CHAPTER 9

Noninvasive assessment of airway inflammation in asthma

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As bronchial asthma is currently considered to be and is defined as being an inflammatory disorder of the airways [1], it seems logical to include an assessment of this inflammation in the diagnosis and follow-up of the disease. In this assessment, a few factors need to be taken into account. Firstly, the inflammation underlying asthma is a complex phenomenon that displays characteristics, not only of the acute phase of an inflammatory process, with increased vascular permeability and plasma exudation, but also of a more subacute inflammatory phase with influx of inflammatory cells and in addition, characteristics of the chronic phase of an inflammatory response which is characterised by structural alterations, coined as airway remodelling [2]. The precise functional role of the various cells and mediators possibly involved in these different phases of the inflammatory process, still remain to be established. In addition, the exact relationship between the various components of the inflammatory process and the clinical characteristics of asthma are also uncertain. It has been argued that asthma symptoms mainly reflect the acute inflammation, caused by the release of pro-inflammatory mediators and resulting in widespread airway narrowing, whereas the functional abnormalities such as bronchial hyperresponsiveness are mainly due to airway remodelling [3, 4]. However, these assumptions are largely based on mathematical models that need to be further proven.

Most of the biopsy studies performed so far, have focused on the eosinophil as the prime marker of the (sub)acute phase of the inflammation. In general, these studies show a weak and inconsistent correlation between eosinophil counts and clinical or functional criteria of disease activity such as symptoms, baseline forced expiratory volume in one second (FEV_1) or peak flow variability [5, 6]. This also applies to the degree of nonspecific bronchial hyperresponsiveness, especially towards a direct acting stimulus such as methacholine or histamine [7, 8]. The correlation with markers of indirect airway responsiveness such as exercise or adenosine is better, but still relatively weak [8]. Similarly, the degree of subepithelial fibrosis as a marker of airway remodelling is not consistently related to the degree of airway responsiveness [9, 10]. These observations imply that clinical and lung function criteria cannot be used as noninvasive indirect markers of the underlying inflammation, but that a more direct assessment is required reflecting the acute and the chronic phase of the inflammatory process. Endobronchial biopsies would enable a direct assessment of the inflammatory process, but the invasiveness of the technique precludes its use in daily clinical practice. Furthermore, it is difficult to evaluate the degree of cellular activation using histochemical techniques and the quantification of inflammatory cells is difficult. What also needs to be borne in mind is that the composition of the airway inflammation in asthma is a dynamic phenomenon,

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that can be influenced by external factors such as intensity of allergen exposure or alterations in the treatment regimen [11, 12]. As a consequence, evaluation of the inflammation should not be a snapshot in time, but performed repeatedly. It would therefore seem that the ideal biomarker should not only offer a noninvasive way to quantify the airway inflammation, but in addition, should be cost-effective and easy to perform repeatedly in a clinical setting.

Noninvasive markers of airway inflammation in asthma

A number of markers have been and are being considered as noninvasive markers of airway inflammation. Examples include blood eosinophil counts or serum eosinophil cationic protein (ECP), urinary eicosanoid metabolites, exhaled gasses, mediators in breath condensate or induced sputum (table 1).

As for any outcome measure, when considering the potential usefulness of any of these markers in the monitoring of disease activity, one of the first elements to be addressed is the reliability and the validity of the markers. Elements of reliability include interobserver consistency and repeatability. In the assessment of validity, a distinction is made between criterion validity or conformity to the gold standard measurement which is agreed to be airway inflammation as assessed in bronchial biopsies and content validity which includes evaluation of the disease specificity of a given marker and the responsiveness to intervention. These various elements have been evaluated to a variable degree for different possible markers of airway inflammation.

Blood markers

Eosinophils and eosinophil cationic protein. Measurement of blood eosinophil counts and serum ECP levels if correctly performed, are reproducible and consistent [13]. However, blood eosinophil counts have been shown to correlate weakly to eosinophil

Table 1. – Biomarkers in asthma

Blood/serum
Eosinophils
Eosinophil cationic protein (ECP)
EPO
sIL-2R
Urine
9α,11β-PGF_2
LTE_4
EPX
Exhaled air
Nitric oxide
Carbon monoxide
Hydrocarbons
Breath condensate
Hydrogen peroxide
LT metabolites
8-isoprostane
Nitrotyrosine
Induced sputum
Cell differential
Soluble mediators (ECP, cysLT's)

EPO: eosinophil peroxidase; sIL-2R: soluble interleukin-2 receptor; LT: leukotriene; cysLT's: cysteinyl leukotriene.

numbers in biopsies [14] and have a poor disease specificity. The correlation of serum ECP with the number of eosinophils in biopsies is variable: although in some studies, a correlation has been reported, this has not been invariably confirmed [14–16]. Serum ECP also lacks disease specificity. Increased levels of ECP can be found in various diseases including cystic fibrosis, whereas conversely, a large degree of overlap exists between normal and asthmatic individuals with varying severity [17–19]. Both eosinophil counts and ECP levels respond to factors known to influence the degree of airway inflammation such as changes in treatment or allergen exposure [20–22]. The sensitivity of ECP to these changes in comparison to other possible biomarkers has not been extensively investigated. From the data available, it would seem that ECP is somewhat more sensitive than mere eosinophil counts but less than sputum eosinophil counts [23]. A striking observation is that the response to treatment can be influenced by additional external factors, such as the smoking habits of the patients [24].

Other markers. Other circulating markers have been proposed, including soluble interleukin (IL)-2 receptor (CD25). However, these have not been extensively evaluated [25, 26].

Urinary markers

Urinary eosinophil peroxidase (EPX) offers an even less invasive alternative to serum ECP [21, 27], especially for children. Another line of investigation is to measure eicosanoid metabolites in urine such as leukotriene (LT)E_4 or 9α,11$\beta$$PGF_2$ [28]. These measurements are reliable but require skilled expertise. How precisely they reflect ongoing inflammation in the airways needs to be further evaluated as increased urinary LTE_4 levels are not limited to asthma and they do not discriminate between asthma and normal subjects [29]. However, urinary markers respond to therapeutic interventions, illustrating their potential usefulness in the long-term monitoring of the disease [21, 30].

Exhaled air

Nitric oxide. To date, of the gases present in exhaled air, nitric oxide (NO) has been most extensively investigated [31]. Recommendations have been issued on how to perform NO measurements, thus adding to the reliability of the technique [32, 33]. Weak correlations have been found between exhaled nitric oxide (eNO) and the number of eosinophils in biopsies or in sputum [34–36]. Exhaled NO is increased in nonsteroid treated asthma [37, 38], albeit the increase is not disease specific [39]. In established asthma, a relationship was found between eNO and asthma symptoms or β_2-agonist use [40, 41]. Exacerbations, both in children and in adults are also accompanied by increased NO levels [42]. In a comparative study, eNO proved to correlate better with asthma severity than serum ECP or soluble IL-2 receptor [43].

As part of the criterion validity assessment, eNO has been shown to respond to factors that are known to influence the degree of inflammation in asthma. Exhaled NO increases in response to allergen exposure. This response is sufficiently sensitive to detect naturally occurring changes in allergen exposure over the pollen season [44]. The response to nonallergic stimuli such as pollutants is less consistent [45, 46]. Treatment with short-acting inhaled β_2-agonists does not influence eNO [47]. This is consistent with the observation that these agents do not influence chronic inflammation in asthma, and validates the use of eNO to assess inflammation, independent of airway calibre. Anti-inflammatory compounds such as antileukotrienes, but especially inhaled steroids reduce eNO levels [48, 49]. Exhaled NO has proven to be extremely sensitive to steroid

treatment. Reduction in eNO may be seen within 6 h after a single dose of nebulised steroids [50] or within 2–3 days following treatment with inhaled steroids [49]. Concordantly, steroid-induced changes in NO precede improvement in symptoms, baseline FEV_1 or sputum eosinophilia [35, 49].

Carbon monoxide. Other gases that have been measured in exhaled air include carbon monoxide (CO) and hydrocarbons such as ethane and pentane [51, 52]. Both are considered to be representative of the level of oxidative stress. Similar to eNO, comparison with normal subjects indicates that exhaled CO is increased in nonsteroid but not in steroid-treated asthma [53, 54]. It is unclear to what extent exhaled CO and NO differ in their steroid sensitivity. The observation that children with persistent asthma, despite treatment with steroids which reduces their NO levels, have significantly higher exhaled CO compared with those with infrequent episodic asthma has led to the proposal that exhaled CO is less steroid-sensitive than eNO [52].

Hydrocarbons. The volatile hydrocarbons ethane and pentane are among the numerous end-products of lipid peroxidation of peroxidised polyunsaturated fatty acids that can be measured by gas chromatography from single breath samples. Increased levels have been measured in nonsteroid treated asthma. Others have described elevated pentane levels during episodes of acute asthma that returned to normal once the acute asthma subsided [55]. Of note is that smoking also increases ethane levels [56].

Breath condensate

Another approach is to measure nonvolatile mediators in condensate of exhaled air. Exhaled breath condensate is collected by cooling or freezing of exhaled air. As the procedure is totally noninvasive and does not influence airway calibre, a major advantage of this technique is that it is extremely well tolerated even by patients with severe airway obstruction and children (figs. 1 and 2). The most common approach is for the subject to breathe *via* a mouthpiece through a nonrebreathing valve block in which inspiratory and expiratory air is separated. During expiration, the breathing air flows through a condenser, which is cooled to -20°C. Preventing saliva contamination by swallowing or

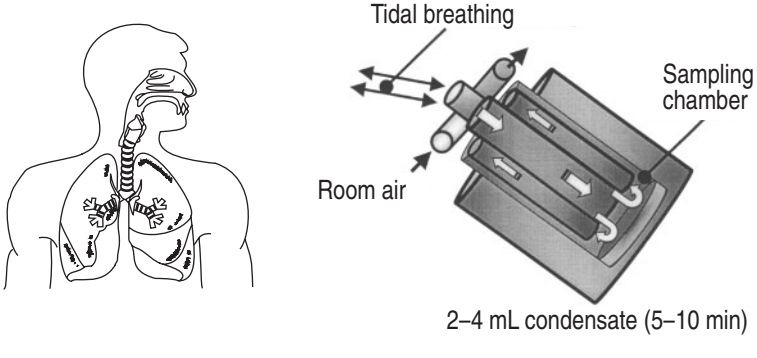

Fig. 1. – Diagram of the apparatus for measuring exhaled breath condensate.

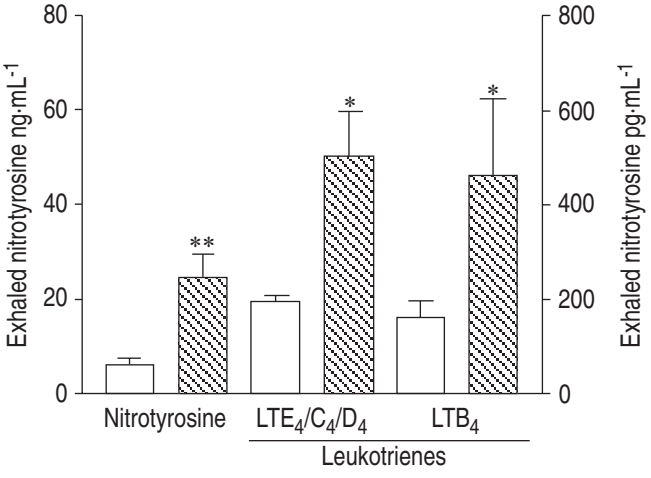

Fig. 2. – Exhaled nitrotyrosine and leukotrienes before and after steroid withdrawal in patients with moderate asthma. □: stable asthma; ▨: unstable asthma. *: $p<0.05$; **: $p<0.01$.

rinsing the mouth and standardising condensate collection by volume or respiratory rate improves the reproducibility of the results. Exhaled condensate is usually analysed by gas chromatography and/or extraction specrophotometry, or by immunoassays. Differences in condensate chemistry are thought to reflect changes in the airway lining fluid caused by inflammation and oxidative stress. Condensate from asthmatic subjects contains increased levels of leukotriene $B_4/C_4/D_4/E_4$ in addition to several markers of oxidative stress including hydrogen peroxide, nitrotyrosine and 8-isoprostane [57–61]. Measurement of cytokines has proven less successful to date. Although the analysis of these various molecules in breath condensate remains to be fully validated, it would seem that they could provide useful information in the disease monitoring. Significant differences in hydrogen peroxide levels were observed between controlled and noncontrolled asthma [58], whereas others have shown that 8-isoprostane levels are less sensitive to steroid treatment than eNO or exhaled ethane [60]. As such, these markers might offer complementary information to the very sensitive NO measurements [59].

Induced sputum

To date, assessment of the reliability and validity of induced sputum has mainly focused on the percentage of eosinophils in the sample. In asthmatics, sputum eosinophil counts have a high interobserver consistency and repeatability, irrespective of the processing technique used [62–64]. In addition, a large number of studies have evaluated the validity of induced sputum. When assessing criterion validity, an element which needs to be borne in mind, is that sputum samples the airway lumen, extending from the central to the peripheral airways with increasing induction time [65–68]. Not unexpectedly therefore, sputum eosinophil counts correlate better with those in bronchoalveolar lavage (BAL) or bronchial wash than with eosinophil numbers in bronchial biopsies [69–72]. This probably reflects, at least in part, the kinetics of the inflammatory process in the airways. Due to differential cell trafficking, the inflammatory cell distribution in the airway lumen can be different from that observed in the airway mucosa. In addition, biopsies offer a snapshot in time of the mucosal inflammation, whereas sputum sample

cells that might have accumulated in the airway lumen over a longer time period. These differences need to be taken into account when using sputum for specific purposes. As for any sample derived from the airway lumen, sputum would seem less appropriate than biopsies for studies focusing on the pathogenesis of asthma. This does however not diminish the potential of sputum eosinophil counts in the diagnosis and clinical follow-up of asthma.

It has been shown that in subjects with obstructive airway disorders, an increased sputum eosinophil percentage has a higher sensitivity and specificity for the diagnosis of asthma than blood eosinophil counts or serum ECP [73]. The degree of sputum eosinophilia was shown to correlate with the clinical severity of the disease, in some studies [74]. In addition, preliminary reports indicate that analysis of induced sputum could help in diagnosing associated conditions such as gastrointestinal reflux by identifying lipid-laden macrophages [75] or associated left heart failure by screening for haemosiderine-laden macrophages recognised by Prussian-blue staining [76]. The possible role of sputum in diagnosing eosinophilic bronchitis as a cause of nonproductive cough has also been highlighted [77].

An important characteristic of induced sputum is its responsiveness to interventions known to affect the degree of inflammation in asthma. As for eNO, allergen exposure increases the per cent eosinophils and metachromatic cells in sputum. This was initially demonstrated following exposure to high doses of allergen given under laboratory conditions to elicit dual asthmatic reactions, which also caused an increase in circulating eosinophil counts [78]. Subsequent studies have illustrated that sputum analysis is sensitive enough to reflect more subtle changes in the degree of airway inflammation. It was shown that repeated exposure to an ~10-fold lower dose of allergen also induced an increase in sputum eosinophil numbers, whereas only a very small increase in blood eosinophil was noted on the last of the five challenge days [79]. Moreover, a significant increase in sputum eosinophils has been documented to occur over the pollen season in subjects with pollen-induced asthma and rhinitis [80]. Similarly, occupational exposure in the workplace can also influence the cellular composition of sputum, without effect on serum ECP [81]. Sputum also responds to nonallergen stimuli including pollutants, such as ozone or diesel exhaust, which have both been shown to increase the number of neutrophils in sputum [46, 82, 83].

Treatment can also influence sputum eosinophil percentages. Monotherapy with short-acting inhaled β_2-agonists has been shown to increase eosinophil counts [84]. However, this effect is not observed when β_2-agonists, either short- or long-acting, are given in combination with inhaled steroids [84, 85]. Anti-inflammatory asthma treatment decreases sputum eosinophil numbers. This has been illustrated for theophylline [86, 87], antileukotrienes [88], but especially for steroids [89–93]. Recent studies indicate that sputum eosinophil counts respond to modulations of the dose of steroids, which are insufficient to influence serum markers such as ECP [23]. An important aspect that needs to be fully established is the dose-response relationship of sputum eosinophilia compared with other biomarkers to changes in the steroid dose. JATAKANON *et al.* [94] compared the effect of a 4-week treatment with budesonide 100, 400 or 1600 $\mu g \cdot day^{-1}$ on exhaled NO, sputum eosinophilia and airway responsiveness to methacholine. The effect on exhaled NO reached a plateau from a dose of ≥ 400 μg, whereas for sputum eosinophilia and airway responsiveness a significant dose-response relationship was observed throughout the different doses [94]. This aspect is of particular interest for disease monitoring. It can be argued that the very high sensitivity of eNO to the effect of steroids, combined with the nonspecific nature of the response limits the usefulness of exhaled NO in disease monitoring and that sputum eosinophilia offers more accurate information when titrating steroids to the minimal dose required to maintain asthma control.

Prospects for disease monitoring

Of particular interest is the observation that as for biopsies, the composition of sputum correlates poorly with other indices of disease activity such as symptom score, baseline FEV_1 or methacholine responsiveness. Although again, a better correlation is noted with indirect markers of airway responsiveness [35, 95–100]. Similarly, the response of sputum eosinophils to intervention is not always paralleled by changes in clinical outcome measures [89–91,101]. This implies that combining sputum analysis to other outcome measures could offer additional information, instead of merely reduplicating existing data, thus possibly improving disease monitoring.

However, before propagating the use of induced sputum in clinical asthma management, several elements need to be further clarified.

In line with the observation that sputum eosinophils correlate poorly with clinical characteristics of the disease, most cross-sectional studies conducted so far illustrate an important variability in sputum eosinophils, even when the samples are obtained from patients with a very similar clinical profile. To date, it is largely unknown whether sputum eosinophil counts in patients that otherwise seem well controlled, are important. Recent reports indicate that patients with high eosinophil counts in their sputum are more likely to lose asthma control, if their maintenance dose of steroids is reduced [102, 103]. Of note is that in these studies, eNO levels had no predictive value. In contrast, in a prospective study involving 31 steroid-treated well-controlled asthmatics the level of sputum eosinophilia was shown not to predict the likelihood of developing spontaneous exacerbations over a 1-yr follow-up period [104]. Based on these somewhat conflicting data, it is therefore unclear whether treament strategies aimed at reducing sputum eosinophils in addition to controlling symptoms will result in long-term outcome of the disease.

Even if this would prove to be the case, a related question is as to what constitutes a clinically significant reduction in eosinophil counts. Recent studies in adults and children indicate that the normal range of sputum eosinophils does not exceed 2.5% [105–107]. Whether one of the goals of asthma treatment should be to maintain sputum eosinophils beneath this or another threshold value is again unknown. Preliminary data indicate that the level of clinical-asthma control over 1-yr is not significantly different in patients who irrespective of treatment have a median eosinophil count above or below 2.5% [108]. These issues need to be further evaluated in properly powered studies that examine in a prospective way whether treatment adaptations based on sputum eosinophils in addition to clinical criteria will result in a different level of long-term control. This type of study will also allow for the evaluation of whether following sputum eosinophilia is better or perhaps complementary to including bronchial hyperresponsiveness in the monitoring of the disease. Although bronchial hyperresponsiveness correlates weakly and inconsistently to inflammatory parameters in biopsies [8], recent data have rekindled interest in this parameter as a potentially useful overall marker of asthma severity. LEUPPI *et al.* [103] reported that in contrast to exhaled NO, the combined measurement of direct and indirect bronchial hyperresponsiveness predicted loss of asthma control when reducing the steroid dose [103]. In addition, SONT *et al.* [10] have shown that compared to standard treatment based on symptoms and lung function, including reduction of bronchial hyperresponsiveness as an additional treatment aim results in a reduced exacerbation rate. Short-term studies indicate that although both bronchial hyperresponsiveness and sputum eosinophils respond to a 1-month treatment with fluticasone propionate 1000 $\mu g \cdot day^{-1}$, the changes in both parameters are not correlated [91]. Whether both markers could therefore prove complementary, again needs to be evaluated.

Another question is whether treatment should be diversified based on the cellular

composition of sputum samples in asthmatics. An increasing number of reports indicate that in asthma roughly two different patterns of inflammation can be distinguished: one in which eosinophils predominate and another that is not eosinophilic, but neutrophilic. This has initially been described in asthma exacerbations [109, 110], but also seems to apply in persistent steroid-treated asthma, irrespective of severity [111–113]. It has been suggested that this has therapeutic implications, as sputum eosinophilia might predict a favourable response to steroids [114–116]. However, although tempting, these recommendations remain to be confirmed in larger scale studies.

Analyses on induced sputum

To date, analysis of induced sputum has focused on the cell fraction, eosinophils in particular. In view of the complexity of the airway inflammation in asthma, complementing this analysis by the measurement of soluble mediators in the sputum supernatants might offer an even more accurate assessment of the inflammatory process. A range of molecules has been detected in supernatant including markers of increased vascular permeability such as fibrinogen or albumin [62, 63, 117], pro-inflammatory mediators including eicosanoid metabolites [118,119] and a variety of cytokines [113, 120, 121]. In general, the soluble markers have been less well validated. This is at least in part due to methodological problems associated with the collecting and processing of the sample as well as interference in the assay with mucus components [122].

What would seem to be of particular interest is to complement eosinophil counts with assessment of a marker that reflects airway remodelling, the more chronic phase of airway inflammation in asthma. As already indicated, theoretical models highlight the contribution of remodelling to the altered airway behaviour in asthma. Monitoring an index of remodelling in the follow-up of asthma therefore appears relevant. The ideal marker of remodelling remains to be identified. Possible candidates include growth factors or enzymes such as elastase or matrix metalloproteinase that are present in increased concentrations in the sputum supernatant of asthmatics [123, 124]. However, the exact functional role of these various molecules in the remodelling process is largely unknown, thus hampering the validation process.

A final point that needs to be further addressed is the feasibility of sputum processing. Provided proper attention is paid to the procedure, sputum induction has proven to be safe in asthma, even in the more severe forms of the disease [110, 125, 126]. Provided the sample is then processed according to a validated technique, the results are reliable [127]. However, it has to be realised that the samples need to be processed within 2 h after induction, in order to avoid deterioration of cell morphology. In addition, the overall procedure is time consuming and requires highly qualified laboratory technicians. Analysis of induced sputum is therefore expensive. Hence, if sputum analysis is to become a tool that is accessible to most clinicians, this technique needs to be simplified to become less labour intensive. Several approaches are currently being developed in this respect. This includes freezing or simultaneous homogenisation and fixation of sputum immediately after producing the sample [128]. This improves the preservation of cell morphology and allows for a longer time delay between induction and analysis of the sample. In addition, this would also enable automated cytometry. Another approach that has been proposed consists of lysing the cell pellet obtained after homogenisation and centrifugation of the sputum sample, in order to release eosinophil associated ECP. ECP in the cell lysate was found to correlate strongly with the absolute numbers of eosinophils in the cell pellet. The ratio of ECP in supernatant over ECP in the lysed pellet would even offer a marker of the degree of activation of eosinophils in the sputum sample [129]. This

technique has the advantage that the measurements can be automated, saving costs. Albeit theoretically appealing, these various approaches need further validation. Another potential disadvantage of induced sputum is that the induction process with nebulised hypertonic saline induces an inflammatory response, so that it is not advisable to make repeated measurements in less than 24 h [128]. While this may not be a limitation in long-term disease monitoring, it limits research studies of kinetic factors.

Conclusion

Analysis of induced sputum offers a relatively noninvasive direct marker of airway inflammation in asthma. Including sputum analysis in the diagnosis but especially in the follow-up of the disease, could offer additional information to clinical-outcome measures. Measurement of exhaled biomarkers is also very promising and is feasible in children and patients with severe disease. Whether incorporating these new measurements into disease monitoring will result in improved long-term clinical control of asthma, or allow for the diversification of treatments now needs to be addressed in carefully designed studies.

Summary

Bronchial asthma is currently considered and defined as an inflammatory disorder of the airways. It therefore seems logical to include a direct marker of airway inflammation in the diagnosis and follow-up of the disease. Several relatively noninvasive biomarkers have been and are being considered in this respect. Examples that have been most extensively investigated, as to their reliability and validity for the assessment of airway inflammation in asthma, include blood eosinophil counts or serum eosinophil cationic protein, urinary eicosanoid metabolites, exhaled gasses, mediators in breath condensate or induced sputum. From the studies performed to date, it would appear that biomarkers correlate poorly with other indices of disease activity, such as symptoms, baseline forced expiratory volume in one second or methacholine responsiveneness. As such, including biomarkers in the diagnosis, but especially follow-up of the disease, could offer additional information to clinical-outcome measures. Whether this attitude in disease monitoring will result in improved long-term clinical control of asthma or allow for the diversification of treatments, now needs to be addressed. In addition, the routine use of these techniques in daily practice requires simplification of the methodology involved.

Keywords: Asthma, biomarker, breath condensate, exhaled nitric oxide, induced sputum.

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