

Biomarkers in asthma

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Purpose of review

The purpose of this review is to highlight seminal and current literature that informs our understanding of the clinical and investigative utility of biomarkers in asthma.

Biomarkers derive from a variety of sources [bronchiolar lavage (BAL), sputum, exhaled breath, and blood], and have widely variant performance characteristics, and applicability.

Recent findings

Increasing attention is given to biomarkers in exhaled breath, both gaseous (exhaled nitric oxide) and higher molecular weight moieties [in exhaled breath condensate (EBC)]. Current research in EBC analysis has focused on validation, standardization, and technical considerations, whereas research on exhaled nitric oxide (ENO) has moved to testing its predictive value in clinical situations. The use of advanced biostatistical techniques, and combinatorial analyses has led to additional advances in the utility of biomarkers.

Summary

To date, the best validated, and best performing biomarkers for clinical asthma appear to be measures of inflammation in induced sputum, and measures of ENO. Some trials using ENO appear particularly promising for early clinical use. EBC metrics are at present too inchoate for clinical purposes. However, not all important clinical and research questions can be addressed with sputum, EBC, or ENO metrics, leaving an important place for BAL, bronchial biopsy, and perhaps EBC measurements in the research arena.

Keywords

asthma, biomarker, phenotype, prediction

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Introduction

Asthma is a complex syndromic disorder characterized by airway inflammation, airway hyperresponsiveness, and variable airway obstruction. The spectrum of asthma in clinical presentation is broad, and underlying pathogenic mechanisms may be quite different. In addition, responsiveness to therapies is not uniform across the asthma spectrum, nor is it necessarily consistent in individual patients over time. There is considerable temporal variability in objective measures of asthma control, related to both known (viral infections, allergen exposures) and unknown or unrecognized triggers of asthma exacerbation. The vast majority of these worsenings are associated with increased levels of airway inflammation. Consequently, measurement of objective metrics of airway inflammation in asthma could be of significant value to clinicians who manage patients with asthma. Such measurements could inform the proper choice of therapy, provide an objective measure for adjusting asthma medication dose, allow separation of asthma phenotypes that are clinically otherwise indistinguishable, and might offer objective classification of asthma for prospective clinical

trials. The most direct measures of airway inflammation, however, are too invasive and have limited clinical use.

The purpose of this review is to summarize the existing base of knowledge in the field of biomarkers in asthma, highlight key strengths and limitations of each approach, review recent contributions to the field, analyze their clinical implications, and suggest directions for future research.

The value of a biomarker, or surrogate marker, is maximized when the surrogate is cheaper, less invasive, technically simpler, or is more broadly available than a 'gold standard'. In addition, there should be biologic plausibility to link the putative surrogate to the metric of interest. (An ideal biomarker is cheaper, less invasive, technically simpler, and is more broadly available than the 'gold standard' with a strong biologic plausibility link to the metric of interest and clinical validity.) For purposes of this review, we will consider the 'gold standard' of assessing airway inflammation to be bronchial biopsy. Bronchial biopsy is expensive, invasive, technically complex, and not widely available to physicians and

researchers who treat and study asthma. There is also considerable variability due to sampling locale and technical factors and hence there would be great utility in identifying and validating a surrogate marker for airway inflammation.

Systemic markers of airway inflammation

Although measures of cells and proteins in peripheral blood have been associated with features of asthma, there are few data to suggest that these measures could provide meaningful insights regarding airway inflammation.

Urine biomarkers

Urinary cysteinyl leukotrienes have been identified as a potential marker of response to leukotriene receptor antagonists, especially in aspirin intolerant individuals [1]. Cai *et al.* [2] noted that among 48 asthma patients in China, patients with a positive clinical response to montelukast therapy had higher pretreatment urinary leukotriene E₄ (LTE₄) levels than nonresponders (224 vs. 175 pg/mg respectively, $P < 0.05$). Although this difference was not sufficiently large to be useful as a clinical predictor of response, the authors state that individuals with urine LTE₄ levels of 200 pg/mg creatinine or more, endows a 3.5-fold greater chance of responding to montelukast than individuals with urine LTE₄ levels less than 200 pg/mg creatinine. This compares to a previous study in children [3] where a cutoff of 100 pg/mg creatinine for baseline urine LTE₄ levels corresponded to a 3.2-fold increased response rate to montelukast. Smoke exposure increases rescue albuterol use, and response to montelukast, suggesting that the smoking phenotype may be somewhat more favorable for leukotriene therapy. Despite these observations, routine measurement of urinary leukotrienes does not appear to have gained widespread acceptance and currently a therapeutic trial of leukotriene antagonists seems to be a simpler option.

Exhaled nitric oxide

Since the discovery of nitric oxide in exhaled air of humans in 1991 [4], and its studied association in allergen induced airway inflammation [5], exhaled nitric oxide (ENO) is the most widely studied and standardized noninvasive biomarker that evaluates airway inflammation in asthma [6,7]. ENO has been well established as a marker of eosinophilic airway inflammation. Its utility to assess response to anti-inflammatory therapy has also been well documented [8,9].

Nitric oxide concentration in expired air is dependent on the flow rate, spatial ventilation distribution [10] and axial diffusion of nitric oxide [11]. The standardized single breath constant expiratory flow (50 ml/s) technique requires the patient to maintain a positive expiratory

pressure and a low flow rate to accentuate the nitric oxide signal [12]. This excludes nasal and oropharyngeal nitric oxide by closing the velum and ensures reproducibility. However, lower flow rates increase the transit time in the airways and diffusion of nitric oxide thus reducing the output. In 2005, the American Thoracic Society and the European Respiratory Society made recommendations regarding the standardized method to measure ENO [13].

ENO correlates with sputum eosinophil count, airway hyperresponsiveness, bronchodilator response, serum IgE levels, allergen skin prick testing, asthma symptoms and lung function [14,15]. ENO levels are affected by age, height, weight, gender, and race [16,17]. Unlike functional assessment, ENO is generally free of day-to-day and diurnal variation and hence is highly reproducible (intraclass correlation coefficient 0.99) [18]. Smoking, alcohol use, spirometric testing, sputum induction, and exertion reduce exhaled nitric oxide. Ozone exposure and *L*-arginine supplements increase ENO.

Exhaled nitric oxide is a reliable and sensitive screening tool to diagnose asthma in high-risk untreated patients. The sensitivity of ENO levels more than 20 parts per billion, in diagnosing asthma among patients with suggestive symptoms was 88% (compared with 0–47% for spirometric and peak flow measurements) with a negative predictive value of 92% [19]. The European Community Respiratory Health Survey II study showed that ENO of 18.5 parts per billion or more had a sensitivity (69.2%) and negative predictive value of 95%, for the diagnosis of asthma, confirming the role of ENO as a screening tool [20]. ENO has a significant clinical role in the evaluation of chronic cough, noninvasively identifying patients with airway hyperresponsiveness and cough variant asthma [21•]. ENO levels do not correlate with disease severity in asthma but complement symptom and spirometric assessments in persistent asthma patients [22••]. Serial measurements of ENO aid in monitoring disease activity, studying asthma control [23,24], and in assessing compliance and response to therapy [9,25•]. ENO levels rise before drop in PEF is noted. Thus ENO shows great promise in identifying impending exacerbations [26]. ENO identifies relapse in patient under clinical remission [24,27] and potentially reduces subclinical inflammation that leads to remodeling and progressive loss of lung function. The clinical utility of ENO measurement in the settings of the emergency room and in-hospital has been studied but remains to be confirmed [28].

A recent cost effectiveness study estimated a saving of 30–120 Euros per patient per year in the ENO guided treatment group. In the USA, the cost of a single test is estimated at \$165, but Medicare and Medicaid services approved reimbursement is only \$19. Till to date, com-

mercial insurers do not reimburse for office measurements. However in a recent trial, asthmatics in the ENO guided treatment group used significantly lower maintenance dose of inhaled corticosteroids than patients in the conventional guidelines based treatment group without significant increase in exacerbation rates [29^{••}]. In the long run incorporating ENO might reduce the healthcare utilization costs by identifying patients who may be steroid resistant and aid in back titration of steroids in well controlled patients. This hypothesis is currently being testing in the context of the United States Asthma Clinical Research Network (ACRN) in the BASALT (Best Adjustment Strategy for Asthma in the Long Term) Trial.

ENO continues to be an outcome marker in clinical trials for current and newer therapies for asthma [30]. The availability of reduced cost portable handheld nitric oxide analyzers, ease of use, real time results will expedite the reach of ENO in to standard of care of asthma management and in to the homes of patients [31].

Exhaled breath condensate

Exhaled breath condensate (EBC) analysis is a noninvasive method to evaluate airway inflammation through various inflammatory mediators and markers, which helps in the understanding of the pathophysiology of asthma. EBC is obtained by collecting warm breath in a cold condensing tube. The condensate is rich in volatile and nonvolatile inflammatory mediators and allows simultaneous analysis through high output methods. EBC reflects changes in the airway lining fluid.

EBC collection is easy and can be used in children and in office settings without much training. EBC collection procedures are not standardized and to date remains a research tool. EBC parameters are influenced by smoking, alcohol consumption, equipment, exercise, mode and rate of breathing, nasal and salivary contamination, environmental temperature and humidity, and assays used [32[•],33–35] leading to undesirable variability. The influence of age, sex, circadian rhythm, and infection remain unknown.

EBC markers increased in asthmatics are adenosine [36], markers of oxidative stress such as hydrogen peroxide [37], isoprostanes [38], nitrogen oxides [39], pH [40], ammonia, prostanoids and leukotrienes [41,42,43[•]]. EBC pH is lower in asthmatics and correlates well with sputum eosinophilia, total nitrate/nitrite, and oxidative stress. EBC pH showed good repeatability in asthmatics (ICC 0.97) over a 1-year period without seasonal variation, but did not reflect the clinical status of the patients [40]. EBC markers that correlate with disease severity, response to treatment, or both are

hydrogen peroxide, leukotrienes [44], 8-isoprostane, nitrate, and nitrite [45[•]]. The concentrations of various mediators studied are very low, requiring highly sensitive assays. These pitfalls limit the clinical validity and utility of EBC. Additionally, biomarkers in EBC do not correlate with biomarkers in bronchoalveolar lavage (BAL) fluid [46[•]].

Future research should focus on standardization of collection procedures, validation of sensitive analyses and establishing reference values [47,48]. The ease of collection of EBC makes it an attractive substrate for proteomics and metabolomics, in efforts to identify new biomarkers, improve disease phenotyping and to identify new targets for therapy.

Expectorated sputum

Sputum induction is a relatively noninvasive method of sampling the airway milieu and hence is an attractive source of biomarkers. In addition, induced sputum analysis has the potential to be done in a clinical setting. Elements of both the cell phase and the supernatant have been utilized to this end.

Sputum-cell phase

Elucidation of the predominant cell type in sputum has allowed asthma patients to be categorized on this basis. Sputum eosinophilia as a marker of asthma severity and subsequent steroid responsiveness is well established. Patients with increased sputum eosinophils have greater basement membrane thickening and a positive response to steroid therapy [49[•],50], but lack a correlation with airway hyper-responsiveness [51]. Green *et al.* [52] and Jayaram *et al.* [53] have shown that a protocol based on suppressing sputum eosinophilia, compared with standardized guideline based therapy resulted in less exacerbations and steroid bursts (as total steroid usage remains the same). This suggests that sputum eosinophilia reflects inadequate control of airway inflammation; increasing the risk of subsequent exacerbation. This hypothesis was supported by the work from other laboratories that found sputum eosinophilia to be a predictor of exacerbation in asthmatic individuals after a graded inhaled steroid withdrawal [54[•]].

Sinus disease as a possible confounder to this was investigated by Mehta *et al.* [55]. Two hundred and one asthmatic patients had both sinus computed tomography (CT) and sputum eosinophil analysis performed. The severity of sinus disease as determined by a CT scoring system was positively correlated with both peripheral and sputum eosinophilia, suggesting a relationship of sinus disease with systemic inflammatory markers.

Sputum induction and subsequent cell count remains labor intensive and requires both skilled staff and sufficient experience to reduce the rate of inadequate sample expectoration and to reduce interobserver error in plate reading. Rheologic properties of sputum currently prohibit automated cell counts. In Europe, however, where the margins between clinical care and research are less distinct than in the United States, induced sputum counts are rapidly becoming an essential tool in the management of patients with oral corticosteroid-dependant asthma. The relationship between eosinophilic cationic protein and eosinophil counts may provide a means of obtaining similar information with a relatively simple assay [56].

The clinical utility of finding sputum neutrophilia remains a step behind. In an admirable example of attempting to tailor therapy to a clinical phenotype of severe asthma, Simpson *et al.* [57[•]] targeted patients with refractory asthma (by ATS workshop criteria) and added clarithromycin to standard therapy in a randomized double-blind placebo-controlled trial. Overall, there were significant reductions in both neutrophils and neutrophil-derived biomarkers (matrix metalloprotease-9 and interleukin-8), but not in the clinical endpoints. Subgroup analysis suggested that this benefit was restricted to individuals with noneosinophilic (either paucigranulocytic or neutrophilic predominant) sputum. The numbers in this latter subgroup were small (14 in both active and control). The results warrant further confirmation in a study powered to detect clinical endpoints and excluding eosinophilic asthma.

Sputum supernatant

A number of potential biomarkers have been examined in induced sputum supernatant. Urokinase plasminogen activator and plasminogen activator inhibitor [58[•]] are raised in asthma individuals over controls, and Neurokinin A [59] rise markedly during exacerbations. Neither has reached the stage of clinical utility. Contrary to expectations, 8-isoprostane was found not to be raised in newly diagnosed asthmatics [38]. Saha *et al.* [60] measured sputum and endobronchial biopsy IL-13. Although IL-13 positivity in sputum was more common in asthmatics than controls, it was neither a consistent finding (occurring in less than half of asthmatic individuals) and bore no relationship to asthma severity. There was, however, a suggestion that IL-13 positive cells were increased in the submucosa of all asthmatic patients and in the airway smooth muscle bundle of severe asthma patients compared with all other individuals. Numbers in this study were low and these findings need to be confirmed.

Bronchoalveolar lavage

The technical aspects of BAL have been extensively investigated for more than two decades [61]. The major strength of bronchoscopic evaluation of airways is the

recovery of biological samples in the form of cellular and soluble materials from medium and small airways, and alveolar spaces. Good tolerance of these procedures in asthma patients of mild and moderate and severe levels of disease have been well documented [22^{••},62,63]. BAL has aided in the identification of a gamut of mediators currently thought to have a significant role in the pathophysiology of asthma (e.g. IL-1, IL-2, IL-4, IL-5, IL-6, IL-10, IL-13, GM-CSF, TNF- α , ICAM-1) [64].

The clinical utility of BAL fluid biomarkers is questioned by the lack of standardization from laboratory to laboratory in reporting the levels of various markers, making comparisons between laboratories difficult. Further, as disease state changes, and volume recovery changes, interpretation of serial evaluation in the same individual is troublesome. Procedural complications of bronchoscopy and BAL are rare.

BAL studies of stable asthmatics with mild disease have generally shown no significant difference in the cellular constitution of BAL fluid when compared with normal volunteers. However, eosinophil predominance is suggestive of asthma. Patients with moderate asthma not treated with corticosteroids have higher percentages of eosinophils in BAL fluid. However, in severe steroid resistant asthma, a neutrophil predominant phenotype is noted [22^{••}]. The cellular and mediator characterization of asthmatic airways through BAL have failed to show consistent correlation with clinical severity. The invasive nature of the procedure has thus far limited its use to research purposes only.

Bronchial brushing

Bronchial brushing provides fresh single epithelial cells, which are unobtainable through BAL or biopsies. Bronchial brushes and cell cultures from harvested cells have demonstrated the inflammatory role of epithelial cells in asthma pathogenesis and variation in response to bronchodilators.

A bronchial epithelial cell forms the first line of defense in the airways and is the communicator between the environment and inflammatory cells. These cells are central to the pathogenesis of allergen sensitization and viral infections related to asthma [65]. These cells from asthmatics exhibit structural and functional differences from normal individuals, such as cytokeratin profiles, augmented release of anti-inflammatory mediators (IL-6, PGE₂, EGF, 15-HETE), faster doubling time, mucus overproduction and increased susceptibility to apoptosis [66]. In steroid treated asthmatics, the epithelial metachromatic cells reflect clinical severity [67]. Bronchial epithelial cells are potential source of nitric oxide and thus play an integral part in airway inflammation [68]. Damage to bronchial epithelial cells and

subsequent repair process, activates the myofibroblasts in the lamina reticularis which leads to a cascade of events resulting in airway hyperresponsiveness and remodeling [69].

Acceptable safety of the procedure has been well documented in asthmatics. The viability is limited by the specimen procuring and processing techniques. Epithelial cell cultures are models to study various mechanisms *in vitro*. However, results from these experiments must be interpreted with caution, because in-vitro systems differ greatly from in-vivo cellular responses to stimuli due to lack of communication with other compartments and neural and humoral feedbacks.

Bronchial biopsy

Biopsies provide morphological information on various anatomical compartments of airways, such as epithelium, basement membrane, submucosa and smooth muscle. Accordingly, it is appropriate to consider bronchial biopsy as the 'gold standard' for assessing airway inflammation. Tissue components are evaluated by light microscopy, electron microscopy, immunohistochemical stains, fluorescence, in-situ hybridization, or PCR for mRNA expression for cytokines and adhesion molecules.

Normal individuals have intact epithelium and lack cellular infiltrates in the submucosa. Bronchial biopsies from asthmatics show evidence of epithelial shedding, goblet cell hyperplasia, thickened lamina reticularis and submucosal eosinophilic and lymphocytic infiltration. Thickened basement membrane due to increased collagen deposition in the reticular layers and proliferation of myofibroblasts are the hallmarks of airway remodeling leading to obstruction [70]. Asthmatic airways have increased granulation of mast cells and high affinity IgE receptor bearing cells [71]. Steroids and other anti-inflammatory interventions have been shown to reduce T-cell and eosinophil infiltration of the lamina propria [72]. Also increased is the expression of IL-4, IL-5, endothelin, eotaxin and ICAM-1; these studies of protein and mRNA expression have added to the understanding of pathogenesis of asthma. A recent study using bronchial biopsy tissues have identified enhanced IL-8 and IFN- γ expression, also IL-5 and eotaxin expression and reduced IL-4 expression of severe asthma compared with moderate disease [73]. Changes in inflammatory mediators and cellular profile precede clinical deterioration, underscoring the need for biomarker monitoring to effectively manage asthma. Patients with reduced FEV1/FVC ratio had thicker basement membranes suggesting airway remodeling.

Studies utilizing bronchial biopsies to quantify inflammation are limited by normal anatomic variability, patchy focal inflammation, sample size, and number of biopsy specimens and quantification of cellular infiltrate. Studies

evaluating the variability and reproducibility of data obtained from bronchial biopsies of asthmatics suggest a sample size of 8–25 individuals to study individual cell types and 13–48 individuals per arm in parallel design drug studies are required to establish adequate statistical power. However, equivocal results in studies conducted on small number of patients may just reflect type II statistical errors. Consequently, it is important to evaluate bronchial biopsy studies for size, reproducibility, and replication.

Novel analytical methods

Preceding sections have summarized the strengths, limitations, and interpretation of biomarkers of airway inflammation in asthma. What is clear is that most single surrogates, having significant sensitivity and specificity, and some predictive value, generally are more useful as population-based tools, rather than for phenotyping an individual subject, or predicting response to therapy for an individual patient. Given the complex pathogenesis of asthma, and its syndromic nature, it is not surprising that no single biomarker has emerged. A novel strategy is to use combinatorial analysis and bioinformatics techniques to extract information from a dataset, the individual components of which may not be particularly informative. In one such approach, we evaluated biomarkers of airway inflammation (cytokines in bronchoalveolar lavage fluid) in individuals with severe asthma, and compared those with nonsevere asthma. Using unsupervised (and unbiased) cluster analysis, an increasingly common bioinformatics approach, we identified four molecular phenotypes, one of which was significantly enriched for individuals with severe asthma. Three other molecular phenotypes distinguished asthmatic individuals who were otherwise clinically indistinguishable, suggesting that powerful bioinformatic methods may provide useful information from datasets that are individually ambiguous [74^{*}]. Moreover, these approaches have yielded predictive data on airway hyperresponsiveness, airway eosinophilia, and other important clinical metrics. Accordingly, these powerful approaches are likely to be more broadly utilized to extract maximal information from increasingly complex datasets.

Conclusion

To date, the best validated surrogates for airway inflammation are ENO, and measurements of components of induced sputum. There are published clinical applications using ENO to guide asthma management, but whether these approaches can be extrapolated from these trials, conducted under very controlled circumstances, to clinical practice is more uncertain. Induced sputum likewise shows predictive value in several circumstances related to asthma management, but the

practical difficulties of sputum induction in patients, and technical variation in sputum processing and analysis limit the applicability of these metrics in the clinic. At present, biomarkers in urine and circulating blood have insufficient predictive power to be of clinical value.

In the research arena, however, each of these techniques provides complementary information. Invasive metrics arguably provide the most biologically relevant information for disease pathogenesis. Mediator and cytokine concentrations can only be measured in fluid phase (BAL, expectorated sputum, and EBC). Structural considerations can only be addressed with biopsy material. Brushings and BAL can provide cellular material for functional studies. Clearly, additional validation and application work will be necessary to advance the field of biomarkers in asthma to the level that respiratory clinicians caring for patients can use them in a predictive, cost-effective manner.

References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- of special interest
- of outstanding interest

Additional references related to this topic can also be found in the Current World Literature section in this issue (pp. 80–81).

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