



HAL
open science

Biomarkers in asthma and allergic rhinitis

Z. Diamant, J.D. Boot, E. Mantzouranis, R. Flohr, P.J. Sterk, R. Gerth van
Wijk

► **To cite this version:**

Z. Diamant, J.D. Boot, E. Mantzouranis, R. Flohr, P.J. Sterk, et al.. Biomarkers in asthma and allergic rhinitis. *Pulmonary Pharmacology & Therapeutics*, 2010, 23 (6), pp.468. 10.1016/j.pupt.2010.06.006 . hal-00699040

HAL Id: hal-00699040

<https://hal.science/hal-00699040>

Submitted on 19 May 2012

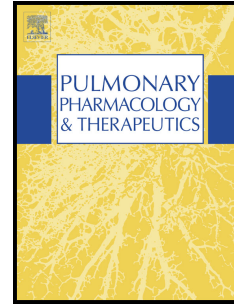
HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Accepted Manuscript

Title: Biomarkers in asthma and allergic rhinitis

Authors: Z. Diamant, J.D. Boot, E. Mantzouranis, R. Flohr, P.J. Sterk, R. Gerth van Wijk



PII: S1094-5539(10)00079-9

DOI: [10.1016/j.pupt.2010.06.006](https://doi.org/10.1016/j.pupt.2010.06.006)

Reference: YPUPT 1020

To appear in: *Pulmonary Pharmacology & Therapeutics*

Received Date: 14 May 2010

Accepted Date: 23 June 2010

Please cite this article as: Diamant Z, Boot JD, Mantzouranis E, Flohr R, Sterk PJ, Gerth van Wijk R. Biomarkers in asthma and allergic rhinitis, *Pulmonary Pharmacology & Therapeutics* (2010), doi: 10.1016/j.pupt.2010.06.006

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Biomarkers in asthma and allergic rhinitis

Z. Diamant¹, J.D. Boot², E. Mantzouranis³, R. Flohr⁴, P.J. Sterk⁵, R. Gerth van Wijk⁶.

- 1) *Erasmus Medical Center, Depts of Allergology and Pulmonology, Rotterdam, The Netherlands*
- 2) *HAL Allergy, Leiden, The Netherlands*
- 3) *Division of Allergy, Immunology, Respiratory, Dept of Pediatrics, University of Crete, Greece*
- 4) *Master student Life Science and Technology, Faculty of Science, University of Leiden, Leiden, The Netherlands.*
- 5) *Academic Medical Center, Amsterdam, The Netherlands*
- 6) *Erasmus Medical Center, Dept of Allergology, Rotterdam, The Netherlands*

Version 12, 120510-final

Corresponding author:

Zuzana Diamant, MD PhD
Erasmus Medical Center
Depts of Allergology and Pulmonology
P.O.Box 2400
3000 CA Rotterdam
The Netherlands
Email: z.diamant@gems.demon.nl

Abstract

A biological marker (biomarker) is a physical sign or laboratory measurement that can serve as an indicator of biological or pathophysiological processes or as a response to a therapeutic intervention. An applicable biomarker possesses the characteristics of clinical relevance (sensitivity and specificity for the disease) and is responsive to treatment effects, in combination with simplicity, reliability and repeatability of the sampling technique. Presently, there are several biomarkers for asthma and allergic rhinitis that can be obtained by non-invasive or semi-invasive airway sampling methods meeting at least some of these criteria.

In clinical practice, such biomarkers can provide complementary information to conventional disease markers, including clinical signs, spirometry and PC₂₀methacholine or histamine. Consequently, biomarkers can aid to establish the diagnosis, in staging and monitoring of the disease activity/progression or in predicting or monitoring of a treatment response. Especially in (young) children, reliable, non-invasive biomarkers would be valuable.

Apart from diagnostic purposes, biomarkers can also be used as (surrogate) markers to predict a (novel) drug's efficacy in target populations. Therefore, biomarkers are increasingly applied in early drug development.

When implementing biomarkers in clinical practice or trials of asthma and allergic rhinitis, it is important to consider the heterogeneous nature of the inflammatory response which should direct the selection of adequate biomarkers. Some biomarker sampling techniques await further development and/or validation, and should therefore be applied as a "back up" of established biomarkers or methods. In addition, some biomarkers or sampling techniques are less suitable for (very young) children. Hence, on a case by case basis, a decision needs to be made what biomarker is adequate for the target population or purpose pursued.

Future development of more sophisticated sampling methods and quantification techniques, such as -omics and biomedical imaging, will enable detection of adequate biomarkers for both clinical and research applications.

Pathophysiology of allergic airways disease

The pathogenesis of asthma and allergic rhinitis is complex. The expression of either or both disorders in an individual largely depends on interactions between several susceptibility genes and environmental factors [1-3]. Atopy is the key factor predisposing for the development of allergic airways disease [4]. Despite modern technologies enabling to unravel several inflammatory mechanisms of allergic airway disease, presently, still many etiological and pathophysiological questions remain unanswered [5].

Overall, the allergic inflammation within the bronchial and nasal tissues shows many similarities with some local differences (Figure 1) [6,7]. Exposure to a new allergen results in uptake and processing by dendritic cells (DCs). Subsequent presentation of the processed allergen by DCs to naïve T helper (Th) cells induces the development of Th2 cells in genetically predisposed individuals [8]. The Th2 cells then release interleukins (IL)-4 and IL-13, causing the differentiation of B cells into allergen-specific immunoglobulin (Ig)-E-producing plasma cells [9]. The newly synthesized IgE binds to high affinity IgE receptors (FcεRI) on the surface of mast cells and basophils, inducing sensitization ('priming'). Upon re-exposure, the allergen binds to the cell surface-bound IgE, which results in cross-linking of the FcεRIs and subsequent degranulation of the mast cells, causing the release of preformed mediators (histamine, chymase and tryptase) and *de novo* synthesis of other pro-inflammatory substances (leukotrienes, prostaglandins, platelet activating factor and bradykinin) [9]. Recent evidence suggests that the airway epithelium also plays an important role in the induction of allergic airway responses by the release of thymic stromal lymphopoietin (TSLP), an IL-7-like cytokine that has been shown to activate DCs to induce Th2-cell responses and to promote the differentiation of TH-17 cells [10-12].

The observation of many varieties within the "asthma syndrome" in terms of clinical presentation, triggers and underlying immunological mechanisms, resulted in the concept of different disease-entities and the definition of distinct asthma phenotypes or endotypes [13,14]. In view of the disease heterogeneity, traditional disease markers, such as clinical symptoms and lung function parameters, appeared inadequate to differentiate across the various subsets or to monitor disease activity and the response to (targeted) therapy, since they appeared poorly correlated with the underlying airway inflammation [15]. In addition, several factor and cluster analyses revealed that symptoms and lung function, markers of airway inflammation and airway hyperresponsiveness provide complementary information on the severity and activity of asthma in both adults and children and can help to differentiate into different asthma phenotypes [16-19]. In this respect, the development of non-invasive

airways sampling methods and detection techniques, allowing identification of several components of the airway inflammation including the determination of useful biomarkers, has greatly contributed to our current insight into the inflammatory cascades within several asthma subsets and the link to customized, targeted therapies [13,20,21].

Biomarkers in asthma and allergic rhinitis: definitions and criteria

A biological marker (biomarker) is a physical sign or laboratory measurement that can serve as an indicator of biological or pathophysiological processes or a response to a pharmacological intervention [22]. There is an ongoing exploration of new biomarkers and initially, all biological compounds of the inflammatory cascade could be eligible candidates. Ideally, a biomarker should have the following characteristics [22]:

- Clinical relevance: indicating a clear relationship between the biomarker and the pathophysiological events in a disorder, causing a clinical endpoint.
- Sensitivity and specificity for intervention effects.
- Reliability and repeatability: the biomarker should be measured in a precise and reproducible way.
- Simplicity of sampling methodology and measurement/detection technique to promote widespread use.

Biomarkers can be employed for various purposes, including diagnosis, staging and monitoring of disease activity/progression or predictors c.q. monitors of a treatment response. In addition, they can provide complementary information to traditional disease markers, such as clinical signs and symptoms or pathophysiological measures. Validated biomarkers are of major value in early clinical trials to establish “proof of mechanism” or “proof of efficacy” of novel drugs in target populations [23]. Implementation of adequate, validated biomarkers in early drug development has several advantages and is being advocated by regulatory authorities, including the EMEA and the FDA [24]. Apart from their clinical implications, biomarkers also enable exploration of pathophysiological mechanisms through targeted drug interventions. When implementing biomarkers in clinical trials or monitoring of asthma and/or allergic rhinitis, it is important to consider the heterogeneous nature of the inflammatory response which may have implications on the selection of adequate biomarkers [25]. In general, one single biomarker may capture only a small

fraction of the intervention effect and, therefore, it is important to sample multiple biomarkers whenever possible. In addition, it is important to ensure that changes in the selected biomarker(s) translate into a meaningful clinical sign or symptom translating into a clinically relevant improvement. Overall, samplings of the biomarker should preferably be conducted in the most relevant environment, *i.e.* the target organs, being the lungs and/or the nose, instead of *e.g.* serum or urine. In this mini-review, we aim to provide a general overview of biomarkers of allergic airways disease, focusing on the less invasive sampling methods of the upper and lower airways. In addition, we will address some potential biomarkers and applicable airway sampling methods applicable in children.

Biomarkers of asthma

Sampling techniques of the lower airways

Overall, there are three types of sampling methodologies of the lower airways: invasive sampling requiring flexible bronchoscopy, semi-invasive sampling by induced sputum and non-invasive sampling of the volatile inflammatory components in exhaled air.

Invasive sampling techniques

Invasive airway samplings include submucosal or transbronchial bronchial biopsies, bronchial brushes and bronchoalveolar lavage (BAL) that may be performed in combination. These sampling techniques are useful tools to address pathophysiological issues as they can provide more complete information on several histopathological features and immunological aspects of asthma and allow differentiation across the different asthma-phenotypes [26-28]. Although bronchial biopsies may provide additional or even superior information on the components (and their interrelationship) of airway inflammation and airway remodeling in asthma, in drug efficacy trials they have largely been substituted by the less invasive sampling techniques, such as induced sputum and exhaled air [5,29]. Moreover, there is ample evidence that specimen obtained with different sampling techniques may be complementary as they provide information on different parts of the bronchial tree [30,31].

Semi-invasive sampling techniques

Induced sputum (IS)

Sputum is defined as secretion originating from the lower airways. Sputum induction by inhalations of hypertonic saline promoting expectoration is a validated method both for research and diagnosis. Generally, the induction protocol is performed with inhalations of 4.5% NaCl during 3x5 minutes, although other protocols using different NaCl solutions (0.9-7%) and/or induction times (up to 30 min) have been employed as well [32]. The thus obtained sputum samples can be processed according to the “entire expectorate” technique or the “selected plug” method [33]. Both methods yield reproducible data, but are not interchangeable [34]. Although splitting the sample requires certain skills, it has several advantages, as it contains less squamous cells, yields cells in overall better condition (higher viability) and higher concentrations of soluble markers (less dilution) [35]. Following centrifugation, the processed samples can be divided into a ‘solid’ phase or cell pellet, consisting of cells, and a ‘fluid’ phase containing soluble mediators. Both components can be quantified to assess the presence and activity of inflammatory components. Sputum induction can be described as a semi-invasive procedure and is safer, cheaper and generally easier to perform than bronchial biopsy or BAL, although more troublesome than exhaled nitric oxide (eNO) or exhaled breath condensate (EBC). Over the last fifteen years, a vast amount of research has contributed to validation and standardization of the technique. An ERS Task Force document has been issued relating on recommendation and guidelines for standardized induction, collection, processing and analysis of sputum [36].

Biomarkers in solid phase

Sputum cell counts are reproducible and validated markers of lower airway inflammation, when performed according to ERS guidelines [34,36]. This especially holds for the eosinophil and neutrophil counts [37]. Eosinophils (and neutrophils in severe persistent asthma) are considered key effector cells in the asthmatic airway inflammation, as their numbers are related to disease severity [38-42]. (Increased) eosinophil counts have been demonstrated in sputum samples of asymptomatic asthmatics with (further) increases during spontaneous exacerbations and in exacerbation models of asthma (e.g. allergen-induced late response and tapering off corticosteroids) [41,43]. Alternatively, sputum eosinophils are the best predictors of the clinical response to corticosteroids in asthma [44] and (pre)treatment with these drugs has been shown to reduce sputum eosinophils both

following allergen challenge and in 'wild type' asthma [45-50]. In most clinical studies, the reduction in sputum eosinophils was accompanied by an improvement in symptoms scores and lung function parameters. Green *et al.* achieved superior asthma control applying a treatment regimen targeting sputum eosinophils *versus* the standard strategy aimed at improving symptom scores and lung function parameters [51]. In general, sputum eosinophil and neutrophil counts are validated biomarkers of airway inflammation in asthma/COPD applicable in both clinical settings (e.g. diagnostic in "refractory asthma") and in early drug development (proof of efficacy), if handled by "experienced hands".

Biomarkers in fluid phase

Presently, numerous inflammatory mediators (including a variety of granulocyte proteins, proteases, cytokines, chemokines, eicosanoids and leakage markers) can be quantified in the fluid phase of sputum ('supernatant'). However, the validity and reproducibility of several techniques has not yet been established. Apart from the induction technique, there are at least three other reasons that can account for this. First, processing of sputum may affect mediator measurements. According to most processing protocols, dithiothreitol (DTT) should be added to the sputum sample for the recovery of mediators by dispersing the mucus layer through cleavage of the disulphide bonds [52]. However, DTT may also affect the disulphide bonds within the mediators [53]. Second, variable dilutions may account for inaccurate quantifications among samples and presently there is not yet a validated factor to adequately correct for dilution [54]. Third, certain mediators may remain below the detection limit of widely used commercial assays; hence, more sensitive detection techniques are required [55]. Eosinophil cationic protein (ECP) as an activation marker of eosinophils has been intensely investigated. In sputum of asthmatics, (increased levels of) ECP have been found to be well-correlated with the eosinophil cell counts [56]. In addition, anti-inflammatory treatment decreases both the eosinophils and ECP within the airways [48,57]. Unfortunately, myeloperoxidase (MPO) as an activation marker of neutrophils seems to be affected by sputum induction and/or processing technique and therefore immunoassays are not always reproducible [53,58,59]. In sputum supernatant it is also possible to measure proteases involved in the process of extracellular matrix degradation. In asthma, increased levels of matrix metalloproteinase-9 (MMP-9) have been found in sputum, BAL and bronchial biopsies [60-64]. In addition, several investigators reported an imbalance between MMP-9 and its counterpart, tissue inhibitor of metalloproteinases (TIMP), resulting in a disease-severity dependent increase of the MMP-9/TIMP ratio [60,61,65]. In conclusion, MMP-9/TIMP ratio in

sputum is a potential marker for monitoring effects of interventions directed against airway remodelling.

Many inflammatory mediators including cytokines and chemokines are degraded by DTT. Several research groups have investigated modified sputum processing techniques to optimize biomarker recovery [66-68]. However, these processing techniques are not fully validated and most of them prevented recovery of other mediators from the samples. As an exception, IL-8, a potent neutrophil chemoattractant, seems less affected and can be quantified by a validated immunoassay [53,69]. In several studies, increased levels of IL-8 have been demonstrated during asthma exacerbations and in more severe disease [69,70]. Eicosanoids are involved in the pathophysiology of asthma [71,72]. Increased levels of cysteinyl leukotrienes (Cys-LTs) can be detected in several body fluids of asthmatic subjects, including sputum [73,74]. Moreover, sputum concentrations of Cys-LTs were found to correlate with disease-severity and failed to be unaffected by corticosteroids [75]. 8-Isoprostane is the most extensively studied eicosanoid and reproducible levels have been measured in sputum and exhaled breath condensate (EBC) of both healthy controls and asthmatic patients, with increased levels in more severe disease and during asthma exacerbations [76].

Recommendations

Sputum induction is a semi-invasive sampling procedure of the lower airways allowing to explore components of airway inflammation. Although not fully interchangeable with BAL and bronchial biopsies, it has been shown to provide useful and consistent information on several inflammatory markers whilst being safer, cheaper and generally easier to perform [30,31,77]. Nevertheless, many subjects experience this procedure as a burden. Another drawback holds that the overall percentage of analysable sputum samples, even in specialized centers, fails to reach 100% [78]. Finally, many inflammatory markers in sputum supernatant are affected by the (standard) processing techniques and more sensitive (sophisticated) assays are needed for optimal biomarker detection [67].

However, many advantages by far outweigh those few cons of induced sputum. A major advantage of sputum sample analysis is the possibility of evaluating multiple inflammatory biomarkers. In the solid phase (*i.e.* the cell pellet), inflammatory cell differentials can be evaluated. The predominant inflammatory cell types (eosinophils or neutrophils) can be reproducibly measured in both the entire and the selected expectorate and help to

characterize the asthma phenotype [25,34]. In addition, sputum eosinophils provide information on the inflammatory status within the airways and can also predict responsiveness to corticosteroids and subsequently be used to monitor treatment effects [44,51,79]. More recently, RT-PCR allowed extraction of mRNA from sputum cells [80,81]. Compared with healthy controls, an increased expression of several inflammatory cytokines (IL-4, 5 and 13) was shown in sputum cells from asthmatics, with further increase in this inflammatory profile expression following low dose allergen exposure, that could be blocked by inhaled corticosteroids [80,81]. In the fluid phase of sputum, several inflammatory mediators are readily measurable, whilst some measurements are unreliable due to the denaturant effects of sputum processing with DTT and/or limited sensitivity of most traditional detection assays. Erin *et al* developed a dialysis technique, in which the DTT was removed from the sputum sample, thus enhancing the recovery of DTT-sensitive cytokines and chemokines [55]. A more simple alternative is to perform mechanical homogenization of the samples (by ultra-centrifugation), which results in a good recovery of spiked cytokines and chemokines [67]. A drawback of this unrefined technique arises from the disruption of cells and subsequent spilling of the intracellular content into the homogenate – which of course, can partly account for higher biomarker concentrations [54]. Similarly to RNA expression profiling in the sputum cell pellet, recovery and quantification of multiple inflammatory markers from sputum supernatant is a valid method to study several aspects of the airway inflammation in asthma. Applying this multi-faceted approach, Brasier *et al* were able to identify distinct asthma phenotypes based on cytokine expression patterns in BAL fluid [82]. Applying optimized processing and detection methods, comparable data can be obtained from sputum supernatant.

In conclusion, induced sputum is a useful semi-invasive sampling tool which allows concomitant evaluation of multiple components of the lower airways inflammation. Prior to implementation of this technique, appropriate biomarkers should be selected that are insensitive to the processing techniques and readily detectable or validation of a novel processing or detection technique is required.

Non-invasive sampling techniques

Exhaled breath condensate (EBC)

Collection of exhaled breath condensate (EBC) is a fully non-invasive sampling technique of the lower airways. Exhaled breath consists of two phases: the gaseous phase, containing volatile substances, such as nitric oxide (NO) and carbon dioxide (CO₂), and a liquid phase containing nonvolatile components, including various water-soluble inflammatory markers [83]. The non-volatile ions and proteins originate from the airway lining fluid. When aerosolized due to local turbulence, these biological entities become liquid constituents of EBC [84]. So far, there is no complete standardization of EBC sample collection or analysis hampering its clinical applicability. However, an ATS/ERS task force addressed several issues resulting in novel EBC guidelines [85]. Several collectors and condensers are currently available [86-90]. All devices are easy to use and subsequent exhaled breath collection can be simply obtained from both young and elderly individuals. Following acclimatization, subjects breathe through a mouthpiece into a non-rebreathing valve connected to a tube for approximately 15–30 min by tidal breathing [85]. During the procedure, the exhaled breath travels through the tube that serves as a cooling chamber and the thus formed condensate is collected (usually around 2 mL/sample) in a cooled collection chamber. Cooling of the samples is advised to preserve “thermo-labile” markers [85]. Subsequently, samples can be directly analyzed or frozen pending analysis.

Biomarkers in EBC

Multiple biomarkers have been measured in EBC. So far, H₂O₂, leukotrienes (LTs), 8-isoprostane and pH have shown the most consistent results. Reproducibly increased EBC-concentrations of H₂O₂, biomarker of oxidative stress, have been measured in active smokers and patients with more severe asthma [91-94]. In steroid-naïve patients with uncontrolled allergic asthma, an inverse correlation has been demonstrated between FEV₁ and/or PC₂₀histamine and exhaled H₂O₂ [92]. Alternatively, in patients with similar asthma characteristics, anti-inflammatory therapy with ICS effectively reduced exhaled H₂O₂ along with improvement in FEV₁ [91,95]. The eicosanoids LTs and 8-isoprostane can be measured in EBC by an enzyme immunoassay (EIA) or by gas chromatography/mass spectrometry [96]. Increased levels of Cys-LTs have been detected in EBC of asthmatic patients. In agreement with sputum data, Cys-LTs levels in EBC appeared to be correlated with disease severity [97] and were effectively reduced by anti-inflammatory drugs [98]. Being a stable and well-detectable biomarker both in health and disease, 8-isoprostane is the most

extensively studied prostanoid in EBC [99]. In asthma, 8-isoprostane levels appeared to correlate with disease severity [100]. Unlike eNO, 8-isoprostane is not completely suppressed by corticosteroid treatment and thus, may be a potential indicator for ongoing airway inflammation despite anti-inflammatory treatment [98-101]. Moreover, recent data suggest a link between 8-isoprostane concentration in EBC and small airways inflammation [102]. Using different collection devices, several research groups found an average pH of 7.8 in the EBC of healthy subjects, whereas in asthma the average pH was found below 7.5 [103-107]. Asthma exacerbations have been shown to result in further decline of pH with reversal following corticosteroid treatment [107]. The low costs, good reproducibility in combination with the availability of reference values are advantages of pH measurements in EBC over the other inflammatory markers. Most other inflammatory markers measured in EBC, including cytokines and chemokines, showed poor reproducibility so far.

Recommendations

EBC is an appealing method enabling repeated samplings from the lower airways in a completely non-invasive and patient-friendly fashion [108]. Presently, commercially available devices (so far, most widely used are the EcoScreen (CardinalHealth) and the RTube (Respiratory Research)) may help to overcome drawbacks arising from the use of the early 'self-made' collectors using different collecting protocols. An ATS/ERS taskforce issued guidelines aimed at standardization of collecting procedures allowing comparison across research centers [85]. So far, studies comparing commercially available devices have shown mixed data. Following identical collection, levels of total protein, eotaxin and cysteinyl leukotrienes were found to be significantly higher in EBC samples collected with the EcoScreen collector compared to the RTube device [109,110]. In addition, the volume of EBC collected with the EcoScreen was found to be consistently higher compared to the RTube samples (1.8 ± 0.1 and 1.4 ± 0.1 mL, respectively) [109,110]. This may be due to the differences in cooling the exhaled air: the EcoScreen has a refrigeration device at a constant temperature of -20°C , while the RTube uses a cooling sleeve (at -20°C), that heats up to 15°C after a 10 minute collection period. This 'warming process' may cause the degradation of heat labile substances, which may also account for the differences in protein and lipid levels found between the two devices. A clear advantage of the RTube is its small size, which enables a more universal application.

Apart from these sampling issues, problems with detection/quantification of inflammatory biomarkers in EBC is of even greater concern [108,111]. This may be due to a limited sensitivity of the ELISA technique to measure inflammatory compounds in the EBC [112].

Novel, sensitive multiplexed immunoassays should allow increased detection of biomarkers in EBC [113]. Furthermore, metabolomic analysis of EBC may be another analytical approach both in adults and children [114-116]. This detection technique, using high-resolution proton nuclear magnetic resonance (NMR) spectroscopy or mass spectroscopy, enables characterization of the metabolic compounds in even small EBC volumes, by producing a 'fingerprint' of the individual samples. This approach seems promising since it can distinguish across the heterogeneous spectrum of asthma and help to predict a drug's clinical efficacy.

In addition, several techniques have been studied to improve the sample biomarker yield, e.g. coating of the collecting tube or employing glass tubing. Tufvesson *et al* found that coating the plastic surfaces with Tween 20 detergent or BSA improved the detection of eicosanoids and cytokines, respectively [117]. However, these coating substances potentially interfere with several detection assays, and therefore, a superior approach may be to employ a glass condenser, as has been shown in a study in healthy volunteers [118]. In this study, significantly more EBC volume yielding detectable biomarkers was recovered using an optimized glass condenser compared to a silicone condenser and the EcoScreen collector [118].

Conclusively, despite several attempts in recent years aimed at optimization of the EBC technique, in terms of collection and biomarker detection, this sampling technique still awaits full validation and standardization before it can be reliably implemented into research or clinical practice. For this purpose, it is worthwhile to incorporate EBC along with more established biomarker sampling techniques in clinical trials and asthma management to aid the development and validation of this promising non-invasive sampling technique.

Exhaled nitric oxide (eNO)

In 2005, the American Thoracic Society (ATS) published recommendations for the measurements of nitric oxide (NO) from the upper and lower respiratory tract [119]. Although various methods have been reported, the online measurement during a single-breath exhalation against a fixed resistance is currently the recommended sampling technique. This highly reproducible and repeatable sampling method can be performed by the stationary chemiluminescence analyzers (Niox Flex, Ecomedics) and the more versatile hand-held electrochemical device (Niox Mino) and is now widely used in both adults and children [120,121].

Exhaled NO as a biomarker

eNO is a sensitive marker of acute airway inflammation in (allergic) asthma, which can be indicative of loss of disease control or exacerbation. Allergen challenge, especially the late asthmatic response (LAR), is a well-known inducer of airway inflammation [122]. A clear correlation has been shown between the size of the allergen-induced LAR and the increase in eNO at 8-10 h post-allergen [123]. Similarly, several studies have demonstrated that loss of asthma control is associated with an increase in eNO [51,124,125]. These studies also demonstrated that the change in eNO is a better predictor for loss of asthma control than baseline eNO *per se*. However, Leuppi *et al* found no increase in eNO during asthma exacerbations as a result of tapering off inhaled corticosteroids (ICS) [126]. This aberrant observation may be due to measuring eNO offline in contrast with online measurements used in other studies.

eNO is very responsive to anti-inflammatory therapy. ICS and other anti-inflammatory therapies for asthma, including leukotriene receptor antagonists (LTRA) and omalizumab (anti-IgE), have been shown to reduce eNO both in children and adults [127-130]. Furthermore, several studies found a correlation between eNO and other markers of airway inflammation and/or airway hyperresponsiveness in asthma which adds to its applicability as a valid, non-invasive biomarker for clinical monitoring and early drug development. Jatakanon *et al.* [131] showed significant correlations between eNO, sputum eosinophils and the provocative concentration causing a 20% fall in FEV₁ (PC₂₀)methacholine in steroid naïve patients with mild persistent asthma. In contrast, this correlation between the different markers of airway inflammation and airway hyperresponsiveness is lost in asthmatics using ICS [132,133]. This is probably due to a fast decrease of eNO attaining a maximal response even on low dose ICS therapy, resulting in almost normal eNO levels, while airway inflammation and hyperresponsiveness are still present. Therefore, eNO should probably not be used as the sole marker of airway inflammation in asthmatics using corticosteroids.

The mostly applied single flow technique cannot discriminate from what part of the bronchial tree the eNO originates. Alternatively, if measured at multiple expired flow rates, eNO can be portioned into NO from the central bronchial parts *versus* NO from the more peripheral (alveolar) compartment. It has already been demonstrated that alveolar NO is increased in severe asthma in comparison with mild to moderate persistent asthma, while there is no difference in eNO between the latter groups [134]. In the same study it was also shown that alveolar NO is refractory to inhaled corticosteroids, but responsive (*i.e.* decreased) to oral

corticosteroids. Another study found a decrease in alveolar NO and a reduction in air trapping after treatment with a small-particles ICS-formulation [135]. In a recent study in asthmatic children, increases in the distal NO fractions (CANO) revealed a distinct asthma phenotype, related to poor asthma control and morbidity independent of other disease markers, including spirometry or atopic status [136]. These data suggest that alveolar NO is a potential marker of distal airway inflammation and sensitive to (systemic) anti-inflammatory therapy.

Recommendations

Exhaled NO is widely perceived as a potential biomarker of inflammatory airways disease, particularly of allergic asthma. Major advantages of standardized eNO samplings are reproducible, non-invasive, online measurements achievable in almost all patients of over 4-5 years [137]. The drawbacks consist of many (endogenous and exogenous) factors affecting NO measures [119, 138]. Another important disadvantage of eNO measurements is the bulkiness and costs of the equipment. In this respect, the recently introduced hand-held and relatively inexpensive NO electrochemical analyzer (MINO®) seems an asset, promoting widespread use of eNO in both clinical and research settings [139]. Exhaled NO values measured with the MINO® were found to be reproducible and in agreement with the stationary units [139-141]. Conclusively, most technical issues surrounding eNO measurements appear to be resolved or manageable and the remaining question is the clinical relevance (and disease specificity) of this biomarker [142].

When compared to induced sputum or EBC, the clear disadvantage is that only one component from the airways is sampled even though this single biomarker is related to the underlying airway inflammation [143-147]. Baseline eNO levels can also aid to establish the diagnosis of asthma. A cut-off value of >20 ppb has a sensitivity and specificity of approximately 70% which is superior to spirometry (FEV₁) measurements [148,149]. Nevertheless, in day-to-day asthma management the role of eNO is controversial [142]. On one hand, it seems that low levels of eNO can predict a successful dose reduction in inhaled corticosteroids while maintaining asthma control [145]. In children, a treatment regimen based on eNO and symptoms, compared to symptoms alone, resulted in a significant reduction in disease-related parameters, including the severity of airway hyperresponsiveness, with a concomitant (but non-significant) reduction in asthma exacerbations requiring oral prednisone [150]. Alternatively, a recent study reported that addition of eNO as an indicator of asthma control on top of standard disease monitoring resulted in the prescription of higher doses of inhaled corticosteroids, without additional

clinically relevant improvements in asthma control [151]. The multiple flow technique is laborious and has not been fully standardized but in the future measuring NO at different flow rates may further refine this biomarker.

Overall, eNO could serve as a biomarker of allergic airway inflammation in clinical trials. In clinical practice, it can help to establish the diagnosis of asthma. However, its applicability as a guide to optimal asthma control is open for debate [142,152,153].

Electronic nose: exhaled molecular profiles

Exhaled air contains a complex mixture of organic compounds derived from systemic as well as local metabolic, inflammatory and oxidative activity [154-156]. These volatile organic compounds (VOCs) may be used to monitor pulmonary or even systemic diseases. The technique is completely non-invasive and allows high-throughput metabolomic analysis. The standard detection technique of molecular compounds in exhaled air is gas chromatography coupled to mass spectrometry (GC-MS) [157,158]. This identifies individual molecular constituents in exhaled air. This technique is suitable for pathophysiological research. For diagnostic assessment, powerful empirical approaches can be applied using pattern recognition algorithms aimed at providing a signature or fingerprint of exhaled mixtures of biomarkers in particular diseases.

Pattern recognition of complex VOC mixtures can also be obtained by using handheld and (close to) real-time electronic noses [159]. eNoses are using an array of sensors with partially different sensitivities for multiple VOCs based on various technologies: conducting polymers, metal oxide, metal oxide field effect transistors, surface or bulk acoustic waves, optical sensors, colorimetric sensors, ion mobility spectrometry, infrared spectroscopy, gold nanoparticles, or even GC-MS [159,160]. eNoses cannot distinguish individual VOCs, but can provide a fingerprint (breathprint) of complex VOC mixtures. Clinical application of eNoses is emerging [159,161,162] along with rapid instrumental and statistical developments.

Breath collection is critical for eNose assessments and includes standardization of expiratory flow, expired volume, water vapour, either or not filtering inspired air with VOC-filter and total *versus* late expired sampling [163,164]. The data analysis uses normalisation methods, followed by pattern recognition algorithms and classification techniques such as principal component analysis [165]. This is essentially integrative, coming close to 'system medicine'

[166]. The downside is that it is essential to carefully deal with the risk of false discoveries, for which explicit recommendations have to be obeyed [167]. Finally, there is still an unmet need of mapping between eNoses [168].

Cross-sectional studies using eNoses have shown discriminative power in respiratory medicine. This holds for lung cancer patients *versus* controls [169-172] and *versus* patients with COPD [173]. Interestingly, asthmatics can also be discriminated from healthy controls and COPD patients (cross-validated accuracy 80-100%) [163,164,174]. In addition, eNoses are an attractive screening method for infectious diseases [175,176]. It is important to notice that all these data are based on cross-validation procedures in so-called 'training sets'. According to the STARD Guidelines for establishing diagnostic accuracy, the next step needs to be external validation [177-179]. Preliminary data using external 'validation sets' of patients with asthma and COPD have only recently become available, and are showing successful identification of newly recruited patients [180]. This suggests that eNoses can have a role in differential diagnosis of respiratory diseases.

Biomarkers in allergic rhinitis

The signs and symptoms of allergic rhinitis are the result of an IgE-mediated allergic reaction involving different cells, mediators, cytokines, chemokines, neuropeptides, chemokines and other components in a complex immunological network [1]. In clinical practice or trials of allergic rhinitis, most evaluation methods of clinical symptoms (by composite symptom scores) and measurements techniques of nasal patency (by rhinomanometry and acoustic rhinometry) are hampered by the lack of validation, a limited reproducibility, due to patient- and observer-related factors and/or equipment-related factors [181]. Assessment of the nasal inflammation by biomarkers offers a more objective and direct read-out that can contribute to our understanding of the mechanisms of allergic rhinitis, to monitor disease severity and to evaluate the effects of (novel) treatments. Although similar sampling methods are being applied as in the lower airways, most of these techniques and biomarkers still await validation.

Sampling techniques of the upper airways

Several tools and techniques are available for sampling of the upper airway biomarkers. Similarly to the lower airways, there are 3 fractions that can be sampled for biomarkers: cellular, soluble and volatile fractions.

Overview of sampling techniques

Soluble substances such as mediators and cytokines can be obtained by nasal lavage (NAL) techniques. Two methods are being used to obtain NAL fluid: first, the head-back method introduced by Naclerio [182]. In this method, NaCl 0.9% is instilled into the nose while the subject is closing off the nasopharynx. Another NAL technique is the so-called “head-forward” method where a nasal pool device is used to instill saline into the nose [183]. When comparing the methods, the first has been shown to yield more reproducible ECP levels, while the latter allows a higher and more reproducible recovery of cell counts [184]. Overall, with the exception of IgE, NAL-biomarkers show substantial intra- and intersubject variability and most inflammatory markers remain below the detection limit of the commonly applied quantification assays [184,185]. Attempts to improve the biomarker yield have been undertaken by increasing the dwelling time of the lavage fluid in the upper airways [186], by reducing the dilution factor using a filter paper [187] or a synthetic absorptive matrix (SAM) [188] for the absorption of nasal secretions/epithelial lining fluid or by optimizing the nasal fluid collection by a nasal secretion collector with polyurethane absorption foams [189] and by the development of more sophisticated detection techniques including multiplex, mRNA analysis, metabolomics and proteomics. However all techniques have their specific limitations and most of them await further validation.

Although cells can be found in the NAL fluid, cellularity, cellular profiles including mRNA patterns can be more accurately assessed by nasal brushes (NAB) and nasal biopsies. Nasal brushing is a simple, relatively patient-friendly method to obtain cells from the nasal mucosa. And despite variability in the individual cell counts, NAB may be particularly suitable for studies in children, large groups and pathophysiological or intervention studies requiring multiple samplings. Furthermore, NAB enables to pick up signals from inflammatory stimuli, including nasal allergen challenge, and may therefore be a valuable tool in the assessment of the effects of anti-inflammatory interventions [185].

Nasal biopsies provide more reproducible information than nasal brushings on the nasal epithelium and the musosa, and additionally on the submucosa as well, however, the methodology does not allow frequently repeated samplings within one individual [190]. Moreover, the methodology requires specialized centers with ample experience. In analogy to the lower airways, more recently attempts have been made to assess nasal inflammation by measuring nasal nitric oxide (nNO) [191,192].

Overview of biomarkers in allergic rhinitis

Mast cell-derived markers

Histamine is the most prominent mediator released from mast cells and basophils during the early phase allergic reaction (Figure 1). This release is reflected by a peak in the NAL level of histamine which is maximal at 15-20 minutes after nasal challenge [193]. A late peak can be found during the late phase reaction between 6-8 hours post-challenge [194]. Unfortunately, high baseline levels of histamine (along with substantial variability) preclude its use as a biomarker of disease severity. Therefore, pre-nasal allergen challenge, nasal washings are needed to remove pre-existent histamine [195].

Other mast cell-derived mediators present in nasal lavage during the early reaction include tryptase [185,195], PGD₂ [182], and leukotrienes [196] (Figure 1). These mediators are probably more stable and hence more reliable markers of mast cell degranulation. More recently, chymase along with its inhibitor, cleaved secretory leucocyte protease inhibitor (cSLPI), have been quantified in NAL fluid of allergic rhinitics with increased levels following nasal allergen challenge as compared to sham challenge [197]. In this study, cSLPI appeared to reflect the activity of chymase recovered from the NAL and sputum of patients with allergic rhinitis and asthma, respectively [197].

Eosinophil derived markers

Eosinophils can be found in the cell pellet of the NAL fluid. In addition, NAB and biopsies are a source of BMK13 positive (activated) eosinophils [198]. Soluble markers of eosinophil activation are among other ECP and EPX. These mediators appear in the NAL fluid approximately 6-10 hours post-nasal allergen challenge [199]. Despite a substantial inter-subject variability, the rise in ECP levels after nasal grass pollen challenge has been shown

to correlate with nasal symptoms during pollen season ($r=0.53$) [200]. Moreover, ECP in the NAL fluid is increased in allergic patients during season compared with an out-season assessment [194]. In addition, using ECP post-challenge allows to study the efficacy of topical corticosteroids. Treatment with intranasal fluticasone resulted in 76% reduction in the late phase nasal symptoms and 83% reduction in ECP levels in NAL of patients with allergic rhinitis [195]. While an early increase in LTB_4 and LTC_4 in the NAL fluid reflects mast cell degranulation [193], a late increase in LTC_4 points at activation of eosinophils and possibly basophils as well.

Markers of nasal permeability

Albumin and α_2 macroglobulin are leakage markers indicative of nasal permeability following allergen challenge [201]. Albumin has been used to characterize the early and late phase nasal response [195,196,202]. However, albumin is also produced by nasal glands [203]. Therefore, α_2 macroglobulin might be a more specific leakage marker of the nasal allergic response. Plasma exudation or leakage is a result of inflammatory mediators promoting nasal permeability. Efficacy of drugs targeting components of inflammation (including these mediators) can be evaluated by albumin and α_2 macroglobulin levels. Antihistamines effectively suppress the α_2 macroglobulin peaks in NAL fluid following nasal allergen challenge [204]. Topical corticosteroids reduce the recovery of α_2 macroglobulin and albumin in NAL fluid during active disease [205] and following nasal allergen challenge [195,206]. In a more recent nasal allergen challenge study, vascular endothelial growth factor (VEGF) has been found in the NAL during the early phase of the nasal allergic reaction [207]. This growth factor is a potent inducer of endothelial cell growth and angiogenesis and is responsible for increased capillary permeability [208].

Various biomarkers of upper airway inflammation

Although several studies have demonstrated clinically relevant cytokines and chemokines (e.g. GM-CSF, IL-1, IL-3, IL-5, IL-6, IL-8, RANTES, MIP-1) in NAL fluid of patients with allergic rhinitis, these data are difficult to interpret due to variability of the samplings and different detection techniques [194]. For this purpose, nasal biopsies may allow a more accurate cytokine profiling of the upper airways.

Nasal nitric oxide (nNO)

Similarly to exhaled NO in asthma, nasal NO (nNO) has been thought to be a useful marker of upper airways inflammation in allergic rhinitis. Standard operation procedures have been established to measure NO in both upper and lower airways [119]. More recently, nNO measurements by the portable NO-analyzer, MINO, were validated against the gold standard chemiluminescence NO-analyzer in both healthy volunteers and patients with AR [209]. Hence, this totally non-invasive, simple, fast and repeatable upper airways sampling methodology could be added to the existing diagnostic and research tools.

Normal levels of nasal nNO range from approximately 400 to 900 ppb [210,211]. Paranasal sinuses substantially contribute to nNO measurements by a continuous production of high levels of nNO (up to 25 ppm) by inducible NO-synthases expressed in the epithelium [212]. The role of NO in the sinuses is likely to increase local host defense by direct inhibition of pathogen growth and by stimulation of mucociliary activity. In contrast, conditions with a low nNO production, including cystic fibrosis and primary ciliary dyskinesia (PCD), are associated with a high susceptibility to sinus infections [212]. In addition, local application of an NO-synthase inhibitor to a healthy volunteer was found to be associated with a drop in nNO levels and the development of a maxillary sinusitis 3 days later [212].

Apart from the endogenous source, ambient NO may also substantially affect nNO measurements [212,213]. Both endogenous and exogenous "high-output" nNO sources may interfere with the interpretation of nNO measurements.

Overall, (active) allergic inflammation induces higher NO production and several studies report increased nNO levels in both symptomatic and asymptomatic allergic rhinitics as opposed to non-allergic controls [210,214]. In contrast, low(er) nNO levels may be found in conditions such as nasal blockage and nasal polyps [212,215].

In daily practice, nNO measurement seems a less attractive candidate for disease monitoring or treatment evaluation due to substantial variability in long-term intra-subject nNO levels (as a result of the aforementioned endogenous and exogenous factors) in combination with only a marginal effect of anti-inflammatory therapy reported by some researchers [216,217].

In clinical trials involving nasal allergen challenge, nNO levels can be reliably measured after the massive nasal congestion and rhinorrhoea present in the early phase have subsided [191].

In conclusion, apart from assessments of clinical signs and symptoms, various biomarkers can be obtained by several more or less non-invasive sampling methods to evaluate the nasal allergic response and disease activity in allergic rhinitis. So far, none of the assessment methods or biomarkers has been validated and both endogenous and exogenous factors introduce a substantial variability. Presently, nasal biomarkers cannot be readily implemented in the daily clinical practice. However, some of these biomarkers may be useful for evaluation of the efficacy of novel treatment modalities in early clinical studies of allergic rhinitis. Nasal lavage and nasal brushings can be relatively easily implemented in nasal provocation studies. The applicability and long-term reproducibility of nNO awaits further investigation.

Biomarkers in childhood asthma

Like in adults, asthma in children is characterised by chronic airway inflammation, based on evidence from bronchial biopsies [218], BAL [219] and sputum [220]. Even during asymptomatic disease episodes, airway inflammation can be demonstrated [221]. A Dutch bronchial biopsy study demonstrated chronic airway inflammation in asymptomatic adolescents, who were thought to have outgrown their early childhood asthma, possibly indicating a risk of disease relapse later in life [222]. Therefore, monitoring of airway inflammation by adequate biomarkers can aid the diagnosis and hence, may positively affect clinical outcomes.

In general, samplings of airway inflammation in (very young) children must be non-invasive, reproducible and easy to perform [223]. Collection of exhaled breath condensate (EBC) e.g. for detection of leukotriene E₄ (LTE₄) [224] and measurements of fractional exhaled nitric oxide (FeNO) are totally non-invasive biomarker sampling techniques that can be easily performed already in very young children [225,226]. In contrast, bronchial biopsies and BAL are too invasive for the assessment of airway inflammation, especially in young children. Similarly, bronchoprovocation tests to assess airway hyperresponsiveness or hypertonic saline-induced sputum to demonstrate airway eosinophilia require a patient's collaboration and hence, cannot be performed in very young children.

Assessment of airway hyperresponsiveness (AHR) to direct stimuli such as methacholine (PC₂₀methacholine) or histamine (PC₂₀histamine) can be performed from the age of 5 years. In children, interpretation of the bronchoprovocation tests (AHR) depends on the child's age.

In asthmatic children under the age of 12 years, AHR is mainly associated with airway inflammation (increased FeNO), while in children older than 12 years, AHR possibly reflects airway remodelling [227].

Sputum can be induced in children of >6 years with a success rate varying from 68 to 100% [228]. Although not fully validated in this patient population, sputum eosinophil counts may provide additional diagnostic information and can predict exacerbations in asthmatic children [229]. Furthermore, sputum eosinophils appeared to be correlated with disease severity in steroid-naïve children with asthma and in severe persistent asthma [228]. In children with moderate to severe persistent asthma, a modest agreement has been found between FeNO and eosinophils in sputum and BAL but a poor correlation between FeNO and eosinophils in distal bronchial biopsies [230,231].

In asthmatic children treated with moderate doses of ICS, FeNO showed a weak correlation with sputum eosinophils, but related well to sputum ECP and urinary EPX levels [232]. Another study in adolescents diagnosed with mild persistent asthma, reported a (better) relationship between FeNO and sputum eosinophils [233]. In this study (population), FeNO appeared to be a useful indicator of atopy and airway inflammation with a negative predictive value for asthma of 83% and a positive predictive value of 54%; this is consistent with most other diagnostic tests for asthma [233].

Consequently, FeNO has often been used as a surrogate marker of (eosinophilic) airway inflammation in children (>4 years) with asthma [234,235], e.g. to diagnose worsening of disease control or exacerbation after discontinuation of ICS [234,235] or to monitor the effect of anti-inflammatory therapy [150]. In the past years, several randomized, controlled studies examined the utility of FeNO to guide management strategies. A study in asthmatics (12-75 years), showed that tailoring ICS on FeNO levels in this cohort was associated with overall fewer exacerbations and a lower mean ICS dose compared to standard strategy based on symptoms [236]. In a study in asthmatic children (6-18 years), titrating ICS on FeNO levels *versus* conventional strategy resulted in improved airway responsiveness to methacholine, less airway inflammation and fewer severe exacerbations in the FeNO group, with no differences in ICS doses and symptom scores between the two strategy arms [150]. However, not all studies using FeNO to guide asthma management resulted in improvement in disease control [237]. In children with clinically stable, atopic asthma and elevated FeNO levels despite ICS, further increase in ICS dose failed to reduce FeNO [238].

A recent Cochrane review evaluated the results of 6 studies (2 in adults and 4 in children/adolescents) tailoring the dose of ICS according to FeNO levels *versus* clinical symptoms [153]. The meta-analysis did not show any significant differences in asthma exacerbations, clinical symptoms, FeNO level or spirometry between the two strategy groups. However, a post-hoc analysis of the paediatric studies revealed a significant ICS-increase in the FeNO arm *versus* the conventional strategy arm, leading to the conclusion that, at this stage, FeNO cannot be routinely recommended to tailor the ICS dose in children [153].

In patients with acute or chronic rhinosinusitis nasal nitric oxide (nNO) levels are significantly decreased. Nasal NO has been proposed as a functional test to evaluate sinus ventilation. It is significantly reduced in primary ciliary dyskinesia and can be a screening tool for this condition [239].

Exhaled breath condensate (EBC) can be easily collected and is a totally non-invasive airway sampling method. Therefore, this methodology seems promising for application in children [240]. However, like in the adults, so far, EBC awaits further evaluation and validation [241]. Similarly, the electronic nose seems a promising tool for future evaluation of a disease's activity or even for diagnostic purposes [159].

Recommendations

In children, measurements of inflammatory markers are inconsistent across the different (sampling) techniques, possibly reflecting disease heterogeneity, methodological limitations or varying sensitivity of the biomarker detection techniques. Hence, at this stage, biomarkers cannot be generally recommended as reliable tools to evaluate or treat an asthmatic child. Nevertheless, measurements of (at least some) airway inflammatory markers can aid diagnosis, monitoring and/or management of asthma, even if it is yet unclear which inflammation marker is most useful. Despite the aforementioned limitations, repeated FeNO measurements in individual patients may offer valuable information in specialized settings [231]. EBC and electronic nose are promising non-invasive airway sampling techniques awaiting further evaluation and validation in children.

Overall conclusion

Non-invasive and semi-invasive sampling methods of the upper and lower airways offer a large variety of potential biomarkers of asthma and allergic rhinitis. In view of the complex inflammatory airway response in both asthma and allergic rhinitis, multiple biomarkers should be sampled, whenever possible. Biomarkers can be useful tools in both clinical practice (diagnosis, disease monitoring) and clinical research including drug development. Further development and validation of sophisticated non-invasive sampling methods and biomarker detection techniques is warranted and should enable general application across target populations of all ages.

References

1. Bousquet J, Khaltaev N, Cruz AA, Denburg J, Fokkens WJ, Togias A et al. Allergic Rhinitis and its Impact on Asthma (ARIA) 2008 update (in collaboration with the World Health Organization, GA(2)LEN and AllerGen). *Allergy* 2008; 63 Suppl 86:8-160.
2. Global Initiative for Asthma (GINA). Global strategy for asthma management and prevention. Bethesda/Maryland: NHLBI/WHO workshop report. Last updated: 2009; www.ginasthma.com.
3. Vercelli D. Gene-environment interactions in asthma and allergy: the end of the beginning? *Curr Opin Allergy Clin Immunol* 2010; 2010 Apr;10(2):145-8.
4. Von Mutius E. Gene-environment interactions in asthma. *J Allergy Clin Immunol* 2009; 123(1):3-11.
5. Boot JD, Panzner P, Diamant Z. A critical appraisal of methods used in early clinical development of novel drugs for the treatment of asthma. *Review. Pulm Pharmacol Ther* 2007;20(3):201-19.
6. Bousquet J, van Cauwenberge P, Khaltaev N. Allergic rhinitis and its impact on asthma. *J Allergy Clin Immunol* 2001; 108(5 Suppl):S147-S334.
7. Jeffery P, Haahtela T. Allergic rhinitis and asthma: inflammation in a one-airway condition. *BMC Pulmonary Medicine* 2006; 6(Suppl 1):S5 (12 pages).
8. Kaiko GE, Horvat JC, Beagley KW, Hansbro PM. Immunological decision-making: how does the immune system decide to mount a helper T-cell response? *Immunology* 2008 Mar;123(3):326-38.
9. Poulsen LK, Hummelshoj L. Triggers of IgE class switching and allergy development. *Ann Med*. 2007;39(6):440-56.
10. Holgate ST. Has the time come to rethink the pathogenesis of asthma? *Curr Opin Allergy Clin Immunol*. 2010 Feb;10(1):48-53.
11. Ziegler SF, Artis D. Sensing the outside world: TSLP regulates barrier immunity. *Nat Immunol* 2010 Mar 19;11(4):289-93.
12. Tanaka J, Watanabe N, Kido M, Saga K, Akamatsu T, Nishio A, Chiba T. Human TSLP and TLR3 ligands promote differentiation of Th17 cells with a central memory phenotype under Th2-polarizing conditions. *Clin Exp Allergy* 2009 Jan;39(1):89-100.
13. Bradding P, Green RH. Subclinical phenotypes of asthma. *Curr Opin Allergy Clin Immunol* 2010 Feb;10(1):54-9.
14. Anderson GP. Endotyping asthma: new insights into key pathogenic mechanisms in a complex, heterogeneous disease. *Lancet* 2008; Sep 20; 372(9643):1107-19.
15. Luskin AT. What the asthma end points we know and love do and do not tell us. *Journal of Allergy and Clinical Immunology* 2005; 115(4, Supplement 1):S539-S545.
16. Rosi E, Ronchi MC, Grazzini M, Duranti R, Scano G. Sputum analysis, bronchial hyperresponsiveness, and airway function in asthma: Results of a factor analysis. *Journal of Allergy and Clinical Immunology* 1999; 103(2):232-37.
17. Leung TF, Wong GW, Ko FW, Lam CW, Fok TF. Clinical and atopic parameters and airway inflammatory markers in childhood asthma: a factor analysis. *Thorax* 2005; 60(10):822-26.
18. Haldar P, Pavord ID, Shaw DE, Berry MA, Thomas M, Brightling CE, Wardlaw AJ, Green RH. Cluster analysis and clinical asthma phenotypes. *Am J Respir Crit Care Med* 2008 Aug 1;178(3):218-24.

19. Moore WC, Meyers DA, Wenzel SE, Teague WG, Li H, Li X, D'Agostino R Jr, Castro M, Curran-Everett D, Fitzpatrick AM, Gaston B, Jarjour NN, Sorkness R, Calhoun WJ, Chung KF, Comhair SA, Dweik RA, Israel E, Peters SP, Busse WW, Erzurum SC, Bleecker ER; National Heart, Lung, and Blood Institute's severe asthma Research Program. Identification of asthma phenotypes using cluster analysis in the Severe Asthma Research Program. *Am J Respir Crit Care Med* 2010 Feb 15;181(4):315-23.
20. Diamant Z. Should pathophysiology have implications on the management of asthma? (Invited state of art) *Int J Respir Care* 2005;1(spring):22-7.
21. Diamant Z, Boot D, Kamerling I, Bjermer L. Methods used in clinical development of novel antiasthma therapies. *Respir Med* 2008;102(3):332-38.
22. Lesko LJ, Atkinson AJ. Use of biomarkers and surrogate endpoints in drug development and regulatory decision making: Criteria, Validation, Strategies 1. *Annual Review of Pharmacology and Toxicology* 2001; 41(1):347-66.
23. Atkinson AJ, Colburn WA, DeGruttola VG, DeMets DL, Downing GJ, Hoth DF *et al.* Biomarkers and surrogate endpoints: Preferred definitions and conceptual framework*. *Clin Pharmacol Ther* 2001; 69(3):89-95.
24. Critical Path Initiative; FDA 2010;
www.fda.gov/ScienceResearch/SpecialTopics/CriticalPathInitiative
25. Wenzel SE, Schwartz LB, Langmack EL, Halliday JL, Trudeau JB, Gibbs RL *et al.* Evidence That Severe Asthma Can Be Divided Pathologically into Two Inflammatory Subtypes with Distinct Physiologic and Clinical Characteristics. *Am J Respir Crit Care Med* 1999; 160(3):1001-8.
26. Adelroth E. How to measure airway inflammation: bronchoalveolar lavage and airway biopsies. *Can Respir J* 1998 Jul-Aug;5 Suppl A:18A-21A.
27. Balzar S, Wenzel SE, Chu HW. Transbronchial biopsy as a tool to evaluate small airways in asthma. *Eur Respir J* 2002 Aug;20(2):254-9.
28. Macedo P, Hew M, Torrego A, Jouneau S, Oates T, Durham A, Chung KF. Inflammatory biomarkers in airways of patients with severe asthma compared with non-severe asthma. *Clin Exp Allergy* 2009 Nov;39(11):1668-76.
29. Murugan A, Prys-Picard C, Calhoun WJ. Biomarkers in asthma. *Curr Opin Pulm Med* 2009 Jan;15(1):12-8.
30. Silkoff PE, Trudeau JB, Gibbs R, Wenzel SE. The relationship of induced-sputum inflammatory cells to BAL and biopsy. *Chest* 2003 Mar;123(3 Suppl):371S-2S.
31. Lemièrè C, Ernst P, Olivenstein R, Yamauchi Y, Govindaraju K, Ludwig MS, Martin JG, Hamid Q. Airway inflammation assessed by invasive and noninvasive means in severe asthma: eosinophilic and non-eosinophilic phenotypes. *J Allergy Clin Immunol* 2006 Nov;118(5):1033-9.
32. Paggiaro PL, Chanez P, Holz O, Ind PW, Djukanović R, Maestrelli P, Sterk PJ. Sputum induction. Review. *Eur Respir J Suppl.* 2002 Sep;37:3s-8s.
33. Efthimiadis A, Spanevello A, Hamid Q *et al*, Methods of sputum processing for cell counts, immunocytochemistry and in situ hybridisation *Eur Respir J Suppl.* 2002 Sep;37:19s-23s.
34. Pizzichini E, Pizzichini MM, Efthimiadis A *et al*, Indices of airway inflammation in induced sputum: reproducibility and validity of cell and fluid-phase measurements. *Am J Respir Crit Care Med* 1996 Aug;154(2 Pt 1):308-17.

35. Pizzichini E, Pizzichini MM, Efthimiadis A, Hargreave FE, Dolovich J. Measurement of inflammatory indices in induced sputum: effects of selection of sputum to minimize salivary contamination. *Eur Respir J* 1996 Jun;9(6):1174-80.
36. Djukanovic R, Sterk PJ, Fahy JV, Hargreave. Standardised methodology of sputum induction and processing. *Eur Respir J* 2002 Jul 1;20(37_suppl):1S-2.
37. Spanevello A, Migliori GB, Sharara A, Ballardini L, Bridge P, Pisati P, *et al.* Induced sputum to assess airway inflammation: a study of reproducibility. *Clin Exp Allergy* 1997 Oct;27(10):1138-44.
38. Corrigan CJ, Kay AB. T cells and eosinophils in the pathogenesis of asthma. *Immunol Today* 1992 Dec;13(12):501-7.
39. Wenzel S. Mechanisms of severe asthma. *Clin Exp Allergy* 2003 Dec;33(12):1622-8.
40. Louis R, Sele J, Henket M, Cataldo D, Bettiol J, Seiden L, *et al.* Sputum eosinophil count in a large population of patients with mild to moderate steroid-naive asthma: distribution and relationship with methacholine bronchial hyperresponsiveness. *Allergy* 2002 Oct;57(10):907-12.
41. Louis R, Lau L, Bron A, Roldaan A, Radermecker M, Djukanovic R. The relationship between airways inflammation and asthma severity. *Am J Respir Crit Care Med* 2000 Jan 1;161(1):9-16.
42. Jatakanon A, Lim S, Barnes PJ. Changes in sputum eosinophils predict loss of asthma control. *Am J Respir Crit Care Med* 2000 Jan 1;161(1):64-72.
43. in't Veen JC, Smits HH, Hiemstra PS, Zwinderman AE, Sterk PJ, Bel EH. Lung function and sputum characteristics of patients with severe asthma during an induced exacerbation by double-blind steroid withdrawal. *Am J Respir Crit Care Med* 1999 Jul;160(1):93-9.
44. Brightling CE, Green RH, Pavord ID. Biomarkers predicting response to corticosteroid therapy in asthma. *Treat Respir Med* 2005;4(5):309-16. Review.
45. Djukanovic R, Wilson SJ, Kraft M, Jarjour NN, Steel M, Chung KF, *et al.* Treatment with Anti-immunoglobulin E Antibody Omalizumab on Airway Inflammation in Allergic Asthma. *Am J Respir Crit Care Med* 2004 Sep 15;170(6):583-93.
46. Gauvreaux GM, Sulakvelidze I, Watson RM, Inman MD, Rerecich TJ, O'Byrne PM. Effects of once daily dosing with inhaled budesonide on airway hyperresponsiveness and airway inflammation following repeated low-dose allergen challenge in atopic asthmatics. *Clin Exp Allergy* 2000 Sep;30(9):1235-43.
47. Inman MD, Watson RM, Rereich T, Gauvreaux GM, Lutsky BN, Stryzak P, *et al.* Dose-dependent Effects of Inhaled Mometasone Furoate on Airway Function and Inflammation After Allergen Inhalation Challenge. *Am J Respir Crit Care Med* 2001 Aug 15;164(4):569-74.
48. Bacci E, Di Franco A, Bartoli ML, Carnevali S, Cianchetti S, Dente FL, *et al.* Comparison of anti-inflammatory and clinical effects of beclomethasone dipropionate and salmeterol in moderate asthma. *Eur Respir J* 2002 Jul 1;20(1):66-72.
49. Godon P, Boulet LP, Malo JL, Cartier A, Lemiere C. Assessment and evaluation of symptomatic steroid-naive asthmatics without sputum eosinophilia and their response to inhaled corticosteroids. *Eur Respir J* 2002 Dec 1;20(6):1364-69.
50. Jayaram L, Pizzichini E, Lemiere C, Man SFP, Cartier A, Hargreave FE, *et al.* Steroid naive eosinophilic asthma: anti-inflammatory effects of fluticasone and montelukast. *Thorax* 2005 Feb 1;60(2):100-5.

51. Green RH, Brightling CE, McKenna S, Hargadon B, Parker D, Bradding P, et al. Asthma exacerbations and sputum eosinophil counts: a randomised controlled trial. *The Lancet* 2002 Nov 30;360(9347):1715-21.
52. Hamid Q, Kelly MM, Linden M, Louis R, et al. Methods of sputum processing for cell counts, immunocytochemistry and in situ hybridization, *Eur Respir J* 2002 Jul 1;20 (37-suppl)19S-23S.
53. Stockley RA, Bayley DL. Validation of assays for inflammatory mediators in sputum. *Eur Respir J* 2000 Apr 1;15(4):778-81.
54. Keatings V, Leigh R, Peterson C, Shute J, et al. Analysis of fluid-phase mediators. *Eur Respir J* 2002 Jul 1;20 (37_suppl)24S-39S.
55. Erin EM, Jenkins GR, Kon OM, Zacharasiewicz AS, Nicholson GC, Neighbour H, Tennant RC, Tan AJ, Leaker BR, Bush A, Jose PJ, Barnes PJ, Hansel TT. Optimized dialysis and protease inhibition of sputum dithiothreitol supernatants. *Am J Respir Crit Care Med* 2008 Jan 15;177(2):132-41.
56. Efthimiadis A, Pizzichini MM, Pizzichini E, Dolovich J, Hargreave FE. Induced sputum cell and fluid-phase indices of inflammation: comparison of treatment with dithiothreitol vs phosphate-buffered saline. *Eur Respir J* 1997 Jun 1;10(6):1336-40.
57. Perng DW, Huang HY, Lee YC, Perng RP. Leukotriene Modifier vs Inhaled Corticosteroid in Mild-to-Moderate Asthma: Clinical and Anti-inflammatory Effects. *Chest* 2004 May 1;125(5):1693-9.
58. Cianchetti S, Bacci E, Ruocco L, Bartoli ML, Ricci M, Pavia T, et al. Granulocyte markers in hypertonic and isotonic saline-induced sputum of asthmatic subjects. *Eur Respir J* 2004 Dec 1;24(6):1018-24.
59. in 't Veen JC, de Gouw HW, Smits HH, Sont JK, Hiemstra PS, Sterk PJ, et al. Repeatability of cellular and soluble markers of inflammation in induced sputum from patients with asthma. *Eur Respir J* 1996 Dec 1;9(12):2441-7.
60. Boulay ME, Prince P, Deschesnes F, Chakir J, Boulet LP. Metalloproteinase-9 in induced sputum correlates with the severity of the late allergen-induced asthmatic response. *Respiration* 2004 May;71(3):216-24.
61. Cataldo DD, Bettiol J, Noel A, Bartsch P, Foidart JM, Louis R. Matrix Metalloproteinase-9, but Not Tissue Inhibitor of Matrix Metalloproteinase-1, Increases in the Sputum From Allergic Asthmatic Patients After Allergen Challenge. *Chest* 2002 Nov 1;122(5):1553-9.
62. Hoshino M, Nakamura Y, Sim J, Shimojo J, Isogai S. Bronchial subepithelial fibrosis and expression of matrix metalloproteinase-9 in asthmatic airway inflammation. *J Allergy Clin Immunol* 1998 Nov;102(5):783-8.
63. Ko FWS, Diba C, Roth M, McKay K, Johnson PRA, Salome C, et al. A Comparison of Airway and Serum Matrix Metalloproteinase-9 Activity Among Normal Subjects, Asthmatic Patients, and Patients With Asthmatic Mucus Hypersecretion. *Chest* 2005 Jun 1;127(6):1919-27.
64. Mattos W, Lim S, Russell R, Jatakanon A, Chung KF, Barnes PJ. Matrix Metalloproteinase-9 Expression in Asthma: Effect of Asthma Severity, Allergen Challenge, and Inhaled Corticosteroids. *Chest* 2002 Nov 1;122(5):1543-52.
65. Vignola A, Riccobono L, Mirabella A, Profita M, Chanze P, Bellia V, et al. Sputum Metalloproteinase-9/Tissue Inhibitor of Metalloproteinase-1 Ratio Correlates with Airflow

- Obstruction in Asthma and Chronic Bronchitis. *Am J Respir Crit Care Med* 1998 Dec 1;158(6):1945-50.
66. Berry MA, Parker D, Neale N, Woodman L, Morgan A, Monk P, et al. Sputum and bronchial submucosal IL-13 expression in asthma and eosinophilic bronchitis. *J Allergy Clin Immunol* 2004 Nov;114(5):1106-9.
 67. Hadjicharalambous C, Dent G, May RD, Handy RL, Anderson IK, Davies DE, et al. Measurement of eotaxin (CCL11) in induced sputum supernatants: validation and detection in asthma. *J Allergy Clin Immunol* 2004 Apr;113(4):657-62.
 68. Kelly MM, Leigh R, Carruthers S, Horsewood P, Gleich GJ, Hargreave FE, et al. Increased detection of interleukin-5 in sputum by addition of protease inhibitors. *Eur Respir J* 2001 Oct;18(4):685-91.
 69. Gibson PG, Simpson JL, Saltos N. Heterogeneity of airway inflammation in persistent asthma: evidence of neutrophilic inflammation and increased sputum interleukin-8. *Chest* 2001 May 1;119(5):1329-36.
 70. Norzila MZ, Fakes K, Henry RL, Simpson J, Gibson PG. Interleukin-8 secretion and neutrophil recruitment accompanies induced sputum eosinophil activation in children with acute asthma. *Am J Respir Crit Care Med* 2000 Mar 1;161(3):769-74.
 71. Diamant Z, Sampson AP. Anti-inflammatory mechanisms of leukotriene modulators. *Clin Exp Allergy* 1999 Nov;29(11):1449-53.
 72. Dworski R. Oxidant stress in asthma. *Thorax* 2000 Oct 1;55(90002):51S-53S.
 73. McFarlane AJ, Dworski R, Sheller JR, Pavord ID, Barry KA, Barnes NC. Sputum cysteinyl leukotrienes increase 24 hours after allergen inhalation in atopic asthmatics. *Am J Respir Crit Care Med* 2000 May 1;161(5):1553-8.
 74. Pavord ID, Ward R, Woltmann G, Wardlaw AJ, Sheller JR, Dworski R. Induced sputum eicosanoid concentrations in asthma. *Am J Respir Crit Care Med* 1999 Dec 1;160(6):1905-9.
 75. Lex C, Payne DN, Zacharasiewicz A, Li AM, Wilson NM, Hansel TT, et al. Sputum induction in children with difficult asthma: safety, feasibility, and inflammatory cell pattern. *Pediatr Pulmonol* 2005 Apr;39(4):318-24.
 76. Wood LG, Garg ML, Simpson JL, Mori TA, Croft KD, Wark PAB, et al. Induced Sputum 8-Isoprostane Concentrations in Inflammatory Airway Diseases. *Am J Respir Crit Care Med* 2005 Mar 1;171(5):426-30.
 77. Grootendorst DC, Sont JK, Willems LN, Kluin-Nelemans JC, Van Krieken JH, Veselic-Charvat M et al. Comparison of inflammatory cell counts in asthma: induced sputum vs bronchoalveolar lavage and bronchial biopsies. *Clin Exp Allergy* 1997; 27(7):769-779.
 78. Vlachos-Mayer H, Leigh R, Sharon RF, Hussack P, Hargreave FE. Success and safety of sputum induction in the clinical setting. *Eur Respir J* 2000; 16(5):997-1000.
 79. Petsky HL, Kynaston JA, Turner C, Li AM, Cates CJ, Lasserson TJ et al. Tailored interventions based on sputum eosinophils versus clinical symptoms for asthma in children and adults. *Cochrane Database Syst Rev* 2007;(2):CD005603.
 80. de Kluijver J, Evertse CE, Schrupf JA, van der Veen H, Zwinderman AH, Hiemstra PS et al. Asymptomatic Worsening of Airway Inflammation during Low-Dose Allergen Exposure in Asthma: Protection by Inhaled Steroids. *Am J Respir Crit Care Med* 2002; 166(3):294-300.

81. Truyen E, Coteur L, Dilissen E, Overbergh L, Dupont LJ, Ceuppens JL et al. Evaluation of airway inflammation by quantitative Th1/Th2 cytokine mRNA measurement in sputum of asthma patients. *Thorax* 2006; 61(3):202-8.
82. Brasier AR, Victor S, Boetticher G, Ju H, Lee C, Bleecker ER et al. Molecular phenotyping of severe asthma using pattern recognition of bronchoalveolar lavage-derived cytokines. *J Allergy Clin Immunol* 2008; 121(1):30-37.
83. Mutlu GM, Garey KW, Robbins RA, Danziger LH, Rubinstein I. Collection and analysis of exhaled breath condensate in humans. *Am J Respir Crit Care Med* 2001;164(5):731-7.
84. Scheideler L, Manke HG, Schwulera U, Inacker O, Hammerle H. Detection of nonvolatile macromolecules in breath. A possible diagnostic tool? *Am Rev Respir Dis* 1993;148(3):778-84.
85. Horvath I, Hunt J, Barnes PJ, On behalf of the ATS/ERS task force on exhaled breath condensate. Exhaled breath condensate: methodological recommendations and unresolved questions. *Eur Respir J* 2005;26(3):523-48.
86. Ferreira IM, Hazari MS, Gutierrez C, Zamel N, Chapman KR. Exhaled nitric oxide and hydrogen peroxide in patients with chronic obstructive pulmonary disease: effects of inhaled beclomethasone. *Am J Respir Crit Care Med* 2001;164(6):1012-5.
87. Hunt J. Exhaled breath condensate: an evolving tool for noninvasive evaluation of lung disease. *J Allergy Clin Immunol* 2002;110(1):28-34.
88. van Beurden WJ, Harff GA, Dekhuijzen PN, van den Bosch MJ, Creemers JP, Smeenk FW. An efficient and reproducible method for measuring hydrogen peroxide in exhaled breath condensate. *Respir Med* 2002;96(3):197-203.
89. Hoffmeyer F, Raulf-Heimsoth M, Harth V, Bünger J, Brüning T. Comparative analysis of selected exhaled breath biomarkers obtained with two different temperature-controlled devices. *BMC Pulm Med* 2009 Nov 30;9:48.
90. Martin AN, Farquar GR, Jones AD, Frank M. Human breath analysis: methods for sample collection and reduction of localized background effects. *Anal Bioanal Chem* 2010 Jan;396(2):739-50.
91. Antczak A, Kurmanowska Z, Kasielski M, Nowak D. Inhaled glucocorticosteroids decrease hydrogen peroxide level in expired air condensate in asthmatic patients. *Respir Med* 2000;94(5):416-21.
92. Emelyanov A, Fedoseev G, Abulimity A, Rudinski K, Fedoulov A, Karabanov A, et al. Elevated concentrations of exhaled hydrogen peroxide in asthmatic patients. *Chest* 2001;120(4):1136-9.
93. Van Hoydonck PGA, Wuyts WA, Vanaudenaerde BM, Schouten EG, Dupont LJ, Temme EHM. Quantitative analysis of 8- isoprostane and hydrogen peroxide in exhaled breath condensate. *Eur Respir J* 2004;23(2):189-92.
94. Nowak D, Antczak A, Krol M, Pietras T, Shariati B, Bialasiewicz P, et al. Increased content of hydrogen peroxide in the expired breath of cigarette smokers. *Eur Respir J* 1996;9(4):652-7.
95. Jobsis Q, Raatgeep HC, Hermans PW, de Jongste JC. Hydrogen peroxide in exhaled air is increased in stable asthmatic children. *Eur Respir J* 1997;10(3):519-21.
96. Cap P, Chladek J, Pehal F, Maly M, Petru V, Barnes PJ, et al. Gas chromatography/mass spectrometry analysis of exhaled leukotrienes in asthmatic patients. *Thorax* 2004;59(6):465-70.

97. Csoma Z, Kharitonov SA, Balint B, Bush A, Wilson NM, Barnes PJ. Increased leukotrienes in exhaled breath condensate in childhood asthma. *Am J Respir Crit Care Med* 2002;166(10):1345–9.
98. Baraldi E, Carraro S, Alinovi R, Pesci A, Ghio L, Bodini A, et al. Cysteinyl leukotrienes and 8-isoprostane in exhaled breath condensate of children with asthma exacerbations. *Thorax* 2003;58(6):505–9.
99. Montuschi P, Barnes PJ, Ciabattoni G. Measurement of 8-isoprostane in exhaled breath condensate. *Methods Mol Biol* 2010;594:73-84.
100. Montuschi P, Corradi M, Ciabattoni G, Nightingale J, Kharitonov SA, Barnes PJ. Increased 8-isoprostane, a marker of oxidative stress, in exhaled condensate of asthma patients. *Am J Respir Crit Care Med* 1999;160(1):216–20.
101. Baraldi E, Ghio L, Piovan V, Carraro S, Ciabattoni G, Barnes PJ, et al. Increased exhaled 8-isoprostane in childhood asthma. *Chest* 2003;124(1):25–31.
102. Battaglia S, Den Hertog H, Timmers MC, Lazeroms SPG, Vignola AM, Rabe KF, et al. Small airways function and molecular markers in exhaled air in mild asthma. *Thorax* 2005;60(8):639–44.
103. Kostikas K, Papatheodorou G, Ganas K, Psathakis K, Panagou P, Loukides S. pH in expired breath condensate of patients with inflammatory airway diseases. *Am J Respir Crit Care Med* 2002; 165(10):1364–70.
104. Vaughan J, Ngamtrakulpanit L, Pajewski TN, Turner R, Nguyen TA, Smith A, et al. Exhaled breath condensate pH is a robust and reproducible assay of airway acidity. *Eur Respir J* 2003;22(6): 889–94.
105. Carpagnano GE, Barnes PJ, Francis J, Wilson N, Bush A, Kharitonov SA. Breath condensate pH in children with cystic fibrosis and asthma: a new noninvasive marker of airway inflammation? *Chest* 2004;125(6):2005–10.
106. Carraro S, Folesani G, Corradi M, Zanconato S, Gaston B, Baraldi E. Acid-base equilibrium in exhaled breath condensate of allergic asthmatic children. *Allergy* 2005;60(4):476–81.
107. Hunt JF, Fang KE, Malik RA, Snyder AS, Malhotra NE, Platts-Mills TA, et al. Endogenous airway acidification. Implications for asthma pathophysiology. *Am J Respir Crit Care Med* 2000;161(3):694–9.
108. Effros RM, Dunning MB, III, Biller J, Shaker R. The promise and perils of exhaled breath condensates. *Am J Physiol Lung Cell Mol Physiol* 2004; 287(6):L1073-L1080.
109. Czebe K, Barta I, Antus B, Valyon M, Horvath I, Kullmann T. Influence of condensing equipment and temperature on exhaled breath condensate pH, total protein and leukotriene concentrations. *Respiratory Medicine* 2008; 102(5):720-5.
110. Soyer OU, Dizdar EA, Keskin O, Lilly C, Kalayci O. Comparison of two methods for exhaled breath condensate collection. *Allergy* 2006; 61(8):1016-18.
111. Van Hoydonck PGA, Wuyts WA, Vanaudenaerde BM, Schouten EG, Dupont LJ, Temme EHM. Quantitative analysis of 8-isoprostane and hydrogen peroxide in exhaled breath condensate. *Eur Respir J* 2004; 23(2):189-92.
112. Borrill ZL, Roy K, Singh D. Exhaled breath condensate biomarkers in COPD. *Eur Respir J* 2008; 32(2):472-86.
113. Sack U, Scheibe R, Wötzel M et al, Multiplex analysis of cytokines in exhaled breath condensate. *Cytometry A* 2006 Mar;69(3):169-72.

114. Carraro S, Rezzi S, Reniero F, Heberger K, Giordano G, Zanconato S et al. Metabolomics applied to exhaled breath condensate in childhood asthma. *Am J Respir Crit Care Med* 2007; 175(10):986-90.
115. de Laurentiis G, Paris D, Melck D, Maniscalco M, Marsico S, Corso G et al. Metabonomic analysis of exhaled breath condensate in adults by nuclear magnetic resonance spectroscopy. *Eur Respir J* 2008; 32(5):1175-83.
116. Carraro S, Giordano G, Reniero F, Perilongo G, Baraldi E. Metabolomics: a new frontier for research in pediatrics. *J Pediatr* 2009 May;154(5):638-44.
117. Tufvesson E, Bjermer L. Methodological improvements for measuring eicosanoids and cytokines in exhaled breath condensate. *Respir Med* 2006; 100(1):34-38.
118. Rosias PP, Robroeks CM, Kester A, den Hartog GJ, Wodzig WK, Rijkers GT et al. Biomarker reproducibility in exhaled breath condensate collected with different condensers. *Eur Respir J* 2008; 31(5):934-942.
119. ATS/ERS Recommendations for Standardized Procedures for the Online and Offline Measurement of Exhaled Lower Respiratory Nitric Oxide and Nasal Nitric Oxide, 2005. *Am J Respir Crit Care Med* 2005 Apr 15;171(8):912-30.
120. 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 Mar 1;21(3):433-8.
121. Silkoff PE, Stevens A, Pak J, Bucher-Bartelson B, Martin RJ. A method for the standardized offline collection of exhaled nitric oxide. *Chest* 1999 Sep;116(3):754-9.
122. Diamant Z, Boot JD, Kamerling I, Bjermer L. Methods used in clinical development of novel anti-asthma therapies. *Respir Med* 2008;102(3):332-8. Review.
123. Kharitonov SA, O'Connor BJ, Evans DJ, Barnes PJ. Allergen-induced late asthmatic reactions are associated with elevation of exhaled nitric oxide. *Am J Respir Crit Care Med* 1995 Jun;151(6):1894-9.
124. 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 Sep 1;164(5):738-43.
125. Zacharasiewicz A, Wilson N, Lex C, Erin EM, Li AM, Hansel T, Khan M, Bush A. Clinical use of noninvasive measurements of airway inflammation in steroid reduction in children. *Am J Respir Crit Care Med* 2005 May 15;171(10):1077-82.
126. Leuppi JD, Salome CM, Jenkins CR, Anderson sd, Xuan W, Marks GB, et al. Predictive Markers of Asthma Exacerbation during Stepwise Dose Reduction of Inhaled Corticosteroids. *Am J Respir Crit Care Med* 2001 Feb 1;163(2):406-12.
127. Jatakanon A, Kharitonov S, Lim S, Barnes PJ. Effect of differing doses of inhaled budesonide on markers of airway inflammation in patients with mild asthma. *Thorax* 1999 Feb;54(2):108-14.
128. Kharitonov SA, Donnelly LE, Montuschi P, Corradi M, Collins JV, Barnes PJ. Dose-dependent onset and cessation of action of inhaled budesonide on exhaled nitric oxide and symptoms in mild asthma. *Thorax* 2002 Oct 1;57(10):889-96.
129. Sandrini A, Ferreira IM, Gutierrez C, Jardim JR, Zamel N, Chapman KR. Effect of montelukast on exhaled nitric oxide and nonvolatile markers of inflammation in mild asthma. *Chest* 2003 Oct;124(4):1334-40.

130. Straub DA, Moeller A, Minocchieri S, Hamacher J, Sennhauser FH, Hall GL, *et al.* The effect of montelukast on lung function and exhaled nitric oxide in infants with early childhood asthma. *Eur Respir J* 2005 Feb 1;25(2):289-94.
131. Jatakanon A, Lim S, Kharitonov SA, Chung KF, Barnes PJ. Correlation between exhaled nitric oxide, sputum eosinophils, and methacholine responsiveness in patients with mild asthma. *Thorax* 1998 Feb;53(2):91-5.
132. Leuppi JD, Salome CM, Jenkins CR, Koskela H, Brannan JD, Anderson SD, *et al.* Markers of airway inflammation and airway hyperresponsiveness in patients with well-controlled asthma. *Eur Respir J* 2001 Sep 1;18(3):444-50.
133. van Rensen ELJ, Straathof KCM, Veselic-Charvat MA, Zwinderman AH, Bel EH, Sterk PJ. Effect of inhaled steroids on airway hyperresponsiveness, sputum eosinophils, and exhaled nitric oxide levels in patients with asthma. *Thorax* 1999 May 1;54(5):403-8.
134. Berry M, Hargadon B, Morgan A, Shelley M, Richter J, Shaw D *et al.* Alveolar nitric oxide in adults with asthma: evidence of distal lung inflammation in refractory asthma. *Eur Respir J* 2005; 25(6):986-91.
135. Cohen J, Douma WR, ten Hacken NH *et al.* Ciclesonide improves measures of small airway involvement in asthma. *Eur Respir J* 2008; 31: 1213–20.
136. Puckett JL, Taylor RW, Leu SY, Guijon OL, Aledia AS, Galant SP, George SC. Clinical patterns in asthma based on proximal and distal airway nitric oxide categories. *Respir Res* 2010 Apr 28;11(1):47.
137. Pijnenburg MW, de Jongste JC. Exhaled nitric oxide in childhood asthma: a review. *Clin Exp Allergy* 2008; 38(2):246-59.
138. Piacentini GL, Bodini A, Peroni DG, Miraglia del Giudice M, Jr., Costella S, Boner AL. Reduction in exhaled nitric oxide immediately after methacholine challenge in asthmatic children. *Thorax* 2002; 57(9):771-73.
139. Alving K, Janson C, Nordvall L. Performance of a new hand-held device for exhaled nitric oxide measurement in adults and children. *Respir Res* 2006; 7:67.
140. Menzies D, Nair A, Lipworth BJ. Portable exhaled nitric oxide measurement: Comparison with the "gold standard" technique. *Chest* 2007; 131(2):410-14.
141. Pizzimenti S, Bugiani M, Piccioni P, Heffler E, Carosso A, Guida G *et al.* Exhaled nitric oxide measurements: correction equation to compare hand-held device to stationary analyzer. *Respir Med* 2008; 102(9):1272-75.
142. 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 Jun;9(2):122-6.
143. Boot JD, De Haas S, Tarasevych S, Roy C, Wang L, Amin D, Cohen J, Sterk PJ, Miller B, Paccaly A, Burggraaf J, Cohen AF, Diamant Z. Effect of an NK1/NK2 receptor antagonist on airway responses and inflammation to allergen in asthma. *Am J Respir Crit Care Med* 2007; 175(5):450-57.
144. Jones SL, Kittelson J, Cowan J, 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(5):738-743.
145. Taylor DR, Pijnenburg MW, Smith AD, Jongste JCD. Exhaled nitric oxide measurements: clinical application and interpretation. *Thorax* 2006; 61(9):817-827.

146. Jatakanon A, Kharitonov S, Lim S, Barnes PJ. Effect of differing doses of inhaled budesonide on markers of airway inflammation in patients with mild asthma. *Thorax* 1999; 54(2):108-14.
147. 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(4):453-59.
148. Fortuna AM, Feixas T, Gonzalez M, Casan P. Diagnostic utility of inflammatory biomarkers in asthma: exhaled nitric oxide and induced sputum eosinophil count. *Respir Med* 2007; 101(11):2416-21.
149. 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(4):473-78.
150. 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(7):831-36.
151. 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(9643):1065-72.
152. 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(2):93-7.
153. Petsky HL, Cates CJ, Li AM, Kynaston JA, Turner C, Chang AB. Tailored interventions based on exhaled nitric oxide versus clinical symptoms for asthma in children and adults. *Cochrane Database Syst Rev* 2009;Oct 7(4):CD006340.
154. Pauling L, Robinson AB, Teranishi RR, Cary P. Quantitative analysis of urine vapor and breath by gas-liquid partition chromatography. *Proc Natl Acad Sci USA* 1971;68:2374-76.
155. Phillips M, Greenberg J. Method for the collection and analysis of volatile compounds in the breath. *J Chromatogr* 1991;564:242-49.
156. Moser B, Bodrogi F, Eibl G, et al. Mass spectrometric profile of exhaled breath. Field study by PTR-MS. *Respir Physiol Neurobiol* 2005;145:295-300.
157. Phillips M. Method for the collection and assay of volatile organic compounds in breath. *Anal Biochem* 1997;247:272-8.
158. D'Amico A, Di Natale C, Paolesse R, Macagnano A, Martinelli E, Pennazza G et al. Olfactory systems for medical applications, *Sens Actuators B* 2008;130:458-65.
159. Röck F, Barsan N, Weimar U. Electronic nose: current status and future trends. *Chem Rev* 2008;108:705-13.
160. Lewis NS. Comparisons between mammalian and artificial olfaction based on arrays of carbon black-polymer composite vapor detectors. *Acc Chem Res* 2004;37:663-72.
161. Thaler ER, Hanson CW. Medical application of electronic nose technology. *Expert Rev Med Device* 2005;2:559-66.
162. Friedrich MJ. Scientists seek to sniff out diseases: electronic "noses" may someday be diagnostic tools. *JAMA* 2009;301:585-6.

163. Dragonieri S, Schot R, Mertens BJ, Le Cessie S, Gauw SA, Spanevello A, Resta O, Willard NP, Vink TJ, Rabe KF, Bel EH, Sterk PJ. An electronic nose in the discrimination of patients with asthma and controls. *Allergy Clin Immunol*. 2007;120:856-62.
164. Montuschi P, Santonico M, Pennazza G, Mondino C, Mantini G, Martinelli E, Capuano R, Ciabattini G, Paolesse R, Di Natale C, Barnes PJ, D'Amico A. Diagnostic performance of an electronic nose, fractional exhaled nitric oxide, and lung function testing in asthma. *Chest* 2010;137:790-6.
165. Scott SM, James D, and Ali. Data analysis for electronic nose systems. *Microchim Acta* 2006;156:183-207.
166. Auffray C, Chen Z, Hood L. Systems medicine: the future of medical genomics and healthcare. *Genome Med* 2009;1:2.
167. Broadhurst DI, Kell DB. Statistical strategies for avoiding false discoveries in metabolomics and related experiments. *Metabolomics* 2006;2:171-96.
168. Skaham O, Carmel L, Harel D. On mappings between electronic noses. *Sensors Actuators B* 2005;106:76-82.
169. Machado RF, Laskowski D, Deffenderfer O, Burch T, Zheng S, Mazzone PJ, Merkhail T, Jennings C, Stoller JK, Pyle J, Duncan J, Dweik RA, Erzurum SP. Detection of lung cancer by sensor array analysis of exhaled breath. *Am J Respir Crit Care Med* 2005;171:1286-91.
170. Mazzone PJ, Hammel J, Dweik R, Na J, Czich C, Laskowski D, Mekhail T. Diagnosis of lung cancer by analysis of exhaled breath with a colorimetric sensor array. *Thorax* 2007;62:565-8.
171. Peng G, Tisch U, Adams O, Hakim M, Shehada N, Broza YY, Billian S, Abdah-Bortnyak R, Kuten A, Haick H. Diagnosing lung cancer in exhaled breath using gold nanoparticles. *Nature Nanotechnol* 2009;4:669-73.
172. D'Amico A, Pennazza G, Santonico M, Martinelli E, Roscioni C, Galluccio G, Paolesse R, Di Natale C. An investigation on electronic nose diagnosis of lung cancer. *Lung Cancer* 2010;68:170-6.
173. Dragonieri S, Annema JT, Schot R, van der Schee MPC, Spanevello A, Carratu P, Resta O, Rabe KF, Sterk PJ. An electronic nose in the discrimination of patients with non-small cell lung cancer and COPD. *Lung Cancer* 2009;64:166-70.
174. Fens N, Zwinderman AH, van der Schee MP, de Nijs SB, Dijkers E, Roldaan AC, Cheung D, Bel EH, Sterk PJ. Exhaled breath profiling enables discrimination of chronic obstructive pulmonary disease and asthma. *Am J Respir Crit Care Med* 2009;180:1076-82.
175. Fend R, Kolk AHJ, Bessant C, Buijtelts P, Klatser PR, Woodman AC,. Prospects for clinical application of electronic nose technology to early detection of Mycobacterium tuberculosis in culture and sputum. *J Clin Microbiol* 2006;44:2039-45.
176. Thaler ER, Huang D, Giebeig L, Palmer J, Lee D, Hansen JW, Cohen N. Use of an electronic nose for detection of biofilms. *Am J Rhinol* 2008;22:29-33.
177. Knottnerus JA, Muris JW. Assessment of the accuracy of diagnostic tests: the cross-sectional study. *J Clin Epidemiol* 2003;56:1118-28.
178. Bossuyt PM, Reitsma JB, Bruns DE, Gatsonis CA, Glasziou PP, Irwig LM, Moher D, Rennie D, de Vet HC, Lijmer JG. The STARD statement for reporting studies of diagnostic accuracy: explanation and elaboration. *Ann Intern Med* 2003;138:W1-12.
179. Gluud C, Gluud LL. Evidence based diagnostics. *BMJ* 2005;330:724-6.

180. Fens N, Rodaan AC, Bel EH, Sterk PJ. External validation of exhaled breath molecular profiling in identifying COPD and asthma. *ATS Meeting May 2010 (abstract); in press.*
181. Nathan RA, Eccles R, Howarth PH, Steinsvåg SK, Togias A. Objective monitoring of nasal patency and nasal physiology in rhinitis. *J Allergy Clin Immunol.* 2005 Mar;115(3 Suppl 1):S442-59. Review.
182. Naclerio RM, Proud D, Togias AG, Adkinson NF, Jr., Meyers DA, Kagey-Sobotka A, et al. Inflammatory mediators in late antigen-induced rhinitis. *N Engl J Med* 1985; 313:65-70.
183. Greiff L, Pipkorn U, Alkner U, Persson CG. The 'nasal pool' device applies controlled concentrations of solutes on human nasal airway mucosa and samples its surface exudations/secretions. *Clin Exp Allergy* 1990; 20:253-9.
184. Belda J, Parameswaran K, Keith PK, Hargreave FE. Repeatability and validity of cell and fluid-phase measurements in nasal fluid: a comparison of two methods of nasal lavage. *Clin Exp Allergy* 2001; 31:1111-5.
185. Boot JD, Chandosing P, de Kam ML, Mascelli MA, Das AM, Gerth van Wijk R, de Groot H, Verhoosel R, Hiemstra PS, Diamant Z. Applicability and reproducibility of measurements of biomarkers in allergic rhinitis. *J Investig Allergol Clin Immunol* 2008;18(6):433-42.
186. Grunberg K, Timmers MC, Smits HH, de Klerk EP, Dick EC, Spaan WJ et al. Effect of experimental rhinovirus 16 colds on airway hyperresponsiveness to histamine and interleukin-8 in nasal lavage in asthmatic subjects in vivo. *Clin Exp Allergy* 1997; 27(1):36-45.
187. Erin EM, Zacharasiewicz AS, Nicholson GC, Tan AJ, Higgins LA, Williams TJ et al. Topical corticosteroid inhibits interleukin-4, -5 and -13 in nasal secretions following allergen challenge. *Clin Exp Allergy* 2005; 35(12):1608-14.
188. Chawes BL, Edwards MJ, Shamji B, Walker C, Nicholson GC, Tan AJ, Følsgaard NV, Bønnelykke K, Bisgaard H, Hansel TT. A novel method for assessing unchallenged levels of mediators in nasal epithelial lining fluid. *J Allergy Clin Immunol.* 2010 Mar 19. [Epub ahead of print]
189. Lü FX, Esch RE. Novel nasal secretion collection method for the analysis of allergen specific antibodies and inflammatory biomarkers. *J Immunol Methods* 2010 Apr 30;356(1-2):6-17.
190. Godthelp T, Holm AF, Fokkens WJ, Doornenbal P, Mulder PG, Hoefsmit EC et al. Dynamics of nasal eosinophils in response to a nonnatural allergen challenge in patients with allergic rhinitis and control subjects: a biopsy and brush study. *J Allergy Clin Immunol* 1996; 97(3):800-11.
191. Boot JD, Chandosing P, De Kam ML, Mascelli MA, Miller B, Gerth van Wijk R, De Groot H, Cohen AF, Diamant Z. Nasal nitric oxide: longitudinal reproducibility and the effects of a nasal allergen challenge in patients with allergic rhinitis. *Allergy* 2007;62(4):378-84.
192. Struben VM, Wieringa MH, Feenstra L, de Jongste JC. Nasal nitric oxide and nasal allergy. *Allergy.* 2006 Jun;61(6):665-70. Review.
193. Naclerio RM, Meier HL, Kagey-Sobotka A, Adkinson NF, Jr., Meyers DA, Norman PS, et al. Mediator release after nasal airway challenge with allergen. *Am Rev Respir Dis* 1983; 128:597-602.

194. Howarth PH, Persson CG, Meltzer EO, Jacobson MR, Durham SR, Silkoff PE. Objective monitoring of nasal airway inflammation in rhinitis. *J Allergy Clin Immunol* 2005; 115:S414-41.
195. de Graaf-in't Veld C, Garrelds IM, Jansen AP, Van Toorenenbergen AW, Mulder PG, Meeuwis J, *et al.* Effect of intranasal fluticasone propionate on the immediate and late allergic reaction and nasal hyperreactivity in patients with a house dust mite allergy. *Clin Exp Allergy* 1995; 25:966-73.
196. van Wijk RG, Zijlstra FJ, van Toorenenbergen AW, Vermeulen A, Dieges PH. Isolated early response after nasal allergen challenge is sufficient to induce nasal hyperreactivity. *Ann Allergy* 1992; 69:43-7.
197. Belkowski SM, Boot JD, Mascelli MA, Diamant Z, de Garavilla L, Hertzog B, *et al.* Cleaved secretory leucocyte protease inhibitor as a biomarker of chymase activity in allergic airway disease. *Clin Exp Allergy* 2009; 39:1179-86.
198. Jacobson MR, Juliusson S, Lowhagen O, Balder B, Kay AB, Durham SR. Effect of topical corticosteroids on seasonal increases in epithelial eosinophils and mast cells in allergic rhinitis: a comparison of nasal brush and biopsy methods. *Clin Exp Allergy* 1999; 29:1347-55.
199. Terada N, Hamano N, Kim WJ, Hirai K, Nakajima T, Yamada H, *et al.* The kinetics of allergen-induced eotaxin level in nasal lavage fluid: its key role in eosinophil recruitment in nasal mucosa. *Am J Respir Crit Care Med* 2001; 164:575-9.
200. Di Lorenzo G, Mansueto P, Melluso M, Candore G, Colombo A, Pellitteri ME, *et al.* Allergic rhinitis to grass pollen: measurement of inflammatory mediators of mast cell and eosinophils in native nasal fluid lavage and in serum out of and during pollen season. *J Allergy Clin Immunol* 1997; 100:832-7.
201. Persson CG, Erjefalt JS, Greiff L, Andersson M, Erjefalt I, Godfrey RW, *et al.* Plasma-derived proteins in airway defence, disease and repair of epithelial injury. *Eur Respir J* 1998; 11:958-70.
202. de Graaf-in't Veld C, Garrelds IM, Koenders S, Gerth van Wijk R. Relationship between nasal hyperreactivity, mediators and eosinophils in patients with perennial allergic rhinitis and controls. *Clin Exp Allergy* 1996; 26:903-8.
203. Raphael GD, Druce HM, Baraniuk JN, Kaliner MA. Pathophysiology of rhinitis. 1. Assessment of the sources of protein in methacholine-induced nasal secretions. *Am Rev Respir Dis* 1988; 138:413-20.
204. Greiff L, Persson CG, Svensson C, Enander I, Andersson M. Loratadine reduces allergen-induced mucosal output of alpha 2-macroglobulin and tryptase in allergic rhinitis. *J Allergy Clin Immunol* 1995; 96:97-103.
205. Meyer P, Andersson M, Persson CG, Greiff L. Steroid-sensitive indices of airway inflammation in children with seasonal allergic rhinitis. *Pediatr Allergy Immunol* 2003; 14:60-5.
206. Svensson C, Andersson M, Greiff L, Blychert LO, Persson CG. Effects of topical budesonide and levocabastine on nasal symptoms and plasma exudation responses in seasonal allergic rhinitis. *Allergy* 1998; 53:367-74.
207. Choi GS, Park HJ, Hur GY, Choi SJ, Shin SY, Ye YM, *et al.* Vascular endothelial growth factor in allergen-induced nasal inflammation. *Clin Exp Allergy* 2009; 39:655-61.
208. Ferrara N. VEGF: an update on biological and therapeutic aspects. *Curr Opin Biotechnol* 2000; 11:617-24.

209. Maniscalco M, de Laurentiis G, Weitzberg E, Lundberg JO, Sofia M. Validation study of nasal nitric oxide measurements using a hand-held electrochemical analyser. *Eur J Clin Invest* 2008; 38(3):197-200.
210. Arnal JF, Didier A, Rami J, M'Rini C, Charlet JP, Serrano E, et al. Nasal nitric oxide is increased in allergic rhinitis. *Clin Exp Allergy* 1997; 27(4):358-362.
211. Maniscalco M, Sofia M, Carratu L, Higenbottam T. Effect of nitric oxide inhibition on nasal airway resistance after nasal allergen challenge in allergic rhinitis. *Eur J Clin Invest* 2001;31:462-66.
212. Lundberg JO. Nitric oxide and the paranasal sinuses. *Anat Rec (Hoboken)* 2008 Nov;291(11):1479-84.
213. Gehring U, Oldenwening M, Brunekreef B, Wieringa MH, Kerkhof M, Smit HA, et al. The impact of ambient NO on online measurements of exhaled and nasal NO: the PIAMA study. *Pediatr Allergy Immunol* 2009; 20:665-72.
214. Kharitonov SA, Rajakulasingam K, O'Connor B, Durham SR, Barnes PJ. Nasal nitric oxide is increased in patients with asthma and allergic rhinitis and may be modulated by nasal glucocorticoids. *J Allergy Clin Immunol* 1997; 99(1 Pt 1):58-64.
215. Scadding G. Nitric oxide in the airways. *Curr Opin Otolaryngol Head Neck Surg* 2007; 15:258-63.
216. Barnes ML, Menzies D, Nair AR, Hopkinson PJ, Lipworth BJ. A proof-of-concept study to assess the putative dose response to topical corticosteroid in persistent allergic rhinitis using adenosine monophosphate challenge. *Clin Exp Allergy* 2007; 37(5):696-703.
217. Wilson AM, Dempsey OJ, Sims EJ, Lipworth BJ. Subjective and objective markers of treatment response in patients with seasonal allergic rhinitis. *Ann Allergy Asthma Immunol* 2000; 85(2):111-14
218. Holgate ST. Inflammatory and structural changes in the airways of patients with asthma. *Respir Med* 2000; 94(Suppl D): S3–S6.
219. Keatings VM, Evans DJ, O'Connor BJ, Barnes PJ. Cellular profiles in asthmatic airways: a comparison of induced sputum, bronchial washings, and bronchoalveolar lavage fluid. *Thorax* 1997; 52: 372–4.
220. Gibson P. Use of induced sputum to examine airway inflammation in childhood asthma. *JACI* 1998; 102: S100–S101.
221. Faul JL, Demers EA, Burke CM, Poulter LW. The reproducibility of repeat measures of airway inflammation in stable atopic asthma. *Am J Respir Crit Care Med* 1999; 160(5 Pt 1): 1457–61.
222. van den Toorn LM, Overbeek SE, de Jongste JC, Leman K, Hoogsteden HC, Prins JB. Airway inflammation is present during clinical remission of atopic asthma. *Am J Respir Crit Care Med*. 2001 Dec 1;164(11):2107-13.
223. Corradi M, Zinelli C, Caffarelli C. Exhaled breath biomarkers in asthmatic children.. *Inflamm Allergy Drug Targets*. 2007 Sep;6(3):150-9.
224. Moeller A, Franklin P, Hall GL, Horak F Jr, Wildhaber JH, Stick SM. Measuring exhaled breath condensates in infants. *Pediatr Pulmonol* 2006; 41: 184–7.
225. Franklin PJ, Turner SW, Mutch RC, Stick SM. Comparison of single breath and tidal breathing exhaled nitric oxide levels in infants. *Eur Respir J* 2004; 23: 369–72.
226. Baraldi E, Jongste Jd. Measurement of exhaled nitric oxide in children, 2001. *Eur J Respir Dis* 2001; 20: 223–37.

227. Motomura C, Odajima H, Tezuka J, Murakami Y, Moriyasu Y, Kando N, Taba N, Hayashi D, Okada K, Nishima S. Effect of age on relationship between exhaled nitric oxide and airway hyperresponsiveness in asthmatic children. *Chest* 2009 Aug;136(2):519-25.
228. Gibson PG, Henry RL, Thomas P. Noninvasive assessment of airway inflammation in children: induced sputum, exhaled nitric oxide, and breath condensate. *Eur Respir J*. 2000 Nov;16(5):1008-15. Review.
229. Marguet C, Ghad S, Couderc L, Lubrano M. What methods can we use to measure inflammation in the asthmatic child? *Arch Pediatr*. 2008 Jun;15(6):1139-45.
230. Lex C, Ferreira F, Zacharasiewicz A, Nicholson AG, Haslam PL, Wilson NM, Hansel TT, Payne DN, Bush A. Airway eosinophilia in children with severe asthma: predictive values of noninvasive tests. *Am J Respir Crit Care Med*. 2006 Dec 15;174(12):1286-91.
231. Bush A, Eber E. The value of FeNO measurement in asthma management: the motion for Yes, it's NO--or, the wrong end of the Stick! *Paediatr Respir Rev*. 2008 Jun;9(2):127-31.
232. Mattes J, Storm van's Gravesande K, Reining U, Alving K, Ihorst G, Henschen M, Kuehr J. NO in exhaled air is correlated with markers of eosinophilic airway inflammation in corticosteroid-dependent childhood asthma. *Eur Respir J* 1999 Jun;13(6):1391-5.
233. Thomas PS, Gibson PG, Wang H, Shah S, Henry RL. The relationship of exhaled nitric oxide to airway inflammation and responsiveness in children. *J Asthma*. 2005 May;42(4):291-5.
234. Aydin A, Taira B, Singer A. Measurement of exhaled nitric oxide in the emergency department in patients with asthma. *J Emerg Med Clin North Am* 2008 Nov;26(4):899-904, vii.
235. Pijnenburg MW, Hofhuis W, Hop WC, De Jongste JC Exhaled nitric oxide predicts asthma relapse in children with clinical asthma remission. *Thorax* 2005 Mar;60(3):215-8.
236. 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 May 26;352(21):2163-73.
237. 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 Jun;9(2):122-6.
238. 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 Jul;35(7):920-5.
239. Carraro S, Gottardi G, Bonetto G, Baraldi E. Exhaled nitric oxide in children with asthma and sinusitis. *Pediatr Allergy Immunol*. 2007 Nov;18 Suppl 18:28-30.
240. Carraro S, Rezzi S, Reniero F, Héberger K, Giordano G, Zanconato S, Guillou C, Baraldi E. Metabolomics applied to exhaled breath condensate in childhood asthma. *Am J Respir Crit Care Med*. 2007 May 15;175(10):986-90.
241. Simpson JL, Wood LG, Gibson PG. Inflammatory mediators in exhaled breath, induced sputum and saliva. *Clin Exp Allergy* 2005; 35: 1180–1185.

Table 1:

Pros and cons of non-invasive lower airways sampling techniques

	Induced Sputum	Exhaled NO	Exhaled Breath Condensate	Electronic Nose
Pros	<ul style="list-style-type: none"> • Multiple biomarkers • Reproducible cell differentials on cytopins • Valid tool for diagnosis (e.g. 'refractory asthma') or assessment of anti-inflammatory therapy 	<ul style="list-style-type: none"> • Non-invasive • Reproducible • Inexpensive measurements • Direct results • Allows serial measurements • Tool for diagnosis/assessment of anti-inflammatory therapy in (allergic) asthma 	<ul style="list-style-type: none"> • Non-invasive • Multiple biomarkers • Allows serial measurements • Potential tool for diagnosis and assessment of anti-inflammatory therapy 	<ul style="list-style-type: none"> • Non-invasive and portable • Almost real-time • Uses high-dimensional biomarker signal • Produces individual signature: 'breath'print • Allows serial measurements • Potential tool for diagnosis and monitoring of anti-inflammatory therapy
Contras	<ul style="list-style-type: none"> • Representative samples available in approx. 80-90% of subjects • Soluble markers subject to dilution • Non-repeatable over short time-period (<12-18 h) • Expertise & experience required (staff/lab) • Rescue medication needed • Contraindicated in severe persistent 	<ul style="list-style-type: none"> • Expensive equipment • Many perturbing factors • Longitudinal samplings within 1 patient are more informative than single measurements- 	<ul style="list-style-type: none"> • Detection assays not fully reproducible • Expensive & time-consuming procedure/assays • Soluble markers subject to dilution • Specialized lab needed 	<ul style="list-style-type: none"> • Sensor technology still developing • Mapping between eNoses required • Off-line SPSS- or R-analysis still required • External validation not completed yet

	asthma/copd/active cardiovascular disorders			
Overall assessment	<ul style="list-style-type: none"> Validated tool for monitoring of the effects of (novel) anti-inflammatory drugs Lengthy, expensive procedure requiring expertise/experience Not suitable for patients with severe bronchoconstriction /comorbidities 	<ul style="list-style-type: none"> Validated tool for diagnosis/monitoring of anti-inflammatory drug-effects Patient & researcher-friendly method 	<ul style="list-style-type: none"> Procedure awaits further validation Patient & researcher-friendly method 	<ul style="list-style-type: none"> Patient & researcher-friendly method Promising technique for both clinical and research applications
Refs*	32, 34, 36, 52, 54	119, 153	85, 108	159, 174

* Position papers and reviews

Figure 1. Allergic airway response

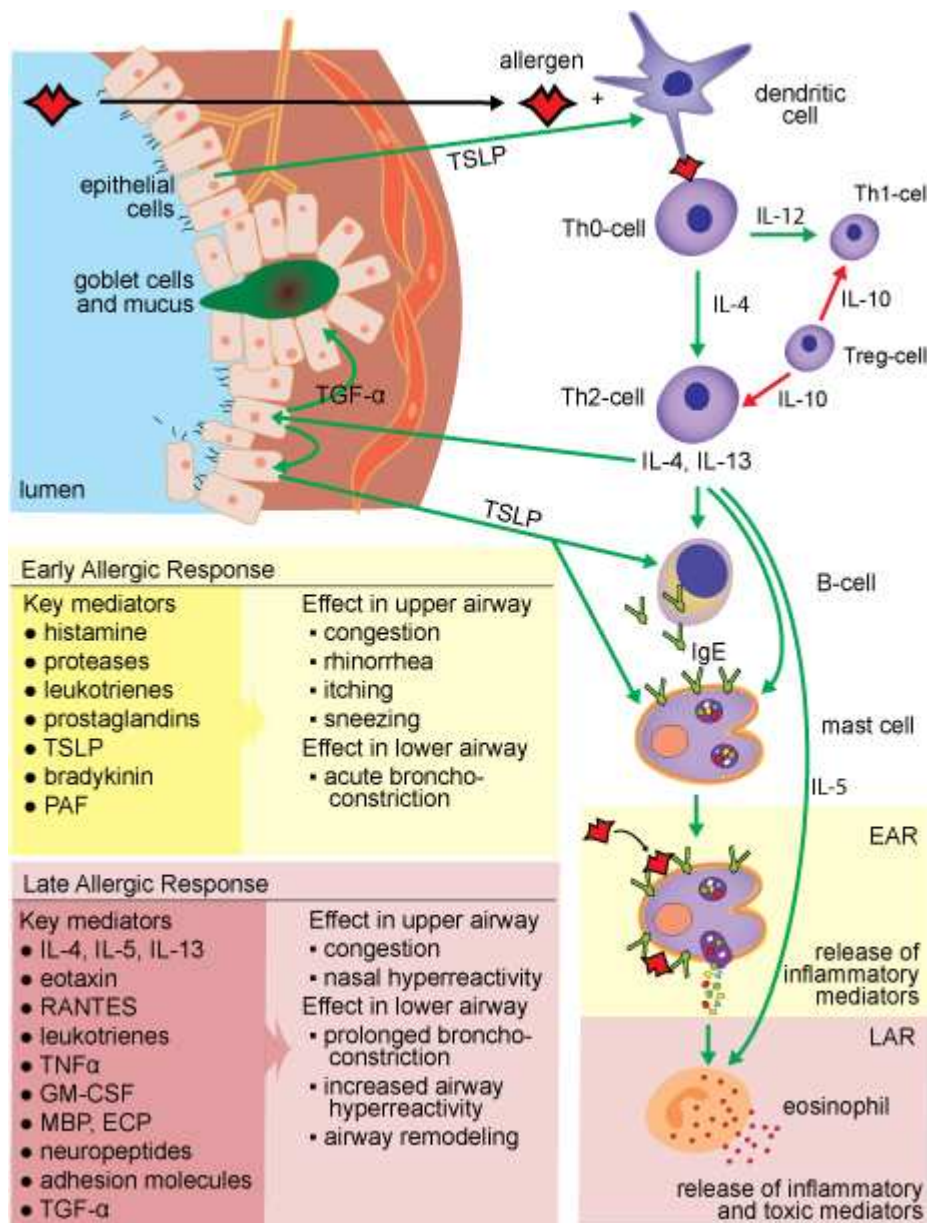


Figure 1: Cells and mediators involved in the allergic responses in asthma and allergic rhinitis. ECP = eosinophilic cationic protein, GM-CSF = granulocyte-macrophage colony stimulating factor, IgE = immunoglobulin-E, IL = interleukin, MBP = major basic protein, PAF = platelet activating factor, TGF- α = transforming growth factor alpha, Th = T helper, TNF α = tumor necrosis factor alpha, TSLP = thymic stromal lymphopoietin (JD Boot, PhD thesis, 2009).