

Chapter 7

Comparison of Different Non-invasive Methods for Detection of Allergic Asthma

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Abstract Non-invasive methods to assess inflammation of lower airways are induced sputum (IS), exhaled nitric oxide (eNO), and exhaled breath condensate (EBC). Here we focused on the assessment of airway inflammation with a panel of non-invasive methods in health care workers (HCWs) with suspected latex allergy with and without current allergic respiratory symptoms about 10 years after the latex ban in German health care facilities. Seventy-seven non-smoking subjects were examined by skin prick test and specific IgE measurements, eNO, IS, and EBC. Sensitivity, specificity, and positive and negative predicted values for relevant biomarkers were calculated using current asthma symptoms as the gold standard. Twenty-nine subjects (38%) reported ongoing asthmatic symptoms (AS). In these subjects the EBC concentrations of nitrogen oxides (NO_x ; $p=0.027$) and leukotriene B_4 ($p=0.025$) were significantly higher than in subjects without AS. In addition, in the subjects with AS the numbers of eosinophils ($p=0.015$) and the concentrations of IL-5 ($p=0.021$) in IS samples were significantly higher than in the subjects without AS. A good correlation between several inflammatory markers in IS was detected. The maximum Youden Index was reached for IS total eosinophils $\geq 3.5 \cdot 10^4$ with a test efficiency of 0.72. In conclusion, non-invasive inflammatory monitoring with EBC and IS may assist the diagnosis of allergic asthma. Self-reported current asthmatic symptoms were reflected by eosinophilic inflammation and the best parameter to support the asthma diagnosis is a total number of eosinophils $\geq 3.5 \cdot 10^4$ in IS.

Keywords Allergic asthma • Diagnosis • Exhaled breath condensate • Exhaled nitric oxide • Induced sputum • Non-invasive methods

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7.1 Introduction

The lung represents the route of entry for many environmental and occupational pollutants which may induce inflammatory processes, hallmarks in the pathophysiology cascade leading to respiratory diseases. To assess these inflammatory processes especially in the lower airways the most widely used non-invasive methods are induced sputum (IS), fractional exhaled nitric oxide (eNO) and exhaled breath condensate (EBC).

In asthma and chronic obstructive pulmonary diseases (COPD), the analysis of cells and mediators in sputum induced by inhalation of a hypertonic saline solution has been widely applied and it has been proved to be reproducible, safe and valid for studying airway inflammation (Quirce et al. 2010). In addition to the determination of the cellular composition of the induced sputum sample an array of mediators can be measured in the cell-free supernatant of IS samples by using immunoassays. The mediators and the cell compositions reflect different aspects of airway inflammation and remodelling including, e.g., eosinophilic activation and or microvascular leakage. In the case of occupational asthma induced by high molecular weight substances, the majority of subjects developed eosinophilic airway inflammation after exposure, e.g., during a specific inhalation challenge with the relevant culprit. In the EAACI Task Force Consensus Paper concerning the relevance of non-invasive methods for the assessment of airway inflammation in occupational settings the authors (Quirce et al. 2010) concluded that an increase in sputum eosinophilic counts greater than 3% after specific inhalation challenge often precedes the occurrence of functional changes on subsequent exposure.

EBC is the liquid phase of the exhaled air sampled by cooling. It contains no cellular components, but metabolic products released by cells from the lungs or substances originating from inflammatory reactions in the airway mucosa reflecting biochemical changes of airway lining fluid (Hoffmeyer et al. 2009). Therefore, inflammatory markers like the concentration of hydrogen ions (pH measurements), metabolites of the arachidonic acid (like leukotriene B₄ or prostaglandins) and cytokines are often measured. In addition, the assessment of oxidative and nitrosative stress (like nitrogen oxide derivatives NO_x) is also possible. As summarized by the authors of the EAACI Task Force Consensus Paper (Quirce et al. 2010), EBC analysis may be useful in occupational studies on the group level and in individuals when serving as their own controls, but the current status of EBC collection and analysis makes the method a good research tool, not yet suitable for the clinical diagnosis setting.

The aim of the study was the implementation of the sputum induction, the collection of EBC, and the marker analysis of the biological material for supporting the diagnosis of allergic asthma. Our study subjects were recruited from health care workers with previously suspected occupational latex allergy who were examined about 10 years after the latex ban in German health care facilities. These subjects (77 non-smoking health care workers) were chosen in order to obtain a non-smoking young adult population with a high prevalence of atopy and asthma and thus to minimize misclassification and confounding factors.

7.2 Methods

7.2.1 Study Design and Subjects

The study was approved by the Ethics Committee of the Ruhr-University (Bochum, Germany) and was conducted in accordance with the Helsinki Declaration of the World Medical Association. All study participants gave written informed consent to the study protocol.

Ninety-one health care workers (HCWs) of the mean age 43.6 years, 15% current smokers, 92% females, 67% with current sensitization to latex (positive SPT or sIgE to latex), and 66% who were atopic (positive sIgE to sx1) were examined in our institute in the scope of a cross-sectional study to test the hypothesis that nationwide preventive measures enable HCWs with latex allergy to work without health risks about 10 years afterwards (Merget et al. 2010). This included physical examination, a detailed questionnaire, lung function test, skin prick test (SPT) to environmental allergens (various manufacturers) and latex (Allergopharma, Reinbek & Stallergenes, Kamp-Lintfort, Germany), and measurements of specific IgE to latex and environmental allergens (sx1) (sIgE; Phadia, Uppsala, Sweden). In skin prick testing, a wheal diameter of at least 3 mm in at least one of duplicate tests was defined as positive. Atopy was defined as a positive skin prick test result to at least one of the common aeroallergens or sx1 of at least class 1. Latex sensitization was defined as either a positive skin prick test with at least one of the two latex extracts or a positive CAP result with latex (≥ 0.35 kU/L). Exhaled nitric oxide (eNO; NIOX Flex, Aerocrine, Bad Homburg, Germany), the collection and analysis of induced sputum (IS), and exhaled breath condensate (EBC) were also performed. Here we focused on the analysis of IS and EBC.

7.2.2 Sputum Induction and Processing

The sputum induction was performed with hypertonic saline solution according to the method of Holz et al. (1998) and Vagaggini et al. (2002). Briefly, lung function was measured before sputum induction. After 10 min the sputum induction started with the inhalation of 3% nebulized saline solution for 5-min periods for up to 30 min. At 10 min intervals, saline concentration was increased from 3% to 4% to 5%. Every 5 min after the start of nebulisation, patients were asked to rinse their mouths and throats carefully to discard saliva and to try to cough sputum into a clean container. Forced expiratory volume in 1 s (FEV₁) was measured and the inhalation of saline was stopped after 30 min or when the FEV₁ decreased by $>20\%$ from baseline values.

Sputum samples were stored for transportation at 4°C. The sputum processing was described in detail by Raulf-Heimsoth et al. (2011). Briefly, the procedure started with selecting all viscid portions (or portions of higher density) from the expectorated samples for minimizing contamination with saliva. The volume of the IS was determined and an equal volume of 0.1% sputulysin (Dithiothreitol) was added. The samples were mixed gently by vortex mixing and incubated for 30 min at 37°C to ensure a complete homogenization. After centrifugation the cell-free supernatants were aliquoted and stored at -80°C under argon protection until further analysis of soluble markers. The cell pellets were re-suspended and the total cell number was determined. For differential cell counts slides were prepared by cytospin (Cytospin 2, Shandon Corp., Pittsburgh, PA) and stained with May-Grünwald-Giemsa and three independent observers counted 200 cells on each slide by light microscopy. Their results were expressed as percentages of the total cell numbers and as the absolute numbers of the cell population (without correction of squamous cells).

7.2.3 Collection of EBC

EBC was collected with the commercially available ECoScreen device (Cardinal Health, Hoechberg, Germany) and described in detail by Hoffmeyer et al. (2007). EBC was collected for 10 min in a Teflon-coated condenser cooled to a temperature of -15°C . The pH value of the EBC was determined using a pH-meter with a glass-electrode (Mettler Toledo, Giessen, Germany) immediately and after deaeration with argon at 2 bar for 10 min, resulting in a stable pH. The samples were aliquoted and stored at -80°C under argon protection until further analysis of soluble markers.

7.2.4 Analysis of Soluble Mediators and Other Markers in IS and EBC

The inflammatory mediators were determined in the thawed cell-free supernatants of IS and EBC samples. All samples underwent only a single freeze-thaw cycle.

In EBC samples, the concentrations of leukotriene B₄ and 8-isoPGF_{2α} were measured with specific enzyme competitive immunoassays (Assay Designs, Ann Arbor, USA; detection limit 11.7 pg/mL for LTB₄ and 6.1 pg/mL for 8-isoPGF_{2α}) and the amount of NO derivatives with a colorimetric assay kit (AlexisTM Cayman Chemicals, Grünberg, Germany) determining the total nitrate/nitrite concentration with a sensitivity of 5 μM and a standard range: 0–35 μM.

In IS samples, the following soluble markers were measured: interleukins (IL-1β, IL-5, IL-6, and IL-8), LTB₄, NO derivatives, and total protein and eosinophilic cationic protein (ECP; using the ImmunoCAP system from Phadia, Uppsala, Sweden). IL-1β was measured with the respective PeliKineTM-Tool set (CLB, Amsterdam, Netherlands) in a standard range of 0.4–300 pg/mL for IL-1β. IL-5 and IL-8 were measured with the OptEIATM ELISAs (BD Biosciences Pharmingen, Heidelberg, Germany) in a standard range of 2–500 pg/mL for IL-5 and in a standard range of 3–200 pg/mL for IL-8. Determination of IL-6 was performed with the DuoSetTM ELISA Development system (R&D Systems, Wiesbaden, Germany) in a standard range of 3–600 pg/mL for IL-6.

Total protein content in the IS samples was determined according to the method of Bradford with bovine serum albumin as standard solution (range 10–100 μg/mL) (Bradford 1976).

7.2.5 Statistical Analysis

Values distribution was assessed using the D'Agostino & Pearson omnibus normality test. Values below the limit of quantification (LOQ) were set 2/3 of the LOQ. Comparisons of unpaired data were performed with a Mann–Whitney test. A two-sided significance level of 0.05 was chosen for all tests. To estimate specific parameters in EBC and IS as suitable diagnostic parameter for the detection of asthmatic symptoms (AS), the self-reported current asthmatic symptoms were taken as the gold standard to calculate sensitivities, specificities, positive predictive values (PPV), negative predictive values (NPV), and test efficiencies. For the evaluation of a parameter, the mediator or cell concentration leading to the maximum Youden Index (sensitivity + specificity – 1) was chosen as the optimal cut-off level (as described by van Kampen et al. 2009). The data were analyzed by using GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego, CA).

7.3 Results

7.3.1 Characterization of the Study Group

In the group of 91 HCWs examined in the scope of the whole study (Merget et al. 2010), exhaled nitric oxide (eNO) was significantly correlated with the atopy status (quantified *via* sIgE to ubiquitous environmental allergens; sx1) ($r=0.438$; $p<0.0001$; Pearson). Seventy-seven of the HCWs (85%) were non-smokers and included in our evaluation. Forty-six (60%) of them were atopic and 40 (52%) had latex sIgE (≥ 0.35 kU/L). Twenty-nine of the non-smoking subjects (38%) reported ongoing asthmatic symptoms and 21 of them reported also rhinitis. For the further evaluation, we divided the 77 non-smokers in two groups: 29 with ongoing asthmatic symptoms (AS) and 48 without current asthmatic symptoms (non-AS). No significant difference between eNO was measured between the AS (median: 16.7 ppm; range: 2.4–114.8 ppm) and the non-AS groups (median: 15 ppm; range: 3.5–102.3 ppm).

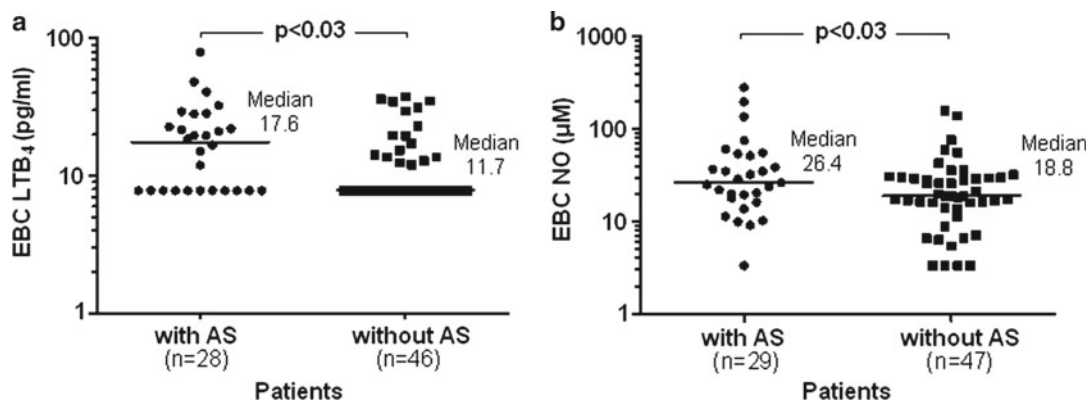


Fig. 7.1 (a) Leukotriene (LT) B₄ and (b) NO_x concentrations in exhaled breath condensate (EBC) in patients with asthmatic symptoms (AS) and without asthmatic symptoms (without AS)

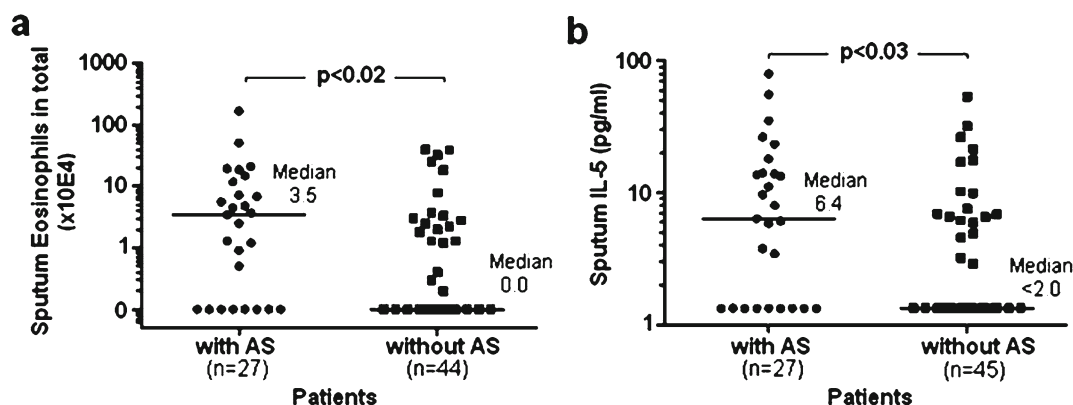


Fig. 7.2 (a) Total numbers of eosinophils and (b) interleukin (IL-5) concentration in induced sputum samples in patients with asthmatic symptoms (AS) and without asthmatic symptoms (without AS)

7.3.2 Analysis of EBC

In subjects with AS, the concentrations of LTB₄ ($p=0.025$; Fig. 7.1a) and nitrogen oxides (NO_x; $p=0.027$; Fig. 7.1b) were significantly higher than in the non-AS subjects. In contrast, the EBC pH values (median pH in AS: 7.04 and in non-AS: 6.91) and the concentrations of 8-isoPGF_{2α} (median 8-isoPGF_{2α} in AS: 198.9 pg/mL and in non-AS: 143.7 pg/mL) were not different. No significant correlation was measured between the pH, 8-isoPGF_{2α}, LTB₄, and NO_x in EBC samples (data not shown).

7.3.3 Analysis of IS

In IS samples of subjects with AS, the numbers of eosinophils ($p=0.015$; Fig. 7.2a), the percentage of eosinophils ($p=0.015$), and the concentrations of IL-5 ($p=0.021$; Fig. 7.2b) were significantly higher than in subjects without AS. In contrast, ECP, IL-8, IL-6 concentrations, and the numbers (and percentages) of neutrophils were not different (data not shown). In addition, in IS samples significant correlations between several cell and soluble parameters were calculated (Table 7.1).

Table 7.1 Spearman (rs; *upper line*) and Pearson (rp; *lower line*) correlation coefficients of cellular and soluble parameters in induced sputum samples of all 77 non-smoking subjects

	Eosinophils (%)	IL-5	ECP	Total protein	IL-8	IL-6	NO _x	IL-1β	LTB ₄
Eosinophils %	1.0	−0.065	0.515***	0.445***	0.526***	0.449***	0.137	−0.016	0.088
		<i>−0.014</i>	0.402***	0.383***	<i>0.115</i>	<i>0.188</i>	<i>0.074</i>	<i>0.005</i>	<i>0.015</i>
IL-5		1.0	0.020	0.041	−0.019	−0.127	−0.164	0.335**	−0.069
			<i>0.090</i>	<i>0.064</i>	<i>0.093</i>	<i>0.037</i>	<i>−0.155</i>	0.351**	<i>0.130</i>
ECP			1.0	0.721***	0.783***	0.596***	0.173	0.372***	0.492***
				0.742***	0.373***	0.416***	<i>0.210</i>	0.279**	<i>0.123</i>
Total protein				1.0	0.714***	0.606***	0.407***	0.252*	0.290*
					0.641***	0.616***	0.398***	0.513***	0.287*
IL-8					1.0	0.737***	0.229	0.351**	0.399***
						0.411***	<i>0.213</i>	0.642***	<i>0.202</i>
IL-6						1.0	0.160	0.245*	0.244*
							0.271*	0.244*	<i>0.128</i>
NO _x							1.0	−0.019	−0.019
								<i>0.078</i>	<i>−0.050</i>
IL-1β								1.0	0.351**
									0.431***
LTB ₄									1.0

Significant values are indicated in bold

*p<0.05; **p<0.01 ***p<0.001

Table 7.2 Estimation of soluble EBC and cellular and soluble IS parameters as optimal test parameter for the detection of asthma

Parameter	Sensitivity (tp/(tp+fn))	Specificity (tn/(tn+fp))	PPV (tp/(tp+fp))	NPV (tn/(tn+fn))	Test efficiency ((tp+tn)/N)	Youden Index at the cut-off level
EBC LTB ₄ ≥17.64 pg/mL	0.50	0.80	0.61	0.73	0.69	0.30
EBC LTB ₄ ≥20 pg/mL	0.39	0.85	0.61	0.70	0.68	0.24
EBC LTB ₄ ≥25 pg/mL	0.25	0.87	0.54	0.66	0.64	0.12
EBC NO ≥25 μM/mL	0.52	0.64	0.47	0.68	0.59	0.16
EBC NO ≥32.1 μM/mL	0.44	0.83	0.60	0.70	0.67	0.27
EBC NO ≥50 μM/mL	0.28	0.89	0.62	0.67	0.66	0.17
Sputum Eos ≥2 %	0.33	0.89	0.64	0.68	0.68	0.22
Sputum Eos ≥3%	0.30	0.90	0.67	0.68	0.68	0.20
Sputum Eos ≥5%	0.04	0.93	0.25	0.61	0.59	−0.03
Sputum Eos total ≥3.5·10 ⁴	0.51	0.84	0.67	0.74	0.72	0.35
Sputum Eos total ≥5·10 ⁴	0.37	0.86	0.63	0.69	0.68	0.25
Sputum Eos total ≥7.5·10 ⁴	0.26	0.86	0.54	0.66	0.63	0.12
Sputum IL-5 ≥5 pg/mL	0.59	0.67	0.52	0.73	0.63	0.26
Sputum IL-5 ≥7.5 pg/mL	0.48	0.80	0.60	0.72	0.68	0.28
Sputum IL-5 ≥10 pg/mL	0.41	0.84	0.61	0.70	0.68	0.25
Sputum Eos ≥3.5·10 ⁴ and Sputum IL-5 ≥7.5 pg/mL	0.22	1.00	1.00	0.68	0.71	0.22
Sputum Eos ≥3.5·10 ⁴ and EBC LTB ₄ ≥17.64 pg/mL	0.30	0.98	0.89	0.70	0.72	0.28
Sputum Eos ≥3.5·10 ⁴ and EBC NO ≥32.1 μM	0.26	1.00	1.00	0.69	0.72	0.26
EBC NO ≥32.1 μM and EBC LTB ₄ ≥7.64 pg/mL	0.30	0.96	0.80	0.69	0.71	0.26

For each parameter results with highest Youden Index are indicated in bold

7.3.4 *Estimation of the Optimal Parameter for the Detection of Asthma*

To estimate the best cut-off value of LTB_4 and NO_x concentrations in EBC, and eosinophils (total and percentage) and IL-5 concentrations in IS, for the detection of asthma sensitivity, specificity, positive predictive value (PPV), negative predictive values (NPV), test efficiencies, and the Youden Index were calculated with different mediator concentrations (Table 7.2). The maximum Youden Index was reached for LTB_4 with a concentration of ≥ 17.64 pg/mL, with test efficiency of 0.69, and for NO_x with a concentration of ≥ 32.1 μM , with a test efficiency of 0.67. The maximum Youden Index was reached for total eosinophils with number of $\geq 3.5 \cdot 10^4$, with test efficiency of 0.72. For IL-5 it was reached with a concentration of ≥ 7.5 pg/mL, with test efficiency of 0.68.

Additionally, IS and EBC parameters with the best outcome (in IS eosinophils $\geq 3.5 \cdot 10^4$ and IL-5 ≥ 7.5 pg/mL, and in EBC $\text{LTB}_4 \geq 17.64$ pg/mL and $\text{NO}_x \geq 32.1$ μM) were combined. The maximum Youden Index was reached for IS total eosinophils $\geq 3.5 \cdot 10^4$ combined with $\text{LTB}_4 \geq 17.64$ pg/mL, with a test efficiency of 0.72, PPV of 0.89, and NPV of 0.70.

7.4 Discussion

Induction of sputum with hypertonic saline was shown to be safe in our subjects and was not associated with any adverse event. Using the ECoScreen device it was possible to collect EBC in a sufficient amount from 76 of the 77 non-smoking subjects of the study group.

The amount of eosinophils and the concentration of IL-5 in IS samples, and the concentrations of LTB_4 and NO_x in EBC were significantly higher in subjects with current asthmatic symptoms. Therefore, these parameters were tested for their suitability to discriminate between ‘with current asthmatic symptoms and without current asthmatic symptoms’ and to support the diagnosis of allergic asthma as predictive values. Our results indicate that although IL-5 in IS and LTB_4 and NO_x concentrations in EBC were adequate parameters, the optimal parameter to support the asthma diagnosis in our study group is the total number of eosinophils $\geq 3.5 \cdot 10^4$ in IS. The present study was performed in non-smoking HCWs with and without current allergic asthmatic symptoms 10 years after the latex ban in German health care facilities. For all these parameters the specificity was >0.8 and therefore sufficient, but the sensitivity ranged between 0.22 and 0.59 which was not adequate to detect all subjects with asthmatic symptoms with these methods. Nevertheless, it has to be considered that all these evaluations are influenced by the selected ‘gold standard’ which is in our case the self-reported asthmatic symptoms. Whereas predictive values depend largely on the prevalence rates of cases under study, sensitivity and specificity are presumably inherent characteristics of the test (Feinstein 1975; van Kampen et al. 2009). Thus, we used the Youden Index, considering sensitivity and specificity equally to define the optimal positivity criterion (cut-off level) for each parameter, because this index should be independent of selection bias.

Our results confirmed the previously reported importance of eosinophilic inflammation in allergic asthma induced by high molecular weight occupational sensitizers (Quirce et al. 2010; Motojima et al. 1993). Interleukin-5, a protein produced by several different cells including CD4^+ T cells, mast cells, and eosinophils, is involved in the development, survival, and activation of eosinophils. Therefore, it is not surprising that we found also a significantly higher IL-5 concentration in sputum samples of subjects with current asthmatic symptoms, but without correlation to the numbers and percentage of IS eosinophils. In contrast to IL-5, the concentrations of the eosinophil granule-derived cationic protein (ECP) in the sputum samples were nearly similar in both groups. Similar to other

studies (Fahy et al. 1993; Motojima et al. 1993; Gibson et al. 1998; Barck et al. 2005) we measured a significant but not very strong correlation between the eosinophils and ECP in the sputum samples ($r_s=0.515$; $r_p=0.402$). In addition, Gibson et al. (1998) and Barck et al. (2005) described that measuring ECP in the sputum cell pellet lysate which reflects the number of eosinophils (in contrast to ECP in the supernatant, which better estimates the eosinophilic activation) is often better correlated to the count of eosinophils in patients with a different airway inflammatory disease to asthma. They recommended the measurement of ECP in the cell pellet lysate as an estimate of eosinophilic inflammation in clinical trials and epidemiological surveys.

Additionally, our data also indicate a significant correlation between the total protein content and eosinophil count as well as all other soluble parameters, with the exception of IL-5 concentrations. Especially for IL-5 concentrations, it has to be kept in mind that more than 50% of the values measured in sputum samples of the subjects without asthmatic symptoms were below the limit of quantification and therefore the parameter was not very robust. Overall, the good correlation of the different markers supported that the inflammatory process is characterized by multiple mediators released by different activated cells. Kulkarni et al. (2010) mentioned that sputum eosinophilia is only present in about 50% of asthmatic subjects across all severities which introduced the possibility that other inflammatory pathways (e.g., with different granulocytic composition) are also important in different asthma subphenotypes. Therefore, one should not focus solely on the assessment of mediators of eosinophilic inflammation, but consider further pathways for the characterization of the asthmatic reaction.

In parallel to the collection and analysis of induced sputum, we also collected and analysed EBC. This method offers the possibility with a safe, non-invasive, and easy to handle diagnostic procedure to indirectly point to biochemical changes that occur in the fluid lining airway surfaces (Cepela and Dodig 2007; Hoffmeyer et al. 2009). Of the available EBC biomarkers, the majority of published data refer to hydrogen peroxide, pH (airway acidity), nitric oxide metabolites, various arachidonic acid metabolites (leukotrienes and prostanoids), and products of the lipid peroxidation. The literature data (summarized in Cepela and Dodig 2007) suggest that the determination of 8-isoprostane and other arachidonic acid products appears to have the highest differential diagnostic potential for the detection of inflammatory processes in asthma and COPD. Our data show that the EBC NO_x and LTB_4 concentrations were significantly different between the patients with and without asthmatic symptoms and that according to the Youden Index LTB_4 concentrations ≥ 17.64 pg/mL seem to be a suitable predictive marker for asthmatic symptoms. Caballero Balanzá et al. (2010) also reported that LTB_4 concentrations in EBC were higher in children with asthma than in healthy controls and in contrast to our data they also found that the 8-isoPGF_{2 α} concentrations were significantly higher in asthmatic than in healthy children.

We found a good correlation between eNO concentrations and the atopy status of our subjects, but no significant eNO difference between the subjects with and without current asthmatic symptoms. Henriksen et al. (2000) reported that eNO is significantly elevated in atopic vs. non-atopic suspected asthmatics and that suspected asthmatics with both airway hyperresponsiveness and atopy have the highest eNO levels. They concluded that measurements of eNO alone are not a useful tool in diagnosing asthma in population surveys.

7.5 Conclusions

The present study demonstrates that the collection of EBC and induced sputum is safe and the analysis of these fluid concerning mediators and cells can be useful in supporting the asthma diagnosis. Although LTB_4 and NO_x concentrations above a defined level are suitable parameters, the optimal parameter to support the asthma diagnosis in our study group was a total number of eosinophils ($\geq 3.5 \cdot 10^4$) in induced sputum.

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