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Asthma outcomes: Biomarkers

Stanley J. Szefler, MD[a] **[coprimary author]**, **Sally Wenzel, MD**[b] **[coprimary author]**, **Robert Brown, MD, MPH**[c], **Serpil C. Erzurum, MD**[d], **John V. Fahy, MD, MSc**[e], **Robert G. Hamilton, PhD**[c], **John F. Hunt, MD**[f], **Hirohito Kita, MD**[g], **Andrew H. Liu, MD**[a], **Reynold A. Panettieri Jr, MD**[h], **Robert P. Schleimer, PhD**[i], and **Michael Minnicozzi, PhD**[j]

[a]National Jewish Health, Denver

[b]University of Pittsburgh

[c]Johns Hopkins University, Baltimore

[d]Cleveland Clinic Foundation

[e]University of California, San Francisco

[f]University of Virginia, Charlottesville

[g]Mayo Clinic Rochester

[h]University of Pennsylvania, Philadelphia

[i]Northwestern University, Chicago

[j]National Institute of Allergy and Infectious Diseases, Bethesda

Abstract

Background—Measurement of biomarkers has been incorporated within clinical research studies of asthma to characterize the population and associate the disease with environmental and therapeutic effects.

Objective—National Institutes of Health institutes and federal agencies convened an expert group to propose which biomarkers should be assessed as standardized asthma outcomes in future clinical research studies.

Methods—We conducted a comprehensive search of the literature to identify studies that developed and/or tested asthma biomarkers. We identified biomarkers relevant to the underlying disease process progression and response to treatment. We classified the biomarkers as either core (required in future studies), supplemental (used according to study aims and standardized), or emerging (requiring validation and standardization). This work was discussed at an National

Corresponding author: Michael Minnicozzi, PhD, Division of Allergy, Immunology, and Transplantation (DAIT), National Institute of Allergy and Infectious Diseases (NIAID), National Institutes of Health (NIH), 6610 Rockledge Drive, Bethesda, MD 20892. minnicozzim@niaid.nih.gov.

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Institutes of Health–organized workshop convened in March 2010 and finalized in September 2011.

Results—Ten measures were identified; only 1, multiallergen screening to define atopy, is recommended as a core asthma outcome. Complete blood counts to measure total eosinophils, fractional exhaled nitric oxide (Feno), sputum eosinophils, urinary leukotrienes, and total and allergen-specific IgE are recommended as supplemental measures. Measurement of sputum polymorphonuclear leukocytes and other analytes, cortisol measures, airway imaging, breath markers, and system-wide studies (eg, genomics, proteomics) are considered as emerging outcome measures.

Conclusion—The working group participants propose the use of multiallergen screening in all asthma clinical trials to characterize study populations with respect to atopic status. Blood, sputum, and urine specimens should be stored in biobanks, and standard procedures should be developed to harmonize sample collection for clinical trial biorepositories.

Keywords

Multiallergen screen; fractional exhaled nitric oxide; sputum eosinophils; total eosinophils; IgE; urinary leukotriene E_4

Asthma clinical research lacks adequate outcomes standardization. As a result, our ability to examine and compare outcomes across clinical trials and clinical studies, interpret evaluations of new and available therapeutic modalities for this disease at a scale larger than a single trial, and pool data for observational studies (eg, genetics, genomics, pharmacoeconomics) is impaired.[4] Several National Institutes of Health (NIH) institutes that support asthma research (the National Heart, Lung, and Blood Institute; the National Institute of Allergy and Infectious Diseases; the National Institute of Environmental Health Sciences; and the Eunice Kennedy Shriver National Institute of Child Health and Human Development), as well as the Agency for Healthcare Research and Quality, have agreed to an effort for outcomes standardization. This effort aims at (1) establishing standard definitions and data collection methodologies for validated outcome measures in asthma clinical research with the goal of enabling comparisons across asthma research studies and clinical trials and (2) identifying promising outcome measures for asthma clinical research that require further development. In the context of this effort, 7 expert subcommittees were established to propose and define outcomes under 3 categories—core, supplemental, and emerging:

- *Core outcomes* are identified as a selective set of asthma outcomes to be considered by participating NIH institutes and other federal agencies as requirements for institute/agency-initiated funding of clinical trials and large observational studies in asthma.
- *Supplemental outcomes* are asthma outcomes for which standard definitions can or have been developed, methods for measurement can be specified, and validity has been proved but whose inclusion in funded clinical asthma research will be optional.
- *Emerging outcomes* are asthma outcomes that have the potential to (1) expand and/or improve current aspects of disease monitoring and (2) improve translation of basic and animal model–based asthma research into clinical research. Emerging outcomes may be new or may have been previously used in asthma clinical research, but they are not yet standardized and require further development and validation.

Each subcommittee used the recently published *American Thoracic Society (ATS)/European Respiratory Society (ERS) Statement: Asthma Control and Exacerbations—Standardizing Endpoints for Clinical Asthma Trials and Clinical Practice*[5] (hereafter referred to as the ATS/ERS Statement) as a starting point and updated, expanded, or modified its recommendations as the subcommittee deemed appropriate. Each subcommittee produced a report that was discussed, modified, and adopted by the Asthma Outcomes Workshop that took place in Bethesda, Md, on March 15 and 16, 2010. The reports were revised accordingly and finalized in September 2011. The recommendations of the workshop participants in regard to asthma biomarkers are presented in this article and summarized in Tables I, II[1–3], and III.

The measurement of biomarkers has been incorporated within clinical studies of asthma to characterize the participant population and to try to associate the disease process with environmental effects and therapeutic interventions.

This summary highlights the current knowledge regarding biomarkers deemed applicable as core measures, specifically the multiallergen screen to define atopy, and supplemental biomarkers, which include measurements of sputum eosinophils, IgE, complete blood count (CBC), fractional exhaled nitric oxide (Feno), and urinary leukotrienes. In addition, several biomarkers are considered emerging measures, such as imaging and cortisol. Future studies will be required to further validate and characterize their roles as outcome measures. We also discuss the issue of proper storage of biologic samples to allow future analyses.

The conclusions of the Asthma Biomarkers Subcommittee extend those included in the ATS/ERS Statement by considering additional publications available since that report and by focusing our comments on the role of biomarkers in clinical research.[5] There are no essential disagreements with the ATS/ERS Statement.

REVIEW OF SPECIFIC CORE AND SUPPLEMENTAL BIOMARKER OUTCOMES

Total and allergen-specific IgE

Summary

- Atopic status is an important phenotype and should be documented in clinical research studies to permit adequate interpretation of study findings. The presence of allergen-specific IgE is a biomarker for atopic asthma.
- The *multiallergen screen* is a single semiquantitative serologic measure of IgE against major allergens. It is considered a core biomarker that permits characterization of the atopic status of a study population in prospective clinical trials and observational studies. It characterizes an individual as atopic but does not specify as to which allergen(s) a person is sensitized.
- Quantitative serologic measures of *individual allergen-specific IgE antibodies* offer information on specific allergen sensitivities and are considered supplemental biomarkers for study population characterization, assessment of efficacy and effectiveness outcomes in intervention studies, and in observational studies, as deemed appropriate by study design.
- Measurement of *allergen-specific IgE by skin prick test*, although widely used in the clinical setting, is considered an emerging biomarker for research because of the variability of the test's performance.

- *Total serum IgE* has been associated with asthma and is considered a supplemental measure for study population characterization, as well as an outcome for intervention and observational studies, as deemed appropriate by study design.

Medical and scientific value—The quantity of total IgE and presence of allergen-specific IgE antibody in serum are both important biomarkers for defining the phenotype of a patient who presents with asthma symptoms.[6] The titers of allergen-specific IgE in serum also may be useful in predicting persistent wheeze and in targeting allergen specificities for allergen avoidance management. Detection of local IgE antibody in the skin and extracts of tissue may aid in adjudicating negative *in vivo* and serologic measures of IgE antibody despite clinical evidence of atopic asthma.[6–11]

Total serum IgE has been associated with asthma.[12–14] Serum IgE levels are highly age-dependent: Atopic infants have an earlier and steeper rise in serum IgE levels than age-matched nonatopic controls.[15,16] Total serum IgE reaches adult levels by age 10 to 15 years and gradually declines from the second decade of life. However, a bigger problem is the considerable overlap in IgE levels between atopic and nonatopic populations, which reduces its utility in identifying atopy.

Allergen-specific IgE defines an individual as having atopic asthma.[6,17,18] It confirms sensitization in support of a clinical history-based diagnosis and aids in identifying allergen triggers. The probability of wheeze and reduced lung function increases with increasing specific IgE levels in serum.[18] Summed levels of mite-, cat-, and dog-specific IgE in 3-year-old children were associated with a 1.33-fold increase in the probability of wheeze by age 5 (95% CI, 1.21–1.47; $P < .0001$) per logarithmic unit increase in IgE antibody, corresponding to an odds ratio of 3.1 at 10 and 4.25 at 30 kUa/L (kilounits of antibody per liter of serum). In contrast, current wheeze was not associated with the size of skin test wheal.[18]

The use of the multiallergen screen for aeroallergens (Phadiatop) in combination with the food allergen mix (fx5) has been more effective than individual allergen-specific IgE measurements in characterizing the atopic status of children.[19,20] When used together to evaluate 4-year-old children, these 2 screening tests exhibited a 97.4% positive predictive value for any suspected allergic disease (asthma, rhinitis, atopic eczema/dermatitis syndrome, and food allergy).[19]

Definition and methodology for measurement—Quantitative measures of *total serum IgE* can be equivalently obtained from any of the US Food and Drug Administration (FDA)–cleared immunoassays from HYCOR (EIA), Phadia (now Thermo Fisher Scientific) (ImmunoCAP), or Siemens (IMMULITE, ADVIA Centaur, and Nephelometer) that are used in clinical laboratories throughout North America.[21] Total serum IgE data from the College of American Pathologists' external proficiency studies display excellent intermethod agreement (coefficients of variation less than 15%) and comparable performance (precision, linearity, analytic sensitivity to 2 IU/mL) for all 5 assays.[22] Thus any of the above total serum IgE assay methods can be used without concerns for comparability. In term of units, 1 IU is equivalent to 2.4 ng of IgE.

Allergen-specific IgE can be measured in serum by 1 of 3 immunoassays or in the skin by using any 1 of the many technical variants of prick and intradermal skin test methods. The skin prick test method has greater diagnostic value, but the lack of full standardization reduces its value in clinical research, especially when cross-study comparisons are considered.[23] Serologic measurements of allergen-specific IgE can be performed using the HY-COR (HYTEC), Phadia (ImmunoCAP), and Siemens (IMMULITE) autoanalyzers. All 3 assays display excellent precision, reproducibility, linearity, and equivalent analytic

sensitivity (0.1 kUa/L).[22] However, the 3 assays measure different levels of IgE antibody to any given allergen specificity. Because the results generated with the 3 assay methods are qualitatively equivalent but not quantitatively identical, the subcommittee recommends the Phadiatop and fx5 (ImmunoCAP) as the IgE antibody analyses for asthma clinical trials. This is because the performance of the Phadiatop multiallergen screen has been the most well documented of the 3 available multiallergen screens.

- One version of the IgE antibody assay is the *multiallergen screen.*[19,20,24,25] It is a single analytic measurement, the *adult* (≥15 years old) version of which simultaneously detects specific IgE antibody to any of 10 aeroallergens that cross allergen groups and includes aeroallergens in the dust mite, pet epidermal, grass, tree and weed pollen, and mold families. Phadia's version of the multiallergen screen, the Phadiatop, generates both a dichotomous positive/negative value and a semiquantitative (kilounits of allergen per liter) estimate of relative positivity. Although the presence of IgE antibody indicates a state of atopy, the precise IgE antibody specificity of a positive result is not defined.[19,20,24,25] The test is proposed as a core biomarker for characterization of the atopic status of study populations for prospective clinical trials and observation studies. The assay's analytic sensitivity was reduced from 0.35 to 0.1 kUa/L in March 2008; however, performance studies to date have continued to use a 0.35 kUa/L positive cut point criterion to define the presence of atopy. The clinical relevance of results in the 0.1 to 0.35 kUa/L range is currently undetermined.
- In *children with asthma* (<15 years of age), the fx5 food allergen mix should be added to the adult Phadiatop because food allergy is more common in this age group and needs to be included when assessing for atopy. The fx5 is a single test that simultaneously detects IgE to any of 6 foods (chicken egg, cow's milk, peanut, soybean, codfish, and wheat) that are the principal sensitizing food allergens for children. This dual test strategy has been selected over the Phadiatop Infant,[19,20,26,27] which also includes food allergens because the paired adult Phadiatop-fx5 combination is a more comprehensive assessment of atopy; performance of the Phadiatop Infant has not been studied in a US population; and unlike the Phadiatop Infant, the adult Phadiatop and fx5 are both FDA cleared.
- Clinics can order specific IgE antibody tests to more than 200 individual allergen specificities, each denoted with a letter-number code (eg, D1 corresponds to *Dermatophagoides pteronyssinus* [dust mite]).[16] Individually performed specific IgE tests have been classified as supplemental biomarkers because the participant's clinical history is needed to identify the target allergens for testing, and more than 1 specific IgE antibody test is generally needed to characterize a particular participant's sensitivities.

In vivo measurements of allergen-specific IgE in the skin are an alternative to serologic assays. Because we could not identify a single generally accepted technique, we have classified skin testing as an emerging biomarker in this report. The following issues need resolution before skin testing can be reclassified as a supplemental biomarker[28]: (1) allergen extract potency, stability, concentration, levels of irritant, and other contaminants need to be more uniform; (2) the technique must be standardized with respect to use of skin prick versus intradermal methods, choice among various skin test devices, number of skin tests performed, reporting scale, use of wheal or erythema as outcome, comparison with saline or histamine controls, skin test spacing, and extent of needle penetration; (3) grading scheme and interpretation strategy need to be clearly defined; and (4) patient variables, such as dermographism and interfering premedications, need to be considered. Finally, the analytic sensitivity of skin testing is unknown.

Repeatability—The results obtained with total and allergen-specific IgE autoanalyzers are highly reproducible, with intra-method interlaboratory coefficients of variation less than 15% based on multilaboratory proficiency survey data.[22] However, a number of nonanalytic factors can alter total and allergen-specific IgE levels over time, including age, seasonal allergen exposure, and parasitic infections. Although originally a concern, pretreatment with anti-human IgE (eg, omalizumab) does not significantly alter total or specific IgE measurements in the ImmunoCAP system.[29]

Skin testing results, in contrast, show inherent variations that are influenced by extract quality, technique, device, experience, and methods of interpretation.[28]

Responsiveness—Total and allergen-specific IgE levels might be affected by pharmacotherapy using standard asthma medications. Corticosteroid administration has been reported to induce IgE synthesis but also to decrease serum IgE levels, especially in allergic bronchopulmonary aspergillosis (ABPA).[30,31] Response to treatment with omalizumab, which increases total IgE but reduces free IgE levels, can be accurately monitored by Immuno-CAP when omalizumab is present in serum.[29] Certain medications can interfere with skin test performance.

Validity—Total serum IgE level is an insensitive indicator of asthma outcome. Allergen-specific IgE antibody levels better reflect the extent of sensitization (atopic state) and might be useful in predicting a predisposition for prolonged wheeze.[17,18] However, as mentioned above, allergen-specific IgE, as measured by serology or skin tests, is an insensitive indicator for assessing clinical asthma outcomes or for predicting future manifestations of asthma control.

Associations—An association between total serum IgE levels and asthma has been reported.[12–14,32] However, asthma heritability is only partly related to the familial aggregation of total serum IgE. In the absence of a parent with asthma, asthma prevalence was significantly higher in children when both parents had total serum IgE levels in the highest tertile.[12] Children with asthma also have higher total IgE levels than predicted by parental IgE levels alone. It has been speculated that total IgE changes in patients with asthma are indirect measures of airway inflammation. However, in most studies this association is weak.

Ina 2001 study involving an American population of children and adolescents (aged 6–18 years) with a history of rhinitis and an asthma prevalence of 59%, the Phadiatop displayed a diagnostic sensitivity of 98% against skin prick test reference and 83.2% against a clinical history reference using a 0.35 kUa/L positive cutpoint.[24] In a 2009 epidemiology study of European children (median age, 2.7 years), in which 122 children were classified as atopic (70%) or nonatopic (30%) by a combined clinical evaluation, skin prick test, and itemized serum IgE antibody analysis, the Phadiatop Infant (which includes principal food allergens) displayed positive and negative predictive values of 95% (95% CI, 89% to 99%) and 94% (95% CI, 89% to 99%), respectively, using a 0.35 kUa/L positive cutpoint.[27] The combined use of the Phadiatop and fx5 has been shown to effectively predict the atopic state (>0.35 kUa/L = IgE antibody positivity) by age 4 years. The combined degree of positivity correlated with the severity of recurrent wheeze and limited peak flows in a pediatric asthmatic population.[19,20]

Increased probability of wheeze and reduced lung function are associated with increasing specific IgE antibody levels as measured in serum but not by skin tests.[18] Increasing summed quantities of mite-, cat-, and dog-specific IgE at age 3 years significantly increased the risk of persistent wheeze by age 5 years.[18]

Practicality and risk—Total and allergen-specific IgE assays are standardized and performed in federally licensed clinical immunology laboratories. Their practicality is enhanced by the fact that serum can be retrieved from long-term storage repositories. Skin testing may be practical in the clinical setting (results immediately available) but less so in clinical research, given that it requires a significant time commitment from both study participants and research staff. Although very small, the risk of adverse reactions is higher with skin testing than with IgE antibody serology. In comparative studies involving aeroallergens, intradermal skin test results add little to the diagnostic evaluation obtained with skin prick test results.[23]

Demographic considerations—Age, gender, and race may affect the levels of total and allergen-specific IgE antibody levels as measured in serum or by skin test.[12–15,32,33]

Priority for NIH-initiated clinical research—The atopic status of patients with asthma can be a determinant of management choices, as well as of the response to therapy and prognosis. Therefore, it is important that the atopic status of a study population is reported. The results of the *multiallergen screen* (Phadiatop), which is a single semiquantitative serologic measure of allergen-specific IgE across major aeroallergens, and in children younger than 15 years, the addition of the fx5 (covering food allergens) are considered core biomarkers that permit characterization of the atopic status of a study population in prospective clinical trial and observational studies. Quantitative serologic measures of *total and individual allergen-specificities of IgE antibody* are considered supplemental biomarker outcomes for study population characterization, for assessment of efficacy and effectiveness in intervention studies, and for use in observational studies. Measurement of allergen-specific IgE by skin test is considered an emerging biomarker because of lack of standardization and the many factors that affect the test's performance and interpretation of results.

Exhaled nitric oxide

Summary

- Feno measured at a constant flow rate is a simple, safe, and reproducible biomarker for use in asthma clinical trials.
- Although Feno values overlap among healthy, atopic, and asthmatic cohorts, changes in Feno values over time in individuals who have asthma are relevant to clinical research studies that seek to measure effects of interventions on airway inflammation, in particular the effects of anti-inflammatory (eg, corticosteroid) therapies.
- Feno levels of less than 25 ppb generally indicate lower likelihood for eosinophilic inflammation and responsiveness to corticosteroids. However, Feno cannot be used interchangeably with sputum eosinophilia as an outcome measure, given that eosinophilic inflammation and Feno levels do not always respond identically to treatment.
- Feno is recommended as a supplemental outcome in clinical trials that seek to evaluate effects of interventions on airway disease and/or to characterize corticosteroid-responsive phenotypes of asthma.

Definition and methodology for measurement

Definition: Measurement of Feno is a quantitative measure of airway nitric oxide (NO), a gaseous mediator produced endogenously in cells by NO synthases. Exhaled NO is

commonly regarded as an indirect marker for airway inflammation. The joint ATS/ERS guidelines for the measurement of Feno are the current standard.[1–3]

Methodology: Exhaled NO is reported in parts per billion in exhaled breath. Measurement methods are well described in the ATS/ERS guidelines.[1–3] Online measures refers to the study participant exhaling directly into the instrument. Offline measures refers to the study participant exhaling from total lung capacity into a nonpermeable gas collection bag that is subsequently sampled for measurement of NO. Either technique is valid and accurate for comparison of NO among populations, but offline measures are not interchangeable with those measured online due to differences in the methodology mostly related to flow dependence. Flow rate of exhalation affects the level of Feno, with faster flow rates resulting in lower Feno levels and slower flow rates resulting in higher Feno levels. An exhalation flow rate of 0.35 L/sec is recommended for offline measures, whereas a flow rate of 0.05 L/sec is recommended for online measures, and the exhalation should be sufficient to obtain an NO plateau for at least 3 seconds. Online Feno measurement, as opposed to offline, is suggested for most clinical trials of asthma as it is more suited to standardization at multiple sites because FDA-approved equipment for online measurement is readily available in most pulmonary function laboratories. However, offline measures of NO may be useful in field studies with remote sample collection or when specific experimental study design calls for investigational assessment of total lung volume NO.

Medical and scientific value—NO is generally accepted as a marker of airway inflammation. Individuals who have asthma have been shown to exhale high levels of NO, which decreased in response to corticosteroids. The testing is noninvasive, easy to perform in children and patients with severe airflow obstruction, and has no risk to patients.

Range of values

Normal ranges: Several publications have reported reference values for Feno in adults[34–38] and children.[39–44] In general, the upper value of normal for online measure of Feno is 25 ppb.

Distribution of NO in individuals with and without asthma: Mean Feno levels in populations without asthma and populations with asthma overlap significantly, but the distribution of Feno in populations with asthma is generally higher than that in a population without asthma. In patients with stable well-controlled asthma, Feno values have been reported to range from 22 to 44 ppb.[45]

Numeric transformation: Feno values are provided directly from NO analyzers in parts per billion.

Repeatability—The coefficient of variation of Feno of healthy individuals is approximately 10% (about 4 ppb),[46–48] whereas the variation in individuals with asthma ranges from 20% to 40%.[46,49]

Variation in results across sites: Using the ATS/ERS standard methods for online measure of exhaled NO will minimize variation across sites.[1,2]

Responsiveness—Many studies describe the correlation of Feno and eosinophilic airway inflammation. Feno is related to eosinophil numbers in bronchoalveolar lavage fluid,[50] bronchial biopsies,[51] and induced sputum.[52–55] In general, however, a low Feno is more likely to exclude airway eosinophilia than a high Feno is likely to predict it. Specifically, in symptomatic adults who have asthma with Feno less than 25 ppb, eosinophilic airway

inflammation is unlikely. Feno greater than 47 ppb suggests eosinophilic inflammation and corticosteroid-responsive asthma, but persistently high Feno in a patient with ongoing asthma symptoms may occur despite adequate anti-inflammatory treatment.[56] A clinically important decrease of Feno is defined as a change of 20% for values over 50 ppb (or a change of 10 ppb for values lower than 50 ppb) that occurs 2 to 6 weeks after initiation of corticosteroid therapy.[3,40,57,58]

Validity—Feno values are generally higher in individuals with asthma than in healthy controls and reflect lower airway inflammation. However, elevated levels may commonly be seen in atopic individuals without asthma.

Associations

Strength and direction of associations: While studies identify the association of Feno and eosinophilic airway inflammation,[52–54,59] the sensitivity and specificity of Feno for sputum eosinophila are only approximately 70%, and the relationship between Feno and eosinophilia may occur independently of asthma control.[60] The relationship between Feno and eosinophilia is not exact, in part because sputum eosinophilia is never found in healthy airways, but NO is present in health and the distribution of values is skewed rightward in the normal range.

Additional information provided by this variable: Feno does not duplicate other outcome measures of inflammation, particularly sputum eosinophilia. For example, corticosteroid therapy reduces Feno values, but anti–IL-5 and anti-IgE therapy for asthma reduce sputum eosinophilia without affecting Feno.[61] Feno is also related to atopy as measured by skin prick test positivity,[62,63] such that well-controlled individuals with asthma and positive allergen skin prick tests have increased Feno levels.[64,65]

Practicality and risk—The test is easy to perform, quick, simple, and well tolerated by participants. Little to no variation in outcomes has been observed across sites using FDA-approved devices under ATS/ERS guidelines. The cost of purchasing and maintaining equipment may be prohibitive for some studies. As less expensive and portable handheld devices are developed, this may be less of a limitation; however, these devices need further evaluation as clinical research tools. No safety issues have been identified.

Demographic considerations—Feno levels are generally lower in children than in adults, whether they have asthma or not[39,40,42–44]; for example, mean Feno levels in children aged 12 years or less are approximately 5 ppb less than those of adults. Men's Feno values are approximately 25% higher than those of women.[37,66,67] Body mass index is inversely associated with Feno in people with asthma, and weight loss may affect levels.[68]

Priority for NIH-initiated clinical research—Feno is recommended as a supplemental outcome for the characterization of study populations, for prospective clinical trials, and for observational studies. Measurements are simple to make, with no risk to participants, and are useful in the assessment of airway inflammation. Feno and sputum eosinophilia are not duplicative outcome measures (ie, reduction in the levels of these biomarkers does not occur in parallel), even though low sputum eosinophilia and low Feno are strongly linked. Feno values overlap between healthy, atopic, and asthmatic individuals and do not generally reflect severity of disease. Additionally, the equipment is expensive, and further information on its biologic relevance is needed. At this point, within-individual changes in Feno values over time may be most relevant to clinical research studies that seek to measure effects of interventions on airway inflammation, in particular effects of corticosteroid-like therapies. Thus although not recommended as a core measure at this time, as data continue to

accumulate regarding the relevance of Feno to clinical outcomes, it is possible that this may become a core biomarker in the future.

Future directions or research questions—The inflammatory factor(s) that promote increased Feno remain unclear, as does the reason for the disconnect between the presence of eosinophilic inflammation and Feno. To optimize the use of this biomarker, future studies that determine the relationship of Feno to asthma control, asthma phenotypes, and airway remodeling in relation to corticosteroid use are needed.

Sputum eosinophils

Summary—Analysis of eosinophil counts in induced sputum identifies patients who have eosinophilic and noneosinophilic phenotypes of asthma. These inflammatory phenotypes can predict response to treatment.

Definition and methodology for measurement—Total and differential counts in induced sputum samples will yield both the eosinophil percentage and the total eosinophil number per milliliter of sputum, which can both be reported. However, the eosinophil percentage outcome is preferred because the percentage transformation controls for the effects of saliva in the sample, which can dilute the concentration of eosinophils.

Two main methods are in use. In the United States, most NIH-funded studies and networks use the whole expectorate method,[69,70] whereas Canadian and European investigators tend to use the sputum plug method.[71] The main elements of these 2 methods are described below:

- The *whole expectorate method* entails the following steps: pretreatment with 4 puffs of albuterol; inhalation of 3% saline for 12 minutes (the duration of sputum induction with this method is standardized at 12 minutes, based on data showing this time length to be optimal for the collection of airway secretions[70]); at a minimum of 2-minute intervals, the study participant spits saliva into one cup before coughing sputum into another; peak flow or FEV_1 is monitored at 2-minute intervals. The whole expectorate is mixed with an equal volume of 10% dithiothreitol and the sample homogenized in a shaking water bath for 15 minutes with intermittent aspiration of the samples by transfer pipette. An aliquot of this sputum is cytocentrifuged to generate cytology slides that can be stained to allow identification of leukocytes. The homogenized sputum also can be centrifuged to yield aliquots of sputum supernatant for measures of inflammatory proteins in the fluid phase.[69]
- The *sputum plug method* consists of the following steps: pretreatment with albuterol; inhalation of 0.9%, 3%, 5%, and 7% saline at 5- to 10-minute intervals. Sputum is expectorated into a container from which the mucus plugs are selected using a wooden spatula.[71] The mucus plug material is processed in dithiothreitol using methods similar to the whole expectorate method, described above.

Few studies have compared data generated by these 2 methods, but available data suggest that the 2 methods yield similar data for sputum eosinophil percentage. Until further comparative studies are available, we recommend that the whole expectorate method be used for NIH-funded studies because that is the current practice.

Medical and scientific value—Airway eosinophilia is a well-defined inflammatory characteristic of a phenotype of asthma likely orchestrated by T_H2 cytokines and known to be responsive to corticosteroid treatment. Eosinophil percentage in induced sputum is a useful marker of airway eosinophilia. Inclusion of sputum cytology as a biomarker of airway

eosinophilia in clinical asthma research will facilitate research on treatment responses and on mechanisms of disease, including mechanisms of disease in noneosinophilic asthma.

Analysis of the cell differential of induced sputum is a useful noninvasive method for evaluating airway inflammation in asthma.[69,71,72] In particular, the analysis of sputum eosinophils has proven valuable and has facilitated studies large enough to allow examination of the relationships between airway eosinophilia and measures of lung function. These studies have shown that sputum eosinophil percentage is related to measures of airflow obstruction and to measures of bronchial hyperresponsiveness.[73–75] Although sputum eosinophil numbers in patients with asthma are along a continuum, a large proportion of people with asthma consistently shows low numbers of eosinophils that are similar to the values found in people who do not have asthma.[73,75] These findings support data from bronchoscopy studies, which also identified patients with eosinophilic or noneosinophilic phenotypes of asthma.[76,77] The presence or absence of sputum eosinophilia can be determined using a 2% cutoff based on published reference values for eosinophils in induced sputum from healthy subjects; that is, subjects with 2% or greater sputum eosinophils have sputum eosinophilia, and subjects with less than 2% sputum eosinophils do not.[78,79] Compared with the noneosinophilic asthma phenotype, the eosinophilic asthma phenotype has more pronounced subepithelial fibrosis and is more responsive to inhaled corticosteroids.[77]

Furthermore, recent studies have provided evidence that T_H2 cytokines orchestrate the eosinophilic asthma phenotype.[80] With the increasing emphasis on accurate phenotyping of asthma to optimize and personalize treatment programs,[81] it is reasonable to propose that sputum eosinophils be quantified in characterizing study participants in asthma research studies. This information will allow a better understanding of how the eosinophil and non-eosinophil phenotypes of asthma influence responses to treatment interventions and also will facilitate mechanistic studies of these distinct phenotypes.

Range of values—Two published studies have provided reference values for sputum eosinophil percentages. In 1 study of 118 healthy nonsmoking subjects, the mean eosinophil percentage was 0.4 with an SD of 0.9, so that the upper range of normal calculated as the mean +2 SD was 2.2%.[78] In another study of 114 healthy subjects, the mean eosinophil percentage was 0.6 with an SD of 0.8, and a mean + 2 SD that was also 2.2%.[79] These data provide the rationale for a cutoff of 2% eosinophils to classify people with asthma as having sputum eosinophilia or not. Using sputum eosinophil analysis in this way has identified subgroups with asthma, with and without sputum eosinophilia.[77,82] The cell counts in sputum are presented as the nonsquamous cell percentage; squamous cells are counted independently to determine sample quality. A squamous cell percentage of greater than 80% is taken to indicate a sputum sample of inadequate quality.[69]

Repeatability—The concordance correlation coefficient for the 1-week repeatability of sputum eosinophils has been reported by the Asthma Clinical Research Network to be 0.82 (95% CI, 0.72–0.88).[83]

Responsiveness—Sputum cell counts can change within hours after an intervention, as has been shown in studies of airway allergen challenge or exposure to ozone.[84–86] The effects of currently available asthma treatments on sputum eosinophils vary. Treatment with corticosteroids consistently decreases sputum eosinophils.[87,88] Although leukotriene receptor antagonists have been shown to reduce sputum eosinophils,[89] these effects are not consistent[88] and are smaller compared with those of corticosteroids[90,91] or omalizumab.[92] Long- or short-acting β-adrenergic agonists are not thought to alter sputum eosinophilia.

Validity—Sputum cell counts are a well-validated method for assessing cellular inflammation of the airways. Validation studies have included comparison with cell counts obtained during bronchoscopy[93] and studies done before and after interventions with corticosteroids,[87] aeroallergens,[84,85] and ozone,[86] as well as repeated measures and safety studies.[83,94]

Associations—Inverse relationships have been observed between percentage of sputum eosinophils and FEV_1, as well as between percentage of sputum eosinophils and PC_{20} to methacholine,[73] but sputum eosinophils alone do not account for all of the variability in these lung function measures among individuals who have asthma; other factors also appear to have strong influences. The relationship between blood and sputum eosinophils is likely complex and has not yet been well described.

Practicality and risk—Although researchers have proposed the use of sputum induction as a research tool in asthma for nearly 20 years, the procedure has not gained wide use in clinical practice because of logistic and practical difficulties. The test is sufficiently complex that it is only used as a clinical test for patient care in a few select centers. In contrast, the use of sputum induction and analysis of induced sputum is much more widespread in the research setting. Experience in research settings has taught us that the test is best used in centers that deploy it frequently and that its use in multicenter studies requires specific training and quality assurance programs.

The hypertonic saline used in sputum induction can cause bronchoconstriction, but pretreatment with albuterol prevents bronchoconstriction in most patients. To guard against excessive bronchoconstriction in some subgroups of patients, it is necessary to monitor lung function (usually by peak flow) during the induction procedure. When such monitoring is done, the procedure has been shown to have acceptable risk.[95]

Demographic considerations—Sputum induction is feasible in children,[96] but because the test requires active participation by the subject, children under the age of 6 will have more difficulty completing it successfully.

Priority for NIH-initiated clinical research—Sputum eosinophil measurement is recommended as a supplemental outcome for the characterization of study populations, for prospective clinical trials, and for observational studies.

The strengths of this method include the fact that sputum cytology provides a direct measure of eosinophilic inflammation in the large airways. With increasing recognition of specific molecular phenotypes of asthma associated with specific cellular profiles, this advantage is becoming even more relevant. Coupled with emerging data that responsiveness to treatment such as corticosteroids or cytokine inhibitors depends on patterns of cellular inflammation in the airway[82,97,98] and that cellular inflammation is associated with specific patterns of airway remodeling,[76,77,80] it becomes clear that mechanism- or treatment-oriented studies in asthma are best done with full knowledge of the airway cytology phenotype.

Weaknesses include participant tolerance, the salty taste of the hypertonic saline, the small risk of bronchoconstriction, the difficulty in obtaining adequate samples in some study participants (particularly children), and the need to participate actively in sputum expectoration. These make the test unappealing to some participants. In addition, whereas the procedure is not very complex, it is probably too demanding for research in some settings, such as doctors' offices. The combined expense of the induction and processing can add significantly to study costs. Finally, technician training is essential, because the test is not automated.

CBC/blood eosinophils

Summary

- Analysis of blood eosinophils by automated CBC provides useful information to characterize study populations for prospective clinical trials and observational studies in asthma.
- Blood eosinophils can be used as a biomarker to monitor systemic biological effects of pharmacologic and immunologic interventions in patients with asthma.

Definition and methodology for measurement—Venous blood is drawn and put into a tube containing EDTA. The CBC is determined using an automated analyzer, such as the Medonic M-Series, Beckman Coulter LH series, Roche Sysmex XE-2100, Siemens ADVIA 120 and 2120, Abbott CELL-DYN series, and Mindray BC series. The total number of white blood cells is multiplied by the percentage of eosinophils to provide the absolute eosinophil count (eosinophils × 10^9/L). The percentage of eosinophils should not be reported unless specific reasons exist for knowing the proportions of eosinophils compared with other cells. Automated counting systems are accurate, but they can produce errors in samples with high blood eosinophil counts. Manual counting is not recommended because of inaccuracy concerns.[99]

Medical and scientific value—The association between eosinophilia and asthma was observed shortly after eosinophils were first described.[100] In patients with asthma, blood eosinophil counts are often, but not always, increased.[101,102] In both children and adults, a direct correlation was observed between blood eosinophil counts and symptom scores,[103,104] and an inverse correlation was found with FEV_1.[104] Furthermore, eosinophil counts in adults correlated with the magnitude of bronchial hyperreactivity and diurnal peak expiratory flow variation.[104] Thus peripheral eosinophil counts may reflect asthma activity in both children and adults.

Historically, eosinophils have been considered effector cells involved in bronchial asthma and allergic diseases.[105] Activated eosinophils release toxic granule proteins and proinflammatory mediators that may cause tissue damage and dysfunction.[106] Eosinophils also may be involved in tissue remodeling and immunoregulation.[107] However, the roles of eosinophils in human asthma are still poorly understood.[108,109]

Range of values—In adults, blood eosinophil counts range from 0.015 to 0.65 × 10^9/L (95% confidence limits).[110] In children 4 to 8 years of age, blood eosinophil counts average 0.206 ± 0.027 × 10^9/L.[111] In children over 12 years of age, the counts are lower: for males, eosinophil counts average 0.180 ± 0.016 × 10^9/L, and for females, they average 0.145 ± 0.012 × 10^9/L.[111]

Repeatability—Blood eosinophil counts vary diurnally in healthy individuals by more than 40%.[112] The counts are inversely related to blood cortisol levels; that is, they are lowest in the morning and highest at night. Therefore blood samples for eosinophil counts should always be collected at the same time of day. Exercise and smoking also increase the blood eosinophil count.[113,114]

Responsiveness—The blood eosinophil count reflects various immunological and inflammatory parameters of asthma, such as blood and tissue levels of cytokines and chemokines. IL-4 and IL-13 play a central role in promoting eosinophil trafficking, whereas IL-5 is the major cytokine promoting eosinophil differentiation, proliferation, and activation.[115] Thus blood eosinophil counts change in response to treatments that affect

these parameters. For example, in patients with asthma, blood eosinophil counts start to decrease within 24 hours after intravenous administration of anti–IL-5 antibody, followed by much greater decreases several days later.[61,116,117] Treatment of asthmatic patients with anti-IgE antibody, leukotriene antagonists, or 5-lipoxygenese (5-LO) inhibitors reduces blood eosinophil counts.[118] Conversely, blood eosinophils can persist in patients with corticosteroid-resistant asthma, perhaps identifying poor corticosteroid responsiveness.[119]

Validity—CBC with automated analyzers is a well-validated method for assessing the number of leukocytes in the blood.

Associations—An inverse correlation exists between the level of pulmonary function and the number of blood eosinophils.[120] Feno and peripheral blood eosinophils are elevated in patients with severe asthma with persistent airflow obstruction. However, no differences have been observed in the numbers of eosinophils between patients with asthma with elevated levels of IgE antibodies and those without elevated levels of IgE antibodies.[121] The relation between blood and sputum eosinophils has not been reported.

Practicality and risk—CBC is a routine, standardized clinical test in medical institutions and clinical practices and is readily available. The variation in results between sites is none to minimal. The cost for the test is $10 to $13. Blood drawing and the blood volume necessary for CBC are considered to be of minimal risk.

Demographic considerations—CBC is feasible in both children and adults. Blood eosinophil counts in 4 ethnic groups (Asian-Indian, black, white, and non-Indian Asian) showed no significant differences.[122]

Priority for NIH-initiated clinical research—Blood eosinophil counts are considered supplemental asthma biomarker measures for characterizing patients and outcome measures in clinical trials and observational studies, depending on the study question and design. This parameter is readily available in medical institutions, and the risk to patients with asthma is minimal. Analysis of eosinophil counts in peripheral blood provides a useful tool for characterizing/phenotyping study populations for prospective clinical trials and observational studies in asthma and for assessing the efficacy of certain pharmacologic and immunologic agents. Although the test has several weaknesses, as discussed below, the relative merit of blood eosinophil counts as an asthma outcome is high, considering the information gained, responsiveness, practical issues, and risk.

Blood eosinophil counts might provide additional unique information for asthma phenotyping, as compared to other asthma biomarkers. For example, in adults, correlations between the blood eosinophil count and the magnitude of airway hyperreactivity are noted irrespective of the presence of specific IgE antibodies.[104] Furthermore, increased blood eosinophil counts were observed in patients with asthma and with extensive sinus involvement by computed tomography (CT) scans.[123]

Eosinophils are primarily tissue-dwelling leukocytes. Thus blood eosinophil counts do not necessarily indicate the extent of eosinophil involvement in affected tissues. The half-life of eosinophils in blood is short (ie, 18.0 ± 2.1 hours).[124] Thus the eosinophil count fluctuates considerably and is influenced by various factors, including exposure to allergens, treatment with inhaled and/or oral corticosteroids, and exposure to infectious agents or stress. After inhalation challenge with an allergen, patients who develop a late-phase reaction show an early decrease in blood eosinophils, followed by an increase.[125] Corticosteroids inhibit the development of eosinophils, although the acute fall in blood eosinophil counts *in vivo* caused by corticosteroids mainly results from the redistribution of the cells in the blood.[126]

The rapid decrease in blood eosinophil counts following infection or stress involves both an increased uptake of eosinophils into tissues and a decreased output of eosinophils from bone marrow.[127]

Urinary leukotriene E_4

Summary

- Urinary leukotriene E_4 (LTE_4) is a validated marker of cysteinyl leukotriene activity and should be considered for incorporation in clinical trials of molecules that may directly or indirectly affect this pathway.

Definition and methodology for measurement—Cysteinyl leukotrienes are eicosanoids produced by a variety of cells associated with allergic inflammation, including eosinophils, mast cells, and basophils. The end metabolite of cysteinyl leukotrienes, LTE_4, can be generally measured in random urine samples. No clear benefit has been shown to measuring 24-hour samples. Mass spectroscopy is recommended as the method of measurement.[128,129]

Medical and scientific value—Urinary LTE_4 is an indirect marker of lung cysteinyl leukotriene activity. Urinary LTE_4 increases with asthma exacerbations, aspirin and allergen challenges, and perhaps at night with nocturnal asthma.[130–133] Drugs that block cysteinyl leukotriene synthesis significantly decrease urinary LTE_4 levels,[134,135] whereas corticosteroids do not.[131,136] Levels have been shown to be greater in individuals with severe asthma whose disease onset occurs after 12 years of age, as compared to those with early onset, perhaps due to the more eosinophilic nature of some phenotypes of adult onset asthma, including aspirin-exacerbated respiratory disease.[137]

Range of values—Values can vary dramatically, depending on methodology. However, both 2-step purification methods (using immunopreciptation and enzyme linked immunoassays) and mass spectroscopy have generally established normal levels to be less than 50 pg/μg (picograms per micrograms) creatinine.[128,129] In aspirin-exacerbated respiratory disease and other eosinophilic forms of severe asthma, levels can be much higher and may be measured in nanograms per microgram.[138,139]

Repeatability—If a study participant's asthma is stable, levels tend to be stable as well.[140] Levels increase following exposure to allergen, aspirin, and other nonsteroidal anti-inflammatory drugs in sensitive individuals, and in exacerbations. The levels decrease in the presence of 5-LO inhibitors.

Responsiveness—Responsiveness to treatment depends on the urinary LTE_4 starting level, with low levels less likely to show measureable responses to 5-LO inhibitors. However, in general, 5-LO inhibitors decrease urinary LTE_4 levels by 40% to 75%.[134,135] Levels increase in asthma exacerbations, as well as with aspirin and allergen challenge.[130,133] Corticosteroids have minimal effects on the levels.[131,136]

Validity—Urinary LTE_4 is modestly associated with lung function measurements over time, as well as with a fall in FEV_1 during aspirin challenge and the degree of airway obstruction during an asthma exacerbation.[130,133,141] While many people who have asthma have increased LTE_4 during asthma exacerbations, the sensitivity and specificity of this measure across all patients with asthma is limited. Although 1 study in children suggested that urinary LTE_4 levels predicted response to leukotriene receptor antagonists, previous studies in adults did not demonstrate such a predictive value.[142]

Associations—Increased basal levels of urinary LTE_4 have been associated with aspirin-sensitive asthma, adult-onset asthma, lung function, and blood eosinophils.[137,141,143,144] However, the associations are not very strong, so LTE_4 cannot be considered a surrogate for these other measures.

Practicality and risk—Whereas collection of urine is simple and of no risk to study participants, the measurement of LTE_4 is not simple. Special equipment and training are required to conduct the recommended measurement approach of mass spectroscopy.[128]

Demographic considerations—Higher urinary LTE_4 levels are seen in adult-onset severe asthma and aspirin-sensitive asthma.[137,143]

Priority for NIH-initiated clinical research—Urinary LTE_4 is recommended as a supplemental outcome for the characterization of study populations, for prospective clinical trials, and for observational studies. The test should be strongly considered for inclusion in any study that attempts to manipulate the eicosanoid pathway. LTE_4 measurement also should be considered for studies that characterize asthma phenotypes, such as aspirin-sensitive, adult-onset, and eosinophilic asthma.

Future directions or research questions—Urinary LTE_4 was collected in most of the early leukotriene-modifying drug trials. However, the relationship of urinary leukotrienes to particular phenotypes, beyond aspirin sensitivity, has never been addressed. It is conceivable that the increased emphasis on asthma phenotyping may increase the importance of urinary LTE_4 measurement. Interventions that affect other aspects of eicosanoid biology (eg, COX) also should consider including measurement of urinary LTE_4 to determine whether this pathway is directly or indirectly affected.

EMERGING BIOMARKERS

Cortisol

Cortisol measures can be used in the following ways:

- Cortisol suppression measures are used primarily as a biomarker to assess inhaled or systemic corticosteroids with respect to the level of systemic exposure and their effect on the hypothalamic-pituitary-adrenal axis.
- Measurement of cortisol levels (particularly 12-hour overnight or 24-hour plasma cortisol) should be considered for the characterization and definition of the therapeutic index of new corticosteroids (asthma effect-to-systemic activity ratio).[57,145,146]
- Salivary cortisol measures can be used in studies to evaluate neuroendocrine effects of stress.[147–149]

The preferred method for cortisol measurement is high-performance liquid chromatography.[57] Immunoassays have a high potential for interference from exogenous corticosteroids. Twenty-four-hour and overnight urinary free cortisol also have been used and are accepted by the FDA as a means of assessing systemic corticosteroid activity. Measurements of salivary cortisol have been used in studies of stress response but have not been standardized and applied to studies comparing systemic effects of oral and inhaled corticosteroids. In addition, some studies directly measure the corticosteroid of interest from plasma or serum to assess bioavailability.[150]

When studying the effect of an inhaled or systemic corticosteroid on the hypothalamic-pituitary-adrenal axis, the degree of cortisol suppression is related to the dose and type of corticosteroid administered, as well as the delivery device for an inhaled corticosteroid.[57,145,150] While cortisol suppression by plasma or urinary free cortisol measurement is a sensitive measure of exogenous corticosteroid administration, the relationship to clinically significant suppression has not been established.[148] To date, cortisol suppression has not been shown to correlate well with clinically relevant measures of corticosteroid adverse effects. Salivary samples also may be used to assess cortisol suppression. Although saliva collection is convenient, especially for pediatric studies, precautions are needed when using this technique for clinical studies due to lack of standardization and lack of data on use for comparative studies.[147]

Measurements of cortisol are considered emerging for asthma clinical trials involving corticosteroids. The strength of plasma or serum cortisol measurement is its sensitivity in identifying the systemic effect of corticosteroid therapy. However, studies are still needed to determine whether the degree of cortisol suppression is associated with long-term risk for clinically relevant adverse effects, such as reduced growth, osteoporosis, or cataracts.[151]

Some directions for future research related to the application of cortisol measurement include the development of convenient and reliable methods to assess cortisol suppression in young children because of the limitations in the volume of blood that can be collected given the frequency of sampling required for currently standardized measures. Therefore defining the relationship between blood and salivary cortisol would be useful, especially in children. Identifying the relationship of cortisol suppression to clinically relevant indicators of adverse corticosteroid effects is also important.

High-resolution CT scanning

High-resolution computed tomography (HRCT) can be used to measure airway lumen (diameter, area), airway wall (thickness, area), parenchymal density, and lung volume. HRCT images are used to measure specific airway narrowing, wall thickening, air trapping, and ventilation inhomogeneity in health and disease. Imaging allows assessment of the structure of the airways and parenchyma not obtainable by any other *in vivo* methods. HRCT airway lumen and wall measurements have been correlated with lung function and severity of asthma. In addition, increased parenchymal lucency has been associated with severe exacerbations of asthma, FEV_1, atopy, and neutrophilic inflammation.[153] HRCT is an emerging outcome measure for NIH-initiated clinical research.

HRCT has no single preferred method or scanner. Multiple scanners, manufacturers, and scanning parameters have been used. HRCT is easy to perform, and the scanners and personnel are readily available. The 1 risk is exposure to ionizing radiation.[152] Validity and reproducibility, particularly for airway measurements, require that, within each study participant, the lungs are scanned at a standard volume.

Pixel counting is the common method of measurement for HRCT. In addition, pixel intensity is used for parenchymal measurements of air content. The various methods have been validated via phantoms of parenchyma and airways of various sizes and density. HRCT measurements have been shown to be repeatable in animal models, although studies in humans with and without lung disease are lacking. The magnitude of change is usually measured as a percentage change in airway luminal size or airway wall thickness or the percentage of parenchymal density measures below a certain threshold (eg, −856 Hounsfield units for air trapping). However, a clinically relevant magnitude of change has not been determined. Furthermore, no normal ranges have been established for any of the HRCT

imaging measurements. The effects of gender, age, or race/ethnicity on HRCT measurements have not been determined.

More validation and reproducibility data are required before HRCT can move from the category of an emerging outcome.

Sputum neutrophils and analytes

Additional emerging outcomes include sputum neutrophil evaluation and various analytes measured in the sputum sol phase. Neutrophilic asthma (generally defined through sputum evaluation) has been proposed as a phenotype associated with more severe disease, lower lung function, corticosteroid use (and poor response to this treatment), asthma exacerbations, and smoking.[154–157] However, sputum neutrophilia is not specific for asthma, being observed in numerous other lung diseases. There is variability in thresholds for neutrophilia, depending on sputum processing and centers, ranging from 40% to 65%.[158,159] A recent study suggested considerable temporal variability without clinical association, while another suggested that when controlling for corticosteroid use and smoking, the phenotype did not exist.[159,160] Similarly, a variety of different analytes have been measured in sputum supernatants. However, to date, the sample sizes are small, and there are no reproducibility studies. For these reasons, sputum neutrophils and measurements of analytes are categorized as emerging biomarkers.

Exhaled breath condensate

Exhaled breath condensate (EBC) is a composite volatile and droplet lung collection that may allow for noninvasive assessment of biochemistry and inflammation, with the relative contributions to EBC from proximal versus distal airways still unclear. Because of the numerous assays available to perform on EBC, with often only a few investigators studying any 1 biomarker, there have been only modest efforts at validation studies on any 1 biomarker. EBC pH is the most technically validated of the assays and focuses on a biochemical disturbance common in inflammatory diseases in general.[161] If airway neutralization therapies prove useful for subgroups of subjects with asthma symptoms, then EBC pH may become a particularly important biomarker. Likewise, careful use of EBC pH may allow identification of acute acid reflux events.[162] Nitrogen oxides, hydrogen peroxide, glutathione, aldehydes, isoprostanes, and pH in EBC may provide more information regarding airway oxidative stresses than other approaches, although confidence in interpretation remains modest. Additionally, assessment of airways inflammation with EBC assays for cytokines, leukotrienes, prostaglandins, adenosine, and others has been reported.[163] Assays for such nonvolatile substances can be improved by controlling for the dilution of airway lining fluid during the collection process.[164] However, these assays remain insufficiently controlled and standardized, and therefore are emerging biomarkers of interest.

Biomarker discovery through genetics and genomic profiling

Biomarker discovery and validation using large clinical trial populations is now possible with the use of genetics and genomic profiling (eg, transcriptomics, proteomics, lipomics, and metabolomics). However, there are several issues to consider relative to the acquisition and storage of samples, as discussed below.

BIOSPECIMEN ACQUISITION AND STORAGE FOR DETECTION OF BIOMARKERS

Most clinical trials archive biospecimens for genetics and genomics (eg, transcriptomics, proteomics, lipomics, and metabolomics); however, the stability of the biospecimens over

extended periods remains unclear. Generally, storage at −80°C immediately upon acquisition confers the greatest stability. Evidence suggests that samples obtained from study participants with asthma are more susceptible to degradation than those obtained from control participants.[165,166] Peripheral blood, the most accessible source for various transcriptomic and proteomic studies, is separated to obtain plasma or serum or processed to obtain the buffy coat for DNA analysis. Limitations of peripheral blood analysis relate to the systemic nature of blood and the extent to which it reflects the lung and/or airway compartment. An excellent resource for biospecimen preparation is the standard operating procedures of the National Cancer Institute's Early Detection Research Network (http://edrn.nci.nih.gov/resources). Blood samples processed with these procedures are stable at −70°C for more than 3 years, although differences in stability between disease and control samples remain to be determined. Clinical blood collection transcriptome analysis remains challenging, with degradation of transcripts and even induction of gene expression by sample handling.[167] The speed of acquisition and maintenance of aliquots at −80°C are likely to be the most important factors in determining stability over time,[165,168] and cloned (c) DNA is considered to be more stable than RNA.

Urine also serves as a convenient and stable platform for the performance of metabolomics, proteomics, and lipidomics. Samples should be spun quickly and snap frozen.[169,170] Unfortunately, although urine is easily acquired, urinary changes may not adequately reflect events occurring in the lung or the airways.

Sputum and EBC, important resources for biomarker discovery, may directly correlate with lung pathology. However, many technical challenges remain before sputum analysis and EBC can be routinely used and sufficiently validated in discovery studies.[165,171–173]

Standard operating procedures for harvesting body fluids are essential to harmonize protocols. Studies are needed to compare approaches for storing blood, urine, sputum, and other airway fluids to determine the optimal approaches to maximize yield without loss of fidelity. Validation and reproducibility studies also are required before stability of biospecimen storage can be assured. The adequate storage and quality assurance of biospecimens should be a priority for collaborative, multicenter studies in asthma. Central storage facilities will likely decrease biospecimen variance and improve quality. Collective research efforts must focus on comparing the quality and stability of biospecimens from large central repositories, such as the National Heart, Lung, and Blood Institute biorepository (Biologic Specimen and Data Repository Information Coordinating Center, or BioLINCC; see https://biolincc.nhlbi.nih.gov/home), to ensure maximal use of these valuable resources. Ethical questions regarding the use of these biospecimens several years after collection need to be thoroughly discussed and consensus reached.

PRIORITY QUESTIONS FOR FUTURE RESEARCH

1. Can exhaled NO best be used as a unique marker of inflammation to predict and monitor response to asthma treatment?
2. Do differences in sputum processing significantly affect the utility of sputum eosinophils to guide anti-inflammatory therapy?
3. Can a simpler surrogate for sputum eosinophils be developed?
4. Will characterization of asthma by atopic status contribute to a better understanding and differentiation of asthma phenotypes?
5. What is the relationship between measures of cortisol suppression and meaningful systemic effects of corticosteroid therapy?

6. Can lung imaging be standardized and used to define airway remodeling in asthma?
7. Can future studies emphasize a design in which the patient population is selected on the basis of biomarker-based phenotypes?

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Abbreviations used

ATS	American Thoracic Society
CBC	Complete blood count
CT	Computed tomography
EBC	Exhaled breath condensate
ERS	European Respiratory Society
FDA	US Food and Drug Administration
Feno	Fractional exhaled nitric oxide
5-LO	5-Lipoxygenase
HRCT	High-resolution computed tomography
LTE_4	Leukotriene E_4
NIH	National Institutes of Health
NO	Nitric oxide

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TABLE I

Recommendations for classifying asthma biomarker outcome measures for NIH-initiated clinical research for adults and children

	Characterization of study population for prospective clinical trials (ie, baseline information)	**Prospective clinical trial efficacy/effectiveness outcomes**	**Observational study outcomes***
Core outcomes	Serologic multiallergen screen (IgE) to define atopic status (also for observational studies)	None	None
Supplemental outcomes	**1** Feno **2** Sputum eosinophils **3** CBC (total eosinophils) **4** Total IgE **5** Allergen-specific IgE **6** Urinary LTE_4	**1** Feno **2** Sputum eosinophils **3** CBC (total eosinophils) **4** Total IgE **5** Allergen-specific IgE **6** Urinary LTE_4	**1** Feno **2** Sputum eosinophils **3** CBC (total eosinophils) **4** Total IgE **5** Allergen-specific IgE **6** Urinary LTE_4
Emerging outcomes	**1** Allergen skin prick testing **2** Sputum neutrophils and analytes† **3** Airway imaging **4** Exhaled breath condensate markers **5** Discovery through genetics and genomics	**1** Allergen skin prick testing **2** Sputum neutrophils and analytes **3** Airway imaging **4** Cortisol measures **5** Exhaled breath condensate markers **6** Discovery through genetics and genomics	**1** Sputum neutrophils and analytes **2** Airway imaging **3** Discovery through genetics and genomics

*Observational study designs include cohort, case-control, cross-sectional, retrospective reviews; genome-wide association studies (GWAS); and secondary analysis of existing data. Some measures may not be available in studies using previously collected data.

†The substance(s) being analyzed.

TABLE II

Methods for measuring and reporting core and supplemental biomarker outcomes

Serologic multiallergen screen	Preferred method: adults—Phadiatop; children— Phadiatop plus fx5 food allergen mix Report as kUa/L (with 0.35 kUa/L as the cutpoint for presence of atopy)
Total serum IgE	Preferred method: any FDA-cleared immunoassay
Allergen-specific IgE	Preferred method: serum immunoassay Preferred assay: ImmunoCAP (Phadia) Alternative assays: HYTEC (Hycor) Immulite (Siemens) Note: skin prick tests have no single generally accepted technique
Feno	Preferred method: ATS/ERS guidelines[1–3] for measurement at 50 L/s Report whether online or offline method was used Report as ppb in exhaled breath (<25 ppb indicates normal value)
Sputum eosinophils	Preferred method: whole expectorate Alternative method: sputum plug Report as: Preferred: eosinophil percentage per mL of sputum Alternative: total eosinophils per μL of sputum
CBC (total eosinophils)	Method: CBC by automated analyzer Report as absolute eosinophil count (eosinophils × 10^9/L) Percentage eosinophils is not recommended
Urinary LTE_4	Preferred method: random urine sample; mass spectroscopy Report as pg/μg creatinine (<50 pg/μg indicates normal value)

TABLE III

Key points and recommendations

1. A multiallergen IgE test is a biomarker for atopic asthma and should be collected for baseline characterization of study participants in all NIH- initiated clinical trials and prospective observational studies.
2. Measurements of total IgE and allergen-specific IgE antibodies are supplemental tests that can be used in baseline characterization of study participants.
3. Feno measured at a constant flow rate is a simple, safe, and reproducible biomarker for use in asthma clinical trials.
4. Although Feno values overlap among healthy, atopic, and asthmatic cohorts, Feno levels <25 ppb generally indicate lower likelihood of eosinophilic inflammation and responsiveness to corticosteroids. However, Feno cannot be used interchangeably with sputum eosinophilia as an outcome measure, given that therapeutics that reduce eosinophilic inflammation do not always affect Feno levels.
5. Measurement of Feno should be considered in clinical trials where effects on T_H2 inflammation are being assessed.
6. Analysis of eosinophil counts in induced sputum identifies participants who have asthma with eosinophilic and noneosinophilic inflammatory phenotypes. Inflammatory phenotypes can predict response to treatment.
7. Analysis of blood eosinophils by automated CBC provides useful information to characterize study populations and potentially to monitor responses to anti-inflammatory therapy.
8. Urinary LTE_4 is a validated marker of cysteinyl leukotriene activity and should be considered for incorporation in clinical trials of molecules that may directly or indirectly affect this pathway.
9. The measurement of cortisol suppression is used primarily as a biomarker to assess the systemic effect of inhaled or systemic corticosteroids on the hypothalamic-pituitary-adrenal axis.
10. HRCT is capable of making structural measurements of the airways and parenchyma.
11. Clinical trials should consider storing blood, sputum, and urine specimens in biobanks.
12. Efforts should be made to standardize procedures to harmonize sample collection for biorepositories from clinical trials.
13. Biomarkers hold considerable promise for advancing personalized medicine. Further research is necessary to establish the specific contributions of different biomarkers in differentiating asthma phenotypes and in predicting and monitoring treatment response.