DOI:
10.1586/14737159.2015.988613

Document Version
Peer reviewed version

Novel biomarkers for asthma stratification and personalized therapy

Journal:	*Expert Review of Molecular Diagnostics*
Manuscript ID:	ERO-2014-0075.R1
Manuscript Type:	Reviews
Keywords:	asthma, biomarker, periostin, eosinophils, nitric oxide

Abstract

A stepwise pharmacological treatment is currently recommended for all asthma patients and is personalized mainly on disease severity, aiming for the lowest step that controls disease. Thus mild intermittent asthma is treated with short acting bronchodilators (step 1). If control is inadequate, therapies of increasing power are introduced (steps 2, 3 and 4) whereas oral steroids (step 5) are reserved for severe asthma.

Nevertheless, asthma comprises several related pathologies with similar clinical manifestations that result from different underlying mechanisms. Therefore novel biomarkers could lead to asthma stratification and thus replace the current stepwise approach.

Promising biomarkers are sputum eosinophils, serum periostin and exhaled nitric oxide. Periostin could differentiate between Th2-high and Th2-Low asthma (Th-2-high patients are more responsive to glucocorticoids) and the less-defined asthma types which often present a therapeutic challenge. Several other biomarkers, mainly cytokines, leukotrienes and exhaled air components, can be quantified in body fluids and exhaled breath and could also be useful for asthma stratification.

Keywords: asthma, biomarker, inflammatory, periostin, eosinophils, exhaled nitric oxide, volatile organic compounds, chitinases, prostaglandins.

Introduction

Asthma is a complex, chronic disease characterized by episodes of reversible airflow obstruction, manifesting in breathlessness, wheezing and cough, with various degrees of airway inflammation, remodelling and bronchial hyperreactivity [1]. Short- and long-acting adrenergic β_2-agonists (SABA/LABA) and inhaled corticosteroids (ICS) have radically improved the lives of many asthma sufferers. Recent years have seen notable progress with regard to the mechanisms involved in asthma immunology and multiple trials of biological drugs, designed to block signaling molecules deemed to be important in its pathology. On the whole, these have met with mixed success, but importantly they provided useful mechanistic insights for post-trial analyses, demonstrating that patients receiving such drugs could be divided into distinct groups of good and poor responders depending on their pre-trial characteristics. This has strengthened the hypothesis that rather than being a single disease, asthma consists of a group of pathologies which converge towards similar clinical manifestations [2].

The current assumption is that asthma could be differentiated into subtypes defined by biomarkers that reflect the predominant pathophysiology in the case of individual patients. Thus, treatment personalization could be achieved using biomarkers [3]. In this review, we outline the most effective strategies for identifying biomarkers in asthma, discuss the current experimental and clinical evidence for their predictive accuracy and assess their potential usefulness in a clinical setting for guiding targeted therapies for this asthma.

Pathophysiology of asthma: not just a Th2-driven disorder

In classic allergic asthma, the first exposure of an organism to an allergen may cause sensitization, a process whereby an inhaled allergen contacts its specific pattern recognition receptor (PRR) on the surface of the airway epithelial cells (AECs), causing them to release interleukin-1 (IL-1) [4]. IL-1 is an autocrine mediator, triggering the release of further cytokines: granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-33 [5] and IL-25 [6]. These mediators, in turn, activate dendritic cells (DCs) and recently discovered [6] group 2 innate lymphoid cells (ILCs) [4]. Subsequently, DCs induce maturation of naive T-cells in lymph nodes into T-helper 2 cells, which produce IL-5, IL-9 and IL-13 (Th-2 type cytokines); whereas allergen-activated ILCs are able to directly produce these three cytokines [7]. In a simplified

description, Th-2 responses can be thought of as B-cell-mediated (humoral immunity), in contrast to Th-1 (T-cell mediated, cellular immunity) responses. In healthy humans, precarious balance exists between Th-1 and Th-2 responses - excessive activity of the former pathways traditionally linked to autoimmune diseases such as multiple sclerosis, while the latter to asthma and allergies.

When re-exposure to the allergen occurs, AECs vigorously resume the production of their ILC-stimulating cytokines, while DCs vastly upregulate their capacity to promote Th-2 cell maturation. Both of these result in a rapid increase in the Th-2 cytokine levels, leading to a substantial increase in the airway populations of mast cells, eosinophils and Th-2 cells themselves, as well as enhanced B-cell IgE class switching [4].

Mast cells are resident tissue cells which play a significant role in sensitization and subsequent allergic response. First encounter with an allergen leads to production of IgE antibodies against it through Th-2 pathways. These antibodies attach to mast cells through FCεRI receptor. On subsequent exposure, antigen molecules bind to the surface-bound IgE molecules, crosslinking them and leading to FCεRI activation, induction of complex phosphorylation cascades and mast cell degranulation [8], which involves liberation of various pre-formed mediators (histamine, serotonin, tryptases). At the same time, there is an upregulation in biosynthesis of freshly produced mediators: lipids such as PGD2 and LTC4, and chemotactic factors IL-4, IL-5, IL-6 [9], leading to inflammatory cell influx and tissue remodelling. MC degranulation can also occur in other ways than antigen binding to its IgE, making MC biology much more complex. For instance, mast cells possess some receptors for IgG antibodies, and a recent study demonstrated that anaphylaxis to peanuts in mice is at least partly mediated by IgG signaling pathways [10]. MCs can also be directly activated by various molecules, such as previously mentioned IL-33 [11]. Basophils are morphologically and functionally similar to mast cells [4].

The functions of eosinophils in normal physiology are controversial, with some authors even suggesting they are entirely dispensable [12]. Despite the early observation that many asthmatic patients have high levels of these cells in their airways, their role in asthma remains largely shrouded in mystery. Once perceived simply as end-stage cells recruited by Th-2 cytokines and responsible for airway remodeling, they are now thought to be at least partly responsible for Th-2 cell maturation through IL-4 release [13].

Taken together, asthma could be viewed a disease where AECs and innate immune cells cooperate to activate adaptive immune cells in form of Th-2 cells, whose cytokines bring about the typically seen pathophysiological changes. However, it has been known for some time that many asthma patients do not conform to this simplified view. For instance, Woodruff et al. in 2009 found that nearly half of the asthmatic patients do not differ from non-asthmatic controls with regard to their Th-2 cytokine levels [32]. This can be partly explained by the yet unknown functions of the innate immune cells, especially neutrophils, which feature prominently in the airways of some patients with severe asthma [14]. Novel T-cell subsets have also been discovered, including Th17, Th9, CD8+, TReg, NKT and T cells, each with their likely role in asthma [7]. Unfortunately, specific immune cell populations investigated in mice (on which the majority of research is carried out) can be difficult to translate to humans. Nevertheless, complex interactions between immune cells likely explain the diversity of asthma; the next section explains the clinical significance of this diversity.

Asthma phenotypes, endotypes and the quest for biomarkers

To date, asthma has been treated using the 'one size fits all' approach, i.e. prescribing medication to control asthma symptoms, without examining the underlying pathophysiology responsible. This has allowed to drastically increase the quality of life of most patients, but important issues such as optimal treatment of exacerbations and stepping down the medication remain unsolved (for detailed discussion of which we recommend a thorough review by Busse et al. [15]). Commonly used ICS have a dampening effect on a broad spectrum of immune pathways and are characterized by mild side effects, but are ineffective in some patients, leading to persistent asthma symptoms or asthma exacerbations. When this happens, clinicians step up the dose or prescribe oral corticosteroids, which possess multiple side effects and hence can only be used for short periods of time. However, the increased knowledge of immunology of asthma (see previous paragraph) has brought about the realization that asthma constitutes a syndrome or group of diseases, sometimes with markedly different pathologies, which often misleadingly present with deceptively similar symptoms. Pathological pathways which predominate in one

patient may be completely silent in another. Learning how to assess the biochemical pathways in asthma in individual patients and introducing drugs able to selectively modify them would enable clinicians to personalize asthma therapy [15].

In order to better classify heterogenous diseases, we can subdivide them into 'phenotypes', which are sets of easily measurable characteristics, such as patient symptoms or clinical measurements. Crucially, knowledge of phenotypes does not require the knowledge of the underlying pathophysiology, relying instead on purely statistical methods, such as cluster analyses (methods for dividing subjects/objects into distinct sets with similar characteristics). 'Endotypes' can be thought of as subtypes of diseases assigned on the basis of elucidated pathophysiology. As such, endotypes are more objective and potentially more useful, and would reflect a higher level of understanding of asthma, which has not yet been achieved – current approaches rely on identifying phenotypes. Various cluster analyses have been performed to distinguish phenotypes, such as that by Moore et al. in 2010, who divided 726 patients into 5 distinct asthma phenotypes [16]. Having analysed a number of similar studies, Wenzel in 2012 divided the known asthma phenotypes into two main groups: Th-2 and non-Th-2 asthma [17]. The former category encompasses classical inflammatory asthma phenotypes, which are amenable to treatment with ICS, such as childhood allergic asthma. The latter contains more mysterious phenotypes such as obesity-associated asthma and neutrophilic asthma, which often arise in adults and are frequently resistant to standard treatment. It is hoped that better understanding of these phenotypes and ultimately elucidating their pathophysiology would help design selective, targeted treatment which could be given to the patients suffering from the particular phenotype, in place of the current stepwise treatment strategy.

The identification of asthma phenotypes necessitates the use of biomarkers whose ideal features are the following:

1) High sensitivity and specificity (and the associated positive and negative predictive values), allowing to reliably identify a given phenotype and exclude other phenotypes.
2) Easily measurable, not requiring complex, expensive and potentially risky interventions.

3) Correlated with treatment responses, e.g. allowing treatment adjustments on the basis of observed trends in biomarker values.
4) Biomarkers should be useful to better understand the underlying asthma pathology (phenotypes could then be replaced by endotypes).
5) Finally, an ideal biomarker should provide quick and direct results to facilitate its clinical use.

Below we discuss the key asthma biomarkers and evaluate which phenotypes they are likely to distinguish between.

Airway biopsies, bronchoalveolar lavage and sputum leukocytes

Airway biopsies provide a good indication of asthma severity, allowing physicians to determine the extent of tissue remodeling and local inflammation. However, their inherent invasiveness and considerable cost limit their application to the most complicated, treatment-refractory asthmatic patients. Bronchoalveolar lavage (BAL) is slightly less cumbersome, but still needs to be performed in a hospital, which restricts its widespread use. In contrast, assessment of induced sputum is less invasive and more cost effective – although still too awkward for routine use [3]. Sputum cellularity is important in order to detect the percentages of neutrophils and eosinophils which are strongly implicated in airway inflammation at all levels of asthma severity.

Simpson et al. in 2006 investigated induced sputum samples of 93 non-smoking asthmatic patients and 42 controls [18]. The aim was to investigate the best way of diagnosing non-T_H2 asthma (which they named NEA, non-eosinophilic asthma) based on sputum cell counts and its variability between patients. For that purpose, they obtained induced sputum samples and quantified the neutrophil and eosinophil levels within these.

The authors found that calculating both absolute and relative levels of sputum eosinophils yielded similar results, allowing them to establish four asthma phenotypes: eosinophilic (raised eosinophil levels), neutrophilic (raised neutrophil levels), mixed granulocytic (raised neutrophils and eosinophils) and paucigranulocytic (normal neutrophil and eosinophil levels). Moreover, 17 out of 18 asthma patients who were originally classed as NEA at the beginning of study retained

their allocation to that group after 4 weeks and 6 out of 7 NEA classified patients who attended a follow up visit approximately 5 years after the original study still maintained their status. Interestingly, the study did not find any significant clinical differences between the patients, except for a higher mean age in the neutrophilic asthma group. This study aimed to achieve asthma stratification as it was one of the first to measure multiple indices in relation with induced sputum eosinophils and neutrophils which are known to be effectors in asthma pathogenesis.

Whether these induced sputum biomarkers will be useful for personalizing asthma treatment is however still unclear. Various trials have demonstrated that sputum eosinophils and neutrophils may be used to guide ICS dosing to reduce the frequency of and predict asthma exacerbations and to indicate asthma severity.

Possibly the most important study in this field was one by Green et al. in 2002 [19]. The authors randomized 74 asthmatic patients to either sputum management group, where management was guided by sputum eosinophil concentrations and severity of symptoms, or to British Thoracic Society group, with treatment guided by this Society's asthma guidelines. When the study was finished after one year, the authors found that the former group had significantly fewer sputum eosinophils (63% lower as measured during 9 visits over 12 months; $P = 0.002$), and more importantly, fewer asthma exacerbations (35 vs 109; $P = 0.01$) than the latter group. The authors surmised that eosinophils have a central role in asthma pathology, including asthma exacerbations, and corticosteroids may possibly act by dampening their activity. However, the authors did not demonstrate any significant correlation between eosinophils and lung function, asthma symptoms, or quality of life. The study was also of small size, and examined a specific patient group of severe, refractory asthmatics. A similar study by Chlumsky et al. produced strikingly similar results – vastly decreased exacerbation rate with no difference in symptoms or lung function [20].

In addition, measurement of induced sputum biomarkers needs to be standardized for the results to be used in any diagnostic form; and even then, the invasiveness and time needed for this test is a significant difficulty.

In response to this problem, study in 2013 by Hastie et al. endeavored to find whether sputum eosinophils and neutrophils could be accurately predicted using less invasive and time

consuming biomarker surrogates [21]. The team measured several biomarkers that had previously been suggested to be associated with sputum eosinophil and neutrophil counts. These biomarkers are currently used but are not validated and have been questioned by other studies regarding their power of prediction [22,23,24,25,26].

These biomarkers include blood eosinophil counts, exhaled nitric oxide (FeNO) and IgE levels to predict sputum eosinophils and age, FEV_1 percent predicted and blood neutrophil counts to predict sputum neutrophil counts. Hastie et al. found that while these surrogate markers do correlate with sputum cell counts but they do not have an accuracy or predictive power much above what we may expect by chance [7]. An example of this is blood eosinophils and neutrophils, which showed a predictive power of 64-69%. This is likely to be explained through biological processes such as cells transmigrating to tissue, which may cause transiently increased levels in the blood cell counts and also due to the large variation seen within atopic asthma patients. By using the aforementioned surrogate biomarkers they were able to allocate 41% of the patients into their correct granulocytic group, however, this again must be improved upon before it can be used within any clinical or diagnostic setting.

Airway remodeling is another cardinal feature of asthma. In 2010 Chakir et al. evaluated the influence of sputum eosinophil count-led vs clinical-led management in terms of the extent of airway remodeling over a 2 year period study involving 20 participants. Airway remodeling was measured by the expression of mucin 5A (MUC5A) and subepithelial collagen layer thickness from bronchial biopsy specimens, collected twice with two year treatment period between collections. After two years, mucin staining in sputum-led group was significantly smaller than in clinical-led group, and the amount of total eosinophils decreased in sputum-led group, but there was no difference between these groups in terms of collagen deposition. This study used a small group of patients, but it is interesting to note that the was no difference between sputum-led and clinician-led management in terms of remodeling, apart from a difference in MUC5A expression. This indicates that the role of eosinophils in airway remodeling in asthma is still largely mysterious.

In summary, sputum eosinophils and neutrophils have increased our understanding of asthma phenotypes and to date, are good predictors of airway T_H2 inflammation and subsequent asthma severity, especially when used in conjunction with age of onset and FEV_1 predicted values.

However, more work is needed to find more efficient and less invasive biomarkers that can accurately predict eosinophil and neutrophil levels within a point-of-care setting and then these cell counts are likely to become a useful adjunct for confirmation.

Periostin

Periostin is a matricellular protein found in numerous tissues. Bornstein et al. in 1995 described matricellular proteins as extracellular proteins which fulfill a non-structural, regulatory role by binding to the cell surface and structural components of the extracellular matrix, such as proteoglycans or collagen [27]. Periostin acts as a ligand for αVβ3 and αVβ5 integrins and appears important for cell migration and extracellular matrix remodelling [28]. Apart from its physiological role, it has also been found to be crucial in a number of pathological processes, including growth and metastasis of multiple cancers [29]. Sidhu et al. proved experimentally in 2010 that periostin is secreted by airway epithelial cells in response to IL-13, a well-known signalling molecule in asthma, and contributes to TGF-β activation and airway remodelling through increased collagen synthesis [30].

In a study investigating the relevance of periostin for measuring the severity of asthma in 2007 Woodruff et al. measured gene expression in airway epithelial cells in healthy subjects, asthmatic patients (before and after ICS therapy) and smokers [31]. 22 genes in asthmatics not receiving corticosteroids were differentially expressed when compared to control patients, although the difference for most genes was less than twofold, with only 5 genes expressed at least 3 times stronger in asthmatics. These genes were: CLCA1 (chloride channel, Ca^{2+}-activated 1; 6.2-fold increase), POSTN (periostin; 4.4-fold increase), PRR4 (proline rich 4 {lacrimal}; 3.9-fold), SERPINB2 (serine peptidase inhibitor B2; 3.5-fold) and CPA3 (carboxypeptidase A3 {mast cell}; 3.3-fold). Here we focus on periostin, as it is supported by comparatively largest body of evidence from studies. Treatment with fluticasone decreased periostin expression 2.1-fold, although only 27 patients finished this arm of the trial. Unfortunately, these changes were calculated from the material biopsied at baseline and one week after the commencement of the trial – a potentially insignificant treatment period, even taking into consideration that the asthmatics enrolled were required to be steroid-free for at least 4 preceding weeks. A strength of the study was that the authors demonstrated that IL-13 increased periostin expression, and

showed that periostin could also be suppressed through use of ICS dexamethasone and budesonide. This seminal work has been valuable in terms of periostin being identified as a potential biomarker for predicting severity and airway remodelling in asthmatics.

(Table 1)

Following on from the above study, Woodruff et al. in 2009 conducted an analysis on the same samples that had been collected two years before [32]. Their previous study had concluded that three genes – POSTN, CLCA1 and SERPINB2 were expressed in a group of asthma patients and that their levels might reflect the extent of T_H2-related inflammation. In this study, the authors performed a simple cluster analysis of 42 asthmatics and 28 controls anddiscovered that in 20 of the asthmatics the T_H2 inflammatory signature (measured by the expression of the three mentioned genes) wasvirtually indistinguishable from that of controls. In other words, 20 asthmatic patients did not significantly overexpress the three T_H2 genes which are related to IL-13 responses. The authors named these patients 'T_H2-low' and established that they had accordinglylow levels of IL-13 and IL-5. In contrast, the remaining 22 asthmatics had significantly higher levels of expression of the three genes and of both cytokines and were dubbed 'T_H2-high'. Notably, individual patients exhibited high levels of correlation between the three mentioned genes (Spearman's rank order correlation {ρ} ranging from 0.77 to 0.88 with $P < 10^{-4}$), indicating that in future measurement of one of them (e.g. periostin in this case) might be sufficient to distinguish between T_H2-high and T_H2-low asthma.

Furthermore, the latter group had greater airway hyperresponsiveness, circulating IgE and eosinophil levels and more extensive airway remodelling than the former group. The researchers also found a difference between these groups in response of FEV_1 to ICS over 2 weeks of treatment: T_H2-high individuals had a robust improvement in FEV_1 (which returned to baseline after discontinuation of therapy), whereas T_H2-low patients paradoxically experienced a worsening of their airway function. This indicates that IL-13 and IL-5 are suggestive of a more severe subtype of asthma, potentially reflected by periostin as biomarker Unfortunately, the authors did not mention the exact cutoffs for POSTN, CLCA1 and SERPINB2 which they used for stratifying patients into 'T_H2-high' and 'T_H2-low' categories.

In addition to periostin (possibly together with CLCA1 and SERPINB2) being used to distinguish between T_H2-high and T_H2-low asthma, it will be important to find out in the future whether periostin can also be used as a biomarker for more specific classifications of T_H2-high asthma endotypes.

Corren et al. in 2011 organized a randomized, double-blind, placebo-controlled, multicentre clinical study investigating immunosuppressive treatment of asthma using lebrikizumab (anti-IL13) [33]. 219 adult patients with suboptimal asthma control, potentially indicating steroid resistance, were enrolled while being treated with ICS. The authors assessed patient Th-2 status by measuring blood IgE and eosinophil levels; serum periostin was measured later. Patients were then randomized to receive lebrikizumab or placebo once monthly for 6 months, and multiple assessments were performed during the study. At 12 weeks, the increase in pre-bronchodilator FEV_1 (from baseline) was significantly larger in lebrikizumab than in placebo group: 9.8% vs. 4.3%. However, when the patients were stratified according to their pre-treatment periostin level, the difference in outcomes was magnified: in high-periostin group, the mean increase in FEV_1 vs. placebo was 14.0% vs. 5.8%, whereas in the low-periostin group, the mean increase in FEV_1 vs. placebo was 5.1% vs. 3.5%. This difference in FEV_1 was apparent from week 1 and continued until week 32, when the study was terminated. Unfortunately, lebrikizumab did not alter asthma symptoms, nor did it diminish the need for the use of rescue medication, although there was a non-significant ($p = 0.10$) decrease in the rate of reported exacerbations. The results of this study are somewhat ambiguous – the rise in FEV_1 was significant, but there were no differences between the antibody and placebo in secondary outcomes, possibly indicating that T_H2-high asthmatics are more responsive to ICS treatment and that IL-13 may only be one effector in the frame of a complex disease.

Noonan et al. in 2013 performed a similar study with the same medication and primary endpoint [34]. Crucially, the patients in this study were well-controlled, and not corticosteroid-resistant asthmatics. Of 212 randomized patients, 117 (56%) were classed as periostin-high, and 93 (44%) as periostin-low. The authors found slight improvements from baseline FEV_1 in periostin-high patients (2.3%-4.8% depending on the dose), but deemed them not statistically significant. They did however find that lebrikizumab drastically lowered treatment failure as defined by the need to commence ICS therapy (27% in placebo group vs. 6% in antibody group). Despite

encouraging results, a significant weakness of this study was in the randomization: placebo recipients were significantly skewed towards periostin-high status (only 18 low-periostin patients received placebo).

Statistical issues aside, the discrepancy between this and Corren's study may be explained by the population differences: in patients not receiving steroids, multiple pathological pathways may be active and hence selective blockade of IL-13 may not be an adequate treatment for some of them. In contrast, in the steroid-resistant patients the effect of IL-13 may be more pronounced and therefore more amenable to treatment. Regardless of this, it indicates the level of complexity of asthma pathophysiology and the need for careful evaluation of the effects of antibodies in different patient cohorts.

A study by Hanania et al. shows a much clearer indication of periostin's suitability as a biomarker [35]. In 2013 they performed a post-factum analysis of their 2011 EXTRA study [36]. This trial enrolled a substantial cohort of 850 patients of various ages, all suffering from poorly-controlled, steroid-resistant, allergic asthma. At the onset of the study, patients were stratified according to the current medication used and the levels of three putative T_H2 inflammation biomarkers were measured in most of the patients throughout the study: exhaled nitric oxide (FeNO), peripheral eosinophil count, and serum periostin. The cut-off between periostin-high and periostin-low groups was selected as a median measured value of 50 ng/ml. A significant advantage of the study was that serum periostin measurements were available from a much greater sample of patients (534) than in any of the studies described above.

The authors found that the use of omalizumab decreased exacerbation rates from 0.93 to 0.66 in periostin-high group, whereas it had no significant effect in the periostin-low group (0.72 vs. 0.73). Therefore, the treatment with this antibody decreased exacerbation rates in periostin-high group below that of periostin-low group. However, no significant changes were observed in FEV_1 or asthma symptoms.

The major disadvantage of periostin at the moment appears to be its limited capability to identify more asthma endotypes within the 'Th2-high' phenotype. Whilst periostin can differentiate between T_H2-high and T_H2-low asthma, additional use of other biomarkers seems to be

necessary in order to detect shed more light on the complex Th2-high asthma. However, a much larger sample population with more control over compounding, external factors such as medications will need to be examined before any such additional biomarkers can be ascertained.

Exhaled nitric oxide (FeNO)

Nitric oxide (NO) is a short-lived, gas free radical that acts as a paracrine mediator in many tissues. Famously identified as the endothelium-derived relaxing factor (EDRF) by Furchgott, Zawadzki and Ignarro [37,38], It subsequently became the founding member of a new family of molecules called gasotransmitters – gaseous mediators responsible for a wide variety of physiological processes [39].NO is produced from a natural amino acid L-arginine by nitric oxide synthase (NOS), which exists in three isoforms [40]. NOS1 (neuronal NOS, nNOS) and NOS3 (endothelial NOS, eNOS) depend on calcium ions and are mostly constitutive, whereas NOS2 (induced NOS, iNOS) is calcium-independent and instead its activity is usually switched on and off by other intracellular signals [41]. It is the latter enzyme that is mostly important for NO production in asthma and other inflammatory conditions [42]. When NO was found to be measureable in the exhaled air of some asthmatic patients, with a reported 2-3 fold increase from baseline when compared to healthy controls [43].,it was realized that the level of this gas in exhaled air (FeNO) might be utilized to assess the extent of airway inflammation in this disease. These hopes were fuelled by the low cost, quickness and non-invasiveness of measuring exhaled FeNO [44]. However, multiple studies on the clinical utility of FeNO have been published since then and the resulting picture is far from clear. This is best demonstrated by the fact that some countries, such as United States, have enthusiastically adopted FeNO as an asthma biomarker, whereas others, such as UK, have been more cautious in this matter. The body of evidence is large, but many studies do not appear to carry much weight due to design problems or insufficient size. Therefore, we have focused on the largest, most recent and interesting studies or meta-analyses both in adults and children.

In 2012 Petsky et al. performed a meta-analysis of two adult and four children asthma studies investigating a total sample population of 1,053 adults and children and utilizing the FeNO biomarker[45]. The authors found that using FeNO values to guide therapy with corticosteroids lead to a daily ICS dose decrease by a mean of 450.03 μg budesonideequivalent in adults, but a

140.18 μg increase in children. There were no differences in other parameters, most importantly the frequency of exacerbations and perceived asthma symptomsOn the whole, this meta-analysis does not support the use of FeNO as a useful biomarker for guiding asthma treatment in adults and children alike, although one might argue that exacerbations and FeNO cut off values were variably defined between the six studies (the latter ranging widely from 20 to 35 ppb)

It is worth looking separately at the two adult asthma studies included in the above meta-analysis. Smith et al. in 2005 constructed a single-blind trial with 97 participants, who were assigned to have their optimal fluticasone (ICS) doses specified based on either FeNO values, or standard clinical algorithms [46]. After the optimal doses were found, the study lasted for 1 year, with continued 2-monthly measurements of FeNO and clinical indices in order to titrate the ICS dose. The authors found that the only significant difference between the FeNO-led and clinical-led groups was the mean daily fluticasone dose, with the latter group taking 370 μg of this corticosteroid, and the latter 641 μg ($P = 0.003$). This indicated that patients assessed on the basis of their clinical indices might have been overtreated, as supported by an earlier meta-analysis demonstrating highest benefit from ICS at the doses up to about 500 μg per day [47]. Interestingly, sputum eosinophil counts in both groups were similar and below 3% threshold defined as clinically important. However, the study was quite small (97 patients), single-blind and importantly did not allow patients to take long-acting beta agonists, an important asthma medication. Interestingly, Shaw et al. conducted a similar study in 2007 on 120 patients and found that there was no statistically significant difference between both groups in terms of cumulative ICS dose used over 12 months, although FeNO-led patients did have a significantly lower final ICS dose (557 vs. 895 ug, $P = 0.028$). As a criticism, this study was also single-blind and used severe asthma patients rather than general asthmatic population. It is generally difficult to compare such studies due to differences in FeNO targets and in management protocols, although general consensus existed at the time that exacerbation rates were not affected by management method in any of these studies.**(Table 2)**

Another study, which failed to find FeNO to be useful as a biomarker was the BASALT study, a recent (2012) randomized controlled trial organized by Calhoun et al. [48]. This study was similar to the ones included in Petsky's meta-analysis, aiming to compare FeNO and daily

asthma symptoms for the prevention of asthma exacerbations compared to the traditional assessment by physician. In this study 342 adults were randomized to three groups, and doses for ICS were adjusted based either on the respective FeNO indication of asthma severity, a physician's assessment every 6 weeks or a daily assessment of generic asthma symptoms. Notably, biomarker-based dose adjustment was not significantly superior to the clinician's assessment-based adjustment – showing an exacerbation rate of 20% vs. 22%. Daily symptom-based assessment was marginally better with an exacerbation rate of 15%, but was still deemed insignificant by the authors. As the rate of exacerbations was lowest in the last group of patients in whom ICS dose was adjusted daily based on symptoms (rather than every six weeks), an improvement could have been made by including a fourth cohort of patients in whom FeNO measurements were performed daily and ICS dose matched on a daily basis to thus obtained FeNO readings. Of course, the use of such strategy would require equipping every patient in the cohort with an FeNO meter or require daily visits to their physician to have their FeNO assessed over several months.

From the methodological point of view the above study is also limited in another way – it assessed only mild-to-moderate asthmatics, in which the degree of underlying airway inflammation is also likely to be only mild to moderate. In contrast, FeNO is postulated to be increased in active, severe airway inflammation, so perhaps it is not surprising that it was not found to be elevated in the above cohort of patients. The real usefulness of FeNO might lie simply in distinguishing between patients with overactive T_H2 responses from these without such inflammation, similar to periostin.

The most convincing piece of evidence for such stratifying power of FeNO comes from the previously discussed EXTRA study of omalizumab, in which FeNO values obtained from 394 patients were available for analysis [20]. Although not nearly as significant as periostin results, in FeNO-high subgroup of patients, there was a substantial 53% reduction in the exacerbation rate, over three times that of FeNO-low subgroup (16%).

Another important study concerned with discrimination between asthma subtypes is one by Amelink et al.[49]. In 2013 they investigated mild-to-moderate and severe adult-onset asthma patients, attempting to describe the differences between these two subgroups and determine potential markers that would in future allow to distinguish between them more easily. Having

stringently excluded all patients with severe co-morbidities or any uncertainties regarding their diagnosis, authors recruited 78 patients suffering from severe and 98 suffering from mild-to-moderate asthma. The authors measured multiple clinical indices during the study, allowing for a comprehensive analysis. Predictably, severe-asthma patients had lower quality of life and higher degree of overall healthcare utilization. More significantly, they were also characterized by less allergy sensitivity (34% vs. 52%), more frequent nasal polyposis (54% vs. 27%) and more significant airflow impairment and air trapping compared to their mild-to-moderate counterparts. Among the multiple factors correlated with disease severity, authors identified FeNO with a significant odds ratio of 1.5. Even though blood neutrophils, nasal polyposis and absence of atopy ranked significantly higher as severity-associated factors, it is important to remember that FeNO is considerably faster, cheaper and more convenient to measure than any of these biomarkers. Sputum eosinophils were also slightly elevated in severe-asthma patients (odds ratio of 1.4), which appears consistent, as NO is abundantly produced not only by endothelial, but also by the airway inflammatory cells [50]. Therefore, FeNO measurement may be potentially useful for distinguishing this adult-onset, severe-asthma phenotype in addition to blood neutrophils and sputum eosinophils to improve accuracy.

Crucially, the above study did not identify obesity or patient gender to be correlated with adult-onset asthma severity. However, a few authors have described another late-onset phenotype, associated with obesity in women, which they claim is different to the neutrophilic phenotype described by Amelink's study. In this phenotype, inflammation is decreased as detectable by diminished FeNO values and airway eosinophilia.

Obesity has a complex relationship to asthma, with higher BMI values predicting worse prognosis in this disease. Moreover, the rise in the prevalence of obesity is concurrent with that of asthma and allergies. Therefore the relationship between BMI, asthma and inflammation may be of some significance. Holguin et al. last year (2013) performed a cross-sectional analysis on a population from the Severe Asthma Research Program (SARP) (see References) to test a putative mechanism responsible for a decrease in FeNO in overweight and obese patients [51].

The authors focused on asymmetric dimethylarginine (ADMA), a substance known to interfere with nitric oxide synthase (NOS), an enzyme crucial for the production of this gaseous mediator. ADMA, same as NO, is produced from a natural amino acid L-arginine and therefore the ratio of

L-arginine to ADMA is informative of the efficiency of the formation of this gas. The researchers found that patients with late-onset asthma exhibited lower L-arginine/ADMA ratio than the patients with early-onset asthma (median of 109 vs. 121). Additionally, the inverse correlation of this ratio to BMI was significantly greater in late-onset vs. early-onset patients (r = −0.4 vs. r = −0.2). This ratio was found to be correlated with log FeNO (r = 0.39) throughout the study population. The authors have also found that in late-onset asthma decrease in L-arginine/ADMA ratio exacerbates FEV_1, but the opposite is observed in early-onset asthma.

The study by Holguin et al. demonstrated how obesity may result in the induction of late onset asthma phenotype. It should be noted here that ADMA is relevant across asthma phenotypes due to its inhibition of nitric oxide synthesis. ADMA is prominent in this section as it appears to be a significant, putative mediator that could explain the inverse relationship of BMI to Fe(NO) in the late-onset, asthma phenotype. In patients afflicted with this asthma phenotype (mainly obese women), uncoupled NOS produces significantly less NO and more reactive oxygen species (ROS), which may impair respiratory function. Both reactive oxygen species' [52] and ADMA levels [53] have been demonstrated to be increased in obese individuals, which supports this theory. The explanation of the molecular underpinning of this asthma phenotype is also particularly attractive due to fact that a deficit in L-arginine is readily corrected with L-citrulline and thus may be a useful adjunct to conventional asthma therapy [54]. Importantly, this study used a pre-determined cohort from SARP study in which the levels of L-arginine and ADMA were measured, and may not necessarily be representative of the general population. This may potentially serve as a useful proof of concept and identifies the directions of future research, but this needs to be improved upon, using a larger trial in future.Finally, another special population of asthmatic patients are smokers, who are more difficult to diagnose and treat with ICS than non-smoking patients [55]. From diagnostic point of view, smokers have been reported to have decreased FeNO levels, diminishing its potential usefulness as a biomarker in this population of patients [56]. To test whether this biomarker could still be used in this population, Spears et al. in 2011 measured FeNO and performed spirometry in 22 smokers and 21 never-smokers both before and after 2 weeks of oral steroid treatment. Obtained values were then mathematically analyzed in a novel way to obtain two indices, alveolar nitric oxide ($C_{alv)}$ and nitric oxide flux (J'_{aw}). The authors found that while FeNO and two indices were decreased in smokers, J'_{aw}, and not FeNO and C_{alv}, was decreased by steroid therapy, potentially raising hopes that J'_{aw} could be

used an asthma biomarker in future. Notably, this was a small unblinded study, with all the inherent consequences – for instance, no relationship between J'_{aw} and ICS dose was found, which could be either due to small study size, or weakness of this index as a biomarker. Nevertheless, this study is interesting as a proof of concept and provides a foundation for future efforts.

In the above section we have discussed a small fraction of the existing clinical evidence for the clinical utility of FeNO as an asthma biomarker. While the clinical utility of this biomarker may not been demonstrated as satisfyingly as that of periostin, it holds the two key advantages over this substance: one is the ease and low cost of measurement, and another, its potential to identify more asthma phenotypes than periostin. It seems that a combination of periostin and FeNO would be more informative for guiding asthma therapy than each of these alone. However, more data will be necessary to firmly establish their use.

Other biomarkers

(Table 3)

Cysteinyl-leukotrienes (Cys-LT) are simple lipids which are known to be important for paracrine signaling and the acknowledgment of their participation in asthma pathophysiology has culminated in the introduction of leukotriene receptor antagonists (LTRA). These medications are characterized by very few side effects, and even though slightly less efficient than long-acting beta agonists when combined with ICS, they nonetheless have their role in the asthma clinic. The discovery that only a small fraction of patients benefit significantly from LTRA, coupled with the fact that LTE_4 can be readily assayed in the urine, has prompted interest in the use of this Cys-LT for predicting therapeutic response to LTRA [1].

Cai et al. in 2007 administered montelukast (a Cys-LT receptor antagonist) to 48 mild-to-moderate asthmatics over a period of 4 weeks, and used strict criteria (improvement of asthma symptoms, FEV_1 and reduction in SABA use) to classify them as responders or non-responders [57]. 25 patients responded to the therapy, and they had a mean LTE_4 concentration of 224.5 ± 34.4 pg/mg creatinine, compared to that of 175.3 ± 37.1 in non-responders. The study used a limited number of patients with moderate disease, however, they were able to demonstrate the

potential of this biomarker for indicating whether to use montelukast or not. Cai et al. showed that patients who measured > 200 pg LTE_4/mg creatinine were 3.5 times more likely to respond to this drug than patients below this threshold. While the collection of urine is non-invasive, assessment of LTE_4 level by mass spectrometry requires appropriately trained staff and relatively costly equipment, although the prices may be expected to decrease.

Prostaglandins' relationship to asthma, specifically allergic asthma, has been investigated over the past decades as their connection to mast cells, a major mediator in bronchoconstriction, eosinophil trafficking and potentially asthma progression [58,59] has been elucidated [60,61]. Specifically, prostaglandin E2 (PGE2) has been linked to mast cell degranulation inhibition and subsequent reduction in bronchoconstriction. Sastre et al. in 2008 showed that asthmatics have a reduced level of PGE2 [62], which may be linked to inhibition through mast cell activation, as recent studies have suggested that the observed reduction in PGE2 may be a contributor in asthma progression [63].

This prostaglandin can be easily measured in urine and given more research into its predictive power and whether the beneficial effects of PGE2 as a muscle relaxant in asthma are physiologically linked to its inhibitory effects on mast cells, may prove to be a beneficial biomarker, or adjunct to, for stratification and guided treatment in allergic asthma.

Volatile organic compounds (VOC) can be measured in the exhaled air, and their profiles could be used to diagnose and distinguish between asthma and COPD and, to a certain degree, give an indication of asthma severity.

Multiple substances have been reported to be elevated in the air exhaled by asthmatics, including pH, isoprostanes (widely used in cardiovascular and respiratory research as markers of oxidative stress) and adenosine [3]. These VOCs are practically utilised to form breath profiles or prints, which can be indicative of airway inflammation using a technology termed the 'electronic nose' that is part of a novel but growing technology group called smell analysis.

Electronic nose technology is being studied extensively in asthma for its specificity and sensitivity in detection and discrimination of airway inflammation. Fens et al. showed in 2011 that the electronic nose had a sensitivity and specificity of 91% and 90%, respectively when discriminating between asthma, chronic obstructive pulmonary disease and healthy control

patients [64]. However, the electronic nose was found to lack sensitivity when discriminating between severe and mild asthmatics, as shown by Dragonieri et al. in 2007 [65].

As well as the detection of asthma, the breath profiling by the electronic nose has also been shown to indicate a patient's responsiveness to steroids with greater accuracy than FeNO or eosinophils [66]. This has potential for improving the treatment in the future as this is largely cost-effective and non-invasive, providing its introduction into clinical use after it becomes commercially available..

Ultimately, the electronic nose lacks some discriminatory power when differentiating asthma phenotypes, however, it may be possible to utilise it as a method for measuring and analysing VOCs as auxiliary asthma biomarkers, pending further refinement and commercial availability. In addition, this technology can aid in reducing misdiagnosis and, when coupled with FeNO, as shown by Montuschi et al. in 2010, can be highly specific for the detection and profiling of VOCs as inflammatory biomarkers [67] Thus, given further research breath profiling may be useful in stratification of asthma types with a high degree of specificity due to the number of components that comprise a breath profile.

Exhaled breath temperature (EBT) represents another potentially useful biomarker, similar to exhaled breath profiling. Kumar et al. in 1998 compared blood flow in the airway mucosa of asthmatic patients and healthy controls utilizing a complex dimethyl ether uptake method [[68]]. They found that blood flow in asthmatic patients was almost twice as high (68.2 ± 7.9 µl/min·ml in receiving glucocorticoids and 55.4 ± 5.3 µl/min·ml not receiving glucocorticoids) as in healthy patients (38.5 ± 5.3 µl/min·ml).Garcia et al. last year (2013) expanded on this and similar reports by investigating the possibility thatthat such increase in blood flow (due to persistent inflammation leading to airway remodelling, including an increase in the density of the mucosal vascular network) may be measurable as an increase in the temperature of the exhaled air69]. They found the exhaled breath temperature (EBT) of uncontrolled asthmatic patients to be increased (mean of 34.9 ± 0.8°C) in comparison to that of controlled asthmatics (33.7 ± 0.8) and controls (33.2 ± 0.2). While it is difficult to envisage the utilization of this potential biomarker for clearly distinguishing between asthma phenotypes, it may be potentially utilized in the future for monitoring the efficacy of asthma treatment.

Chitinases were described in studies by Zhu et al. in 2004 [70] and Chupp et al. in 2007 [71], who have identified and elucidated them as important effecter molecules in airway inflammation. Specifically, increased serum levels of enzymatically inactive chitinase-like protein YKL-40 represent a possible biomarker in severe childhood asthma. This biomarker has been specifically proposed by Konradsen et al. 2013 to be indicative of therapy resistant asthma and was shown by the same team to correlate with exhaled FeNO (P=0.004), blood neutrophils (P<0.001) and significantly, bronchial wall thickening (P=0.01) and have a negative correlation with asthma control (P=0.03) [72].

Interestingly, Konradsen et al. 2013 found that children who possessed a single nucleotide polymorphism in the gene CHI3L1 and were resistant to therapy had the highest levels of YKL-40, while children with this polymorphism and controlled asthma had lower levels. In children who did not have this polymorphism in CHI3L1, no difference was found between resistant and non-resistant asthma. This evidence supports the hypothesis that increased levels of YKL-40, as a result of the polymorphism, are indicative of severe, therapy-resistant asthma and increased markers of inflammation. Whether asthma therapy resistance is due to high levels of YKL-40 or YKL-40 is co-product of the specific asthma type is not yet clear, regardless, YKL-40 is likely a easily attainable biomarker for stratification of severity in childhood asthma. The next step for this biomarker should be to quantify the genetic influence and variation seen in asthma severity and therapy resistance between the polymorphisms in the CHI3L1 gene.

Conclusion and future directions

We described several key asthma biomarkers in terms of their potential to distinguish between various phenotypes of this disease and guide its pharmacotherapy. Despite recent progress, many doctors still diagnose and treat asthma as a single disease, rather than what it currently appears to be: a collection of various, closely related pathologies with similar symptoms.

These difficulties in identifying asthma phenotypes represent perhaps the prime cause of some resistance to asthma stratification and personalized therapy. The next step that we expect to see in the future is changing the current umbrella classification to sufferers of asthmatic symptoms, leading to individual and stratified diagnoses based on biomarkers. This will improve our

understanding of various phenotypes of asthma and how they can be pharmacologically targeted. Currently, the clinician can use sputum, periostin and perhaps FeNO signature to classify patient as T_H2 or non-T_H2, but this is mostly useful for providing prognosis for such patients (non-T_H2 patients will be more likely to be refractory to treatment), rather than guide any specific therapy. We have only scratched the surface of the complexity of asthma phenotypes, and at the moment do not have any medications that would allow us to specifically target individual subtypes. The current clinical utility of asthma phenotypes is therefore marginal but we expect this to change drastically in the near future, leading to more personalized diagnosis and tailored treatment.

Key Issues

- Asthma is a chronic respiratory disorder characterized by episodes of reversible airflow obstruction, manifesting in shortness of breath, wheezing and cough.
- Current diagnosis of Asthma is an "umbrella term" that is employed to diagnose groups of similar symptoms; however, asthma is far more diverse and requires greater stratification. To date, Inhaled Corticosteroids (ICS) are the most common treatment for asthma. Biomarker discovery is currently looking to stratify asthma endotypes to enable specific drug discovery and personalize treatment.
- Eosinophils and neutrophils are closely linked to airway inflammation and are currently measured within induced sputum to stratify asthma into 4 distinct cellular types; eosinophilic (raised eosinophil levels), neutrophilic (raised neutrophil levels), mixed granulocytic (raised neutrophils and eosinophils) and paucigranulocytic (normal neutrophil and eosinophil levels). Eosinophils are most closely linked to severe asthma and indicate the need for ICS use.
- Periostin is a biomarker directly linked to IL-13 and IL-5 secretion and is elevated in asthmatics with extensive airway remodeling. Elevated periostin can be used to distinguish between T_H2-high and T_H2-low asthma and again, indicate more severe asthma subtypes for those classified as T_H2-high.
- Nitric oxide (FeNO) is a short-lived, gaseous, free radical that is present in exhaled breath. It can be measured using a range of devices, including an electronic nose, which has the added ability of giving a profile of multiple volatile organic compounds (VOC) in addition to FeNO from exhaled breath. Asthmatics show elevated levels of FeNO and may show variation in levels depending on the degree of airway inflammation. Multiple studies have found it to be inaccurate for anything more than distinguishing between healthy and asthmatic patients, although researchers are hopeful that it will be refined, in conjunction with VOCs to indicate the risk of exacerbation on a daily basis.
- Future asthma biomarkers include exhaled breath profiling of multiple volatile organic compounds for more specific distinctions between asthma subtypes. Simple lipids such as Leukotriene E-4, which are measurable from urine and have also been found to indicate the responsiveness of a patient to leukotriene receptor antagonists (LTRA), when elevated. LTRA is used to reduce airway inflammation.
- Biomarkers are also merging into genetic testing such as a polymorphism found in CHI3L1, which encodes for a chitin protein YKL-40. The CHI3L1 gene is hypothesised to be indicative of severe, therapy resistant, childhood asthma when it contains a specific single nucleotide polymorphism.

References

Papers of special note have been highlighted as:

• of interest

•• of considerable interest

[1] Holgate ST. Pathogenesis of asthma. Clin Exp Allergy 38(6), 872-97 (2008).

[2] Editorial (no authors given). A plea to abandon asthma as a disease concept. Lancet 368(9537), 705 (2006).

[3]Alexis NE. Biomarker sampling of the airways in asthma. Curr Opin Pulm Med 20(1), 46-52 (2014).

[4] Deckers J, Branco Madeira F, Hammad H. Innate immune cells in asthma. Trends Immunol 34(11), 540-7 (2013).

[5] Willart MA, Deswarte K, Pouliot P *et al.* Interleukin-1α controls allergic sensitization to inhaled house dust mite via the epithelial release of GM-CSF and IL-33. J Exp Med 209(8):1505-17 (2012).

[6] Suzukawa M, Morita H, Nambu A *et al.* Epithelial cell-derived IL-25, but not Th17 cell-derived IL-17 or IL-17F, is crucial for murine asthma. **J Immunol** 189(7), 3641-52 (2012)

[7] Moro K, Yamada T, Tanabe M *et al.* Innate production of T(H)2 cytokines by adipose tissue-associated c-Kit(+)Sca-1(+) lymphoid cells. Nature 463(7280), 540-4 (2010).

[8] Sibilano R, Frossi B, Pucillo CE. Mast cell activation: A complex interplay of positive and negative signaling pathways. Eur J Immunol. Epub ahead of print (2014).

[9] P. Bradding, I.H. Feather, S. Wilson *et al.* Immunolocalization of cytokines in the nasal mucosa of normal and perennial rhinitic subjects. The mast cell as a source of IL-4, IL-5, and IL-6 in human allergic mucosal inflammation. J Immunol 151, 3853-65 (1993).

[10] Arias K, Chu DK, Flader K *et al.* Distinct immune effector pathways contribute to the full expression of peanut-induced anaphylactic reactions in mice. J Allergy Clin Immunol 127(6), 1552-61 (2011).

[11] Allakhverdi Z, Smith DE, Comeau MR, Delespesse G. Cutting edge: The ST2 ligand IL-33 potently activates and drives maturation of human mast cells. J Immunol 179(4), 2051-4 (2007).

[12] Gleich GJ, Klion AD, Lee JJ, Weller PF. The consequences of not having eosinophils. Allergy 68(7), 829-35 (2013).

[13] Jacobsen EA, Zellner KR, Colbert D, Lee NA, Lee JJ. Eosinophils regulate dendritic cells and Th2 pulmonary immune responses following allergen provocation. J Immunol 187(11), 6059-68 (2011).

[14] Nakagome K, Matsushita S, Nagata M. Neutrophilic inflammation in severe asthma. Int Arch Allergy Immunol 158 Suppl 1:96-102 (2012).

[15] Busse WW. Asthma diagnosis and treatment: filling in the information gaps. J Allergy Clin Immunol 128(4), 740-750 (2011)

[16] Moore WC, Meyers DA, Wenzel SE *et al.* Identification of asthma phenotypes using cluster analysis in the Severe Asthma Research Program. Am J Respir Crit Care Med 181(4), 315-23 (2010).

[17] Wenzel SE. Asthma phenotypes: the evolution from clinical to molecular approaches. Nat Med. 18(5), 716-25 (2012).

[18] Simpson JL, Scott R, Boyle MJ, Gibson PG. Inflammatory subtypes in asthma: assessment and identification using induced sputum. Respirology 11(1), 54-61 (2006).

• One of the first studies which measured biomarkers (in this case inflammatory effector cells in induced sputum) for distinguishing between subtypes of asthma.

[19] Green RH, Brightling CE, McKenna S *et al.* Asthma exacerbations and sputum eosinophil counts: a randomised controlled trial. Lancet 360(9347), 1715-21 (2002).

[20] Chlumský J, Striz I, Terl M, Vondracek J. Strategy aimed at reduction of sputum eosinophils decreases exacerbation rate in patients with asthma. J Int Med Res. 34(2), 129-39 (2006).

[21] Hastie AT, Moore WC, Li H *et al.* Biomarker surrogates do not accurately predict sputum eosinophil and neutrophil percentages in asthmatic subjects. J Allergy Clin Immunol 132(1), 72-80 (2013).

[22] Hillas G, Kostikas K, Mantzouranis K *et al.* Exhaled nitric oxide and exhaled breath condensate pH as predictors of sputum cell counts in optimally treated asthmatic smokers. Respirology 16(5), 811-8 (2011).

[23] Thomas RA, Green RH, Brightling CE *et al.* The influence of age on induced sputum differential cell counts in normal subjects. Chest 126(6), 1811-4 (2004).

[24] Ducharme ME, Prince P, Hassan N *et al.* Expiratory flows and airway inflammation in elderly asthmatic patients. Respir Med 105(9), 1284-9 (2011).

[25] Nadif R, Siroux V, Oryszczyn MP *et al.* Heterogeneity of asthma according to blood inflammatory patterns. Thorax 64(5), 374-80 (2009).

[26] Lemière C, Ernst P, Olivenstein R *et al.* Airway inflammation assessed by invasive and noninvasive means in severe asthma: eosinophilic and noneosinophilic phenotypes. J Allergy Clin Immunol 118(5), 1033-9 (2006).

[27] Bornstein P. Diversity of function is inherent in matricellular proteins: an appraisal of thrombospondin 1. J Cell Biol 130, 503–506 (1995).

[28] Gillan L, Matei D, Fishman DA *et al.* Periostin secreted by epithelial ovarian carcinoma is a ligand for alpha(V)beta(3) and alpha(V)beta(5) integrins and promotes cell motility. Cancer Res. 62 (18), 5358–64 (2002).

[29] Wang X, Liu J, Wang Z *et al.* Periostin contributes to the acquisition of multipotent stem cell-like properties in human mammary epithelial cells and breast cancer cells. PLoS One 8(8), e72962 (2013).

[30] Sidhu SS, Yuan S, Innes AL *et al.* Roles of epithelial cell-derived periostin in TGF-β activation, collagen production, and collagen gel elasticity in asthma. Proc Natl Acad Sci U S A 107(32), 14170–14175 (2010).

[31] Woodruff PG, Boushey HA, Dolganov GM *et al.* Genome-wide profiling identifies epithelial cell genes associated with asthma and with treatment response to corticosteroids. Proc Natl Acad Sci U S A 104(40), 15858–15863 (2007).

[32] Woodruff PG, Modrek B, Choy DF *et al.* T-helper Type 2–driven Inflammation Defines Major Subphenotypes of Asthma. Am J Respir Cri Care Med 180(5), 388–395 (2009).

[33] Corren J, Lemanske RF, Hanania NA *et al.* Lebrikizumab treatment in adults with asthma. N Engl J Med 365(12), 1088-98 (2011).

•• This seminal study identified significant differences in outcome of therapy with a modern biological drug (lebrikizumab, anti-IL13) between patients with high- and low-pretreatment levels of periostin. The former group of patients benefited from lebrikizumab therapy, while the latter did not, indicating a potential benefit of using periostin to guide therapy with this medication.

[34] Noonan M, Korenblat P, Mosesova S *et al.* Dose-ranging study of lebrikizumab in asthmatic patients not receiving inhaled steroids. J Allergy Clin Immunol 132(3), 567-574 (2013).

[35] Hanania NA, Wenzel S, Rosén K *et al.* Exploring the effects of omalizumab in allergic asthma: an analysis of biomarkers in the EXTRA study. Am J Respir Crit Care Med 187(8), 804-11 (2013).

•• A large study which examined the effects of omalizumab (anti-IgE, used for treatment of refractory asthma) on allergic asthma patients, with concomitant measurement of periostin, FeNO and eosinophils. The authors established that patients who had high pretreatment levels of any of the three biomarkers were likely to respond to therapy, unlike the patients with low pretreatment levels. In future, this might be important for selecting patients likely to benefit from omalizumab therapy.

[36] Hanania NA, Alpan O, Hamilos DL *et al.* Omalizumab in severe allergic asthma inadequately controlled with standard therapy: a randomized trial. Ann Intern Med 154(9), 573-82 (2011).

[37] Furchgott RF, Zawadzki JV. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. Nature 288(5789), 373-6 (1980).

[38] Ignarro LJ, Buga GM, Wood KS, Byrns RE, Chaudhuri G. Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. Proc Natl Acad Sci U S A 84(24), 9265-9 (1987).

[39] Szabo C. Gaseotransmitters: new frontiers for translational science. Sci Transl Med 2(59), 59 (2010).

[40] Förstermann U, Closs EI, Pollock JS *et al.* Nitric oxide synthase isozymes. Characterization, purification, molecular cloning, and functions. **Hypertension** 23 (6 Pt 2), 1121-31 (1994).

[41] Asano K, Chee CB, Gaston B *et al.* Constitutive and inducible nitric oxide synthase gene expression, regulation, and activity in human lungepithelial cells. Proc Natl Acad Sci U S A. 91(21), 10089-93 (1994).

[42]Dupont LL, Glynos C, Bracke KR, Brouckaert P, Brusselle GG. Role of the nitric oxide-soluble guanylyl cyclase pathway in obstructive airway diseases. **Pulm Pharmacol Ther.** Epub ahead of print (2014).

[43] Alving K, Weitzberg E, Lundberg JM. Increased amount of nitric oxide in exhaled air of asthmatics. Eur Respir J 6(9), 1368-70 (1993).

[44] Stonham C. Use of exhaled nitric oxide in asthma care. Nurs Times 109(42), 22, 24-5 (2013).

[45] Petsky HL, Cates CJ, Lasserson TJ *et al.* A systematic review and meta-analysis: tailoring asthma treatment on eosinophilic markers (exhaled nitric oxide or sputum eosinophils). Thorax 67(3), 199-208 (2012).

[46] Smith AD, Cowan JO, Brassett KP, Herbison GP, Taylor DR. Use of exhaled nitric oxide measurements to guide treatment in chronic asthma. N Engl J Med 352(21), 2163-73 (2005).

[47] Holt S, Suder A, Weatherall M. *et al*. Dose-response relation of inhaled fluticasone propionate in adolescents and adults with asthma: meta-analysis. BMJ 323(7307), 253-6 (2011).

[48] Calhoun WJ, Ameredes BT, King TS *et al.* Comparison of physician-, biomarker-, and symptom-based strategies for adjustment of inhaled corticosteroid therapy in adults with asthma: the BASALT randomized controlled trial. JAMA 308(10), 987-97 (2012).

[49] Amelink M, de Groot JC, de Nijs SB *et al.* Severe adult-onset asthma: A distinct phenotype. J Allergy Clin Immunol 132(2), 336-41 (2013).

•• This study investigated the differences between severe adult-onset asthma and typical early-onset asthma. It was found that FeNO, sputum eosinophils and blood neutrophils were strongly correlated with the former subtype of asthma, which might be potentially used in future for more accurate diagnosis.

[50] Ricciardolo FLM. Multiple roles of nitric oxide in the airways. Thorax 58(2), 175-82 (2003).

[51] Holguin F, Comhair SA, Hazen SL *et al*. An association between L-arginine/asymmetric dimethyl arginine balance, obesity, and the age of asthma onset phenotype. Am J Respir Crit Care Med 187(2), 153-9 (2013).

•• This study provided a possible pathogenesis of obesity-associated asthma, which had been elusive thus far. The authors found that adults suffering from this asthma phenotype had lower plasma L-arginine/ADMA ratio and FeNO than their counterparts, and proposed that uncoupling of nitric oxide synthase might be causing an increase in production of ROS and in effect airway damage.

[52] Komakula S, Khatri S, Mermis J *et al.* Body mass index is associated with reduced exhaled nitric oxide and higher exhaled 8-isoprostanes in asthmatics. Respir Res 8, 32 (2007).

[53] Kocak H, Oner-Iyidogan Y, Gurdol F, Oner P, Esin D. Serum asymmetric dimethylarginine and nitric oxide levels in obese postmenopausal women. J Clin Lab Anal 25(3), 174-8 (2011).

[54] Schwedhelm E, Maas R, Freese R *et al.* Pharmacokinetic and pharmacodynamic properties of oral L-citrulline and L-arginine: impact on nitric oxide metabolism. Br J Clin Pharmacol 65(1), 51-9 (2008).

[55] Tomlinson JE, McMahon AD, Chaudhuri R. *et al.* Efficacy of low and high dose inhaled corticosteroid in smokers versus non-smokers with mild asthma. Thorax 60(4), 282-7 (2005).

[56] Spears M , Weir CJ, Smith AD *et al.* Bronchial nitric oxide flux (J'aw) is sensitive to oral corticosteroids in smokers with asthma. Respir Med 105(12), 1823-30 (2011).

[57] Cai C, Yang J, Hu S, Zhou M, Guo W. Relationship between urinary cysteinyl leukotriene E4 levels and clinical response to antileukotriene treatment in patients with asthma. Lung 185(2), 105-12 (2007).

• This study found that patients with high pre-treatment levels of urinary LTE_4 were more likely to respond to treatment with montelukast (cys-LT receptor antagonist) than the patients with low pre-treatment levels of this biomarker. Montelukast has few side effects, and seems very effective in a small proportion of patients, while being inferior to inhaled corticosteroids in most. In future measurement of urinary LTE_4 could identify patients likely to respond to montelukast.

[58] Frossi B, Gri G, Tripodo C, Pucillo C. Exploring a regulatory role for mast cells: 'MCregs'? Trends Immunol 31(3), 97-102 (2010).

[59] Reuter S, Stassen M, Taube C. Mast cells in allergic asthma and beyond. Yonsei Med J 51(6), 797-807 (2010).

[60] Galli SJ, Tsai M. IgE and mast cells in allergic disease. Nat Med 18(5), 693-704 (2012).

[61] Kay LJ, Yeo WW, Peachell PT. Prostaglandin E2 activates EP2 receptors to inhibit human lung mast cell degranulation. Br J Pharmacol 147(7), 707-13 (2006).

[62] Sastre B, Fernández-Nieto M, Mollá R *et al.* Increased prostaglandin E2 levels in the airway of patients with eosinophilic bronchitis. Allergy 63(1), 58-66 (2008).

[63] Roca-Ferrer J, Garcia-Garcia FJ, Pereda J *et al.* Reduced expression of COXs and production of prostaglandin E(2) in patients with nasal polyps with or without aspirin-intolerant asthma. J Allergy Clin Immunol 128(1), 66-72 (2011).

[64] Fens N, Roldaan AC, Van Der Schee MP *et al.* External validation of exhaled breath profiling using an electronic nose in the discrimination of asthma with fixed airways obstruction and chronic obstructive pulmonary disease. Clin Exp Allergy 41(10), 1371-8 (2011).

[65] Dragonieri S, Schot R, Mertens B *et al.* An electronic nose in the discrimination of patients with asthma and controls. J Allergy Clin Immunol 120(4), 856-62 (2007).

[66] Van der Schee MP, Palmay R, Cowan JO, Taylor DR. Predicting steroid responsiveness in patients with asthma using exhaled breath profiling. Clin Exp Allergy 43(11), 1217-25 (2013).

[67] Montuschi P, Santonico M, Mondino C *et al.* Diagnostic performance of an electronic nose, fractional exhaled nitric oxide, and lung function testing in asthma. Chest 137(4), 790-6 (2010).

• This study found that diagnostic performance in asthma was greatest when FeNO measurement was combined with use of electronic nose, a sensitive assay for volatile organic compounds, thus highlighting the benefit of combining biomarkers for increasing the accuracy of diagnosis.

[68] Kumar SD, Emery MJ, Atkins ND, Danta I, Wanner A. Airway mucosal blood flow in bronchial asthma. Am J Respir Crit Care Med, 158, 153–156 (1998).

[69] García G, Bergna M, Uribe E, Yañez A, Soriano JB. Increased exhaled breath temperature in subjects with uncontrolled asthma. The international journal of tuberculosis and lung disease. 17(7), 969-72 (2013).

[70] Zhu Z, Zheng T, Homer RJ *et al.* Acidic mammalian chitinase in asthmatic Th2 inflammation and IL-13 pathway activation. Science 304(5677), 1678-82 (2004).

[71] Chupp GL, Lee CG, Jarjour N *et al.* A chitinase-like protein in the lung and circulation of patients with severe asthma. N Engl J Med 357(20), 2016-27 (2007).

[72] Konradsen JR, James A, Nordlund B *et al.* The chitinase-like protein YKL-40: a possible biomarker of inflammation and airway remodeling in severe pediatric asthma. J Allergy Clin Immunol 132(2), 328-35 (2013).

Table 1. Summary of asthma studies in which periostin was investigated as a potential stratifying biomarker.

Study	Population	Design	Outcome
Woodruff et al., 2007[16]	42 nonsmoking asthmatics (who did not take inhaled or oral corticosteroids for 4 wks before enrolment), 28 nonsmoking healthy controls, and 16 current smokers without asthma, but with mild to moderate airflow obstruction (disease controls)	12-week RCT; gene expression examined by microarrays and qPCR (from samples obtained by bronchoscopy with airway epithelial brushing collection) at onset and after 1 wk of treatment with placebo or fluticasone 500 µg BD	POSTN gene expressed 4.4x higher in asthmatics than in healthy controls, which decreased 2.1x after fluticasone treatment
Woodruff et al., 2009[17]	*(as above)*	*(as above)*	Approximately half (20 out of 44) asthmatic patients have a high baseline expression of three T_H2-related genes (POSTN, CLCA1, and SERPINB2), whereas remaining asthmatics have a low expression. Treatment with fluticasone decreases the level of expression of these genes in the former group to the level of the latter group.
Corren et al., 2011[18]	219 patients with uncontrolled asthma (ACQ5 symptom-only version score of ≥ 1.5) refractory to ICS (≥ 6 months' use), and 112 healthy controls	Patients randomized to receive lebrikizumab (anti-IL-13) 250 mg or placebo subcutaneously for 24 wks, and regularly assessed until 32nd wk	Lebrikizumab improves FEV_1 (by 8.2 percentage points vs. placebo, P = 0.03) in T_H2-high, but not in T_H2-low patients (increase by 1.6 percentage points vs. placebo, but with P = 0.61), which directly correlates with serum periostin levels; no alteration in asthma symptoms or use of rescue medication

Noonan et al., 2013[19]	212 asthmatics with stable asthma (diagnosis of asthma at least 12 months before treatment, a bronchodilator response, and relative change in FEV_1 before treatment < 15%)	Patients randomized into four approximately equal groups: receiving lebrikizumab (125, 250, or 500 mg) or placebo subcutaneously for 12 wks, and regularly assessed until 20th wk	Lebrikizumab significantly lowers treatment failure rates (6% failure rate for lebrikizumab-treated vs 27% for placebo-treated patients), but has no significant effect on FEV_1 for any lebrikizumab dose; and the effect of its administration does not differ between high- and low-periostin patient groups
Hanania et al., 2013[20]	850 patients (aged 12-75 yrs) with uncontrolled severe persistent allergic asthma recruited for EXTRA study; data on FeNO, blood eosinophils and serum periostin available from 394, 797, and 534 patients, respectively	Post-factum analysis of part of the population of EXTRA study: patients randomized to receive omalizumab (anti-IgE, dose dependent on serum total IgE and patient body weight) or placebo for 48 wks	Omalizumab decreases exacerbation rate in periostin-high (≥ 50 ng/ml) patients by 30% vs placebo (95% CI: 22-51%, P = 0.07), but not in periostin-low (< 50 ng/ml) patients (3% decrease, 95% CI: 24.3-32%, P = 0.94); no improvement in symptoms and FEV_1

Table 2. Summary of the studies investigating exhaled nitric oxide.

Study	Population	Design	Outcome
Petsky et al., 2012[26]	1231 participants (children and adults) completed a total of 9 studies	Meta-analysis of 9 studies (3 adult using sputum eosinophils, and 2 adult and 4 children using FeNO); 4 of them double, and 5 single-blind; marked variability in defining asthma exacerbations (main outcome in all studies), although all treated such exacerbations with oral steroids	Using FeNO for guiding corticosteroid therapy allowed a decrease in daily dose of this medication in adults, compared to control group (mean difference of -450.03 µg, 95% CI: -676.73 to -223.34, P < 0.0001). However, in children the same strategy led to an increase in daily ICS used (mean difference of 140.18 µg, 95% CI: 28.94-251.42, P = 0.014. No significant differences in terms of exacerbations when FeNO was used for guiding treatment.
Calhoun et al., 2012[27]	342 adults with mild-to-moderate asthma controlled by low-dose ICS	RCT of 342 adults evenly assigned to three groups: having their ICS dose adjusted by FeNO values, symptom score, or physician assessment; with primary outcome of time to treatment failure	No significant difference in time to treatment failure among three groups. For physician-based assessment, the failure rate was 22%, for FeNO-based 20%, and for symptom-based 15%. Hazard ratio for physician-based vs biomarker-based assessment was 1.2 (97.5% CI: 0.6-2.3)
Hanania et al., 2013[20]	850 patients (aged 12-75 yrs) with uncontrolled severe persistent allergic asthma recruited for EXTRA study; data on FeNO, blood eosinophils and serum periostin available from 394, 797, and 534 patients, respectively	Post-factum analysis of part of the population of EXTRA study: patients randomized to receive omalizumab (anti-IgE, dose dependent on serum total IgE and patient body weight) or placebo for 48 weeks	Treatment with omalizumab led to a 53% (95% CI: 37-70%, P = 0.001) decrease in exacerbations (vs placebo) in the high-FeNO (≥ 19.5 ppb) subgroup, and to an insignificant 16% (95% CI: -32 to 46%, P = 0.45) decrease in exacerbations in the low-FeNO (< 19.5 ppb) subgroup.

Amelink et al., 2013[28]	176 patients with adult onset asthma, of whom 78 had severe asthma and 90 mild-to-moderate asthma (according to Innovative Medicines Initiative consensus criteria)	Cross-sectional observational study involving two visits; one during which multiple clinical indices were measured, and another specifically for methacholine challenge	Blood neutrophil (OR 7.6, 2.9-19.8, P < 0.01) and sputum eosinophil (OR 1.5, 1.1-2.2, P = 0.02) counts, but also FeNO (OR 1.5, 1.1-2.2, P = 0.02) were found to be associated with severe adult-onset asthma (a distinct asthma phenotype)
Holguin et al., 2013[30]	Severe Asthma Research Programme subgroup in whom plasma levels of L-arginine and ADMA had been measured: 155 patients, 49% of whom had severe, and the rest mild-to-moderate asthma; mean age of onset was 10 years, and duration 22 years; population divided into late- and early-onset asthma (onset at 12 years or older, or lower, respectively)	Cross-sectional study of selected patients from SARP study	In late-onset asthma, plasma L-arginine/ADMA ratio was lower than in early-onset asthma (median of 109, 95% CI: 81-138 vs 121, 95% CI: 94-178, P = 0.02). L-arginine/ADMA (log) found to be inversely correlated to BMI in the late-onset asthmatic group (r = -0.4, P = 0.0006), but more weakly correlated in the early-onset group (r = -0.2, P = 0.07).

Table 3. Summary of the studies investigating other potential biomarkers.

Study	Biomarker	Population	Design	Outcome
Cai et al., 2007[34]	Cysteinyl Leukotriene E4 as an indicator of response to treatment with montelukast.	48 patients with mild to moderate asthma, who were not receiving a concomitant treatment with leukotriene modifiers or oral corticosteroids and not having experienced any change in their asthma parameters in the preceding 10 days	4 wk trial of treatment with leukotriene receptor antagonist montelukast; measurement of various clinical parameters at onset and end of study; patients classified as responders if: (1) experienced reduction of ≥ 20% in mean symptom score; (2) reduction of ≥ 20% in β_2-agonist usage; and (3) a mean improvement of FEV1 of ≥ 10% from baseline value	25 patients classified as responders, and 23 as non-responders; urinary LTE_4 level was significantly higher in responders than in non-responders (224.5 ± 34.4 vs. 175.3 ± 37.1 pg/mg creatinine, $p < 0.05$); patients with urinary LTE_4 > 200 pg/mg creatinine 3.5 times more likely (95% CI: 1.7-15.8) to respond to montelukast than those below this level

Sastre et al., 2008[39]	prostaglandin E2 as an indicator of severity in Eosinophilic Bronchitis.	13 patients with asthma, 13 patients with nonasthmatic eosinophilic bronchitis, and 11 controls (nonsmokers with no history of asthma, allergic diseases, or chronic bronchitis)	Cross-sectional study, which meeasured cytokine mRNA levels (by real time qPCR), proinflammatory mediators, and concentration of eicosanoids (by enzyme immunoassays) in induced sputum	Induced sputum PGE_2 concentrations significantly raised in patients with eosinophilic bronchitis (838 ± 612 pg/ml) compared to asthmatic (7.54 ± 2.14 pg/ml) and healthy subjects (4 ± 1.3 pg/ml); no other significant differences between asthmatic patients and nonasthmatic eosinophilic bronchitis patients
Fens et al., 2011[41]	Profiling of exhaled volatile organic compounds for discrimination of asthma with fixed airways obstruction and chronic obstructive pulmonary disease.	60 asthmatic patients (21 with fixed airways obstruction, and 39 with classic reversible airways obstruction) and 40 COPD patients (GOLD stages II-III)	Cross-sectional study involving sampling of volatile organic compounds (VOC) in exhaled breath using an electronic nose sensor	Breathprints had accuracy of 88% in distinguishing between fixed asthma and COPD (sensitivity 85%, specificity 90%) and 83% for classic asthma (sensitivity 91%, specificity 90%), which was not affected by current smoking status
Dragonieri et al., 2007[42]	Profiling of exhaled volatile organic compounds for discrimination between old and young asthma sufferers.	10 young patients with mild asthma (25.1 ± 5.9 yrs, FEV_1 62.3 ± 23.6), 10 older patients with severe asthma (57.3 ± 7.1 years, FEV_1 108.3 ± 14.7), and matched 10 young and 10 older controls	*(same in principle as above)*	Electronic nose enabled discrimination of both young and old asthma patients from their respective controls, but was less accurate at distinguishing between mild and severe asthma patients

Van der Schee et al., 2013[43]	Volatile organic compound profiling in comparison to exhaled FeNO and eosinophils for the prediction of steroid responsiveness.	25 patients with mild or moderate asthma and 20 controls	6 wk RCT. Discontinuation of steroid treatment in asthmatic patients for 28 days or loss of control, followed by treatment with oral prednisolone 30 mg/day for 14 days; assessment of various indices during steroid-free period with aim of predicting future steroid responsiveness	Analysis of VOC using electronic nose was superior to both FeNO and sputum eosinophils in predicting steroid responsiveness (AUC = 0.883 ± 0.16, P = 0.008; 0.545 ± 0.28, 0.751; and 0.610 ± 0.29, 0.441, respectively)
Montuschi et al., 2010[44]	Volatile organic compopunds, FeNO and lung function for comparative diagnostic performance in asthma.	27 patients with mild or moderate persistent asthma, and 24 healthy controls	Cross-sectional study which compared diagnostic performance of various biomarkers: FeNO, lung function testing, and VOC measured by electronic nose	Diagnostic performance was highest for electronic nose (87.5%), followed by FeNO (79.2%) and lung function testing (70.8%); highest diagnostic performance was obtained by combining electronic nose and FeNO (95.8%)
Garcia et al., 2013[45]	Exhaled breath temperature for the prediction of asthma and discrimination between controlled and uncontrolled asthma.	100 patients with persistent asthma (50 with controlled and 50 with uncontrolled) and 50 healthy controls	Cross-sectional study, which measured lung function by post-bronchodilator forced spirometry, asthma control test and exhaled breath temperature (EBT)	Patients with asthma had significantly increased EBT compared to healthy controls; this was particularly visible in uncontrolled asthmatics (EBT of 34.9 ± 0.8 ^{0}C) compared to well-controlled asthmatics (EBT 33.7 ± 0.8 ^{0}C) and controls (EBT 33.2 ± 0.2 ^{0}C, P < 0.001)

Konradsen et al., 2013[47]	Chitinase-3-like protein 1- YKL-40 (secreted glycoprotein) for the prediction of therapy resistance in children with asthma.	34 children with severe refractory asthma, 39 children with controlled persistent asthma, and 27 healthy controls	Cross-sectional study, involving using ELISA to measure serum YKL-40 levels and other clinical measurements	Children with therapy-resistant asthma had significantly higher serum YKL-40 levels compared with healthy children (19.2 ng/ml vs 13.8 ng/ml, P = 0.03), which in these children correlated with FeNO (r = 0.48, P = 0.004), blood neutrophils (r = 0.63, P < 0.001) and bronchial wall thickening on high-resolution CT (r = 0.45, P = 0.01).