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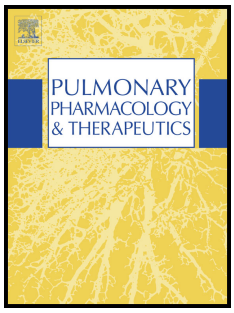

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ACCEPTED MANUSCRIPT

Biomarkers in asthma and allergic rhinitis

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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 PC_{20}methacholine 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.

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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 (FcepsilonRI) 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 FcepsilonRIs 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 outrange 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-facetted 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.

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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, H_2O_2, leukotrienes (LTs), 8-isoprotane and pH have shown the most consistent results. Reproducibly increased EBC-concentrations of H_2O_2, 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 FEV_1 and/or PC_{20}histamine and exhaled H_2O_2 [92]. Alternatively, in patients with similar asthma characteristics, anti-inflammatory therapy with ICS effectively reduced exhaled H_2O_2 along with improvement in FEV_1 [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 ^{0}C, while the RTube uses a cooling sleeve (at -20 ^{0}C), that heats up to 15 ^{0}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 (PC_{20})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 exogeneous) 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 (FEV_1) 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. Clinicall 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 diskinesia (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 E_4 (LTE_4) [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 (PC_{20}methacholine) or histamine (PC_{20}histamine) 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-naieve 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, sofar, 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.

ACCEPTED MANUSCRIPT

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Table 1:

Pros and cons of non-invasive lower airways sampling techniques

	Induced Sputum	**Exhaled NO**	**Exhaled Breath Condensate**	**Electronic Nose**
Pros	• Multiple biomarkers • Reproducible cell differentials on cytospins • Valid tool for diagnosis (e.g. 'refractory asthma') or assessment of anti-inflammatory therapy	• Non-invasive • Reproducible • Inexpensive measurements • Direct results • Allows serial measurements • Tool for diagnosis/assessment of anti-inflammatory therapy in (allergic) asthma	• Non-invasive • Multiple biomarkers • Allows serial measurements • Potential tool for diagnosis and assessment of anti-inflammatory therapy	• Non-invasive and portable • Almost realt-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	• 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	• Expensive equipment • Many perturbing factors • Longitudinal samplings within 1 patient are more informative than single measurements-	• Detection assays not fully reproducible • Expensive & time-consuming procedure/ assays • Soluble markers subject to dilution • Specialized lab needed	• Sensor technology still developing • Mapping between eNoses required • Off-line SPSS- or R-analysis still required • External validation not completed yet

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	asthma/copd/active cardiovascular disorders			
Overall assessment	• 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	• Validated tool for diagnosis/monitoring of anti-inflammatory drug-effects • Patient & researcher-friendly method	• Procedure awaits further validation • Patient & researcher-friendly method	• 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

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Figure 1. Allergic airway response

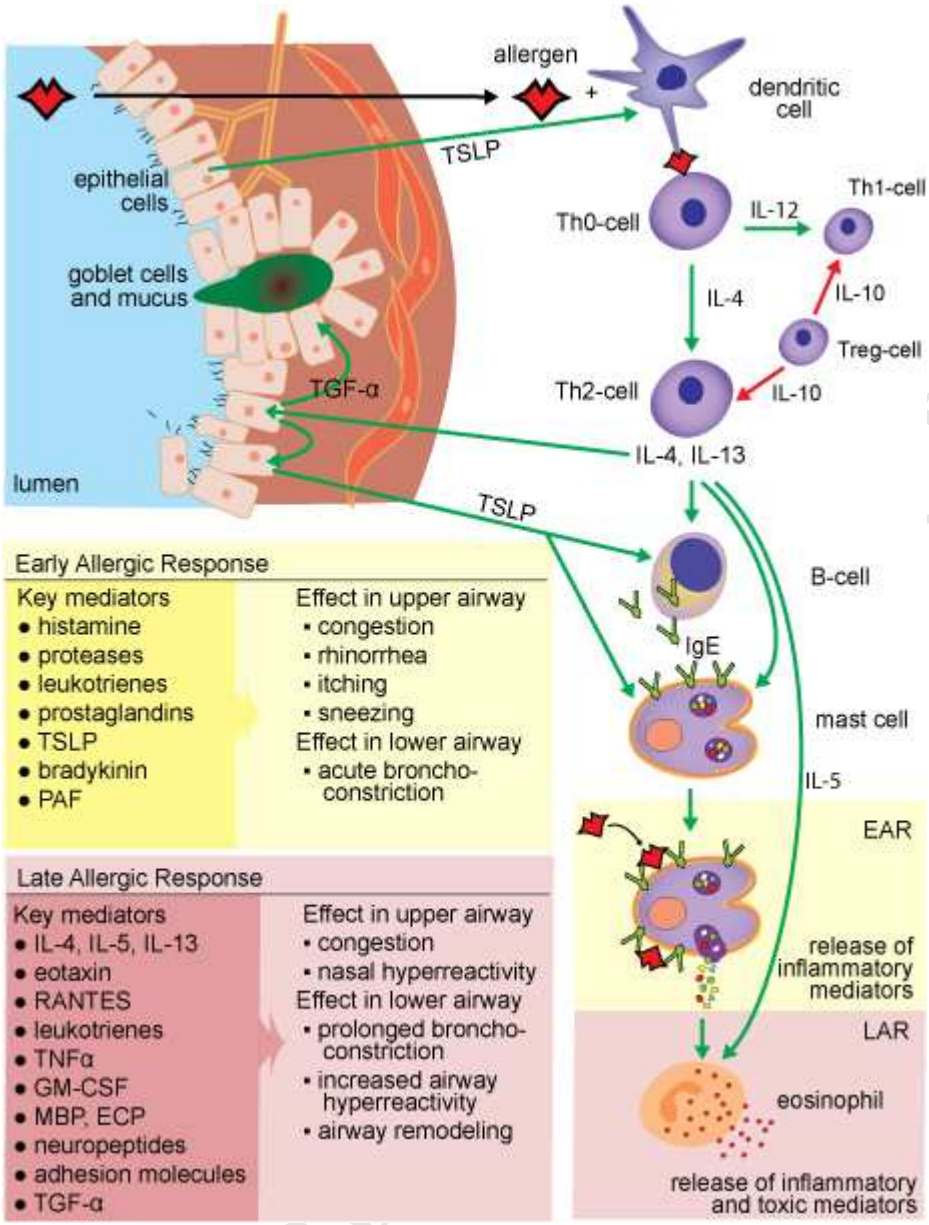

Figure 1: Cells and mediators involved in the allergic responses in asthma and allergic rhinitis. ECP = eosinophilic cationic protein, GM-CSF = granulocyte-macrophage colony stimulating factor, IgE = immunoglobulin-E, IL = interleukin, MBP = major basic protein, PAF = platelet activating factor, TGF-α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