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ASTHMA (WJ CALHOUN AND SP PETERS, SECTION EDITORS)

**Which Biomarkers Are Effective for Identifying Th2-Driven
Inflammation in Asthma?**

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Abstract

Recognition of asthma as a heterogeneous disease revealed different potential molecular targets and urged the development of targeted, customized treatment modalities. Evidence was provided for different inflammatory subsets of asthma and more recently, further refined to T helper (Th)2-high and Th2-low subphenotypes with different responsiveness to standard and targeted pharmacotherapy. Given these differences in immunology and pathophysiology, proof of concept studies of novel treatment modalities for asthma should be performed in adequate, well-defined phenotypes. In this review, we describe both existing and novel biomarkers of Th2-inflammation in asthma that can be applied to classify asthma subphenotypes in clinical studies and for treatment monitoring.

Introduction

Current guidelines acknowledge the heterogeneity of asthma and the potential value of biomarkers to aid diagnosis and adequate treatment [1]. The concept of different asthma phenotypes has been recognized by clinicians since a long time, exemplified by the subdivision into intrinsic (non-allergic) and extrinsic (allergic) asthma [2,3]. In the late 1990s, Rosi and colleagues showed by a factor analysis that clinical parameters, physiological tests and inflammatory biomarkers provide complimentary information and that combining these data could help to define an individual's asthma phenotype and subsequent customized treatment [4]. Ever since a number of cluster analyses have been performed [5,6]. Furthermore, several attempts have been made to define asthma (sub)phenotypes based on clinical or physiological presentations or provoking stimuli, yielding overlapping characteristics. Although none of the proposed phenotyping systems has been validated so far, inflammatory phenotyping (*i.e.*, eosinophilic, neutrophilic, mixed, paucigranulocytic) offers the potential of linking a specific inflammatory phenotype to (targeted) treatment options and disease monitoring [5,7]. More recently, asthma phenotypes have been defined based on their T-helper 2 (Th2)-gene expression, yielding at least two major phenotypes: *i.e.*, "Th2- high" and "Th2-low" subsets [8,9]. Although not fully confirmed, some of these insights have already been successfully implemented into clinical practice, treatment monitoring and clinical trials [10]. In this context, periostin is an emerging biomarker of eosinophilic and Th2-driven inflammation in asthmatic patients [11].

In the current asthma exacerbation model of Th2-driven airway inflammation used to study targeted therapies [Figure 1], inhaled allergen initiates activation of mast cells with subsequent release of pro-inflammatory mediators, including leukotrienes (LTs)

and prostaglandins (PGs) during the early asthmatic airway response (EAR). During the subsequent late asthmatic response (LAR), activated Th2-cells release interleukin (IL)-4, IL-5, IL-13, eotaxin and TARC [12], with subsequent IgE isotype switching in B cells, eosinophil activation including pro-inflammatory mediator release and airway smooth muscle (ASM) cell proliferation [13]. In addition, airway epithelial cells secrete IL-25 and IL-33, thus activating dendritic cells and promoting the release of IL-5 and IL-13 from innate lymphoid cells [14,15,16,17,18,19,20,21]. IL-13 induces the migration and survival of eosinophils [20], activation of macrophages [22], mucus hypersecretion [23], production of inducible nitric oxide (NO) synthase (iNOS) by airway epithelial cells [24], and transformation of airway fibroblasts into myofibroblasts promoting collagen deposition [23]. Apart from its role in the pathophysiology of airway remodelling, IL-13 also has been shown to induce non-specific airways hyperresponsiveness (AHR) [25].

In this review we discuss the most important Th2-derived biomarkers currently used in clinical practice and clinical research of asthma and potential future applications.

Systemic Th2-biomarkers

Blood eosinophils

For decades, peripheral blood eosinophil counts have been used for indirect assessment of airway inflammation and to aid the diagnosis of asthma. Evidence has been provided that total peripheral blood eosinophil counts can guide corticosteroid treatment, to predict asthma exacerbations [26] and to be sensitive markers of fatal asthma [27].

In healthy adults, blood eosinophil counts generally range from 0.015 to $0.65 \times 10^9/L$ [28]. There is a substantial diurnal variation (over 40%), with the lowest counts in the morning and the highest at night [29]. Blood eosinophil counts and serum IL-5 and IL-13 levels were reduced after 16 weeks of treatment with omalizumab (anti-IgE) [30,31].

Similarly, 50 weeks of treatment with mepolizumab (anti-IL-5) significantly decreased blood eosinophil counts, sputum eosinophils and the number of severe exacerbations in patients with severe eosinophilic asthma refractory to high dose of inhaled corticosteroids (ICS) or systemic corticosteroids [32, 33, 34]. Thus, peripheral blood eosinophil counts are believed to reflect Th2-driven inflammation within the airways.

Soluble Th2-markers from peripheral blood

Immunoglobulin E (IgE)

IgE is a key mediator of allergic conditions including asthma and allergy [35]. Hence, total serum IgE level is a specific biomarker of atopy and Th2-driven inflammation. In a multicenter trial, 182 patients with mild-to-severe asthma (approx 30% receiving ICS) were followed for 2.5 years. While correcting for FEV₁, sex, and age, total serum IgE level was the only independent variable tested predicting corticosteroid-induced improvement in AHR [36]. In another study of 562 patients with mild-to-severe allergic asthma, total serum IgE levels were related to asthma severity and inversely correlated with lung function (FEV₁ % predicted) [37]. Additionally, despite a substantial inter-subject variability, there was only minor overlap in serum IgE levels between the asthmatic (554 ± 447 IU/mL) and the control population (69 ± 33 IU/mL). In fact, a cut-off of 200 IU/mL would indicate sensitivity of 93% and specificity of 91% among these subjects.

Eosinophil cationic protein (ECP)

Eosinophil cationic protein (ECP) and eosinophilic peroxidase (EPO) are released during degranulation of eosinophils and, hence, increased serum levels of these products are

indicative of systemic eosinophilic inflammation. Serum ECP levels are often increased in asthmatic subjects, with a sensitivity of 70% and a specificity of 74% (efficiency 73%) and have been indicative for the presence of asthma [38]. Seasonal increases in serum ECP levels have been observed in sensitized, corticosteroid-naïve asthmatics [39]. In this study, the increase in serum ECP levels significantly correlated with changes in parameters of asthma activity: *i.e.*, increases in symptom scores and blood eosinophils, and decreases in PEF values and PD20 methacholine [39]. In another observational study, patients with seasonal allergic rhinitis (AR) who developed asthma-like symptoms over 6 years, initially presented with significantly higher serum ECP levels (16.7 microg/L vs 8.2 microg/L) and higher serum EPO levels (17.9 microg/L vs 8.8 microg/L) compared with rhinitics who did not develop lower airway symptoms over time [40]. Hence, in these subjects with seasonal AR, serum ECP and EPO levels measured outside of season showed a high predictive ability for the development of asthma [40]. In addition, both treatment with ICS [41] and targeted therapies, including omalizumab [42] and mepolizumab [34], decreased serum ECP levels in asthmatic patients.

Eotaxin

The chemokine eotaxin (CCL11) is a potent and selective chemoattractant for human eosinophils [43]. Serum eotaxin levels were higher in asthmatic patients (175.8±49.3 pg/mL) compared to controls (109.6±56.1 pg/mL), and correlated to the ECP levels in the asthmatics [44]. In a study investigating serum samples from 944 individuals of 218 asthma-affected families, eotaxin levels were higher in asthmatic parents than in asthmatic children, but no difference was found between healthy and asthmatic individuals [45]. In addition, serum eotaxin has been useful in predicting the severity of

symptoms that patients develop during steroid-tapering and could therefore be evaluated in guiding asthma treatment [46].

TARC

Thymus and activation-regulated chemokine (TARC; CCL17) is another biomarker suggested to be involved in the Th2-inflammatory response. Mean serum concentrations of TARC has been shown to be primarily increased in atopic dermatitis (325 pg/mL), but in some studies also in bronchial asthma (271 pg/mL) and allergic rhinitis (147 pg/mL; *versus* healthy volunteers (31.9 pg/mL) [47,48].

In asthmatic subjects, serum TARC concentrations were found to correlate with serum eotaxin concentrations [49]. In addition, some authors also report correlations with total IgE [50], while this was not found by others [48,49]. TARC in plasma was also increased in asthmatic children who were sensitized to cat allergen but not in those sensitized to other aeroallergens [50], and increased after allergen challenge [51]. Plasma TARC concentration may be responsive to corticosteroid treatment as increased levels (mean 131.0 pg/mL) have been found in asthmatic children not on inhaled corticosteroids compared with those treated with ICS (97.5 pg/mL) and healthy controls (76.0 pg/mL) [50].

Interleukins (IL) 4,5 and 13

Th2 cytokines such as IL-4, IL-5 and IL-13 play important roles in allergic diseases. However, due to their overall low levels in peripheral blood especially in stable clinical state, it is often difficult to detect them using traditional assays [48]. In some studies, increased serum IL-4 and IL-5 levels were found in asthmatic subjects compared to

healthy controls, but no relationship was found between these cytokine levels and clinical parameters of asthma [52]. In the aforementioned study investigating asthma-affected families [45], serum levels of IL-4 and IL-5 were lower in asthmatic adults compared to asthmatic children, with no difference between healthy and asthmatic individuals. However, IL-5 was the best predictor for extrinsic asthma and allergic rhinitis in children, and frequent asthma exacerbations in children were associated with increased serum IL-5 levels. In a study comparing fluticasone propionate (FP) and salmeterol (Salm) *versus* FP only, inhaled allergen challenge increased mean serum IL-5 from 0.7 to 5.9 pg/mL in sensitized asthmatics. Compared to FP only, pretreatment with the combination (FP/Salm) significantly reduced serum IL-5 and blood eosinophils 1 to 6 h post-allergen challenge [53]. Similarly, treatment with omalizumab reduced serum levels of IL-5 and IL-13 in atopic asthmatics [31].

In a recent study, a combined anti-IL4/13 antibody (dupilumab®) was used to treat patients with moderate-severe asthma with elevated blood eosinophil counts (≥ 300 cells/ μ L) or increased sputum eosinophils ($\geq 3\%$). In addition to a significant clinical effectiveness: *i.e.*, reducing numbers of exacerbations and improving ACQ-5 scores, Th2-associated biomarkers as TARC, 3-eotaxin, IgE, and FeNO were all significantly reduced following dupilumab treatment [54].

Periostin

Periostin is a matrix protein associated with fibrosis; its expression in airway structural cells is upregulated by recombinant IL-4 and IL-13 [9]. Within the airways of asthmatics, periostin expression has been found to correlate with several aspects of airway remodeling [11]. In patients with severe uncontrolled asthma unresponsive to maximal

ICS treatment, increased serum periostin levels were indicative of persistent eosinophilic airway inflammation sampled by sputum and bronchial biopsies. In this study population, serum periostin was the single best predictor of airway eosinophilia compared to other Th2-markers tested: *i.e.*, IgE levels, blood eosinophil counts and fractionated exhaled NO (FENO) levels [11]. In a recent interventional study with lebrikizumab (anti-IL-13), baseline serum periostin levels were used to subphenotype patients [55]. After 12 weeks of treatment, improvements in FEV₁ and asthma exacerbations were more evident in the lebrikizumab-treated group compared with the placebo group, while the increase in FEV₁ after lebrikizumab-treatment was even more pronounced in the high-periostin subgroup compared to the low-periostin group (serum periostin levels above and below the median for the 212 study subjects). Similarly, lebrikizumab treatment was associated with a significant reduction in FENO levels compared with those seen in the placebo group, especially in the high-periostin subgroup [55]. These data suggest that serum periostin levels might be a useful tool for identifying responders to anti-IL-13 therapies. Following a similar subphenotyping of patients with uncontrolled severe persistent allergic asthma, a decrease in exacerbation frequency was seen following treatment with omalizumab in the high *versus* the low group referring to three Th2-biomarkers: FeNO, blood eosinophils and serum periostin (< or >50 ng/mL) [56].

Th2-biomarkers in urine

In asthmatic children, eosinophil protein X (EPX) measurements in urine were applied for the monitoring of eosinophilic inflammation [57]. Similar to other eosinophil-derived markers, excretion of EPX/EDN shows circadian rhythms with the highest levels occurring at night [58]. Increased urine concentrations of EPX/EDN were detected in

asthmatics and reductions were shown following anti-inflammatory (ICS) treatment [59]. Increased urine levels of EPX/EDN (≥ 100 microg/mMol creatinine) in wheezing children during the first year may predict the development of allergic sensitization or asthma in later life [60].

Cysteinyl leukotrienes (CysLTs: LTC₄, LTD₄ and LTE₄) are eicosanoids produced through the 5-lipoxygenase pathway by a variety of cells associated with allergic inflammation, including eosinophils, mast cells, and basophils [61]. CysLTs are excreted in urine as LTE₄ (normal levels about 50 pg/ μ g creatinine), which is an indirect marker of CysLTs activity within the airways. Urinary LTE₄ levels increase with both spontaneous asthma exacerbations, aspirin and allergen challenges, and in nocturnal asthma during the night [62,63,64,65,66]. In contrast to corticosteroids [63], drugs inhibiting CysLTs synthesis, such as leukotriene synthesis inhibitors, significantly reduce urinary LTE₄ levels [67,66]. As anticipated, urinary LTE₄ can help predict the clinical response to leukotriene modifiers [68].

Th2-biomarkers in airway samplings

Eosinophils in bronchial tissue

Approximately 50% of the asthmatics across different severities consistently show airway eosinophilia on airway sampling (bronchial biopsies, sputum) [7]. However, within the eosinophil phenotype, various subphenotypes exist with different responsiveness to gold standard therapy as was shown by a cluster analysis [5]. Eosinophils within the airway mucosa are also known to be increased in Th2-driven, allergic asthma, with further increases following relevant allergen challenge [69] and in patients with aspirin-sensitive asthma [70]. In pollen sensitized allergic rhinitics,

segmental allergen challenge with grass pollen extract increased eosinophils both in the challenged and unchallenged bronchial mucosa as well as in peripheral blood and nasal biopsies [71]. Of interest, the presence of eosinophils in bronchial biopsies may help to predict responsiveness to gold standard therapy with ICS [72]. Alternatively, in allergic asthmatics, anti-leukotriene treatment (montelukast 10 mg QID) for 8 weeks was shown to effectively decrease eosinophils in bronchial biopsies to a similar degree as low-dose fluticasone (2x100 mcg), while fluticasone was significantly superior in reducing serum ECP [73]. Other targeted therapies, such as omalizumab effectively reduced eosinophils both in sputum and in bronchial biopsies of allergic asthmatics following allergen challenge [74].

Soluble markers

Bronchoalveolar lavage & bronchial washings

Several Th2-biomarkers can be quantified in bronchoalveolar lavage (BAL) fluid. The levels of ECP (and EPX) are increased in BAL fluid of asthmatics (increases dependent on asthma severity/activity/treatment) *versus* healthy controls [75,76]. Repeated low doses of cat allergen increased ECP levels in BAL (from mean 0.8 to 3.1 microg/L) and nonspecific AHR in sensitized mild asthmatics, without inducing clinical symptoms [77]. In addition, increased ECP and specific IgE levels in BAL may be related to the pathogenesis of the ragweed-induced late airway response (LAR) in ragweed-sensitized asthmatics [78].

In an observational study of patients with eosinophilic bronchitis, asthmatics and healthy controls, only a minority of subjects yielded baseline eotaxin values above the detection limit (0.21 pg/mL in BAL fluid and 6.25 pg/mL in BW, respectively), and

hence, no difference could be found between the 3 study populations [79]. In a longitudinal study, a sustained increase in BAL eotaxin levels was found in asthmatics *versus* healthy controls regardless of ICS treatment, while BAL IL-5 levels seemed more ICS-responsive [80].

IL-4, IL-5 and IL-13 are typically involved in atopic asthma, and they have all been demonstrated to increase after allergen provocation [81, 82, 83, 84, 85, 86, 87, 88], where the effect on IL-4 persisted for 2 weeks while IL-13 levels were back to baseline after 1 week [89]. A similar effect was seen in allergic rhinitics [81].

Similar to other Th2-cytokines, TARC is increased in BAL following allergen challenge [51,84], and is correlated to both IL-5 and IL-13. Moreover, TARC levels are higher in patients with an allergen-induced LAR compared to those without [90]. More recently, a BAL study revealed a Th2-high phenotype, with severe poorly controlled asthma despite corticosteroids, with upregulation of the PGD2 pathway [91].

Leukotrienes

A limited number of studies have been published on leukotrienes measurements in BAL. Involvement of LTs (*i.e.*, LTB₄ and CysLTs) in the inflammation and the physiology of nocturnal asthma has been demonstrated as increased BAL LTB₄ and CysLT levels were found at 4:00 AM in nocturnal asthmatics compared to healthy controls. In parallel, urinary LTE₄ were also significantly higher in nocturnal asthmatics *versus* controls. In contrast, no differences in BAL LT-levels could be demonstrated in both groups at 4 PM [92]. Following allergen challenge, no difference was found in BAL CysLT levels between patients with a LAR compared to those without a LAR (3,2-8,7 pg/mL) [90], possibly due to the fact that CysLTs can be released during both the early and the late response to allergen, by mast cells and eosinophils, respectively. However, a more pronounced

difference was seen between patients with aspirin-sensitive *versus* aspirin-tolerant asthma, possibly due to the marked airway eosinophilia in the aspirin-sensitive group [93, 94]. In one study, CysLTs have been shown to respond to ICS, as BAL LTC₄ levels of asthmatic subjects were lower after 2.5 years ICS therapy [95], while leukotriene modifiers, as exemplified by the 5-lipoxygenase inhibitor, zileuton, in the aforementioned study by Wenzel et al, effectively blocked all LTs while improving nocturnal manifestations of asthma [92].

Sputum

Given the invasive nature of bronchial biopsies, sputum analysis has offered a valid alternative allowing non-invasive asthma phenotyping and customized treatment [7,96]. Induced sputum is feasible in the majority of patients, yields repeatable cell counts (mainly eosinophils and neutrophils) across all asthma severities [96] and allows mRNA analysis [97]. Across the literature, eosinophilic airway inflammation is defined by at least $\geq 2\%$ sputum eosinophils. Following allergen-induced LAR, repeatable increases in sputum eosinophils have been demonstrated in allergic asthmatics with superior outcomes if expressed as %non-squamous cells *versus* cells/mL [98]. So far, sputum eosinophils have been successfully applied as outcome variables in many proof of concept and (treatment) monitoring studies. The pre-requisite for successful implementation of this technique implies harmonization of standard operating procedures (both during induction and processing) across research centers and adequate analysis in a qualified, central laboratory [99].

Soluble markers

Similarly to BAL, several studies showed increased sputum ECP levels in asthmatics *versus* healthy controls [¹⁰⁰ , ¹⁰¹ , ¹⁰² , ¹⁰³] correlating with asthma activity/severity/treatment status [¹⁰⁴, ¹⁰⁵]. Asthmatics in remission and asthma patients with a slow FEV₁ decline had similar sputum ECP levels [¹⁰⁶], while patients with a fast FEV₁ decline and exacerbating asthmatics had higher ECP levels [103]. Patients with seasonal allergic asthma and rhinitis with nonspecific AHR showed increased sputum ECP levels during season with persistent increases off season [¹²⁰] and inhaled allergen increased sputum ECP levels in sensitized asthmatics [¹⁰⁷, ¹⁰⁸]. Several treatments have been shown to decrease sputum ECP levels: *e.g.* prednisolone [¹⁰⁰], beclometasone [¹⁰⁹], montelukast [¹¹⁰] and roflumilast [¹¹¹].

Sputum Th2-cytokines including IL-4, IL-5, IL-13, eotaxin and TARC are increased in asthmatic subjects as compared with healthy controls, with further increases during both spontaneous and modeled (*e.g.* allergen challenge) exacerbations [¹², ¹⁰¹ , ¹⁰³ , ¹¹² , ¹¹³, ¹²¹]. Sputum TARC levels were increased in asthmatics (1117 pg/mL) as compared with healthy controls (31,8 pg/mL), and were positively correlated with sputum eotaxin. However, in contrast to sputum eotaxin, which showed a strong positive correlation with the sputum ECP, sputum TARC did not correlate with the ECP levels [⁴⁹]. Ample evidence has been provided for a major role of IL-13 in the pathophysiology of airway remodeling [¹¹⁴], which clinically translates into *e.g.* rapid FEV₁ decline and nonspecific AHR [¹¹³]. Smoking and/or its immunological effects evidently affect IL-13 as sputum IL-13 levels were higher in smokers than non-smokers regardless of concomitant asthma [¹⁰¹]. IL-13 production appears to be reciprocally regulated by corticosteroid therapy, since sputum IL-13 levels were overall lower in corticosteroid-treated asthmatics

compared with untreated controls [115]. Similarly, following inhaled allergen challenge, increases in IL-4, IL-5, IL-13, eotaxin and TARC were found in ultracentrifuged sputum of dual responders at 7 and 24 h post-challenge, while these levels were significantly decreased by pretreatment with a short course of high dosed fluticasone, corresponding with complete blockade of the allergen-induced LAR [12]. In this study, ultracentrifugation of sputum (at 35,000 rpm) was applied since Th2-cytokines are usually undetectable if measured under baseline conditions [12].

Sputum CysLTs were increased in asthmatics (median, 9.5 ng/mL) *versus* healthy controls (6.4 ng/mL), and higher in subjects with persistent asthma requiring ICS (median, 11.4 ng/mL) or within 48 h of an acute, severe exacerbation (13 ng/mL) *versus* subjects with episodic asthma (7.2 ng/mL) [116, 117, 118, 119, 120]. In addition, sputum CysLTs and urinary LTE₄ concentrations were higher in asthmatics with eosinophilic airway inflammation [116]. Seasonal (allergic) asthmatics could be differentiated from those with rhinitis with or without AHR by higher sputum CysLT levels [geometric mean: 3.3 (lower 95%-upper 95% confidence interval (CI) of geometric mean: 1.9-5.1) vs. 1.4 (0.9-2.2) and 0.7 (0.3-1.6) pg/microg total protein, respectively] during pollen season, while levels declined off-season [121]. In addition, susceptible asthmatics to exercise-induced bronchoconstriction [122] and aspirin-sensitive asthmatics have increased sputum CysLTs [123]. Fluticasone combined with montelukast produced greater reductions in sputum CysLTs than fluticasone with salmeterol [124], while zafirlukast did not affect sputum CysLTs levels [125].

Exhaled markers

Exhaled nitric oxide

In untreated asthma, FeNO has been shown to correlate with airway eosinophilic inflammation sampled by induced sputum and bronchial biopsies [126]. Based on FeNO levels, Dweik and colleagues identified two asthma phenotypes: high (≥ 35 ppb) and low (< 35 ppb) subphenotype; the FeNO-high subphenotype characterized by longer disease duration, nonspecific AHR, atopy and airway eosinophila [127].

The lack of correlation between sputum eosinophils and FeNO in patients treated with anti-IL-5 (mepolizumab®) indicates that NO production is related to IL-13 and Th2-response rather than to the eosinophilic inflammation *per se* [128]. Indeed, a link exists between serum periostin levels and FeNO, with high FeNO levels being indicative of Th2-inflammation within the airways. In line with these observations, treatment with anti-IL-13 therapy (lebrikizumab®) has shown to reduce FeNO levels in high-periostin and high-FeNO asthmatics [55]. In addition, 12 weeks of lebrikizumab treatment was associated with improvements in lung function and exacerbations in patients with Th2-high phenotype (*i.e.*, high serum periostin and high FeNO at baseline) compared to those with a low Th2-profile [55].

FeNO measured according to ERS/ATS recommendations (50mL/s) mainly reflects NO-production within central airways and is closely associated with the expression of iNOS within bronchial epithelial cells [129]. Alternatively, the origin of NO from the peripheral airways (alveolar NO; CANO) is not completely known. In an *in vitro* study on small airway epithelial cells, the Th1-cytokines (*i.e.*, IL-1beta, TNF-alpha, and IFN-gamma) provided a pronounced iNOS expression compared to IL-13 inducing a modest increase [130]. This is somewhat in contrast to bronchial epithelial cell known to respond very well to the Th2-cytokine IL-13 [131]. In asthmatics *in vivo*, CANO seems to correlate with

distal lung eosinophilic inflammation [72]. Increased CANO levels were found (7.1 ppb) in patients refractory to high doses of ICS, but decreased following treatment with prednisolone (30 mg QID). There was a close correlation between BAL eosinophils and CANO while single FeNO measurements correlated with eosinophil counts in BW [72].

Exhaled breath condensate (EBC)

In theory, EBC allows sampling from the entire airways, including small airways. However, in practice, the method is associated with a number of methodological issues hampering interpretation. Due to their size, proteins, including cytokines, are invariably present in low levels close to or under detection limit. Increased levels of reactive oxidative markers and eicosanoid mediators (8-isoprostane and leukotrienes) can be more readily and reproducibly measured [132,133].

Saliva

Saliva can be easily obtained and biomarkers have been proposed to reflect systemic inflammation making saliva samplings a promising tool for larger population screenings and epidemiological surveys [134]. Even though clearly detectable levels of pro-inflammatory mediators and cytokines can be measured in saliva [135], surprisingly few studies have been published on the topic.

Conclusions

Apart from more traditional biomarkers, including blood and sputum eosinophils, serum IgE and FeNO levels, novel emerging Th2-related markers, such as periostin, TARC and others, have been identified. Combinations of different biomarkers may further help to

refine the Th2-subphenotypes and hence, may serve as useful tools to aid diagnosis and to define clinical phenotypes suitable for Th2-targeted therapies. Already today, there are very promising clinical examples proofing that it is possible to use Th2-related biomarkers to identify responders, with a high degree of accuracy. However, the optimal biomarker or combination of biomarkers awaits validation. In addition, there is an additional need to define the value of adequate biomarkers, not only to identify potential responders to therapy, but also as true primary outcome measures being closely related to disease activity and thereby competing with traditional variables as lung function and disease control.

Compliance with Ethics Guidelines

Conflict of Interest

Zuzana Diamant has served on an advisory board (Aerocrine) and served as a consultant for various companies (Hall Allergy Hexal, Mundipharma, Urogenix, Profess, QPS Netherlands).

Leif Bjerner has served on advisory boards and received payment for giving lectures.

Ellen Tufvesson declares that she has no conflict of interest.

Human and Animal Rights and Informed Consent

This article does not contain any studies with human or animal subjects performed by any of the authors.

References

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- 1 Global Initiative for Asthma (updated 2012), www.ginasthma.com.
- 2 Diamant Z, Boot JD, Mantzouranis E et al: Biomarkers in asthma and allergic rhinitis. *Pulm Pharmacol Ther* 2010, 23(6):468-481.
- 3 Diamant Z, Boot JD, Virchow JC: Summing up 100 years of asthma. *Respir Med* 2007,101(3):378-388.
- 4 Rosi E, Ronchi MC, Grazzini M et al. Sputum analysis, bronchial hyperresponsiveness, and airway function in asthma: results of a factor analysis. *J Allergy Clin Immunol* 1999,103:232-237.
- 5 Haldar P, Pavord ID, Shaw DE, et al: Cluster analysis and clinical asthma phenotypes. *Am J Respir Crit Care Med* 2008,178(3):218-224.
- 6 Moore WC, Meyers DA, Wenzel SE et al: National Heart, Lung, and Blood Institute's Severe Asthma Research Program. *Am J Respir Crit Care Med* 2010,181(4):315-323
- 7 Wenzel SE: Asthma: defining of the persistent adult phenotypes. *Lancet* 2006, 368(9537):804-813.
- 8 Woodruff P G, Boushey HA, Dolganov GM et al: Genome-wide profiling identifies epithelial cell genes associated with asthma and with treatment response to corticosteroids. *Proc Natl Acad Sci U S A*, 2007, 104: 15858–15863.
- 9 Woodruff PG, Modrek B, Choy DF, et al: T-helper type 2-driven inflammation defines major subphenotypes of asthma. *Am J Respir Crit Care Med* 2009,180(5):388-395.
- 10 Ingram JL, Kraft M: IL-13 in asthma and allergic disease: asthma phenotypes and targeted therapies. *J Allergy Clin Immunol* 2012,130(4):829-842.
- 11 Jia G, Erickson RW, Choy DF et al: Bronchoscopic Exploratory Research Study of Biomarkers in Corticosteroid-refractory Asthma (BOBCAT) Study Group. Periostin is a systemic biomarker of eosinophilic airway inflammation in asthmatic patients. *J Allergy Clin Immunol*, 2012,130(3):647-665.
- 12 Ruddy M, Zuiker R, Morelli N, et al: Detection of increased levels of Th2-derived cytokines in ultracentrifuged sputum following allergen challenge and their responsiveness to fluticasone. *Am J Respir Crit Care Med*, 2010:A4043.
- 13 Van der Pouw Kraan TC, Van der Zee JS, Boeijs LC et al: The role of IL-13 in IgE synthesis by allergic asthma patients. *Clin Exp Immunol* 1998, 111(1):129-35.
- 14 Besnard AG, Togbe D, Guillou N et al: IL-33-activated dendritic cells are critical for allergic airway inflammation. *Eur J Immunol* 2011, 41(6):1675-86.
- 15 Smithgall MD, Comeau MR, Yoon BR et al: IL-33 amplifies both Th1- and Th2-type responses through its activity on human basophils, allergen-reactive Th2 cells, iNKT and NK cells. *Int Immunol* 2008,20(8):1019-30.
- 16 Ikeda K, Nakajima H, Suzuki K et al: Mast cells produce interleukin-25 upon Fc epsilon RI-mediated activation. *Blood* 2003, 101(9):3594-6.
- 17 Wang YH, Angkasekwinai P, Lu N et al: IL-25 augments type 2 immune responses by enhancing the expansion and functions of TSLP-DC-activated Th2 memory cells. *J Exp Med* 2007,204(8):1837-47.
- 18 Bartemes KR, Iijima K, Kobayashi T et al: IL-33-responsive lineage- CD25+ CD44(hi) lymphoid cells mediate innate type 2 immunity and allergic inflammation in the lungs. *J Immunol* 2012, 188(3):1503-13.
- 19 Mjösberg JM, Trifari S, Crellin NK et al: Human IL-25- and IL-33-responsive type 2 innate lymphoid cells are defined by expression of CCR2 and CD161. *Nat Immunol* 2011, 12(11):1055-62.
- 20 Pope SM, Brandt EB, Mishra A et al: IL-13 induces eosinophil recruitment into the lung by an IL-5- and eotaxin-dependent mechanism. *J Allergy Clin Immunol* 2001, 108(4):594-601.
- 21 Ramirez-Icaza G, Mohammed KA, Nasreen N et al: Th2 cytokines IL-4 and IL-13 downregulate paxillin expression in bronchial airway epithelial cells. *J Clin Immunol* 2004, 24(4):426-34.

-
- 22 Martinez-Nunez RT, Louafi F, Sanchez-Elsner T: The interleukin 13 (IL-13) pathway in human macrophages is modulated by microRNA-155 via direct targeting of interleukin 13 receptor alpha1 (IL13Ralpha1). *J Biol Chem* 2011,286(3):1786-94.
- 23 Zhu Z, Homer RJ, Wang Z et al: Pulmonary expression of interleukin-13 causes inflammation, mucus hypersecretion, subepithelial fibrosis, physiologic abnormalities, and eotaxin production. *J Clin Invest* 1999, 103(6):779-88.
- 24 Chibana K, Trudeau JB, Mustovich AT et al: IL-13 induced increases in nitrite levels are primarily driven by increases in inducible nitric oxide synthase as compared with effects on arginases in human primary bronchial epithelial cells. *Clin Exp Allergy* 2008, 38(6):936-46.
- 25 Venkayya R, Lam M, Willkom M et al: The Th2 lymphocyte products IL-4 and IL-13 rapidly induce airway hyperresponsiveness through direct effects on resident airway cells. *Am J Respir Cell Mol Biol* 2002,26(2):202-8.
- 26 Horn B R, Robin E D, J. Van Kessel TA: Total eosinophil counts in the management of bronchial asthma. *N Engl J Med*, 1975, 292: 1152–1155.
- 27 Ulrik CS, J. Frederiksen J: Mortality and markers of risk of asthma death among 1,075 outpatients with asthma *Chest* 1995,108:10–15.
- 28 Krause JR, Boggs DR: Search for eosinopenia in hospitalized patients with normal blood leukocyte concentration. *Am J Hematol* 1987, 24(1):55–63.
- 29 Winkel P, Statland BE, Saunders AM et al: Within-day physiologic variation of leukocyte types in healthy subjects as assayed by two automated leukocyte differential analyzers. *Am J Clin Pathol* 1981,75(5):693–700.
- 30 Massanari M, Holgate ST, Busse WW, et al: Effect of omalizumab on peripheral blood eosinophilia in allergic asthma. *Respir Med* 2010,104(2):188–196.
- 31 Noga O, Hanf G, Kunkel G: Immunological and clinical changes in allergic asthmatics following treatment with omalizumab *Int Arch Allergy Immunol* 2003,131:46–52.
- 32 Haldar P, Brightling C E, Hargadon B et al: Mepolizumab and exacerbations of refractory eosinophilic asthma. *N Engl J Med* 2009,360:973–984.
- 33 Nair P, Pizzichini MM, Kjarsgaard M et al.: Mepolizumab for prednisone-dependent asthma with sputum eosinophilia. *N Engl J Med* 2009,360: 985–993.
- 34 Büttner C, Lun A, Splettstoesser T et al. Monoclonal anti-interleukin-5 treatment suppresses eosinophil but not T-cell functions. *Eur Respir J* 2003, 21(5):799-803.
- 35 Burrows B, Martinez F D, Halonen M. et al. Association of asthma with serum IgE levels and skin-test reactivity to allergens. *N Engl J Med* 1989, 320: 271–277.
- 36 Kerstjens HA, Schouten JP, Brand PL, et al: Importance of total serum IgE for improvement in airways hyperresponsiveness with inhaled corticosteroids in asthma and chronic obstructive pulmonary disease. The Dutch CNSLD Study Group. *Am J Respir Crit Care Med* 1995,151(2 PT 1):360-368.
- 37 Ahmad Al Obaidi A H, Mohamed Al Samarai A G, Yahya Al Samarai A K et al: The predictive value of IgE as biomarker in asthma. *J Asthma* 2008,45: 654–663.
- 38 Peona V, De Amici M, Quaglini S et al: Serum Eosinophilic Cationic Protein: Is There a Role in Respiratory Disorders? *Journal of Asthma*, 2010, 47:131–134.
- 39 Vatrella A, Ponticiello A, Parrella R: Serum eosinophil cationic protein (ECP) as a marker of disease activity and treatment efficacy in seasonal asthma. *Allergy* 1996, 51:547–555.
- 40 Nielsen LP, Peterson CG, Dahl R: Serum eosinophil granule proteins predict asthma risk in allergic rhinitis. *Allergy* 2009;64(5):733-737.
- 41 Stelmach I, Jerzynska J, Kuna P: Markers of allergic inflammation in peripheral blood of children with asthma after treatment with inhaled triamcinolone acetonide. *Ann Allergy Asthma Immunol* 2001,87(4):319-326.

-
- 42 Zietkowski Z, Skiepmo R, Tomasiak-Lozowska MM et al: Airway inflammation and eotaxin in exhaled breath condensate of patients with severe persistent allergic asthma during omalizumab therapy. *Adv Med Sci* 2011,56(2):318-322.
- 43 Conroy DM, Jopling LA, Lloyd CM, et al: CCR4 blockade does not inhibit allergic airways inflammation. *J Leukoc Biol* 2003,74(4):558-563.
- 44 Jahnz-Róyk K, Plusa T, Mierzejewska J: Eotaxin in serum of patients with asthma or chronic obstructive pulmonary disease: relationship with eosinophil cationic protein and lung function. *Mediators Inflamm* 2000,9(3-4):175-179.
- 45 Pukelsheim K, Stoeger T, Kutschke D et al: Cytokine profiles in asthma families depend on age and phenotype. *PLoS One* 2010, 13;5(12):e14299.
- 46 Hoffmann HJ, Nielsen LP, Harving H et al: Asthmatics able to step down from inhaled corticosteroid treatment without loss of asthma control have low serum eotaxin/CCL11. *Clin Respir J* 2008, 2(3):149-157.
- 47 Hijnen D, De Bruin-Weller M, Oosting B, et al: Serum thymus and activation-regulated chemokine (TARC) and cutaneous T cell- attracting chemokine (CTACK) levels in allergic diseases: TARC and CTACK are disease-specific markers for atopic dermatitis *J Allergy Clin Immunol* 2004, 113(2):334-340.
- 48 Sugawara N, Yamashita T, Ote Y, et al: TARC in allergic disease. *Allergy* 2002,57:180-181.
- 49 Sekiya T, Yamada H, Yamaguchi M, et al: Increased levels of a TH2-type CC chemokine thymus and activation regulated chemokine (TARC) in serum and induced sputum of asthmatics. *Allergy* 2002,57:173-177.
- 50 Leung TF, Wong CK, Chan IH, et al: Plasma concentration of thymus and activation-regulated chemokine is elevated in childhood asthma. *J Allergy Clin Immunol* 2002,110:404-409.
- 51 Bochner BS, Hudson SA, Xiao HQ et al. Release of both CCR4-active and CXCR3-active chemokines during human allergic pulmonary late-phase reactions. *J Allergy Clin Immunol* 2003,112(5):930-934.
- 52 ten Hacken NH, Oosterhoff Y, Kauffman HF, et al: Elevated serum interferon-gamma in atopic asthma correlates with increased airways responsiveness and circadian peak expiratory flow variation. *Eur Respir J* 1998,11(2):312-316.
- 53 Koopmans JG, Lutter R, Jansen HM, van der Zee JS: Adding salmeterol to an inhaled corticosteroid reduces allergen-induced serum IL-5 and peripheral blood eosinophils. *J Allergy Clin Immunol* 2005,116(5):1007-1113.
- 54 Wenzel S, Ford L, Pearlman D, et al: Dupilumab in Persistent Asthma with Elevated Eosinophil Levels. *N Engl J Med* 2013; (ahead of print).
- 55 Corren j, Lemanske R F, Hanania N A, et al: Lebrikizumab treatment in adults with asthma. *N Engl J Med* 2011, 365: 1088–1098.
- 56 Hanania NA, Wenzel S, Rosén K, et l: Exploring the effects of omalizumab in allergic asthma. *Am J Respir Crit Care Med* 2013,187(8):804-811.
- 57 Venge P: Monitoring the allergic inflammation. *Allergy* 2004,59(1):26-32.
- 58 Wolthers OD, Heuck C: Circadian variations in serum eosinophil cationic protein, and serum and urine eosinophil protein X. *Pediatr Allergy Immunol* 2003,14: 130–133.
- 59 Kristjansson S, Strannegård IL, Strannegård Ö, et al: Urinary eosinophil protein X in children with atopic asthma: a useful marker of antiinflammatory treatment. *J Allergy Clin Immunol* 1996, 97: 1179-1187.
- 60 Oymar K: High levels of urinary eosinophil protein X in young asthmatic children predict persistent atopic asthma. *Pediatr Allergy Immunol* 2001,12: 312–317.
- 61 Diamant Z, Sampson AP: Anti-inflammatory mechanisms of leukotriene modulators. Editorial. *Clin Exp Allergy* 1999,29:1449-1453.

-
- 62 Daffern PJ, Muilenburg D, Hugli TE et al. Association of urinary leukotriene E4 excretion during aspirin challenges with severity of respiratory responses. *J Allergy Clin Immunol* 1999,104:559–564.
- 63 O'Shaughnessy KM, Wellings R, Gillies B et al. Differential effects of fluticasone propionate on allergen-evoked bronchoconstriction and increased urinary leukotriene E4 excretion. *Am Rev Respir Dis* 1993,147(6 Pt 1):1472–1476.
- 64 O'Sullivan S, Roquet A, Dahlen B et al: Urinary excretion of inflammatory mediators during allergen-induced early and late phase asthmatic reactions. *Clin Exp Allergy* 1998, 28(11):1332–1339.
- 65 Green SA, Malice MP, Tanaka W et al: Increase in urinary leukotriene LTE4 levels in acute asthma: correlation with airflow limitation. *Thorax* 2004,59(2):100–104.
- 66 Diamant Z, Timmers MC, van der Veen H, et al: The effect of MK-0591, a novel 5-lipoxygenase activating protein inhibitor, on leukotriene biosynthesis and allergen-induced airway responses in asthmatic subjects in vivo. *J Allergy Clin Immunol* 1995,95:42-51.
- 67 Liu MC, Dube LM, Lancaster J: Acute and chronic effects of a 5-lipoxygenase inhibitor in asthma: a 6-month randomized multicenter trial. Zileuton Study Group. *J Allergy Clin Immunol* 1996,98(5 Pt 1):859–871.
- 68 Cai, C., Yang, J., Hu, S., et al: Relationship between urinary cysteinyl leukotriene E4 levels and clinical response to antileukotriene treatment in patients with asthma. *Lung* 2007; 185: 105-112.
- 69 Wenzel SE. Asthma phenotypes: the evolution from clinical to molecular approaches. *Nat Med* 2012;18(5):716-25.
- 70 Sousa AR, Lams BE, Pfister R, et al: Expression of interleukin-5 and granulocyte-macrophage colony-stimulating factor in aspirin-sensitive and non-aspirin-sensitive asthmatic airways. *Am J Respir Crit Care Med* 1997,156(5):1384-1389.
- 71 Braunstahl GJ, Kleinjan A, Overbeek SE, et al: Segmental bronchial provocation induces nasal inflammation in allergic rhinitis patients. *Am J Respir Crit Care Med* 2000, 161(6):2051-2057.
- 72 Berry M, Hargadon B, Morgan A, et al: Alveolar nitric oxide in adults with asthma: evidence of distal lung inflammation in refractory asthma. *Eur Respir J* 2005,25(6):986-991.
- 73 Overbeek SE, O'Sullivan S, Leman K, et al: Effect of montelukast compared with inhaled fluticasone on airway inflammation. *Clin Exp Allergy* 2004, 34(9):1388-1394.
- 74 Van Rensen EL, Straathof KC, Veselic-Charvat MA, et al: Effect of inhaled steroids on airway hyperresponsiveness, sputum eosinophils, and exhaled nitric oxide levels in patients with asthma. *Thorax* 1999, 54(5):403-408.
- 75 Robinson DS, Assoufi B, Durham SR, et al: Eosinophil cationic protein (ECP) and eosinophil protein X (EPX) concentrations in serum and bronchial lavage fluid in asthma. *Clin Exp Allergy* 1995,25(11):1118-1127.
- 76 Vignola AM, Chanez P, Campbell AM, et al: Airway inflammation in mild intermittent and in persistent asthma. *Am J Respir Crit Care Med* 1998, 157(2):403-409.
- 77 de Blay F, Krieger P, Spirlet F, et al: Repeated inhalation of low doses of cat allergen that do not induce clinical symptoms increases bronchial hyperresponsiveness and eosinophil cationic protein levels. *Int Arch Allergy Immunol* 1999, 120(2):158-165.
- 78 Peebles RS Jr, Hamilton RG, Lichtenstein LM, et al: Antigen-specific IgE and IgA antibodies in bronchoalveolar lavage fluid are associated with stronger antigen-induced late phase reactions. *Clin Exp Allergy* 2001, 31(2):239-248.
- 79 Woodman L, Sutcliffe A, Kaur D, et al: Chemokine concentrations and mast cell chemotactic activity in BAL fluid in patients with eosinophilic bronchitis and asthma, and in normal control subjects. *Chest* 2006, 130(2):37137-8.
- 80 Feltis BN, Reid DW, Ward C et al: BAL eotaxin and IL-5 in asthma, and the effects of inhaled corticosteroid and beta2 agonist. *Respirology* 2004, 9(4):507-13.

-
- 81 Becky Kelly EA, Busse WW, Jarjour NN: A comparison of the airway response to segmental antigen bronchoprovocation in atopic asthma and allergic rhinitis. *J Allergy Clin Immunol* 2003,111(1):79-86.
- 82 Julius P, Hochheim D, Böser K, et al: Interleukin-5 receptors on human lung eosinophils after segmental allergen challenge. *Clin Exp Allergy* 2004, 34(7):1064-1070.
- 83 Kroegel C, Julius P, Matthys H et al: Endobronchial secretion of interleukin-13 following local allergen challenge in atopic asthma: relationship to interleukin-4 and eosinophil counts. *Eur Respir J* 1996,9(5):899-904.
- 84 Pilette C, Francis JN, Till SJ et al: CCR4 ligands are up-regulated in the airways of atopic asthmatics after segmental allergen challenge. *Eur Respir J* 2004,23(6):876-884.
- 85 Teran LM, Carroll MP, Shute JK et al: Interleukin 5 release into asthmatic airways 4 and 24 hours after endobronchial allergen challenge: its relationship with eosinophil recruitment. *Cytokine* 1999,11(7):518-522.
- 86 Thunberg S, Gafvelin G, Nord M, et al: Allergen provocation increases TH2-cytokines and FOXP3 expression in the asthmatic lung. *Allergy* 2010,65(3):311-318.
- 87 Virchow JC Jr, Walker C, Hafner D, et al: T cells and cytokines in bronchoalveolar lavage fluid after segmental allergen provocation in atopic asthma. *Am J Respir Crit Care Med* 1995,151(4):960-968.
- 88 Walker C, Bauer W, Braun RK, et al: Activated T cells and cytokines in bronchoalveolar lavages from patients with various lung diseases associated with eosinophilia. *Am J Respir Crit Care Med* 1994,150(4):1038-1048.
- 89 Batra V, Musani AI, Hastie AT, et al: Bronchoalveolar lavage fluid concentrations of transforming growth factor (TGF)-beta1, TGF-beta2, interleukin (IL)-4 and IL-13 after segmental allergen challenge and their effects on alpha-smooth muscle actin and collagen III synthesis by primary human lung fibroblasts. *Clin Exp Allergy* 2004,34(3):437-444.
- 90 Ali FR, Kay AB, Larché M: Airway hyperresponsiveness and bronchial mucosal inflammation in T cell peptide-induced asthmatic reactions in atopic subjects. *Thorax* 2007,62(9):750-757.
- 91 Fajt ML, Gelhaus SL, Freeman B, et al: Prostaglandin D2 pathway upregulation: Relation to asthma severity, control, and TH2 inflammation. *J Allergy Clin Immunol* 2013, 31(6):1504-1512.
- 92 Wenzel SE, Trudeau JB, Kaminsky DA, et al: Effect of 5-lipoxygenase inhibition on bronchoconstriction and airway inflammation in nocturnal asthma. *Am J Respir Crit Care Med* 1995,152(3):897-905.
- 93 Cowburn AS, Sladek K, Soja J, et al: Overexpression of leukotriene C4 synthase in bronchial biopsies from patients with aspirin-intolerant asthma. *J Clin Invest* 1998,101(4):834-846.
- 94 Szczeklik A, Sladek K, Dworski R, et al: Bronchial aspirin challenge causes specific eicosanoid response in aspirin-sensitive asthmatics. *Am J Respir Crit Care Med* 1996,154(6 Pt 1):1608-1614.
- 95 Oosterhoff Y, Overbeek SE, Douma R, et al: Lower leukotriene C(4) levels in bronchoalveolar lavage fluid of asthmatic subjects after 2.5 years of inhaled corticosteroid therapy. *Mediators Inflamm* 1995,4(6):426-430.
- 96 Bakakos P, Schleich F, Alchanatis M et al.: Induced sputum in asthma: from bench to bedside. *Curr Med Chem* 2011,18(10):1415-1422.
- 97 Sohn SW, Lee HS, Park HW, et al: Evaluation of cytokine mRNA in induced sputum from patients with allergic rhinitis: relationship to airway hyperresponsiveness. *Allergy* 2008, 63(3):268-273.
- 98 Gauvreau GM, Watson RM, Rerecich TJ, et al: Repeatability of allergen-induced airway inflammation. *J Allergy Clin Immunol* 1999,104(1):66-71.
- 99 Clarke GW, Diamant Z, Greenaway SD et al. Launching the BIOSPIT initiative: Harmonizing sputum outcomes in multicenter trials. *Pulm Pharmacol Ther* 2013;26(3):400-401.

-
- 100 Al Obaidi AH, Al Samarai AG, Al-Janabi J et al. The predictive value of eosinophil cationic protein and lactate dehydrogenase in asthma: a comparative study of serum versus sputum. *World Allergy Organ J* 2009, 2(7):144-149.
- 101 Hillas G, Loukides S, Kostikas K, et al: Increased levels of osteopontin in sputum supernatant of smoking asthmatics. *Cytokine* 2013,61(1):251-255.
- 102 Popov TA, Shenkada MS, Tzoncheva AV, et al: Circadian changes in the sputum of asthmatic subjects and healthy controls. *World Allergy Organ J* 2008,1(5):74-78.
- 103 Xu J, Jiang F, Nayeri F, Zetterström O: Apoptotic eosinophils in sputum from asthmatic patients correlate negatively with levels of IL-5 and eotaxin. *Respir Med* 2007,101(7):1447-1454.
- 104 Fujimoto K, Kubo K, Matsuzawa Y et al: Eosinophil cationic levels in induced sputum correlate with the severity of bronchial asthma. *Chest* 1997, 112:1241–1127.
- 105 Hanxiang N, Jiong Y, Yanwei C, et al: Persistent airway inflammation and bronchial hyperresponsiveness in patients with totally controlled asthma. *Int J Clin Pract* 2008, 62(4):599-605.
- 106 Broekema M, Volbeda F, Timens W, et al: Airway eosinophilia in remission and progression of asthma: accumulation with a fast decline of FEV(1). *Respir Med* 2010,104(9):1254-1262.
- 107 Schulze J, Voss S, Zissler U, et al: Airway responses and inflammation in subjects with asthma after four days of repeated high-single-dose allergen challenge. *Respir Res* 2012,19;13:78.
- 108 Lee JH, Park KH, Park JW et al: YKL-40 in induced sputum after allergen bronchial provocation in atopic asthma. *J Investig Allergol Clin Immunol* 2012;22(7):501-507.
- 109 Lazarus SC, Chinchilli VM, Rollings NJ, et al: Smoking affects response to inhaled corticosteroids or leukotriene receptor antagonists in asthma. *Am J Respir Crit Care Med* 2007,175(8):783-790.
- 110 Strauch E, Moske O, Thoma S, et al: A randomized controlled trial on the effect of montelukast on sputum eosinophil cationic protein in children with corticosteroid-dependent asthma. *Pediatr Res* 2003,54(2):198-203.
- 111 Gauvreau GM, Boulet LP, Schmid-Wirlitsch C, et al: Roflumilast attenuates allergen-induced inflammation in mild asthmatic subjects. *Respir Res* 2011,12:140.
- 112 Dente FL, Carnevali S, Bartoli ML et al: Profiles of proinflammatory cytokines in sputum from different groups of severe asthmatic patients. *Ann Allergy Asthma Immunol* 2006, 97:312–320.
- 113 Park SW, Jangm HK, An MH, et al: Interleukin-13 and interleukin-5 in induced sputum of eosinophilic bronchitis: comparison with asthma. *Chest* 2005,128(4):1921-1927.
- 114 Broide DH: Immunologic and inflammatory mechanisms that drive asthma progression to remodeling. *J Allergy Clin Immunol* 2008,121(3):560-570.
- 115 Komai-Koma M, McKay A, Thomson L, et al: Immuno-regulatory cytokines in asthma: IL-15 and IL-13 in induced sputum. *Clin Exp Allergy* 2001,31(9):1441-1448.
- 116 Aggarwal S, Moodley YP, Thompson PJ, Misso NL: Prostaglandin E2 and cysteinyl leukotriene concentrations in sputum: association with asthma severity and eosinophilic inflammation. *Clin Exp Allergy* 2010, 40(1):85-93.
- 117 Delimpoura V, Bakakos P, Tseliou E, et al: Increased levels of osteopontin in sputum supernatant in severe refractory asthma. *Thorax* 2010,65:782–786.
- 118 Pavord ID, Ward R, Woltmann G, et al: Induced sputum eicosanoid concentrations in asthma. *Am J Respir Crit Care Med* 1999,160(6):1905–1909.
- 119 Tseliou E, Bakakos P, Kostikas K, et al: Increased levels of angiopoietins 1 and 2 in sputum supernatant in severe refractory asthma. *Allergy* 2012,67(3):396-402.
- 120 Tufvesson E, van Weele LJ, Ekedahl H, Bjermer L: Levels of cysteinyl-leukotrienes in exhaled breath condensate are not due to saliva contamination. *Clin Respir J* 2010,;4(2):83-88.

-
- 121 Tufvesson E, Aronsson D, Bjermer L: Cysteinyl-leukotriene levels in sputum differentiate asthma from
rhinitis patients with or without bronchial hyperresponsiveness. *Clin Exp Allergy* 2007,37(7):1067-1073.
- 122 Hallstrand TS, Henderson WR Jr: Role of leukotrienes in exercise-induced bronchoconstriction. *Curr
Allergy Asthma Rep* 2009,9(1):18-25.
- 123 Gaber F, Daham K, Higashi A, et al: Increased levels of cysteinyl-leukotrienes in saliva, induced sputum,
urine and blood from patients with aspirin-intolerant asthma. *Thorax* 2008,63(12):1076-1082.
- 124 Pavord I, Woodcock A, Parker D, et al: Salmeterol plus fluticasone propionate versus fluticasone
propionate plus montelukast: a randomised controlled trial investigating the effects on airway
inflammation in asthma. *Respir Res* 2007, 27;8:67.
- 125 Reid DW, Misso NL, Aggarwal S, et al: Tolerance and rebound with zafirlukast in patients with persistent
asthma. *J Negat Results Biomed* 2008, 19;7:3.
- 126 Ricciardolo FL, Di Stefano A, Silvestri M, et al: Exhaled nitric oxide is related to bronchial eosinophilia and
airway hyperresponsiveness to bradykinin in allergen-induced asthma exacerbation. *Int J Immunopathol
Pharmacol* 2012,25(1):175-182.
- 127 Dweik RA, Sorkness RL, Wenzel S, et al: Use of exhaled nitric oxide measurement to identify a reactive,
at-risk phenotype among patients with asthma. *Am J Respir Crit Care Med* 2010, 181(10):1033-1041.
- 128 Nair P, Kjarsgaard M, Armstrong S, et al: Nitric oxide in exhaled breath is poorly correlated to sputum
eosinophils in patients with prednisone-dependent asthma. *J Allergy Clin Immunol* 2010, 126(2):404-
406.
- 129 Redington AE, Meng QH, Springall DR, et al: Increased expression of inducible nitric oxide synthase and
cyclo-oxygenase-2 in the airway epithelium of asthmatic subjects and regulation by corticosteroid
treatment. *Thorax* 2001,56(5):351-357.
- 130 Jiang J, Malavia N, Suresh V, George SC: Nitric oxide gas phase release in human small airway epithelial
cells. *Respir Res* 2009,10:3.
- 131 Suresh V, Mih JD, George SC: Measurement of IL-13-induced iNOS-derived gas phase nitric oxide in
human bronchial epithelial cells. *Am J Respir Cell Mol Biol* 2007,37(1):97-104.
- 132 Liang Y, Yeligar SM, Brown LA: Exhaled breath condensate: a promising source for biomarkers of lung
disease. *Scientific World Journal* 2012,2012:217518.
- 133 Kazani S, Planaguma A, Ono E, et al. Exhaled breath condensate eicosanoid levels associate with asthma
and its severity. *J Allergy Clin Immunol* 2013, Apr 19. pii: S0091-6749(13)00369-2 (in press).
- 134 Rathnayake N, Akerman S, Klinge B, et al: Salivary biomarkers for detection of systemic diseases. *PLoS
One* 2013, 8(4):e61356.
- 135 Blicharz TM, Siqueira WL, Helmerhorst EJ, et al: Fiber-optic microsphere-based antibody array for the
analysis of inflammatory cytokines in saliva. *Anal Chem* 2009,81(6):2106-2114.

Figure1.

Immunological interactions within Th-2 driven airway inflammation in asthma
[69].