

Mechanisms of allergic diseases

Series editors: Joshua A. Boyce, MD, Fred Finkelman, MD, William T. Shearer, MD, PhD, and Donata Vercelli, MD

Using biomarkers in the assessment of airways disease

D. Robin Taylor, MD, FRCP(C) *Dunedin, New Zealand*

A biomarker provides a window on underlying disease activity. This is helpful when the pathology, treatment response, or both are heterogeneous or when trying to interpret nonspecific respiratory symptoms in patients with comorbidities. The successful application of a biomarker result is critically dependent on the specific question being addressed and the performance characteristics of the biomarker in relation to that question in the context of pretest probabilities. Negative prediction might be the best way to use a biomarker, such as a D-dimer, pro-brain natriuretic peptide, and exhaled nitric oxide. In this review the role of biomarkers in airways disease (notably induced sputum eosinophils and exhaled nitric oxide) is considered in relation to risk stratification, identification of treatment responders, identification of a clinical phenotype, monitoring of disease, and new drug development. (*J Allergy Clin Immunol* 2011;■■■:■■■-■■■.)

Key words: Airways disease, asthma, biomarkers, chronic obstructive pulmonary disease, exhaled nitric oxide, induced sputum, performance characteristics, validation

A biomarker is a surrogate measurement designed to characterize and quantify an underlying disease process.^{1,2} The need for high-performance biomarkers in respiratory disease is especially great. For most nonmalignant pulmonary conditions, confirmation of the underlying pathology by means of tissue biopsy is rarely undertaken: the cost/benefit ratio is too high. Instead, imaging is used to assess anatomic abnormalities associated with disease (eg, chest radiography), measurements of respiratory physiology are obtained to identify disturbances of function (eg, spirometry), or both. However, there are significant limitations associated with these conventional methods.³ Changes in structure and function are the consequences of pathology and might occur only intermittently, as in asthma, or later if the process is continuous over time, as in chronic obstructive pulmonary disease (COPD). In disease that is mild, early, or both, the structure and function might appear normal, even though the patient has symptoms. In patients with complex disease, especially if there are

Abbreviations used

AHR:	Airway hyperresponsiveness
BNP:	Brain natriuretic peptide
COPD:	Chronic obstructive pulmonary disease
CV:	Coefficient of variation
FENO:	Fraction of exhaled nitric oxide
ICS:	Inhaled corticosteroid
LR:	Likelihood ratio
NO:	Nitric oxide
NPV:	Negative predictive value
PPV:	Positive predictive value
VTE:	Venous thromboembolism

comorbidities, symptoms are nonspecific and multifactorial, and confirming their exact cause is difficult. Symptoms are not always related to changes in structure or function. These clinical realities are what make a biomarker potentially useful. Indeed, biomarkers of underlying disease activity occupy a different domain to measurements of structure and function⁴ and provide complementary information.

Importantly, the use of biomarkers has the potential to unravel heterogeneity. There is heterogeneity in the pathologies of asthma, COPD, and their overlap syndromes. In turn, this results in heterogeneity as to the clinical phenotype and, even more importantly, treatment response. For example, in asthmatic subjects airway inflammation characterized by sputum eosinophilia is associated with frequent exacerbations (clinical phenotype),⁵ whereas airway inflammation characterized by neutrophilia has relative **steroid resistance** (treatment response).⁶ Being able to distinguish clinical and treatment-response phenotypes using a relevant biomarker has the potential to improve clinical management.

The list of requirements for a good biomarker is demanding (Table I). Rigorous biological and clinical validation is necessary before a biomarker can be endorsed. However, beyond the methodologic issues (limits of detection, precision, accuracy, and reproducibility), there are technical aspects specific to the respiratory tract that add further complexity to biomarker development. In contrast to blood or urine, sampling exhaled breath or airway secretions is problematic and requires standardization. The respiratory tract does not provide a uniform source of “substrate.” Factors such as oropharyngeal contamination, dead-space gas, and expiratory flow affect sampling techniques. There is no easy way around the methodologic complexities. Recent advances have provided a bewildering array of measureable compounds that are related to the pathophysiology of airways disease, and in theory they might be used as biomarkers. However, the transition from “bench to bedside” for a clinically useful biomarker is a protracted exercise, with many false starts and blind alleys.

From the Dunedin School of Medicine, University of Otago, Dunedin, New Zealand.

Disclosure of potential conflict of interest: D. R. Taylor has received lecture fees from Aerocrine AB.

Received for publication February 3, 2011; revised March 21, 2011; accepted for publication March 24, 2011.

Reprint requests: Professor D. Robin Taylor, MD, FRCP(C), Dunedin School of Medicine, PO Box 913, Dunedin, New Zealand. E-mail: robin.taylor@stonebow.otago.ac.nz.

0091-6749/\$36.00

© 2011 American Academy of Allergy, Asthma & Immunology

doi:10.1016/j.jaci.2011.03.051

Terms in boldface and italics are defined in the glossary on page ■■■.

The aim of the present review is to discuss key issues in the application of biomarkers in the treatment of respiratory disease. It is not the purpose of this review to address the technical aspects of biomarker measurement or the specifics of their application. The reader is directed to the recent review by Diamant et al,⁷ which deals with this topic. Likewise, it is not our purpose to provide an account of *exhaled nitric oxide* (NO) as a biomarker in clinical practice. However, given the present author's research experience in that field, we will refer to evidence based on exhaled NO research to illustrate and inform the general concepts under discussion.

USING A BIOMARKER: WHAT ARE THE CLINICAL OBJECTIVES?

The measurement of a biomarker can have several objectives. It is important to understand those that can and, just as importantly, those that cannot be served by making the measurement. There are several domains (Table II), and the proven utility of a biomarker in one domain may not be the case for another.

Risk stratification

Risk stratification has the potential to enhance strategies for disease prevention. This is established practice for cardiovascular disease by using demographic (sex) and comorbidity (diabetes and hypertension) data combined with blood glucose and cholesterol measurements.⁸ Regrettably, there are no examples in respiratory medicine in which biomarkers are used in this way.⁹ However, this ought to be our aim.

In patients with COPD, longitudinal studies have identified that increased inflammatory markers are associated with increased future risk of morbidity and mortality. Dahl et al¹⁰ have reported that a decrease in lung function over a 6-year period was 3 to 4 times greater in relation to a baseline plasma fibrinogen level of greater than 3.3 g/L. Later, these authors reported that mortality in patients with COPD was significantly increased in patients whose baseline measurement of C-reactive protein was greater than 3 mg/L.¹¹ Although in that study mortality was not always caused by COPD, having a marker that, combined with other parameters, identifies subjects at particular risk of end-stage disease is highly desirable, and further work in this area would be rewarding.¹²

There have been previous attempts to assess the risk of childhood asthma based on clinical parameters.¹³ More recently, Caudri et al¹⁴ combined a number of factors to optimize risk stratification for the development of persistent childhood asthma. After obtaining information at age 4 years about wheezing frequency, eczema, and parental atopy, the addition of serum IgE (increased; yes/no) and exhaled NO (as a continuous variable) enhanced the power to predict persistent asthma at age 8 years (Fig 1).¹⁴ Debley et al¹⁵ have also reported that baseline exhaled NO measurement can be used in infants (mean age, 15 months; range, 6-24 months) as a predictor of wheezing requiring intervention over the next 6 months with anti-inflammatory treatment (area under the curve, 0.890), decrease in lung function (area under the curve, 0.830), or both. In that study the performance of exhaled NO measurement was not improved by the addition of other variables as predictors. Further longitudinal studies are required to confirm whether single markers or combinations of clinical

GLOSSARY

ANTI-IL-5: Humanized biologic antibodies blocking IL-5-induced eosinophilopoiesis and trafficking to the lungs, gastrointestinal tract, and skin have been used in therapeutic trials for asthma, hypereosinophilic syndrome, and eosinophilic esophagitis.

ASTHMA CONTROL TEST: A 5-point asthma index that is filled out by the patient and that can indicate incomplete asthma control when scores are 19 or less. The ACT can also be used as a predictive index for response/need for therapy, as well as an index of adequate asthma control.

COEFFICIENT OF VARIATION (CV): The CV is calculated by dividing the SD by the mean and reflects the scatter of a given variable. Although the SD values of 2 variables might be difficult to compare directly, the CV can be used to compare the variability between 2 factors because it is calculated as a proportion. This is also true when comparing the scatter for variables expressed in different units.

EXHALED NITRIC OXIDE (NO): NO is produced by several airway cell types, including epithelial cells, and its levels are increased in patients with atopic asthma and rhinitis. Exhaled NO can be used to predict inhaled corticosteroid response and relapse on discontinuation of therapy.

NEGATIVE PREDICTIVE VALUE (NPV): The NPV is the likelihood that a person with a negative test result actually does not have the disease in question. It is calculated by dividing the number of true-negative results by the total number of negative test results (true-negative plus false-negative results).

POSITIVE PREDICTIVE VALUE (PPV): The PPV is the likelihood that someone with a positive test result actually has the disease in question. The PPV is a reflection of the test and not the disease itself and is calculated by dividing the number of true-positive results by the number

of true-positive plus false-positive results. True-positive results represent patients with a positive test result and disease and false-positive results are patients with a positive test result but without disease.

PRETEST PROBABILITY: The pretest probability is the probability of the disease in the target population before the result of the diagnostic test is known. The calculation is done by calculating the number of patients with the disorder among all of the patients with symptoms of the disorder who both do and do not have the disorder.

PRO-BRAIN NATRIURETIC PEPTIDE (PRO-BNP): Although first isolated from the brain, the cardiac ventricles are the main source of BNP. BNP inhibits renin and aldosterone, decreases sodium retention, and increases glomerular filtration rates. BNP can function as an indicator of increased ventricular mass and a surrogate marker for heart failure.

PROTEOMICS: Proteomic technology is aimed at the discovery of proteins in the pathogenesis or presence of a disease state. Techniques such as 2-dimensional gel analysis, peptide sequencing, or antibody arrays can be used to find both known and unknown proteins.

STEROID RESISTANCE: Glucocorticoid-resistant asthma is defined clinically as continued airflow obstruction and airway inflammation despite adequate glucocorticoid therapy. Mechanisms of resistance include induction of the β isoform of the glucocorticoid receptor (GCR) through alternative splicing. GCR β binds to DNA but does not activate transcription.

TNF- α : TNF- α is produced by dendritic cells, mast cells, eosinophils, CD4⁺ T cells, airway cells (eg, epithelial and smooth muscle cells), and fibroblasts. Increased TNF- α levels are seen in patients with severe refractory asthma, and TNF- α blockade might be a useful therapy in this subset of patients.

The Editors wish to acknowledge Seema Aceves, MD, PhD, for preparing this glossary.

TABLE I. Characteristics of the ideal biomarker

In relation to its measurement (all of the following are required):
1. Is minimally invasive
2. Is easily measured
3. Is reproducible
In relation to clinical application (not all of the following apply to every biomarker):
4. Indicates a key pathophysiologic process in relation to the disease of interest, either as a pathogenic mediator or an epiphenomenon
5. Can be used to confirm a diagnosis or identify a particular clinical phenotype
6. Can be used to identify a treatment response phenotype
7. Is responsive to changes in disease activity
8. Is responsive within a timeframe that precedes changes in clinical status and permits preemptive intervention
9. Is responsive to changes in disease activity that are mediated by treatment intervention
10. Demonstrates a dose-response relationship and thus can be used to guide treatment requirements
11. Demonstrates a dose-response relationship and thus might be used to guide treatment requirements
12. Can be used to predict future clinical or adverse outcomes

TABLE II. The possible clinical domains in which a biomarker can be applied

1. Risk stratification in subjects or populations
2. Screening for undiagnosed or latent disease
3. Diagnosis:
• linking a clinical syndrome to an underlying pathologic process
• staging
4. Disease monitoring:
• current disease activity
• guidance as to treatment requirements and subsequent response to intervention
• prognosis/future risk

and biological variables may be used to distinguish a clinical phenotype (ie, chronic persistent asthma) from among a heterogeneous population of wheezy infants.

Screening for latent disease

Understandably, the role of biomarkers to screen for undiagnosed disease is being pursued vigorously in the field of lung cancer, notably by using *proteomics*,¹⁶ but the challenges are immense, and to date, no candidate or array of candidate markers has been validated. There is also a potential role for biomarkers in patients with occupational asthma. It is recognized that atopy is a risk factor for sensitization, particularly to high-molecular-weight agents. However, sequential testing for agent-specific sensitization, airway hyperresponsiveness (AHR), or both does not appear to add value to simple questionnaires.^{17,18} Perhaps there is a role for a relevant biomarker? Acutely, exhaled NO levels increase after exposure to occupational agents in sensitized subjects.^{18,19} But can changes over time be used to predict the advent of exposure-related sensitization? Recently, Tossa et al²⁰ reported that among at-risk apprentices, a progressive increase in exhaled NO levels from baseline to 15 months was associated with the advent of occupational AHR. The key question is

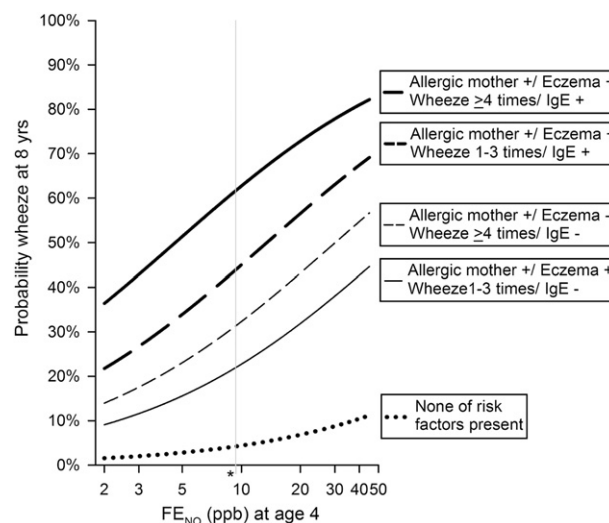


FIG 1. Predicted probability (as a percentage) for wheezing at age 8 years based on FE_{NO} values (parts per billion, log scale) measured at age 4 years, adjusted, and stratified for specific IgE levels, maternal allergy, doctor-diagnosed eczema, and wheezing frequency at age 4 years. Different lines represent children with different subsets of risk factors. From Caudri et al¹⁴ and reproduced with permission from the BMJ publishing group.

whether such findings translate into sufficiently high **positive predictive values** (PPVs) to make interval measurements of the biomarker worthwhile in individual subjects.

Diagnosis, monitoring of disease and treatment requirements, and assessment of prognosis and future risk will be addressed in later sections of this review.

USING A BIOMARKER: UNDERSTANDING PERFORMANCE CHARACTERISTICS AND ASKING THE RIGHT QUESTIONS

Diagnosis

The success with which a biomarker operates in diagnostic decision making depends on its performance characteristics. The terminology is familiar: sensitivity, specificity, PPV and **negative predictive value** (NPV), and likelihood ratios (LRs, both positive and negative). Unfortunately, familiarity with the terms does not guarantee that we understand them, and this often leads to inappropriate expectations and mistaken applications of a diagnostic test.

A sensitive test identifies most subjects with the condition but also some persons without the condition. A sensitive test can rule a condition out when the result is negative (high NPV or low negative LR). In such circumstances the clinician will refrain from diagnosing or intervening unless additional evidence indicates that the test result is false negative. A specific test means that those with a positive result have the condition, but some affected subjects might be missed. A specific test can rule a condition in when the result is positive (high PPV or strong positive LR). In such circumstances a clinician will choose to accept the diagnosis and will consider intervention unless additional evidence indicates that the test result is false positive or the risks of intervention outweigh the benefits. Furthermore, it needs to be decided in relation to the clinical question whether positive or negative prediction is more important and what levels of performance

TABLE III. Factors and questions affecting the interpretation of a biomarker for monitoring disease, assessing treatment requirements, or both

1. The distribution of normal values in a healthy population: the cut points that define normality
2. The distribution of values in an affected population when the disease is well controlled: cut points that define unacceptable levels of disease activity
3. The role of group mean reference values derived from healthy subjects in relation to clinical objectives: Is normalization important?
4. The relationship between changes in disease activity (as measured using the biomarker) and changes in the clinical status of the patient
5. The MCID between 2 measurements obtained at different time points in relation to either underlying disease activity, the clinical status of the patient, or both
6. Is the MCID best measured as absolute values or percentage change?
7. The prognostic significance of changes in the biomarker measurement that exceed the MCID and the lag time between changes in the biomarker and changes in the clinical status of the patient
8. The magnitude and scale of the biomarker-disease relationship (dose-response) in relation to a treatment intervention

MCID, Minimum clinically important difference.

justify using the test result. An NPV or negative LR is of value in excluding a current condition; a PPV or positive LR is of value in future risk assessment. However, is an NPV of 85% adequate, or should it be 95%? There is no general agreement as to what thresholds should be used to determine what is a good test. Although less commonly reported, LRs are increasingly used because their calculation is independent of pretest probabilities. For positive LRs, values of 2 to 5, 5 to 10, and greater than 10 are used to define weak, moderate, or strong (positive) LRs, and values of less than 0.5 and less than 0.1 are used to define low and very low (negative) LRs, respectively.

The measurement of D-dimer and *pro-brain natriuretic peptide* (pro-BNP) provides excellent examples of how these principles can be applied. Both tests are widely used to evaluate acute dyspnea, but their application is limited²¹ because their performance characteristics are only acceptable when results are within the normal range. A normal D-dimer has a high NPV for venous thromboembolism (VTE; >95%), and it helps to rule out VTE. Likewise, a normal pro-BNP value has a high NPV for cardiac failure (>95%), and it helps to rule out cardiac dysfunction as a contributor to dyspnea, especially in patients with comorbidities. Conversely, high D-dimer levels are associated with only modest PPVs, and the test is unreliable as a positive predictor. Taken together, these features determine the questions that can and cannot be answered by using the tests. Increased levels of D-dimer or pro-BNP cannot be used to determine that a patient needs anticoagulant or diuretic therapy: the PPVs for VTE or cardiac failure are much lower, and therefore the tests cannot be relied on to answer these particular questions.^{21,22}

There is an additional caveat when interpreting the performance characteristics of a diagnostic test that concerns **pretest probability**. This is exemplified in a recent D-dimer study. Among 1722 patients with a possible diagnosis of VTE and a normal D-dimer, the incidence of VTE at 3 months' follow-up was 2.3% (95% CI, 1.4% to 3.8%). Overall, this is acceptable as the basis for using a negative D-dimer to justify withholding anticoagulation therapy. However, when the population was stratified according to their Wells score (a pretest probability assessment

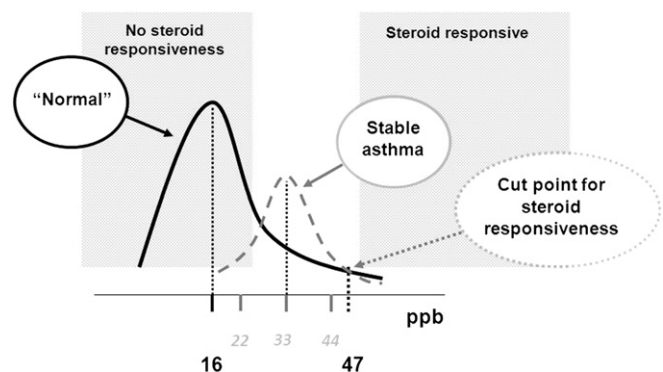


FIG 2. A composite schematic representation based on data from the studies of Smith et al²⁷ and Olin et al.^{28,29} The solid black line represents the distribution of exhaled NO values in healthy nonatopic healthy adults and is skewed to the right. The dotted gray line represents the distribution of FENO measurements in patients with stable asthma, with the 95% CIs on the x-axis (22-44 ppb). Using a cut point of 25 ppb, there is a high negative likelihood of there being active steroid-responsive eosinophilic airway inflammation. In contrast, at levels of greater than 50 ppb, there is a moderate likelihood of steroid responsiveness. Intermediate values need to be interpreted with caution.

tool), those with a high score (VTE likely) had an incidence of proved VTE at 3 months of 9.3% (95% CI, 4.8% to 17.3%) despite a normal D-dimer value at presentation. The authors concluded that "in patients with a 'likely' pre-test probability, there should be limited reliance on the D-dimer result, and patients should undergo further testing."²³

As far as exhaled NO is concerned, the same principles apply. First, clinical decision making should be based on evidence from studies of patients with respiratory symptoms (ie, appropriate pretest probabilities). In the study by Dupont et al,²⁴ the pretest probability of diagnosed asthma was 67%. The PPV of fraction of exhaled nitric oxide (FENO) for diagnosing asthma (at the optimum cut point) was 90%, and the NPV was 90%. In the study by Smith et al,²⁵ the pretest probability was lower at 36%; the PPV for diagnosed asthma was correspondingly lower at 70%, but the NPV was high at 92%. These and similar more recent data²⁶ indicate that FENO values, like D-dimer and pro-BNP values, provide more reliable information when the measured value is low. This does not preclude using high measurements to make clinical decisions, but the weight attached to a high result needs to be appropriately modified in light of different performance characteristics.

Monitoring disease and modifying treatment

The use of a biomarker to guide ongoing disease management is somewhat different to making a diagnosis. There are a number of additional considerations listed in Table III. Experience with NO has highlighted the complexities in this area.

Even where a normal range for a biomarker is derived from a large population, it may be different from the range of values obtained in affected subjects with stable disease. This is the case for exhaled NO (Fig 2).²⁷⁻²⁹ The discrepancy between "normal" and "affected but stable" ranges implies that clinical stability does not necessarily equate with the absence of the underlying disease activity, a normal biomarker level, or both.³⁰ In turn, this means that population-based reference ranges and predicted values might have limited application in diagnosing or

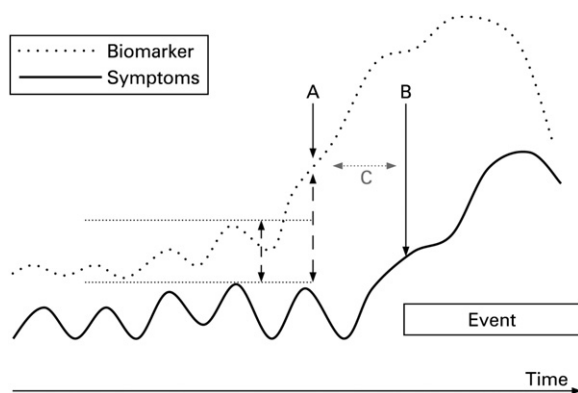


FIG 3. Schematic representation of changes in biomarker values in relation to symptoms, a clinical event, or both. *A*, Point at which biomarker values increase beyond the normal range and the signal is meaningful. *B*, Point at which symptoms become apparent. *C*, Interval of time during which intervention can be applied. This needs to be greater than the time required for an intervention to abort or modify the exacerbation. The time interval can be days or years depending on the disease (eg, asthma or COPD) or the event/outcome (eg, exacerbation or chronic respiratory failure). From Taylor and Pavord⁴⁹ and reproduced with permission of the BMJ publishing group.

monitoring disease. Furthermore, in some situations normalization of the biomarker by means of treatment intervention might not be a desirable therapeutic objective.³¹ Personal best values obtained at a time of clinical stability (preferably on >1 occasion) are more likely to provide an appropriate reference point for interpreting subsequent measurements.³²

Clinical decisions are based on whether an individual result is greater than or less than a chosen cut point for the biomarker. Low/normal values generally indicate the absence of underlying disease activity, and high/abnormal values indicate its presence. However, it is important to know the performance characteristics that apply for each particular cut point (these might differ) and to modify the expectations of the test accordingly. The so-called Asthma Treatment Algorithm studies^{33–39} used 1, 2, or even 3 cut points for FENO values as the basis for changing inhaled corticosteroid (ICS) treatment. The chosen cut points were based on upper limits of normal derived from healthy control subjects^{33,34,36} or patients with stable asthma.³⁸ In the study by Shaw et al,³⁷ a low cut-point, validated against corresponding sputum eosinophil counts, was used to determine the likely absence of underlying disease activity (low FENO value and high NPV for inflammation, and therefore the ICS dose was reduced). In contrast, Hewitt et al³⁵ used a high cut point related to likely steroid responsiveness (high FENO value and moderate PPV for steroid response, and therefore the ICS dose was increased). These inconsistencies point to the immense difficulties in standardizing the application of a biomarker.

Occasionally, what is abnormal is defined by the categorical presence or absence of the biomarker. The presence of sputum eosinophils is always abnormal (even though the accepted cut point defining abnormality is not zero!).⁴⁰ This is simpler and might explain why using sputum eosinophils to guide asthma treatment^{41,42} has proved to be more successful than using exhaled NO levels.⁴³

When using a biomarker to monitor disease over time, it is also important to know the minimal important difference between 2 separate measurements. This is based on 2 considerations. The

first is the *coefficient of variation* (CV) for repeated measurements of the test. Where the difference between 2 measurements is greater than the CV, it is reckoned that a true biological change has occurred. The second consideration is clinical and relates to the magnitude of the change in the biomarker, which equates to a significant change in underlying disease activity or, alternatively, the clinical status of the patient. For exhaled NO, the CV is relatively low in healthy subjects at around 10%.^{44,45} In patients with stable asthma, it is higher at around 20% to 25%,^{44–46} implying that changes in underlying disease activity are not necessarily clinically apparent. In turn, this means that the minimum important difference might be much greater than the CV. This has been confirmed for exhaled NO by Michils et al,⁴⁷ who have reported that a decrease of at least 40% in FENO values is required to identify patients going from poor to satisfactory asthma control (PPV, 83%).

Assessing prognosis and future risk

Assessing future risk overlaps with risk stratification, but the former is more concerned with outcomes in patients with established disease who are being monitored. Assessing future outcomes is an almost intuitive aspect of clinical care, but it has only recently been highlighted as a management objective.⁴⁸ Apart from acute exacerbations or deteriorating asthma control, future risk might include longer-term outcomes, such as accelerated decrease in lung function or even mortality.

In this context there is an additional requirement, namely that the signal from the biomarker (either as an absolute value or as change from baseline) is not only predictive of future events but also occurs sufficiently early to permit adequate intervention. This is demonstrated in Fig 3.⁴⁹

The prognostic significance of both sputum eosinophils and exhaled NO has been tested in asthmatic subjects. Anticipating future events is a situation in which PPVs and LR_s need to be high; negative prediction is unhelpful. In an early study Jones et al⁵⁰ reported the performance of sputum eosinophil and FENO measurements as predictors of loss of asthma control (within 1–7 days). Using a cut point of 3%, the PPV for sputum eosinophils was only 71%. For exhaled NO (either high absolute values or change from baseline), PPVs were around 80%. In another study in which FENO values were used to assess the risk of asthma exacerbations over 18 months, a FENO value of greater than 28 ppb had a PPV of 77%, with a positive LR of 3.3.⁵¹ Leaving aside the pragmatic difficulties of measuring a biomarker regularly in ambulatory patients, it is an open question as to whether PPVs of less than 90% are clinically useful, and therefore assessing future changes in asthma control is not a question that measurement of FENO can address with sufficient reliability.

USING BIOMARKERS TO ASSESS THE RESPONSE TO TREATMENT

Anti-inflammatory therapy in patients with airways disease

The most clinically important role for a biomarker in airways disease management is to predict the response to anti-inflammatory treatment. Current guidelines for asthma recommend a universal step-up regimen rather than a selective approach,⁵² but in fact, the response to ICS therapy is heterogeneous, and nonresponders are common.^{53,54} In the Gaining Optimal Asthma Control study

TABLE IV. The requirements of a biomarker in trials of novel therapies

1. The biomarker should be able to define the pathologic phenotype (qualitative).
 2. The biomarker should, if possible, reflect the intensity of the underlying disease activity (quantitative).
 3. The biomarker should be responsive to changes in the underlying disease activity resulting from treatment intervention (dose response).
 4. The biomarker should, if possible, be used as a secondary end point to determine treatment efficacy.
- Alternatively:
5. The biomarker should be able to define the clinical phenotype (qualitative).
 6. The biomarker should, if possible, reflect the clinical status of the patient (quantitative).
 7. The biomarker should be responsive to changes in a surrogate clinically relevant end point, which is directly related to underlying disease activity.

The assumption is that the intervention is targeted toward a phenotype in which there is a clear relationship between the mode of action of the treatment and the underlying disease.

approximately 30% of patients did not achieve asthma control despite progressive increases in their inhaled fluticasone dose to maximum levels.⁵⁵ A more rational and less empiric approach is needed. A biomarker that prospectively identifies a responder phenotype is also potentially useful when the within-patient pathology is itself heterogeneous and only one aspect of the pathology is treatment responsive. This occurs in patients with mixed asthma/COPD among whom response rates for ICSs are low and unpredictable (at best 25%) and in whom targeted therapy would avoid inappropriate treatment. Such an approach would also liberate clinical decision making from the confusion associated with conventional diagnostic labeling (is it asthma or COPD?).^{49,56}

Both sputum cells and exhaled NO measurements are potentially useful when assessing the need for ICS treatment.⁵⁷ Again, the performance characteristics of these tests need to be carefully considered. In patients with childhood asthma, there is evidence that FENO measurements can be used to predict those who are likely to respond to inhaled fluticasone more favorably than oral montelukast.^{58,59} In adult patients with undiagnosed new-onset respiratory symptoms²⁷ and in patients with COPD,⁶⁰ the NPVs for a low FENO value as a predictor of steroid response are high (91% and 87%, respectively; ie, low FENO values predict nonresponders). In contrast, the PPVs for high FENO values are much lower (82% and 67%, respectively). Interestingly, even in asthmatic patients without sputum eosinophilia (so-called paucigranulocytic asthma), the performance characteristics of the FENO value as a predictor of steroid response are equally good.⁶¹

These studies^{27,58-60} address the following question: “ICS treatment—yes or no?” at first presentation. In ongoing asthma management a different question usually applies: “ICS treatment—more or less?” Particularly in patients with severe asthma with fixed airflow obstruction, comorbidities (eg, obesity, gastroesophageal reflux, or anxiety), or both, it might be difficult to judge whether more anti-inflammatory treatment will be beneficial or, just as importantly, whether a reduction in steroid dose can be achieved. Once started, there is reluctance on the part of clinicians to reduce or withdraw anti-inflammatory treatment, and a biomarker would provide objective reassurance.³⁵ Exhaled NO values may be used to identify patients in whom either reduction or even withdrawal of ICS treatment is safe: low values 2 to 4

weeks after ICS withdrawal indicate that clinical relapse is unlikely, with NPVs exceeding 90%.^{62,63}

Randomized controlled trials designed to test whether FENO-based algorithms can be used to optimize ICS treatment have yielded disappointing results.⁶⁴ These studies addressed the question “ICS treatment—how much?,” and they indicate that FENO measurement is not helpful in addressing this question. However, this does not mean that there is no clinical role for exhaled NO as a biomarker, as some have suggested.^{65,66} Perhaps a less demanding question should have been asked. In a recent study, Perez-de-Llano et al⁶⁷ asked the following question: “More ICS treatment—yes or no?” Among 102 patients with poorly controlled asthma (*Asthma Control Test* score <20), symptoms remained uncontrolled in 49 (48%) after administering maximum doses of inhaled fluticasone for 1 month followed by a course of oral steroid for a further month.⁶⁷ The authors evaluated whether baseline FENO value was a significant predictor of response to the intervention, and it was (AUC, 0.925). At a cut point of 30 ppb or less, the NPV for steroid response was 92%. This study highlights how, when the question is framed correctly, the biomarker’s utility is more readily apparent.

Development of novel therapies

Most patients with asthma are adequately managed with ICS treatment. The challenges arise among patients whose asthma is severe and in whom the underlying pathology and treatment responsiveness are heterogeneous.^{68,69} Ideally, the development of novel treatments should be targeted to patients in whom the mode of action of the intervention is relevant to their pathologic phenotype, as identified with a biomarker. The inclusion of all comers in phase 3 trials risks obscuring a treatment effect when it is in fact present in a more specific, biomarker-defined subgroup. In addition, when a new treatment is aimed at reducing underlying disease activity, then a marker that measures underlying disease activity should be the primary end point. In that context a biomarker is all the more reliable when it is an active mediator and not just a bystander in the pathogenic pathway.

The requirements of a biomarker in drug development are set out in Table IV. The story of *anti-IL-5* and *anti-TNF- α* treatment illustrates some key points. Mepolimumab is an mAb directed against IL-5 that is responsible for the maturation and differentiation of eosinophils, which are integral to the pathogenesis of asthma.⁷⁰ In a small early study, a single dose of anti-IL-5 resulted in a dramatic reduction in blood and eosinophil numbers, although there were no changes in AHR.⁷¹ This apparently successful proof of concept study was followed by a larger study by Flood-Page et al.⁷² These investigators undertook a study in 362 unselected asthmatic patients, and again, the clinical outcomes (symptoms, peak flows, and spirometric indices) were not significant, despite the fact that changes in the biomarker (eosinophil numbers) were striking. These data seemed like the death knell for anti-IL-5 therapy.⁷³ However, long-term studies reporting a reduction in asthma exacerbations with mepolimumab were published later.^{74,75} The “success” of the later studies can be attributed to their biomarker-related design features. The biomarker (1) defined an integral component of the underlying disease process (eosinophil numbers), (2) defined a clinical phenotype (frequent exacerbations), (3) was used to specify selection criteria for the study, and (4) was related to the primary study end point (exacerbations).

Similar issues are relevant to anti-TNF- α treatment for asthma. Preliminary studies indicated that peripheral blood monocyte expression of TNF- α is a feature of refractory rather than ICS-responsive asthma and as such can be used to identify patients who are more likely to respond to anti-TNF- α therapy.⁷⁶ Later experience with anti-TNF- α treatment confirmed that when treatment is tested in small groups of patients targeted on their pathologic phenotype, treatment response phenotype, or both, positive outcomes were obtained.⁷⁶ Large studies comprising an unselected heterogeneous population yielded nonsignificant results, perhaps because a real effect in important subgroups was obscured.^{72,77} These experiences emphasize the role of biomarkers in new drug development.

SUMMARY

The use of biomarkers in respiratory medicine is in its infancy. Successive studies proclaim the relevance of yet another biomarker for the clinical evaluation of airways disease, but the pathway from identification to clinical application is long and complex. Yet there is an urgent need to develop biomarkers that enable clinicians to distinguish one clinical phenotype from another. Even more importantly, biomarkers should be aimed at identifying treatment responders. Targeted therapy is the way forward not only in new drug development but also in how we approach the use of conventional treatments. However much resistance there might be to change, attempts to distinguish asthma from COPD should be set aside in favor of identifying steroid responsiveness as a realistic and more judicious alternative to empiric treatment.

Greater efforts are required in the future to validate biomarkers in the clinical sphere. Journal editors need to spot those studies that adequately report the performance characteristics of a biomarker in relation to key clinical questions. In the meantime, the tests that we already have available should be selectively but more widely used. Above all, the practicing clinician needs to use biomarkers, such as D-dimer, pro-BNP, and exhaled NO, to answer the right questions with informed expectations. These should be based on performance characteristics obtained in study populations for whom pretest probabilities are known. All of this might seem like a tall order, and it is. However, the alternative is for skepticism about biomarkers to prevail and for respiratory medicine to remain in the dark ages characterized by clinical practices that have not changed substantially since the 1970s.

REFERENCES

1. Biomarkers Definitions Working Group. Biomarkers and surrogate endpoints: preferred definitions and conceptual framework. *Clin Pharmacol Ther* 2001;69:89-95.
2. Lesko LJ, Atkinson AJ Jr. Use of biomarkers and surrogate endpoints in drug development and regulatory decision making: criteria, validation, strategies. *Annu Rev Pharmacol Toxicol* 2001;41:347-66.
3. Luskin AT. What the asthma end points we know and love do and do not tell us. *J Allergy Clin Immunol* 2005;115(suppl):S539-45.
4. Rosi E, Ronchi MC, Grazzini M, Duranti R, Scano G. Sputum analysis, bronchial hyperresponsiveness, and airway function in asthma: results of a factor analysis. *J Allergy Clin Immunol* 1999;103:232-7.
5. Bradding P, Green RH. Subclinical phenotypes of asthma. *Curr Opin Allergy Clin Immunol* 2010;10:54-9.
6. Berry M, Morgan A, Shaw DE, Parker D, Green R, Brightling C, et al. Pathological features and inhaled corticosteroid response of eosinophilic and non-eosinophilic asthma. *Thorax* 2007;62:1043-9.
7. Diamant Z, Boot JD, Mantzouranis E, Flohr R, Sterk PJ, Gerth van Wijk R. Biomarkers in asthma and allergic rhinitis. *Pulm Pharmacol Ther* 2010;23:468-81.
8. Jackson R. Updated New Zealand cardiovascular disease risk-benefit prediction guide. *BMJ* 2000;320:709-10.
9. Taylor DR. Risk assessment in asthma and COPD: a potential role for biomarkers? *Thorax* 2009;64:261-4.
10. Dahl M, Tybjaerg-Hansen A, Vestbo J, Lange P, Nordestgaard BG. Elevated plasma fibrinogen associated with reduced pulmonary function and increased risk of chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2001;164:1008-11.
11. Dahl M, Vestbo J, Lange P, Bojesen SE, Tybjaerg-Hansen A, Nordestgaard BG. C-reactive protein as a predictor of prognosis in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2007;175:250-5.
12. Donaldson GC. C-reactive protein: does it predict mortality? *Am J Respir Crit Care Med* 2007;175:209-10.
13. Castro-Rodriguez JA, Holberg CJ, Wright AL, Martinez FD. A clinical index to define risk of asthma in young children with recurrent wheezing. *Am J Respir Crit Care Med* 2000;162:1403-6.
14. Caudri D, Wijga AH, Hoekstra MO, Kerkhof M, Koppelman GH, Brunekreef B, et al. Prediction of asthma in symptomatic preschool children using exhaled nitric oxide, Rint and specific IgE. *Thorax* 2010;65:801-7.
15. Debley JS, Stamey DC, Cochrane ES, Gama KL, Redding GJ. Exhaled nitric oxide, lung function, and exacerbations in wheezy infants and toddlers. *J Allergy Clin Immunol* 2010;125:1228-34.
16. Zhang Z, Chan DW. Cancer proteomics: in pursuit of "true" biomarker discovery. *Cancer Epidemiol Biomarkers Prev* 2005;14:2283-6.
17. Suartha E, Malo JL, Heederik D, Ghezzo H, L'Archeveque J, Gautrin D. Which tools best predict the incidence of work-related sensitisation and symptoms. *Occup Environ Med* 2009;66:111-7.
18. Gautrin D, Malo JL. Risk factors, predictors, and markers for work-related asthma and rhinitis. *Curr Allergy Asthma Rep* 2010;10:365-72.
19. Ferrazzoni S, Scarpa MC, Guarnieri G, Corradi M, Mutti A, Maestrelli P. Exhaled nitric oxide and breath condensate pH in asthmatic reactions induced by isocyanates. *Chest* 2009;136:155-62.
20. Tossa P, Paris C, Zmirou-Navier D, Demange V, Acouetey DS, Michaely JP, et al. Increase in exhaled nitric oxide is associated with bronchial hyperresponsiveness among apprentices. *Am J Respir Crit Care Med* 2010;182:738-44.
21. Singer AJ, Thode HC Jr, Green GB, Birkhahn R, Shapiro NI, Cairns C, et al. The incremental benefit of a shortness-of-breath biomarker panel in emergency department patients with dyspnea. *Acad Emerg Med* 2009;16:488-94.
22. Stein PD, Woodard PK, Weg JG, Wakefield TW, Tapson VF, Sostman HD, et al. Diagnostic pathways in acute pulmonary embolism: recommendations of the PLOPED II investigators. *Am J Med* 2006;119:1048-55.
23. Gibson NS, Sohne M, Gerdes VE, Nijkeuter M, Buller HR. The importance of clinical probability assessment in interpreting a normal d-dimer in patients with suspected pulmonary embolism. *Chest* 2008;134:789-93.
24. Dupont LJ, Demedts MG, Verleden GM. Prospective evaluation of the validity of exhaled nitric oxide for the diagnosis of asthma. *Chest* 2003;123:751-6.
25. Smith AD, Cowan JO, Filsell S, McLachlan C, Monti-Sheehan G, Jackson P, et al. Diagnosing asthma: comparisons between exhaled nitric oxide measurements and conventional tests. *Am J Respir Crit Care Med* 2004;169:473-8.
26. Pedrosa M, Cancelliere N, Barranco P, Lopez-Carrasco V, Quirce S. Usefulness of exhaled nitric oxide for diagnosing asthma. *J Asthma* 2010;47:817-21.
27. Smith AD, Cowan JO, Brassett KP, Filsell S, McLachlan C, Monti-Sheehan G, et al. Exhaled nitric oxide: a predictor of steroid response. *Am J Respir Crit Care Med* 2005;172:453-9.
28. Olin AC, Bake B, Toren K. Fraction of exhaled nitric oxide at 50 mL/s: reference values for adult lifelong never-smokers. *Chest* 2007;131:1852-6.
29. Olin AC, Rosengren A, Thelle DS, Lissner L, Bake B, Toren K. Height, age, and atopy are associated with fraction of exhaled nitric oxide in a large adult general population sample. *Chest* 2006;130:1319-25.
30. van Den Toorn LM, Prins JB, Overbeek SE, Hoogsteden HC, de Jongste JC. Adolescents in clinical remission of atopic asthma have elevated exhaled nitric oxide levels and bronchial hyperresponsiveness. *Am J Respir Crit Care Med* 2000;162:953-7.
31. Pijnenburg MW, Bakker EM, Lever S, Hop WC, De Jongste JC. High fractional concentration of nitric oxide in exhaled air despite steroid treatment in asthmatic children. *Clin Exp Allergy* 2005;35:920-5.
32. Smith AD, Cowan JO, Taylor DR. Exhaled nitric oxide levels in asthma: personal best versus reference values. *J Allergy Clin Immunol* 2009;124:714-8, e4.
33. de Jongste JC, Carraro S, Hop WC, Baraldi E. Daily telemonitoring of exhaled nitric oxide and symptoms in the treatment of childhood asthma. *Am J Respir Crit Care Med* 2009;179:93-7.
34. Fritsch M, Uxa S, Horak F Jr, Putschoegl B, Dehlink E, Szeplafusi Z, et al. Exhaled nitric oxide in the management of childhood asthma: a prospective 6-months study. *Pediatr Pulmonol* 2006;41:855-62.

35. Hewitt RS, Modrich CM, Cowan JO, Herbison GP, Taylor DR. Outcomes using exhaled nitric oxide measurements as an adjunct to primary care asthma management. *Prim Care Respir J* 2009;18:320-7.
36. Pijnenburg MW, Bakker EM, Hop WC, De Jongste JC. Titrating steroids on exhaled nitric oxide in children with asthma: a randomized controlled trial. *Am J Respir Crit Care Med* 2005;172:831-6.
37. Shaw DE, Berry MA, Thomas M, Green RH, Brightling CE, Wardlaw AJ, et al. The use of exhaled nitric oxide to guide asthma management: a randomized controlled trial. *Am J Respir Crit Care Med* 2007;176:231-7.
38. 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* 2005;352:2163-73.
39. Szeffler SJ, Mitchell H, Sorkness CA, Gergen PJ, O'Connor GT, Morgan WJ, et al. Management of asthma based on exhaled nitric oxide in addition to guideline-based treatment for inner-city adolescents and young adults: a randomised controlled trial. *Lancet* 2008;372:1065-72.
40. Reddel HK, Taylor DR, Bateman ED, Boulet LP, Boushey HA, Busse WW, et al. An official American Thoracic Society/European Respiratory Society statement: asthma control and exacerbations: standardizing endpoints for clinical asthma trials and clinical practice. *Am J Respir Crit Care Med* 2009;180:59-99.
41. Green RH, Brightling CE, McKenna S, Hargadon B, Parker D, Bradding P, et al. Asthma exacerbations and sputum eosinophil counts: a randomised controlled trial. *Lancet* 2002;360:1715-21.
42. Jayaram L, Pizzichini MM, Cook RJ, Boulet LP, Lemiere C, Pizzichini E, et al. Determining asthma treatment by monitoring sputum cell counts: effect on exacerbations. *Eur Respir J* 2006;27:483-94.
43. Gibson PG. Using fractional exhaled nitric oxide to guide asthma therapy: design and methodological issues for ASThma Treatment ALgorithm studies. *Clin Exp Allergy* 2009;39:478-90.
44. Ekroos H, Karjalainen J, Sarna S, Laitinen LA, Sovijarvi AR. Short-term variability of exhaled nitric oxide in young male patients with mild asthma and in healthy subjects. *Respir Med* 2002;96:895-900.
45. Kharitonov SA, Gonio F, Kelly C, Meah S, Barnes PJ. Reproducibility of exhaled nitric oxide measurements in healthy and asthmatic adults and children. *Eur Respir J* 2003;21:433-8.
46. Pijnenburg MW, Floor SE, Hop WC, De Jongste JC. Daily ambulatory exhaled nitric oxide measurements in asthma. *Pediatr Allergy Immunol* 2006;17:189-93.
47. Michils A, Baldassarre S, Van Muylen A. Exhaled nitric oxide and asthma control: a longitudinal study in unselected patients. *Eur Respir J* 2008;31:539-46.
48. Expert Panel Report 3 (EPR-3). guidelines for the diagnosis and management of asthma—summary report 2007. *J Allergy Clin Immunol* 2007;120(suppl):S94-138.
49. Taylor DR, Pavord ID. Biomarkers in the assessment and management of airways diseases. *Postgrad Med J* 2008;84:628-34.
50. Jones SL, Kittelson J, Cowan JO, Flannery EM, Hancox RJ, McLachlan CR, et al. The predictive value of exhaled nitric oxide measurements in assessing changes in asthma control. *Am J Respir Crit Care Med* 2001;164:738-43.
51. Gelb AF, Flynn Taylor C, Shinar CM, Gutierrez C, Zamel N. Role of spirometry and exhaled nitric oxide to predict exacerbations in treated asthmatics. *Chest* 2006;129:1492-9.
52. Gina. At a glance asthma management reference. Available at: www.ginasthma.org. Accessed 2010.
53. Szeffler SJ, Martin RJ, King TS, Boushey HA, Cherniack RM, Chinchilli VM, et al. Significant variability in response to inhaled corticosteroids for persistent asthma. *J Allergy Clin Immunol* 2002;109:410-8.
54. Bossley CJ, Saglani S, Kavanagh C, Payne DN, Wilson N, Tsartsali L, et al. Corticosteroid responsiveness and clinical characteristics in childhood difficult asthma. *Eur Respir J* 2009;34:1052-9.
55. Bateman ED, Boushey HA, Bousquet J, Busse WW, Clark TJ, Pauwels RA, et al. Can guideline-defined asthma control be achieved? The Gaining Optimal Asthma Control study. *Am J Respir Crit Care Med* 2004;170:836-44.
56. Maestrelli P. Same role and same treatment for airway eosinophilia in asthma and COPD? *Eur Respir J* 2007;29:831-3.
57. Pavord ID, Shaw DE, Gibson PG, Taylor DR. Inflammometry to assess airway diseases. *Lancet* 2008;372:1017-9.
58. Zeiger RS, Szeffler SJ, Phillips BR, Schatz M, Martinez FD, Chinchilli VM, et al. Response profiles to fluticasone and montelukast in mild-to-moderate persistent childhood asthma. *J Allergy Clin Immunol* 2006;117:45-52.
59. Knuffman JE, Sorkness CA, Lemanske RF Jr, Mauger DT, Boehmer SJ, Martinez FD, et al. Phenotypic predictors of long-term response to inhaled corticosteroid and leukotriene modifier therapies in pediatric asthma. *J Allergy Clin Immunol* 2009;123:411-6.
60. Dummer JF, Epton MJ, Cowan JO, Cook JM, Condliffe R, Landhuis CE, et al. Predicting corticosteroid response in chronic obstructive pulmonary disease using exhaled nitric oxide. *Am J Respir Crit Care Med* 2009;180:846-52.
61. Cowan DC, Cowan JO, Palmay R, Williamson A, Taylor DR. Effects of steroid therapy on inflammatory cell subtypes in asthma. *Thorax* 2010;65:384-90.
62. Zacharasiewicz A, Wilson N, Lex C, Erin EM, Li AM, Hansel T, et al. Clinical use of noninvasive measurements of airway inflammation in steroid reduction in children. *Am J Respir Crit Care Med* 2005;171:1077-82.
63. Pijnenburg MW, Hofhuis W, Hop WC, De Jongste JC. Exhaled nitric oxide predicts asthma relapse in children with clinical asthma remission. *Thorax* 2005;60:215-8.
64. Petsky HL, Cates CJ, Lasserson TJ, Li AM, Turner C, Kynaston JA, et al. A systematic review and meta-analysis: tailoring asthma treatment on eosinophilic markers (exhaled nitric oxide or sputum eosinophils). *Thorax* 2010 [Epub ahead of print].
65. Stick S, Franklin P. NO more dogma. Nitric oxide marker in asthma. *Am J Respir Crit Care Med* 2009;179:87-8.
66. Franklin PJ, Stick SM. The value of FeNO measurement in asthma management: the motion against FeNO to help manage childhood asthma—reality bites. *Paediatr Respir Rev* 2008;9:122-6.
67. Perez-de-Llano LA, Carballada F, Castro Anon O, Pizarro M, Golpe R, Baloiira A, et al. Exhaled nitric oxide predicts control in patients with difficult-to-treat asthma. *Eur Respir J* 2010;35:1221-7.
68. The ENFUMOSA cross-sectional European multicentre study of the clinical phenotype of chronic severe asthma. European Network for Understanding Mechanisms of Severe Asthma. *Eur Respir J* 2003;22:470-7.
69. Wenzel SE. Asthma: defining of the persistent adult phenotypes. *Lancet* 2006;368:804-13.
70. Busse WW, Ring J, Huss-Marp J, Kahn JE. A review of treatment with mepolizumab, an anti-IL-5 mAb, in hypereosinophilic syndromes and asthma. *J Allergy Clin Immunol* 2010;125:803-13.
71. Leckie MJ, ten Brinke A, Khan J, Diamant Z, O'Connor BJ, Walls CM, et al. Effects of an interleukin-5 blocking monoclonal antibody on eosinophils, airway hyper-responsiveness, and the late asthmatic response. *Lancet* 2000;356:2144-8.
72. Flood-Page P, Swenson C, Faiferman I, Matthews J, Williams M, Brannick L, et al. A study to evaluate safety and efficacy of mepolizumab in patients with moderate persistent asthma. *Am J Respir Crit Care Med* 2007;176:1062-71.
73. O'Byrne PM. The demise of anti IL-5 for asthma, or not. *Am J Respir Crit Care Med* 2007;176:1059-60.
74. Haldar P, Brightling CE, Hargadon B, Gupta S, Monteiro W, Sousa A, et al. Mepolizumab and exacerbations of refractory eosinophilic asthma. *N Engl J Med* 2009;360:973-84.
75. Berry MA, Hargadon B, Shelley M, Parker D, Shaw DE, Green RH, et al. Evidence of a role of tumor necrosis factor alpha in refractory asthma. *N Engl J Med* 2006;354:697-708.
76. Brightling C, Berry M, Amrani Y. Targeting TNF-alpha: a novel therapeutic approach for asthma. *J Allergy Clin Immunol* 2008;121:5-12.
77. Wenzel SE, Barnes PJ, Bleecker ER, Bousquet J, Busse W, Dahlen SE, et al. A randomized, double-blind, placebo-controlled study of tumor necrosis factor-alpha blockade in severe persistent asthma. *Am J Respir Crit Care Med* 2009;179:549-58.