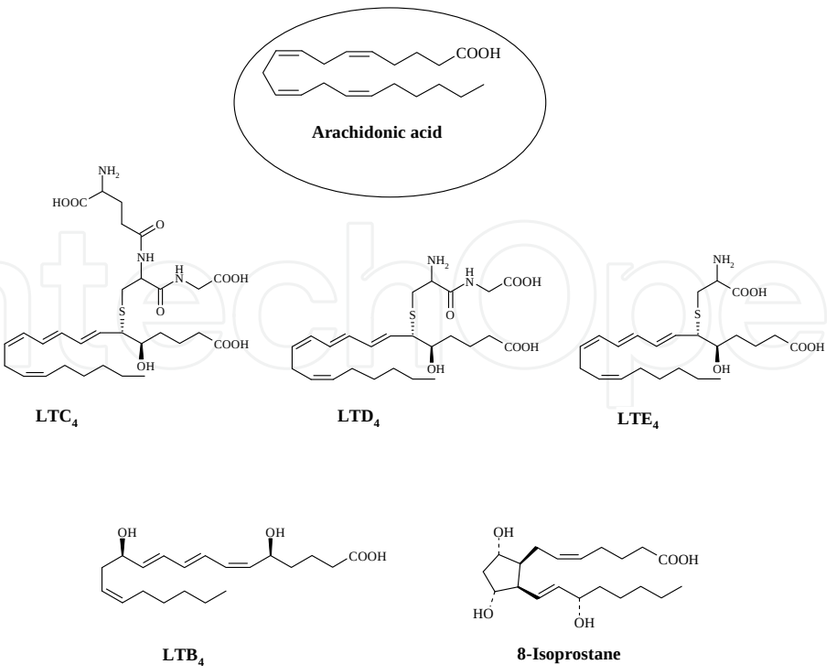

Fig. 4. Arachidonic acid derivatives – potential biomarkers of bronchial asthma

Leukotrienes, in particular cys LTs (LTC_4, LTD_4, LTE_4) have a potentially important role in asthma biomarker based diagnostics (Kazani & Israel, 2010). Concentrations of cys LTs in EBC are elevated in patients with asthma when compared to healthy controls. Their levels often decrease after LT directed anti-inflammatory therapy. The values of cys LTs in asthmatics and also controls are rather contradictory. On the other hand, the majority of works have found reasonably different values in asthma patients and healthy controls. The levels of LTB_4 and its relation to asthma are rather conflicting. Elevated levels in asthmatics were reported (97.5 pg/mL) when compared to the control (32.3 pg/mL) (Kostikas et al., 2005) and also by other authors (Csoma et al., 2002; Montuschi & Barnes, 2002a). On the other hand, no difference in the levels between well-controlled asthmatics (4.6 pg/mL) and controls (4.3 pg/mL) were referenced either (Carraro et al., 2005).

Isoprostanes are formed by the free-radical lipid peroxidation of arachidonic acid, representing *in-vivo* markers of oxidative stress (Janssen, 2001). The most studied is 8-isoprostane (8-iso prostaglandin $F_{2\alpha}$). Elevated levels of 8-isoprostane in asthmatics (42.4 pg/mL) when compared with controls (32.3 pg/mL) were observed by several authors (Montuschi, 2009; Zhao et al., 2008).

Several other biomarkers have been assessed in EBC, including adenosine (Csoma et al., 2005), aldehydes (Corradi et al., 2004), glutathione (Csoma et al., 2005), thiobarbituric acid reactive substances (TBARs) (Nowak et al., 1999), ammonia (Gessner et al., 2003), several cytokines, such as interleukin–4 (IL-4), interleukin–6 (IL-6), tumour necrosis factor-α (TNF-α), interferon-γ (IFN-γ) (Koutsokera et al., 2008) etc. However, it is believed that the pursuit of normal/pathological (asthma) concentration values for these biomarkers is not yet feasible and further studies focusing on standardization of measurement are needed in that direction.

4. Cysteinyl leukotrienes – The most prominent biomarkers of bronchial asthma

Leukotrienes (LTs) are a family of inflammatory lipid mediators synthesized from arachidonic acid ((5Z,8Z,11Z,14Z)-5,8,11,14-eicosatetraenoic acid) by a variety of cells (eosinophils, mast cells, basophils and macrophages). The name leukotriene is derived from their main producer, leukocytes, and from the specific structure of these substances, i.e. three conjugated double bonds. An overproduction of LTs is a major cause of inflammation in bronchial asthma. The biosynthetic pathway can be triggered in the organism by a variety of stimuli, including antigens, microbes, cytokines, immune complexes, and toxins. These stimuli activate signal transduction cascades that in turn activate LTs, forming enzymes, i.e. phospholipase and lipooxygenase (Peters-Golden & Brock, 2003).

Arachidonic acid is a twenty-carbon polyunsaturated ω-6 fatty acid bound in the cell membrane phospholipids, mostly in phosphatidylinositol in the C_2 position. Arachidonic acid is released into the organism by the enzyme phospholipase (Fig. 5 and Fig. 6). The phospholipase pathway is active in leukocytes, including mast cells, eosinophils, neutrophils, monocytes, and basophiles. The split of arachidonic acid from phospholipids may occur in three ways (Fig. 6). The first direct pathway, producing arachidonic acid and lysophospholipid, is catalyzed by phospholipase A_2.

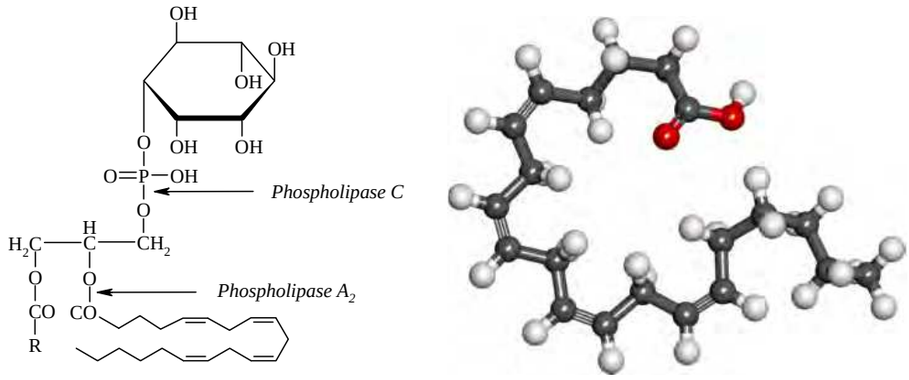

Fig. 5. Phosphatidylinositol with arachidonic acid in the most common position (C_2) with arrows pointing at hydrolyzes sites for phospholipase enzymes (left); arachidonic acid structure (right)

The other two metabolic processes lead to an intermediate, 1,2-diacylglycerole, which is produced by the action of phospholipase C enzyme. 1,2-diacylglycerol can be split into arachidonic acid and monoacylglycerols by diacylglycerollipase. In addition, diacylglycerolkinase can also convert it to phosphatidic acid which is further split by phospholipase A_2 to arachidonic acid and lysophosphatidic acid. In the organism, arachidonic acid is metabolized by three reaction ways: (1) it is transformed to leukotrienes by 5-lipoxygenase enzyme (5-LO); (2) it is converted to prostaglandins by cycloxygenase and (3) it is nonenzymatically converted to isoprostane (arachidonic acid is attacked by reactive oxygen species). Leukotrienes are synthesized in the cells from free arachidonic acid by the action of 5-lipoxygenase enzyme. The catalytic mechanism involves the insertion of an oxygen species at the C-5 position of the carbon skeleton of arachidonic acid to form an intermediate 5-HPETE (5-hydroxyperoxy-6,8,11,14-eicosatetraenoic acid) which is

spontaneously reduced to 5-HETE (5-hydroxy eicosatetraenoic acid). The enzyme 5-LO participates in the next step of the transformation of 5-HETE to the reactive epoxide leukotriene A_4 (LTA_4). The efficient utilization of endogenous arachidonate by 5-LO requires activation of a protein termed "5-lipoxygenase activating protein" (FLAP).

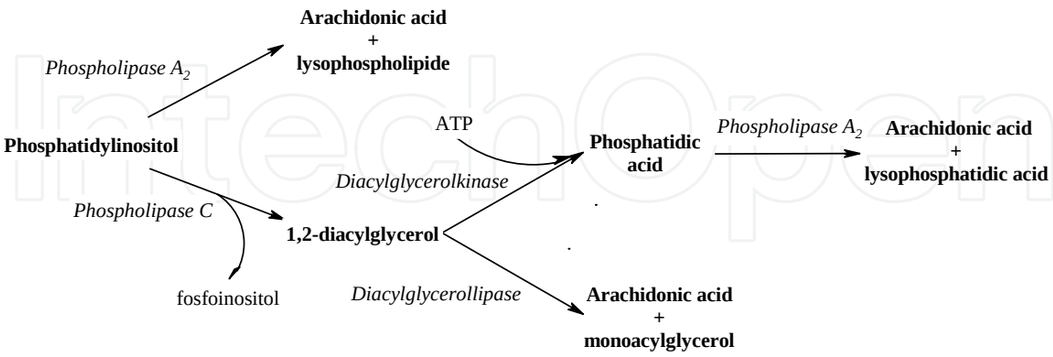

Fig. 6. Different pathways of generation of arachidonic acid from membrane phospholipids

LTA_4 can be further transformed by one of two possible enzymatic pathways. During inflammation, the levels of LTB_4 are elevated by the action of LTA_4 hydrolase, while the second pathway is dominant during allergic reactions and produces cys LTs. The first member of the family of cys LTs, LTC_4, is produced by coupling of LTA_4 and reducing glutathione. This transformation is catalysed by LTC_4 synthetase. The next members of the cys LT series (LTD_4 and LTE_4) are produced by a gradual transformation occurring sequentially ($LTC_4 \rightarrow LTD_4 \rightarrow LTE_4$) by a consecutive action of γ-glutamyl transpeptidase enzyme ($LTC_4 \rightarrow LTD_4$) and dipeptidase ($LTD_4 \rightarrow LTE_4$) (Montuschi, 2004) (Fig. 7). The biological effect of cys LTs is mediated *via* specific receptors classified as Cys-LT receptors. Their two subtypes, Cys-LT_1 and Cys-LT_2 receptors, are mainly located on smooth muscle cells, eosinophils and other cells throughout the body. Binding of leukotrienes to Cys-LT_1 receptors located in the lungs and the airways leads to the constriction of bronchi and bronchioles. It can also cause mucosal oedema and an increased secretion of viscous mucus, which leads to narrowing of the airway lumen and repetitive episodic states of dyspnoea expiratory wheezing. The Cys-LT_2 receptor is responsible for the constriction of blood vessels in the lungs (Barnes & Smith, 1999; Izumi et al., 2002). These effects can even be caused by very low concentrations of cys LTs with values around 10 mol/L. In excess, cys LTs can induce anaphylactic shock. (Brocklehurst, 1960). For this reason, cys LTs are known as "slow reacting substances of anaphylaxis (SRS-A)"

In order to properly handle and monitor cys LTs in body fluids, the knowledge of their physico-chemical properties is of vital importance. The temperature and light stability in different environments (e.g. EBC, solvents) is a crucial point for an analytical/diagnostic method development. Although the effect of daylight on the stability of cys LTs was experimentally tested, none was actually found. On the contrary, temperature was found to be the parameter with the most significant effect on the stability of cys LTs. It was demonstrated that the composition of EBC with regard to cys LTs was rapidly changed at ambient temperature (25 °C) as a result of (1) their enzymatic inter-conversion and (2) their limited temperature stability. Fig. 8 depicts the behaviour of cys LTs in EBC and in organic solvents (mixture of acetonitrile and water 70 : 30 – v/v was used as the mobile phase in LC-MS) where

the negative effect of temperature (25 °C) on the composition of individual cys LTs was demonstrated. In the matrix of EBC, besides the degradation of individual substances (very apparent in the mobile phase), inter-conversion of individual cys LTs was observed in the series of $LTC_4 \rightarrow LTD_4 \rightarrow LTE_4$, evidently catalyzed by the present enzymatic systems. At the temperature of -80 °C, EBC samples could be stored without any detectable change in the cys LTs content for a period of 3 months (similarly, the temperature of -20 °C seemed to be acceptable) (Ohanian et al., 2010). On the other hand, the temperature of 4 °C was not sufficient for the storage of the EBC sample. Although the inter-conversions as well as the degradations were slowed at this temperature (in comparison to 25 °C), the degradations of all the studied biomarkers still occurred in a period of hours in both experimentally studied matrices (EBC, organic solvent). Effects of sample handling, time and storage conditions on cys LTs in EBC have also been studied by other authors (Ohanian et al., 2010; Beyer, 1987).

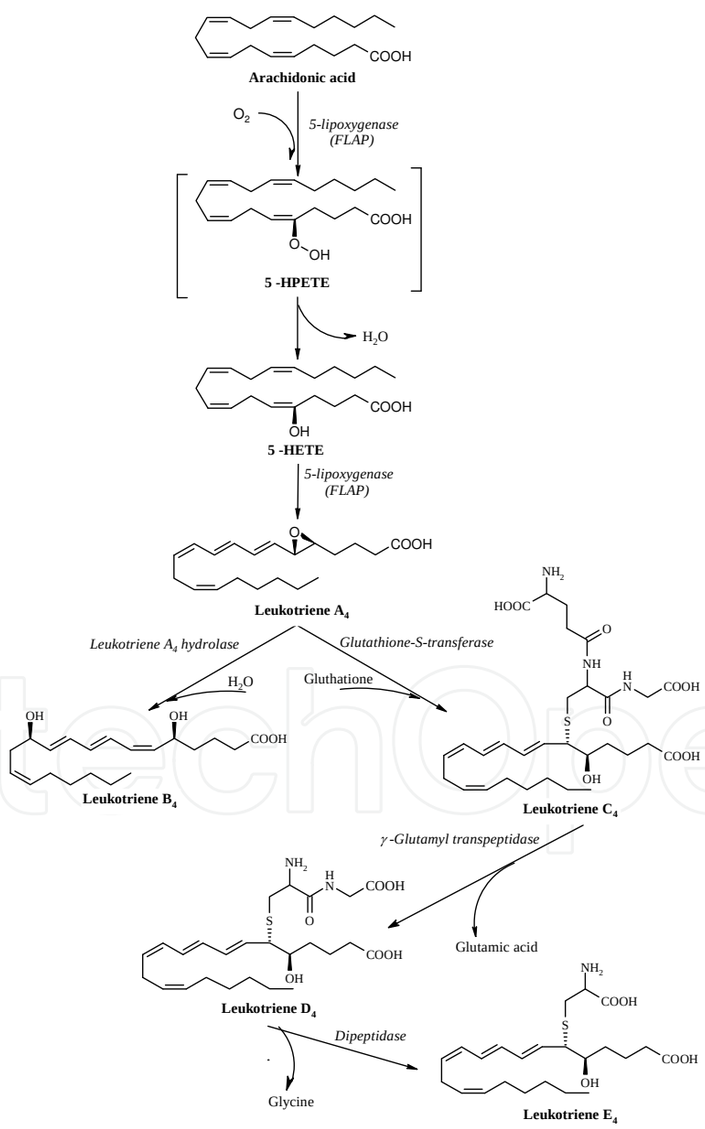

Fig. 7. Synthesis of leukotrienes

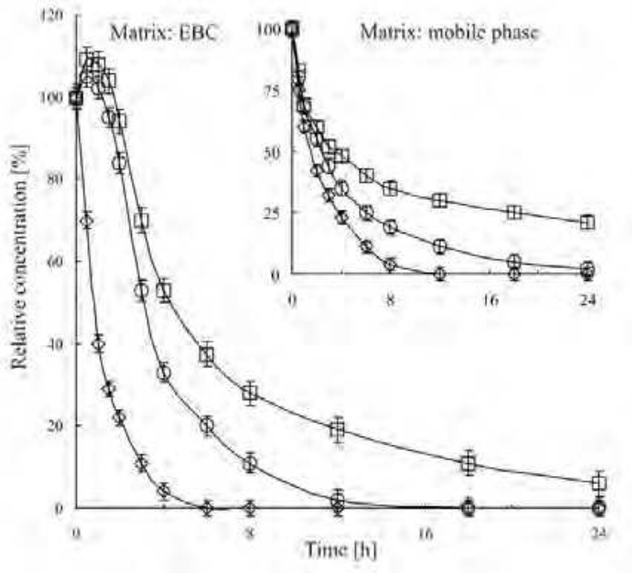

Fig. 8. Stability of cys LTs in EBC matrix and in organic solvent; LTC_4 (◇), LTD_4 (○) and LTE_4 (□)

It was also essential to map the behaviour of the biological system with respect to the production of cys LTs during the circadian biorhythm. The intra-day physiological variations were determined by analyzing EBC samples collected from volunteers (age 18 ± 1, non-smokers, without the diagnosis of bronchial asthma). EBC samples were obtained at different times throughout the day (6, 12, 18 and 24 h). An identical trend was demonstrated in the concentration profiles of cys LTs in all subjects, increasing during the day and returning to its original level during the night. The level of cys LTs increased by an average of 15.4 %. The inter-day physiological variations of the method were assessed by analyzing samples of healthy volunteers obtained from each subject in one week period. Regarding the inter-day physiological variations in a group of 10 healthy people, the total level of cys LTs did not differ by more than 6.6 % for each individual over a period of 5 consecutive days. The clinical studies should respect the circadian biorhythm and collect EBC clinical samples in a similar day-time (e.g. all samples should be collected between 8 and 12 a.m.).

5. Methods for determination of cysteinyl leukotrienes

Since the discovery and elucidation of the structures of leukotrienes and other eicosanoids (prostaglandin and thromboxane) and their metabolites, numerous analytical techniques have been developed for their analysis in complex biological matrices. Various publications have described the determination of leukotrienes in the blood plasma (Shindo et al., 1997; Henden et al., 1993) urine (Armstrong et al., 2009; Misso et al., 2004; Higashi et al., 2004), EBC (Syslova et al., 2011; Brussino et al., 2010; Biko et al., 2010) and saliva (McKinney et al., 2000; Tufvesson et al., 2010). At present, immunochemical methods are used for the quantitative determination of leukotrienes formed *in vivo*, namely Enzyme Linked Immunosorbent Assay (ELISA, sometimes also denoted as Enzyme Immunoassay – EIA) (Samitas et al., 2009; Csoma et al., 2002; Baraldi et al., 2003; Antczak et al., 2002; Chappell et

al., 2011) or instrumental analytical methods based on gas or liquid chromatography (GC, LC) coupled to a variety of detectors, preferentially mass spectrometer (MS), (Sanak et al., 2010; Čáp et al., 2004; Montuschi & Barnes, 2002b; Syslová et al., 2011) but also ultra-violet detector (UVD), fluorescence detector (FLD), or electrochemical detector (ECD).

Immunochemical methods offer simple, rapid, robust yet sensitive, and easily automated methods for routine analyses in clinical laboratories. Immunoassays are based on highly specific binding between an antigen and an antibody. An epitope (immunodeterminant region) on the antigen surface is recognized by the antibody's binding site. The type of antibody and its affinity and avidity for the antigen determines the sensitivity and specificity of the assay. Depending on their format, immunoassays can be qualitative or quantitative. They are able to measure low levels of disease biomarkers and therapeutic or illicit drugs in a patient's blood, serum, plasma, urine, or saliva.

Antigen is a substance of natural or synthetic (artificial) origin. When exposed to antigens, the immune system reacts by synthesis of antibodies in plasmatic cells (lymphocytes). The antibodies then bond to the antigen molecules through weak interactions (hydrogen bonds, non-polar hydrophobic interactions, van der Waals forces, Coulomb forces, London dispersion attractive forces and steric repulsive forces) and block the antigen effects. In the organism, antibodies are represented by glycoproteins denoted as immunoglobulins (Ig). The Y-shaped immunoglobulin molecule consists of two identical heavy H-chains and two light L-chains (Fig. 9). The heavy chain is connected to the light chain by one disulfide bridge, while the heavy bridges are interconnected by multiple disulfide bridges. Apart from these, each chain has its own intrachain disulfide bonds (Daussant & Desvaux, 2007).

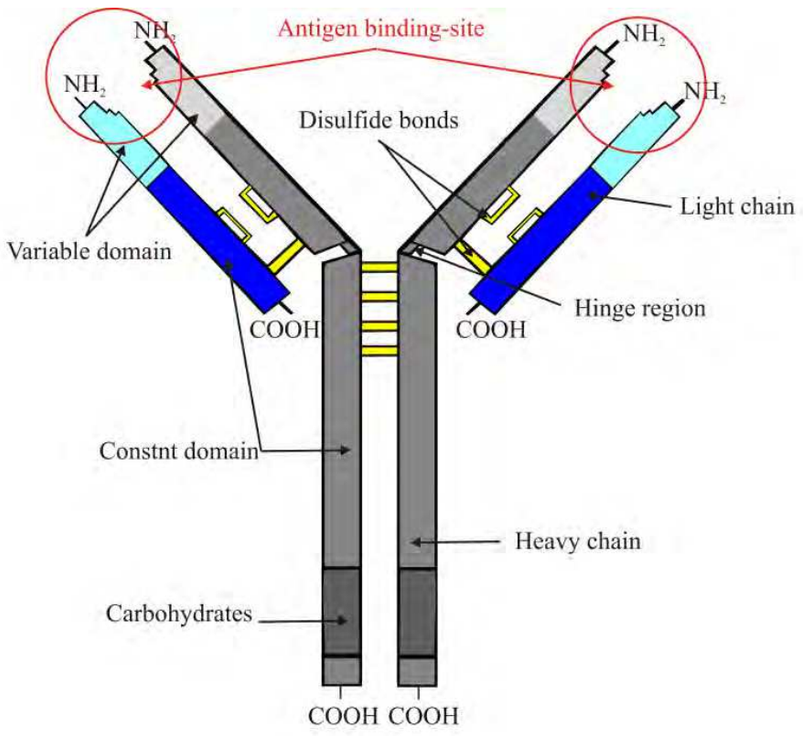

Fig. 9. Structure of an immunoglobulin monomer.

According to the types of H-chains, immunoglobulins can be divided into five main groups: IgA, IgD, IgE, IgG a IgM. The most abundant in the blood serum are IgG, representing about 75 % of the total Ig amount. Both heavy and light chains can be subdivided into their functional domains: while the part closer to the amino-end is termed variable (V), the fragment containing a carboxyl group is said to be constant (C). The variable (heterogeneous) moiety forms a unique part of immunoglobulin which is complementary to antigen *via* a highly specific key-lock mechanism between the antigen and antibody. The immunoglobulin molecules can be cleaved into so-called hinge regions by the action of papain (a plant enzyme) and it is thus possible to split the antibody molecule into three parts: two Fab-fragments containing arms (i.e. the entire light chain and a part of the heavy chain) and one crystalline Fc-fragment. The latter contains the remaining parts of both H-chains interconnected by disulfide bridges. The Fab-fragments allow the bonding of antigens and the Fc-fragments bond to receptors on the surface of leukocytes or can be employed in antibody immobilization upon various surfaces without losing the functionality of the antigen binding place (immunochemical methods) (Daussant & Desvaux, 2007).

ELISA (Enzyme Linked Immunosorbent Assay), also called EIA (Enzyme ImmunoAssay), belongs to the most frequently used methods applicable in the quantitative analysis of antigens. This method comes in a range of modifications which are all based on a highly specific interaction of antigen and antibody. One of these binding partners is covalently bound to an enzyme (usually peroxidase, acetylcholinesterase or alkaline phosphatase) whose role is the catalytic conversion of the added substrate to a coloured product. The colour intensity, determined spectrophotometrically or fluorimetrically, directly or indirectly reflects the amount of antigen present in the sample. A common attribute of all ELISA methods is the immobilization (*via* adsorption or a covalent bonding) of the antibody (when the antigen is determined) on a solid support such as microtiter plate, which facilitates the separation of immunochemically bond molecules. Either direct or direct sandwich ELISA can be utilised for the antigen detection (Fig. 10). In direct ELISA methods, antigens (e.g. cys LTs as biomarkers of bronchial asthma) from biological matrices (EBC, blood plasma, urine) bind to the immobilized antibodies (mouse monoclonal anti cys LTs IgG). Direct ELISA relies upon competitive binding of cys LTs from the biological sample and cys LTs bound to an enzyme (acetylcholinesterase). After the incubation period (usually overnight), during which the antigen-antibody complex is formed, sample removal and washing of the complex, a mixture of substrates (acetylcholine and 5,5′-dithio-bis(2-nitrobenzoic acid) is added. Acetylcholine is cleaved by an enzyme to thiocholine, further reacting with 5,5′-dithio-bis(2-nitrobenzoic acid) and yielding yellow 5-thio-2-nitrobenzoic acid, which can be determined spectrophotometrically. In sandwich ELISA, only cys LTs contained in the biological sample bind to the anchored antibodies. Colour assignment is allowed as a result of the substrate conversion by the enzyme linked to free antibodies against cys LTs added to the solution. The antibodies with enzyme form a sandwich complex with anchored cys LTs (the antibody captured with an enzyme uses other interaction with the antigen than the immobilized antibody). As in the direct method, enzyme cleaves the substrate into a detectable coloured product.

Radioimmunoassay (RIA) works on a similar principle as the direct competitive ELISA where the main difference lies in the use of a labelled antigen. The enzyme on antigen is replaced by a tyrosine moiety containing a γ-radioactive iodine isotope. The γ-radiation is

then monitored for the non-bonded labelled antigen present in the sample. However, operating with radioactive species can only be carried out at specialized facilities with appropriate equipment which represents a relevant disadvantage and explains the less frequent utilization of RIA in practice. At present, this method is not used for the determination of cys LTs (Lindgren et al., 1983).

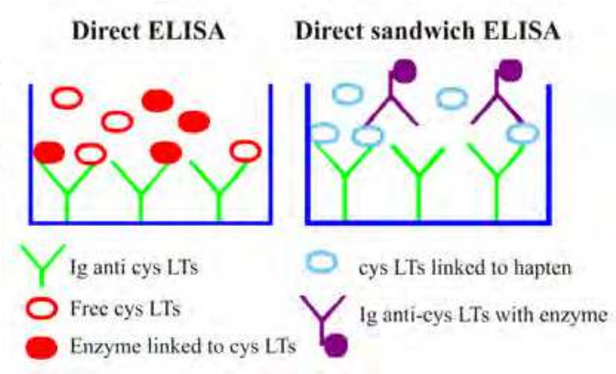

Fig. 10. The principles of direct ELISA and direct sandwich ELISA

Gas chromatography coupled with mass spectrometry (GC-MS) can be used for the analysis of leukotrienes giving information both about their structures and quantities. This analytical method takes advantage of its (1) high separation selectivity determined by the type of capillary columns used and (2) high specificity and sensitivity enabled by the integration of a mass spectrometric detector. Therefore, GC-MS method allows the quantification of substances in biological matrices on ng/mL-level. The most significant disadvantage is the need of a sufficient volatility and thermal stability of analytes in the sample, which is not the case of leukotrienes. To resolve it, pre-treatment procedures (extraction and derivatization) are necessary to be included in this particular case prior to quantitative and qualitative analysis. Derivatization is a chemical reaction of an analyte with a suitable derivatization reagent which changes its physical and chemical properties (in this case mainly volatility and thermal stability). Additionally, derivatization prior to a GC-MS analysis is carried out to improve the sensitivity of MS detection by enabling a better fragmentation in the detector. Leukotrienes contain several functional groups which can be employed in the derivatization reaction. The following are among the most popular: esterification of the carboxyl group, etherification of the hydroxyl group or hydrogenation of the double bonds. However, when cys LTs are hydrogenated on a rhodium catalyst (reaction time 20 min, temperature 0 °C), the cysteinyl moiety is cleaved off by a parallel desulfuration and thus only a sum of cys LTs can be determined by a GC-MS analysis. (Fauler et al., 1991; Balazy & Murphy, 1986). When other reactions are utilized, such as esterification by ethyl chloroformate (Čáp et al., 2004), pentafluorobenzyl ester-trimethyl silyl ether (PFB-TMS) (Awad et al., 1993; Ferretti et al., 1992), pentalfuorophenyl diazoalkanes (Hofmann et al., 1990), or etherification by *N*,*O*-bis(trimethylsilyl) trifluoroacetamide (BSTFA), followed by suitable extraction steps, individual cys LTs can be quantified by this method. Although there are only few different derivatization reactions used together with the analysis of leukotrienes by GC–MS, many variations of these reactions have been reported with regards to the type of a solvent, amount (excess) of derivatizing agent, temperature, reaction time and the storage of the samples to be injected into the GC–MS.

High Performance Liquid Chromatography (HPLC) in combination with mass spectrometry is generally used for the analysis of low volatile and thermally labile substances and thus it is highly suitable for the analysis of leukotrienes. The high selectivity of separation is achieved by a suitable choice of chromatographic phase systems, i.e. the liquid and stationary phase. Reversed-phase HPLC is the most commonly used with the stationary phase consisting of silica gel modifiable by non-polar octadecyl groups and the polar liquid phase usually consisting of water, acetonitrile or methanol, optionally with addition of buffers. For the detection, usually UV, fluorescence, electrochemical or MS methods are used. Nowadays, the combination of HPLC and MS allows facile separation and parallel detection of even very low analyte concentrations present in complex matrices. Since the remaining detectors mentioned above do not allow the quantification of analytes and lack the high specific structural information, HPLC-MS is becoming the first choice method for the analysis of substances in biological matrices. Therefore, the analysis of complex body fluids on a picogram scale is viable using HPLC-MS and also suitable for the future routine practice. In order to increase the detector precision and sensitivity, the following is advisable prior to the HPLC-MS analysis: (1) addition of an isotopically labelled internal standard and (2) use of a pre-treatment method (immunoextraction, solid phase extraction and lyophilisation) to remove undesired species and concentrate the sample.

When an MS detection is utilized, the analytes need to be evaporated and ionized. As this can be carried out at atmospheric pressure (API – atmospheric pressure ionization), it is also feasible with thermally labile substances (leukotrienes). Electrospray ionization (ESI) is one of the most frequently used API techniques. It belongs to the soft ionization techniques characterized by the preservation of a molecular ion peak with minimal fragmentation of the analyzed molecule. Depending on the molecule charge of a measured analyte, two measurement modes can be distinguished, i.e. positive electrospray ionization (ESI^+) in which protonated molecular ion $[M+H]^+$ is produced and negative electrospray ionization (ESI^-), where the molecule is deprotonated $[M-H]^-$. Ionization of cys LTs is possible in both modes but the $[M-H]^-$ ion is preferred especially because of a higher response in the detector. The combination of ESI ionization and a triple-stage quadruple analyzer (TSQ) is a suitable detection technique for the quantification of the given analytes. The first and the third quadruples (Q1 and Q3) are identical and capable of using the same scan modes. On the contrary, the second quadruple (Q2) is different both in its construction and function, allowing fragmentation of the analyte upon elastic collision with an inert gas (argon). Therefore, it is often referred to as the collision cell. A mass spectrometer equipped with a triple quadruple uses a highly selective single reaction monitoring mode (SRM) for the quantification and structural identification of substances. In the case of cys LTs, Q1 isolates the deprotonated $[M-H]^-$ molecular ions (LTC_4 m/z = 624 Da, LTD_4 m/z = 495 Da and LTE_4 m/z = 438 Da, see Fig. 11 – MS/MS spectra of cys LTs), which are further used as precursor ions for the subsequent collision-induced dissociation (CID) in Q2. In the collision cell, the molecule selectively degrades and yields product ions which are analyzed on quadrupole Q3, giving MS/MS spectra. For the quantification of cys LTs, the following SRM transitions are used: LTC_4 - 624.1 → 351.2 (collision energy 27eV); LTD_4 - 495.2 → 477.3 (21 eV) and LTE_4- 438.2 → 333.1 (19 eV).

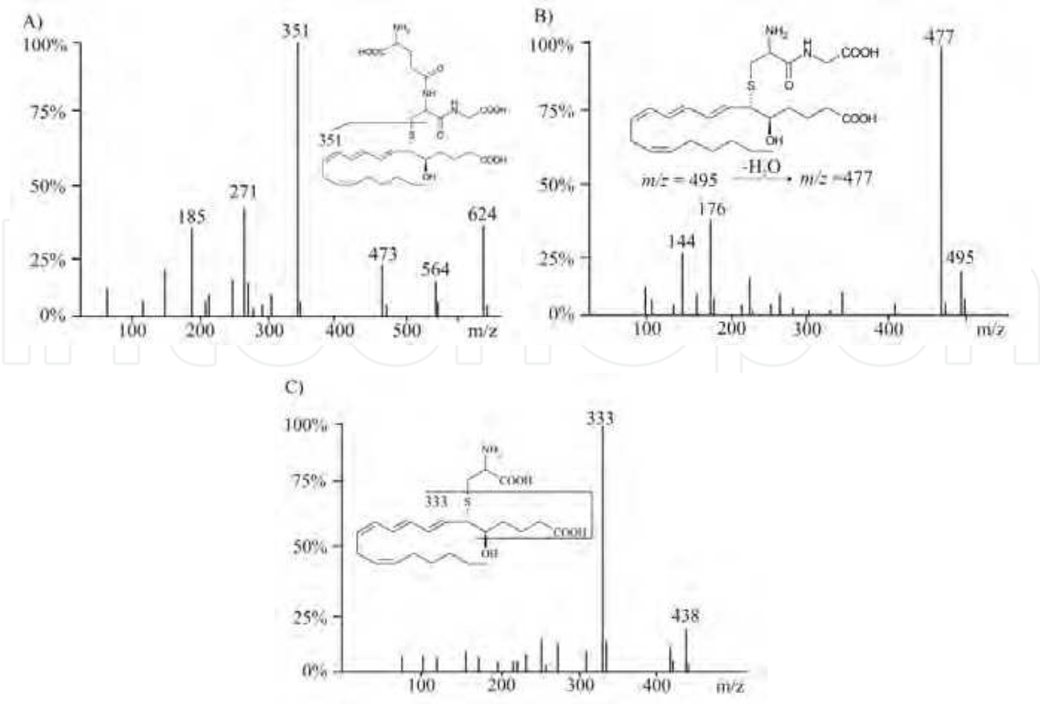

Fig. 11. ESI- collision spectra of cys LTs (A –LTC_4, B-LTD_4, C-LTE_4)

Metabolomic techniques ("breathomics"), based on nuclear magnetic resonance (NMR), have also been applied in the EBC analysis, leading to the characterization of the biochemical fingerprints of airways (Carraro et al., 2007). Selected signals from NMR spectra offered a slightly better discrimination (linear discriminant analysis (LDA) of approximately 86 %) for asthmatics *versus* healthy children, as compared to the success rate of approximately 81 % for the combination of exhaled nitrogen oxide (ENO) and the forced expiratory volume at one second (FEV_1). The selected NMR variables were derived from the region of 3.2 – 3.4 ppm, indicative of oxidized compounds, and from the region of 1.7 - 2.2 ppm, indicative of acetylated compounds.

Introduction of internal standards may increase the precision of a quantification analysis. To achieve this, it is desirable to select a substance with physical and chemical properties identical to the observed endogenous cys LTs. It should act in the same manner not only during the detection but also in all preceding pre-treatment steps (extraction, derivatization). With some methods (ELISA, RIA), inner standards cannot be used as they are not separable from the endogenously present substance. Other methods (LC with UV, fluorescence or electrochemical detection) require using substituted analogues of cys LTs such as derivatized cys LTs. However, the use of these substances only partially resolves the drawbacks of separation and quantification methods. A successful MS detection requires the usage of ideal internal standards meeting the condition of identical physical and chemical properties and differing only in their molecular weight by the substitution of ^{1}H, ^{12}C or ^{16}O by corresponding stable isotopes ^{2}H, ^{13}C or ^{18}O. The minimal difference in the molecular weight between an endogenous cys LT and an isotopically labelled cys LT is 3 Da. Cys LTs labelled with ^{2}H and ^{13}C are nowadays commercially available and easily used in the quantification of cys LTs (Syslová et al., 2011; Montuschi, 2009).

6. Pre-treatment methods used for isolation and concentration of biomarkers

With only minor exceptions, the first step of analytical methods (including LC-MS, ELISA) in analysis cys LTs is their extraction from a biological matrix (EBC, urine, plasma, cerebrospinal fluid etc.). In principle, only few extraction techniques have been reported for the separation of cys LTs from EBC samples. These methods include immunoaffinity extraction (IAE) (Syslová et al., 2011), solid phase extraction (SPE) (Mizugaki et al., 1999; Ohanian et al., 2010; Chappell et al., 2011; Yang et al., 2009; Debley et al., 2007; Syslová et al., 2011), lyophilization (LYO) (Debley et al., 2007; Syslová et al., 2011) and solvent extraction (Huwyler & Gut, 1990). The applied methods varied primarily in their selectivity related to the given analytes. IAE showed the highest selectivity for cys LTs, which was determined by the highly specific reaction between the antigen (cys LTs) and the antibody (mouse monoclonal antibody against leukotriene C_4, D_4 and E_4 covalently bound to Sepharose 4B). This highly specific interaction not only enabled the separation of organic metabolites from salts contained in EBC, which was similar to SPE, but also cys LTs from other compounds present in EBC and especially from arachidonic acid derivatives. Identical specificity was achieved when immunoaffinity columns with antibodies immobilized to cys LTs were utilized. When SPE was used, the salts were eliminated together with potentially present other substances with a markedly different retention factor at the applied stationary phase of the SPE column (i.e. the eliminated substance was not retained within the stationary phase or it could have remained sorbed on it during the elution of cys LTs). The substances with a similar retention index (e.g. LTB_4, isoprostanes, thromboxanes) were contained in the solution together with cys LTs even after the SPE. For the separation of cys LTs, various stationary phases have been described in a number of papers including octadecyl (C18) (Syslová et al., 2011; Mizugaki et al., 1999) and various other kinds of copolymers (Ohanian et al., 2010; Chappell et al., 2011; Yang et al., 2009; Debley et al., 2007). SPE method enabled to supplement a less precise method of separation, i.e. the liquid–liquid extraction using the following solvents: water/propan-2-ol/dichloromethane (Huwyler & Gut, 1990; Clancy & Hugli, 1983). The liquid-liquid extractions are time-consuming since multi-step extractions may be needed in some cases or large volumes of organic solvents may be necessary for an efficient extraction. In addition, liquid-liquid extraction has not yet been demonstrated to be efficient for leukotrienes (Salari & Steffenrud, 1986). LYO is a useful method for concentrating non-volatile (or slightly volatile) substances dissolved in water. Cys LTs contained in EBC fall into this category. During the freeze-drying of the sample, no temperature stress of labile cys LTs occurred, which could have led to preferring freeze vacuum drying over IAS, SPE and solvent extraction (in this case, samples, when stored, had to be defrosted and processed at laboratory temperature). However, in contrast to this temperature-related advantage, there was no selectivity at all for the given analytes resulting in a deteriorated ionization during the mass-spectrometric determination even with the preceding liquid chromatography step. Apparently, this was caused by the presence of large quantities of substances including salts (Debley et al., 2007; Syslová et al., 2011). The methods of SPE, solvent extraction and LYO cannot eliminate possible analyte isomers potentially affecting the detectable level of cys LTs and thus there are higher requirements on the analysis, especially on the chromatographic part of the method.

Using the pre-separation methods based on antigen immunoextractionis connected with only minor errors and more accurate substance quantification is achieved (Table 2). Commercially available immunoaffinity sorbents and columns are burdened with a number

of drawbacks, such as (1) time consumption (processing one sample takes approx. two hours), making the integration of non-compliance with the stability of cys LTs at room temperature more difficult, (2) the method labour input (in sorbents, it is especially difficult to separate EBC from an immunoaffinity sorbent and it is necessary to perform a series of mechanical operations, i.e. vortexing and centrifugation) and (3) limited repetitive use (the binding capacity in immunoaffinity columns is rapidly deteriorated by regeneration as a consequence of washing of antibodies from the support, while immunoaffinity sorbents exhibit a poor mechanical separation from an aqueous environment since boundless layers of water–sorbent are produced).

Analyte	Precision RSD (%)	Accuracy RE (%)
IAS		
LTC_4	9.7	-9.6
LTD_4	8.7	-8.0
LTE_4	8.1	-7.0
SPE-C18		
LTC_4	10.1	-10.4
LTD_4	9.2	-8.5
LTE_4	8.7	-8.2
LYO		
LTC_4	11.2	-14.2
LTD_4	9.9	-11.5
LTE_4	9.7	-9.3

Table 2. Validation parameters (precision and accuracy) for various pre-treatment methods (IAS, SPE and LYO) combined with LC-MS (n = 5)

Efforts to immobilize antibodies on more suitable supports by producing magnetic immunoparticles or encapsulating antibodies to polymeric matrices (e.g. polyvinyl alcohol) strive to minimize the above mentioned drawbacks. The very basic element of every magnetic immunoparticle (Fig. 12) is the magnetic core (1) composed of a magnetic material (e.g. magnetite, maghemite, cobalt, nickel, etc.). The magnetic core is ensphered in a functionalized outer coating (2), which usually stabilizes it (e.g., chitosan, carbon, polyethylene glycol, polyvinyl alcohol, etc.). On the surface, a cross-linker (3) is commonly bound (glutaraldehyde, carbodiimide, hydroxymethyl phosphine, etc.), which allows anchoring of an antibody. Such a prepared immunoparticle can be used for the separation of biomarkers from complex matrices. The produced antigen-antibody complex is separated from the solution using an external magnetic field (Fig. 12). The covalent bond between a magnetic particle and an antibody allows carrying out the regeneration of immunoparticles without any significant loss of the binding capacity.

By encapsulating antibodies to polyvinylalcohol (PVA) hydrogel (consisting of a mixture of two polymers: PVA with the function of a supporting matrix and polyethylene glycol (PEG) as a plasticizer) and producing porous immunolenses (Fig. 13), a rapid, accurate and very easy-to-handle tool is achieved for the separation of biomarkers from EBC. It was found experimentally that even after a repeated regeneration, no reduction occurred in the binding capacity of the immunolenses.

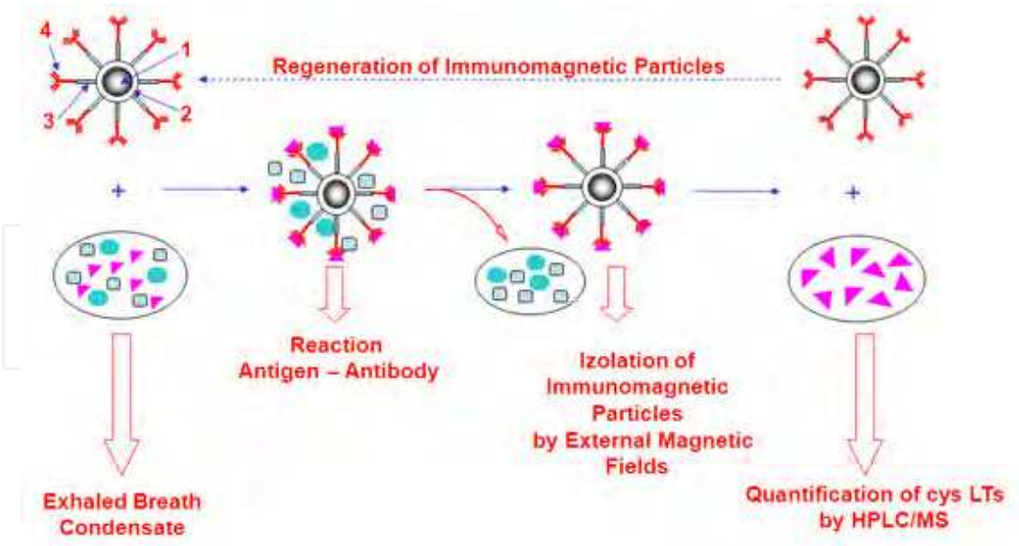

Fig. 12. Scheme of magnetic immunoparticles and their utilization for separation of biomarkers from EBC

Fig. 13. Polymeric immunoparticles with encapsulated antibodies

7. Detection based on mass spectrometric methods and comparison with others

The following analytical methods are currently used for the quantitative determination of cys LTs in EBC: EIA (ELISA), HPLC-MS and GC-MS. These individual methods can be optimally compared on the basis of method validation parameters (including pre-treatment methods and derivatization), i.e. precision, accuracy, limit of detection (LOD) and limit of quantification (LOQ), which are listed in the table 3. The least utilized method is the derivatization of cys LTs with a consecutive GC-MS detection (Čáp et al., 2004). The method is accompanied by several shortcomings: (1) the choice of derivatization reagent (ethylchloroformate) which causes the degradation of LTC_4, while the referenced authors provide that LTC_4 is not quantified in clinical studies, even though its presence in EBC has been demonstrated using LC-MS method; (2) unsuitable use of an internal standard, i.e. arachidonic acid, which is endogenously present in EBC and its physico-chemical properties are significantly different from cys LTs for both its derivatization step and the very GC-MS analysis; (3) GC-MS analysis using thermal transfer of substances to the gas phase, which is

not an appropriate method for the thermally labile cys LTs. In the literature, we have encountered different LOQs for immunochemical methods, while other validation parameters have not been provided (owing to the manufacturer's validation of an EIA/ELISA kit being applicable only to a urine matrix). Using immunochemical methods with a monoclonal antibody for cys LTs, only Σ cys LTs can be determined (contrary to LC-MS methods permitting precise quantifications of individual leukotriene levels). Furthermore, with this method, one can encounter a false positive or negative effect on the results owing to a cross-reaction with other substances contained in EBC. The specificity of antibodies against cys LTs are different and vary with each EIA and ELISA kit. We have encountered, for instance, the following values in immunoglobulin specificity: 1) LTC_4 – 100 %, LTD_4 – 100 %, LTE_4 – 79 %, LTD_5 – 61 %, LTC_5 – 54 %, LTE_5 – 41 %, N-acetyl LTE_4 = 10.5 % (Cysteinyl Leukotriene EIA kit, Cayman Chemical Company, Ann Arbor), 2) LTC_4 – 100 %, LTD_4 – 100 %, LTE_4 – 65 %, N-methyl LTC_4 – 124 %, LTB_4 - < 0.01 % (Cysteinyl Leukotriene Express EIA kit, Cayman Chemical Company, Ann Arbor). Such variable values of specificity may be the source of inconsistent results reported in the literature projected in the values for clinical studies. Due to antibody's low specificity, acquired structural (qualitative) information cannot be selectively attributed to the detected cys LTs using EIA. The determined concentration of cys LTs in EBC are significantly lower using immunochemical methods than using LC-MS. This may be due to disrespecting the thermal stability of the analyte, because the reaction between antigen and antibody are incubated for about 12 hours at room temperature or at 4 °C. At these temperatures, a relatively rapid decomposition of cys LTs occurs (see section 4). Most of the shortcomings of GC-MS and EIA (ELISA) methods are resolves by the unification of liquid chromatography with mass spectrometry. LC-MS method in a highly selective and accurate SRM mode (see section 5) affords both quantitative and qualitative information about the monitored biomarkers. Liquid chromatography can be used in both HPLC mode (high performance liquid chromatography) and UHPLC mode (ultra-high performance liquid chromatography). In UHPLC, the separation of substances is carried out at higher flow rates of the mobile phase (1 mL/min) on LC columns with a smaller average particle size of the stationary phase (diameter of particles 2 μm), 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 ((19,19,20,20,20-$^2H_5$) LTC_4, (19,19,20,20,20-$^2H_5$) LTD_4, (19,19,20,20,20-$^2H_5$) LTE_4). Immediately after the collection, a known amount of isotopically labelled internal standard is added to 1 mL of EBC sample (in order to monitor the substance amount from the very beginning and eliminate the error of cys LTs disintegration during the sample handling), which has the identical physico-chemical properties as the endogenously present biomarkers with the only exception of a different molecular weight. This is beneficially utilized in MS detection and both substance resolution (deuterium-labelled cys LTs are characterized by identical retention times in the LC separation, such as the unlabelled cys LTs, but they can be well separated in the mass spectrometer). The disadvantage of the LC-MS analysis of cys LTs still remains the inclusion of the pre-treatment step (SPE, immunoaffinity extraction), during which EBC sample is exposed to room temperature. This problem is resolved using 2D technology for liquid chromatography. In the first dimension, it is possible to carry out an on-line SPE using the SPE column Hypersil GOLD (20x 2.1 mm, particle size 12 μm, Thermo Scientific, USA) and the subsequent dimension using the LC chromatography.

Methods	LOQ	References	Note
EIA	78.1 pg/mL	Hoffmeyer et al, 2009	determined as cys LTs
EIA	13 pg/mL	Debley et al., 2007	determined as cys LTs
EIA	13 pg/mL	Piotrowski et al., 2007	determined as cys LTs
EIA	13 pg/mL	Antczak et al., 2002	determined as cys LTs
EIA	7.8 pg/mL	Goldbart, et al., 2006	determined as cys LTs
EIA	5 pg/mL	Leung et al., 2006	determined as cys LTs
ELISA	14 pg/mL	Soyer et al., 2006	determined as cys LTs
GC-MS	1pg/mL	Čáp et. al., 2004	only LTD4, LTE4; determined separately for each LT
LC - MS	<10 pg/mL	Syslová et al., 2011	determined separately for each LT

Table 3. Limit of quantification (LOQ) for detection methods for cys LTs

8. Clinical experience in biomarker-based diagnostics of occupational bronchial asthma

Occupational asthma is an occupational condition defined as: "A disease characterized by variable airflow limitation and/or airway hyper-responsiveness due to causes and conditions attributable to a particular occupational environment and not stimuli encountered outside the workplace". The proper diagnosis of occupational asthma is important for the prognosis of the disease and can be based on several tests. Out of these, controlled exposure to suspected occupational allergens in the laboratory is considered to be the gold standard. Occupational allergens are either applied with a special bronchoprovocation device or the patients are exposed to allergens in the challenge chamber under conditions reproducing the workplace exposure. An integral part of the specific bronchoprovocation tests is the monitoring of subjective complaints, objective findings and the repeated lung function measurement over 24 h. Exposure to allergens can also be performed directly in the workplace. Nevertheless, it is not always possible to make an unequivocal diagnosis of occupational asthma. It may pose difficulty especially in the case of a borderline decrease in ventilatory parameters, or, on the other hand, in patients with advanced or persisting obstructive ventilatory defect, in whom the specific bronchoprovocation tests cannot be performed. If the specific test is not feasible, other tests (and their combinations) like non-specific bronchoprovocation tests, skin prick tests, serum-specific imunoglobuline E (IgE) can be helpful in the determination of final diagnosis (each with various specificity and sensitivity compared to the specific bronchoprovocation test) (Klusáčková et al., 2008). Patients on permanent antiasthmatic treatment whose reaction to allergens is partially mitigated by the treatment represent another diagnostic problem. Therefore, a need arises to look for other markers (in addition to the ventilatory parameters), which would enable the verification of the reaction to the occupational allergen. Induced sputum analysis is one of the procedures previously reported to be helpful in occupational asthma diagnosis. However, it has the disadvantage that, despite the induction of sputum by hypertonic saline, some people do not produce a satisfactory sample of sputum for analysis. Multiple studies reported eosinophils increase after a bronchoprovocation test

(Lemiere, 2007). Exhaled nitric oxide could be another helpful marker in occupational asthma diagnosis. A definite advantage is that its collection is non-invasive but the sticking point is the lack of unambigouos data after the alergen testing. Exhaled breath condensate (EBC) contains many substances reflecting changes in the airways and it offers further possibilities for the diagnosis and monitoring of occupational asthma and other respiratory diseases. Collection of the EBC enables to study the concentration of leukotrienes directly in the airways and to detect individual cys LTs – LTC_4, LTD_4, LTE_4. The other parameters of interest for the monitoring during the bronchoprovocation are leukotriene B_4 and 8-isoprostane, known to be elevated in asthmatics (Hatipoglu & Rubinstein, 2004).

The aim of our study was to monitor changes in EBC concentrations of cys LTs in patients with occupational asthma diagnosis (without any lung pharmacotherapy) and healthy subjects. The pilot clinical study was performed in a group of 20 subjects with a given diagnosis (56–64 years old, male, nonsmokers) and with the equal number in the control group (52–66 years old, male, non-smokers). EBC samples collected during the clinical study were worked up by the IAS (pre-treatment method used for LC-MS determination) (Syslová et al., 2011) and the results were statistically evaluated. A significant difference was found between the control group and the group with the diagnosis in the values of concentration of LTC_4 (57.4 ± 8.6 pg/mL of EBC in the control group and 80.1 ± 6.2 pg/mL of EBC in those diagnosed with bronchial asthma) and LTE_4 (25.3 ± 5.1 pg/mL in the control group *versus* 35.7 ± 4.8 pg/mL in the bronchial asthma group). The difference in the levels was highlighted in the sum values of cys LTs, where the control group reached values of 77.8 ± 13.8 pg/mL of EBC and the asthmatic group reached 133.2 ± 20.1 pg/mL of EBC. Although a difference in the levels of LTD_4 was observed in its average value as well, differing from 19.9 ± 4.3 pg/mL of EBC in positively diagnosed subjects to 13.8 ± 5.2 pg/mL of EBC in the healthy subjects, the significance of this biomarker for the differential diagnostics of bronchial asthma was not entirely proven as the difference in the confidence intervals was statistically insignificant (Fig. 14).

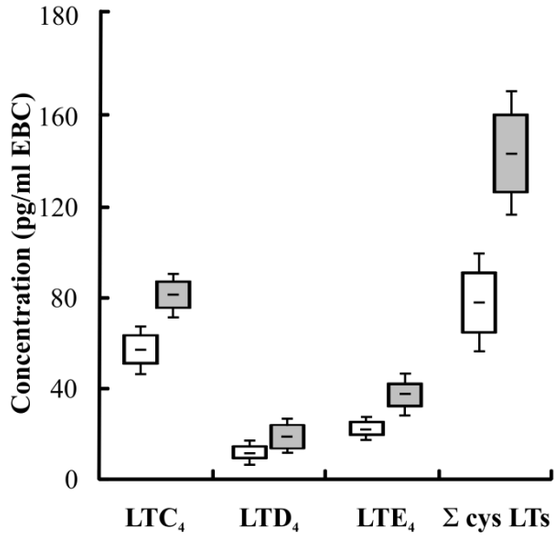

Fig. 14. Clinical study – concentration of cys LTs in EBC; bronchial asthma (grey), healthy subjects (white)

9. Bronchial asthma multimarker screening in exhaled breath condensate

Increased concentration levels of cys LTs in EBC do not always signify a proven diagnosis of bronchial asthma. High levels of cys LTs in EBC could also point to other lung diseases such as cystic fibrosis (Lammers, 2008), COPD (chronic obstructive pulmonary disease) (Usery et al., 2008) or a mere interindividual variation can be involved, where the increased concentration of cys LTs may represent the physiological level compared to other healthy individuals. For this reason, it is even convenient to monitor other substances that have been detected in EBC. Besides cys LTs, other low-molecular substances, considered as relevant biomarkers of other diseases, were monitored in EBC: 8-isoprostane (damage of phospholipids membrane, biomarker of oxidative stress, potential biomarker of bronchial asthma), leukotriene B_4 (damage of phospholipids membrane, biomarker of inflammation, potential biomarker of bronchial asthma), *o*-tyrosine (damage of amino acids, biomarker of oxidative stress), nitrotyrosine (damage of amino acids, biomarker of oxidative stress), 8-hydroxyguanosine (damage of RNA, biomarker of oxidative stress), 8-hydroxy-2′-deoxy-guanosine (damage of DNA, biomarker of oxidative stress), 5-hydroxymethyluracil (damage of DNA, biomarker of oxidative stress), malondialdehyde (damage of phospholipids membrane, potential biomarker of bronchial asthma, biomarker of oxidative stress) and 4-hydroxy-*trans*-nonenale (damage of phospholipids membrane, biomarker of oxidative stress). For their detection, 2D LC-MS method was developed. In the first dimension, on-line SPE (Hypersil GOLD column; 20 x 2.1 mm, particle size 12 μm, Thermo Scientific, USA) was carried out. Water was employed as the mobile phase for the column activation and a mixture of water : acetonitrile (30 : 70) was used for its regeneration. The separation of eluted components from the SPE column was performed on an analytical column (Hypercarb 100 x 2.1 mm column, particle size 5 μm, Thermo Scientific, USA) utilizing gradient elution (0:00 – 70 % A → 10.00 – 70 % A → 25:00 – 5 % A → 55: 00 to 5 % A → 60:00 – 70 % A). Here, solvent A consisted of water with pH adjusted to 10.5 with ammonia and solvent B was composed of methanol and acetonitrile in the ratio 60 : 40 (v/v) with an addition of ammonia (0.1 %). Using an MS detector, the developed method allows to separate and subsequently quantify not only the substances mentioned above but also diastereomers and several other prostaglandins (prostaglandin D_2 and E_2), as shown in the sample chromatogram. Fig. 15 shows the resulting chromatographic record. Using this method, a pilot study was carried out monitoring the levels of substances in healthy individuals (n = 10, age = 66 ± 1.5) and in people with occupational asthma (n = 11, age = 67 ± 2.7). Fig. 16 shows the clinical study results where a statistically significant difference was determined in addition to Σ cys LTs for LTB_4, malondialdehyde, 4-hydroxy-*trans*-nonenal and 8-isoprostane.

EBC analyses in healthy subjects and patients with a diagnosis of occupational asthma using high-resolution mass spectrometry (HRMS; LTQ Orbitrap Velos, Thermo, USA) or nuclear magnetic resonance (NMR) and processing of the obtained data by the so-called principal component analysis (PCA) can reveal and determine differences in individual samples and thus identify substances statistically significant for the given disease (Wan et al., 2008). In the context of PCA, the data can be regarded as a multivariate statistical problem where the metabolite or protein concentrations represent the true variables. Spectra are divided into "bins" of discrete spectral width (ppm in the case of NMR, m/z in the case of MS) and the areas under the curve (AUC) in these "bins" are integrated and serve as *pseudo*-variables. PCA therefore reduces a large number of (usually correlated) "true" variables into a smaller

number of (uncorrelated) variables, the so-called principal components (PC). PCA results in the decomposition of raw data into "scores", which reveal the relationship among samples and into "loadings" that show the relationships among the variables. The first PC interprets the greatest variability in data, the second PC (independent of/orthogonal from the first) interprets the second best and so on. If one assumes the data space can be reduced to a cube, PCA resembles turning the cube in different angles and identifying planes in the data space, separating the investigated and compared groups (typically control and case).

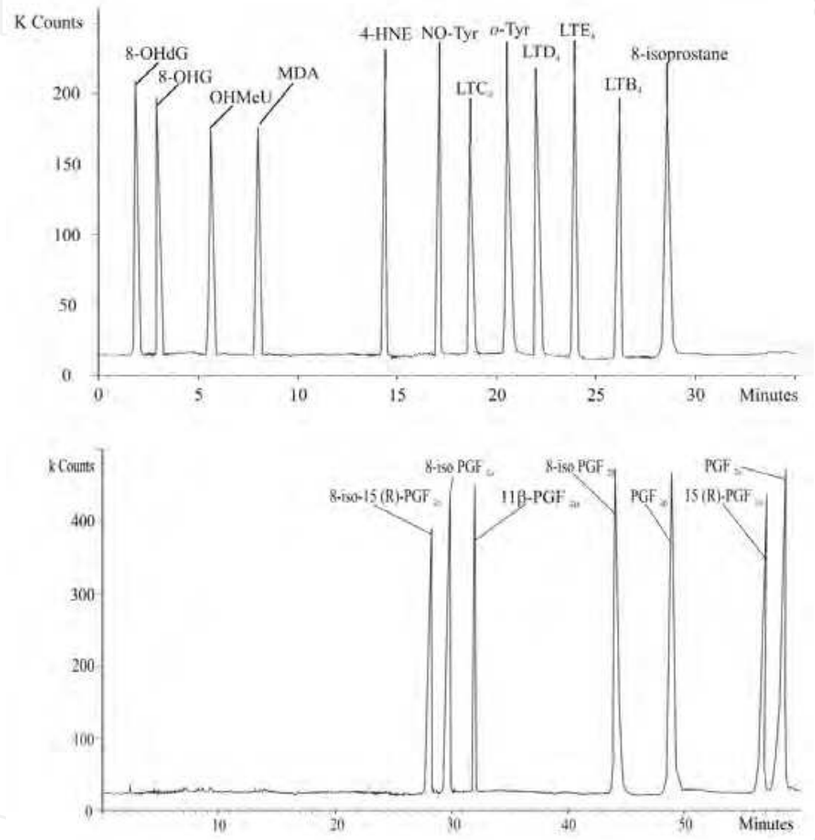

Fig. 15. Chromatogram of detected biomarkers (above) and chromatogram of diastereomers of 8-isoprostane (below)

The above-mentioned approach will be combined with a recent significant advance in chemometric modelling, which uses full-resolution data sets that represent each data point in the spectrum rather than binned data that represent summed segments of a spectrum. The use of full-resolution (all computer points in spectrum) data allows the spectral structure to be retained, and this together with models of orthogonal partial least squares discriminant analysis (O-PLS-DA) incorporate the correlation weight of the variables, enabling plotting of the loadings that are colour-coded and easily interpretable. O-PLS-DA modelling together with a back-scaling step can be successfully applied to determine the metabolic and variable allergenic consequences in multiple bio-fluid compartments.

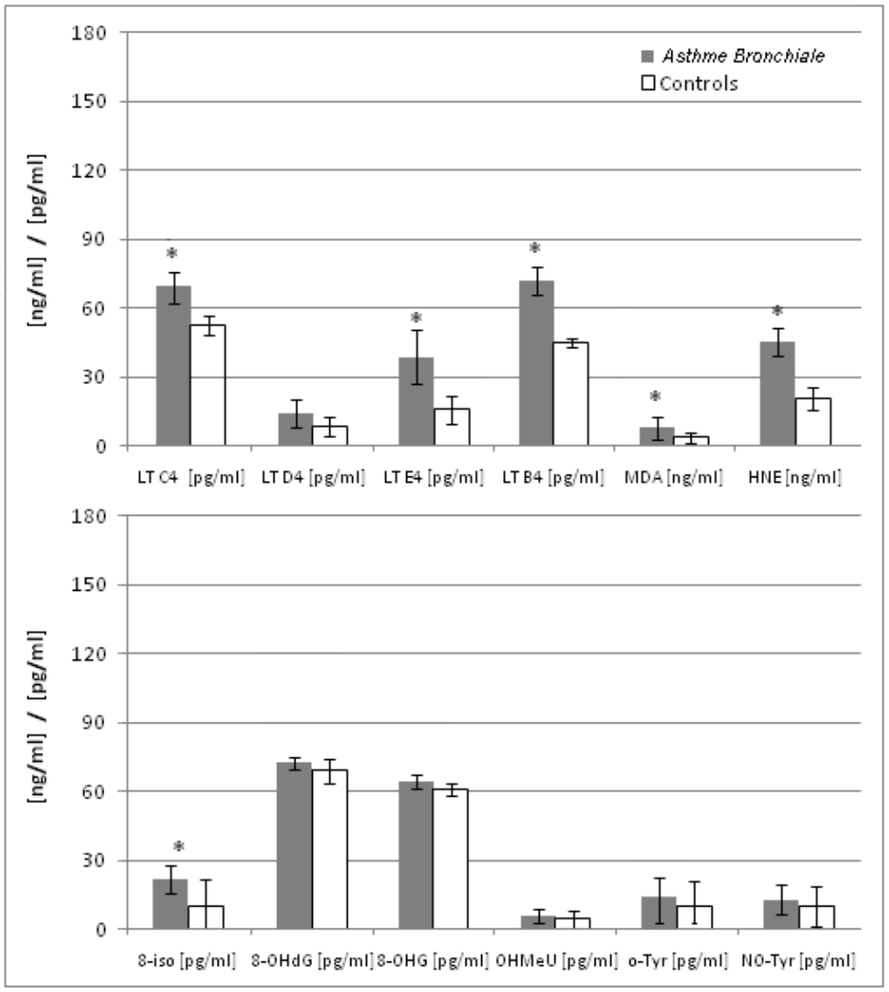

Fig. 16. Concentration of biomarkers in EBC; bronchial asthma (grey), healthy subjects (white)

Alternative approach to the multimarker analysis of both leukotrienes and other biomarkers including cytokines, chemokines as well as genetic profiling is based on a relatively novel technology, i.e. microarrays, or very recently, nanoarrays. Microarrays are two-dimensional platforms, typically based on a glass microscope slide, upon which specific biological probes are anchored, using either deposition or *in-situ* synthesis, in a high-density (tens of thousands to millions of probes) matrix in a predetermined spatial order. The analytical principle is based on a selective hybridization of the immobilized probes with targets contained in the biological sample, in this case EBC, using most commonly fluorescence or color detection. In the case of leukotrienes, antibody microarrays with specific probes to cys LTs are commercially available. Nevertheless, commercially available antibody as much as protein microarrays, have exhibited a rather low or very variable sensivity for each probe and thus further development was required to increase the sensitivity for clinical and research applications (Kricka et. al, 2002). The very novel platform though, nanoarrays, produced by nanolitographic methods (Bearingers et al., 2009; Rosa & Liang, 2009), e.g. the patented dip pen nanolithography (Lee et al., 2006) is exhibiting a principally much better

and consistent sensitivity. This technology has already been verified by several applications exhibiting high sensitivity in analyses of e.g. interleukins and THF-α markers. More applications are yet to be developed and validated, while it can be predicted that this platform may well function and be validated for cys LTs in the near future. I.e. nanoarrays thus can be anticipated to eventually create a solid multimarker alternative suitable for a routine screening, providing to have accomplished necessary validations.

10. Conclusions: Future perspective of biomarkers in exhaled breath condensate

Measurements of asthma biomarkers in EBC offer a novel way of monitoring lung inflammation and provide insight into the pathophysiology of the disease. The diagnostics based on determination of biomarkers present in EBC is completely non-invasive, well accepted by the patients, applicable to children, suitable for longitudinal studies, patient follow-up and potentially useful for monitoring drug therapy. From the medicinal point of view, biomarkers present in EBC reflect lungs information, rather than they give systemic information. Characterisation of selective profiles of exhaled biomarkers (cys LTs, LTB_4, 8-isoprostane, etc.) might be relevant to the differential asthma diagnosis. However, EBC analysis requires standardisation and validation of the whole diagnostic process including sample collection, sample pre-analysis handling (e.g. storing, internal standardisation, pre-treatment method application) and uniform analytical technique application.

Future research should be oriented on the identification of reference values for the different asthma biomarkers in healthy subjects (children and adults), on large longitudinal studies to ascertain if sequential measurements in the individual patient reflect asthma severity and the degree of lung inflammation, studies on relationship between the concentrations of asthma biomarkers and its symptoms, lung function, and other indices and results from other diagnostic methods. Unavoidable will also be to conduct controlled studies in order to establish the usefulness of EBC analysis for guiding pharmacological treatment in asthma and the effect of drugs on asthma markers present in EBC. Studies to determine the usefulness of EBC analysis for predicting treatment response and assessment of new therapies should also be carried out. Last but not least is also the knowledge on the feasibility of gene expression analysis in EBC. From the point of complex approach to EBC analysis it will also be important to determine the biomarker concentrations and profiles in regard to different lung diseases as well as to identify other inflammation mediators. This represents a great deal of work remaining. The truth is that EBC analysis is currently used in various clinical trials and studies. On the other hand, it is important to proclaim that the fact whether and when EBC analysis will be applicable to the clinical settings is still difficult to predict.

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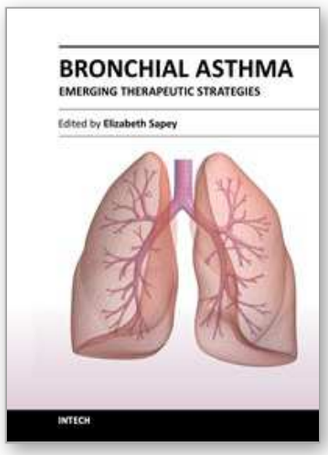

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Asthma remains a serious health concern for millions of people globally. Despite continuing research interest, there have been few advancements that impact clinically on patient care, potentially because asthma has been treated as a homogeneous entity, rather than the heterogeneous condition it is. This book introduces cutting-edge research, which targets specific phenotypes of asthma, highlighting the differences that are present within this disease, and the varying approaches that are utilized to understand it.

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