Chapter 2

Non-invasive Biomarkers in Asthma: Promises and Pitfalls

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Additional information is available at the end of the chapter

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Abstract

The asthma concept has evolved throughout the years: one major step in asthma management is the recognition of the chronic (airway) inflammation; another major step is further understanding of asthma heterogeneity and subsequent development of targeted therapies. While the concept of chronic inflammation, airway structural changes and their variability over time are widely accepted, their measurement and monitoring have gone through many hardships.

In this chapter, we discuss the need for applicable biomarkers in asthma management and focus on the currently available and most promising totally non-invasive samplings and detection techniques, ranging from single biomarkers to biomarker panels and composite signatures, including molecular high-throughput "omics" technologies outcomes. Limitations of these biomarkers are compared with minimal-, semi- and invasive techniques. Additionally, we discuss the benefits of an integrative systems medicine approach, considering asthma phenotypes based on cluster analysis of multidimensional biomarker datasets and its contribution to recent developments towards the promise of better understanding asthma and personalised asthma management.

Keywords: asthma, biomarkers, composite signature, phenotype, personalised medicine

1. Introduction

According to the concurrent paradigm, asthma should not be regarded as a single disease, but rather as a complex of multiple, overlapping syndromes. The heterogeneity of asthma has been

recognised already for over a century, for instance, as intrinsic and extrinsic ("allergic") asthma [1].

The introduction and subsequent validation of hypertonic saline-induced sputum analysis revealed different inflammatory asthma phenotypes: i.e. eosinophilic versus non-eosinophilic [2]. Asthma phenotypes comprise shared similar observable characteristics, produced by the interactions of an individual's genetic make-up and the environment that can be affected by several triggers and respond to treatment. However, phenotypes may vary over time and do not directly link to the underlying pathophysiology. Factor analyses involving various disease characteristics and biomarkers, including fractional exhaled nitric oxide (FeNO) levels and sputum cell differentials, helped to further define asthma (sub)phenotypes [3, 4].

In the 1990s, in analogy with animal models, asthma was thought to be a typical T-helper (Th)2- and immunoglobulin E (IgE)-driven disease, and hence, the proof of clinical effectiveness of potential asthma therapeutics was tested in the allergen challenge model. More recently, genomics and other sophisticated "omics" techniques enabled further characterisation of various inflammatory cells and other biomarkers, and helped to link asthma subphenotypes or endotypes to specific cellular and molecular pathways. For instance, gene expression profiling revealed two major subtypes: i.e. "Th2-high" and "Th2-low" asthma providing evidence for responders and non-responders to Th2-targeted therapies [5, 6]. Apart from the involvement of the adaptive immune responses, pathognomonic for parasites and allergens, more recent insight showed the major involvement of the innate system (ILC2s: innate lymphoid type 2 cells) in some asthma endotypes [7]. Interestingly, both Th2 cells and ILC2s produce type 2 cytokines (i.e. interleukin (IL)-4, IL-5 and IL-13) and these type 2 responses are mainly mediated by eosinophils. However, the underlying "upstream" mechanisms differ: while allergens mainly drive Th2-responses [8], viruses and pollutants are common triggers for ILC2-mediated type 2 responses that involve epithelial cells and IL-25, IL-33 and thymic stromal lymphopoietin [9]. Presently, it is not fully clarified how exactly both type 2 response pathways interrelate.

Apart from disease typing, the discovery of new inflammatory pathways and related biomarkers resulted into the development of endotype-specific, individualised asthma treatment.

In this review, we aim to highlight the key non-invasive and semi-invasive biomarkers currently used in the management of asthma.

2. Do we need biomarkers in asthma?

Given the heterogeneity of asthma and the evidence that standard therapy is not (fully) effective in all patients, especially in those with more severe disease and those at risk for frequent exacerbations, the need for appropriate biomarkers allowing the identification and subsequent targeted treatment of these patients has been increasingly recognised. Since asthma is multidimensional and thus presents at several different levels including clinical, physio-

logical, histological, cytological and molecular, various approaches have been developed to identify effective biomarkers (**Table 1**) [10]. In addition, given the complexity of the disease, (unbiased) biomarker clustering within different asthma populations has been performed by several research groups, which revealed different disease subphenotypes with varying disease course and/or response to treatment [3, 4].

Disease level	Parameters/biomarkers
Clinical	Age of onset Frequent exacerbators Therapy resistance Cofactors, including allergy, nasal polyps, recurrent viral infections, air pollutants including passive and/or active tobacco smoke, obesity
Physiological	Lung function (normal, reversible, fixed obstruction) Airway hyperresponsiveness
Cytological	Inflammatory cells and soluble markers in: Sputum (central airways); BAL, bronchial wash/brushings (peripheral airways)
Histological	(Trans)bronchial biopsies (inflammatory and structural cells and structures)
Exhaled air	FeNO (fractional exhaled nitric oxide) EBC (exhaled breath condensate) VOCs (volatile organic compounds: eNose) EBT (exhaled breath temperature)
Systemic biomarkers	Peripheral blood: eosinophils, CRP, IgE, periostin, cytokines
Molecular	Genomic SNP analysis (i.e. the large-scale genotyping of single nucleotide polymorphisms) Transcriptomic analysis (i.e. the measurement of all gene expression values in a cell or tissue type simultaneously) Proteomic analysis (i.e. the identification of all proteins present in a cell or tissue type) Metabolomic analysis (i.e. the identification and quantification of all metabolites present in a cell or tissue type; eNose)

BAL: bronchoalveolar lavage; CRP: C-reactive protein; eNose: electronic nose; IgE: immunoglobulin E; SNP: single nucleotide polymorphism

Table 1. Clinical and biological biomarkers in asthma.

Using a systems biology approach in large cohorts of patients, researchers within the Innovative Medicines Initiative Severe Asthma Project U-Biopred have been collecting data, including molecular analyses, tissue, exhaled air and blood samplings, as well as clinical and lung function data, and patient-reported symptoms [11]. By combining this information, the researchers aimed to generate a "handprint", i.e. a combination of clinical and biological

characteristics (biomarkers) indicative of a specific asthma subphenotype/endotype. Subsequent studies are being undertaken to test if one's "handprint" can predict the disease course and can indicate a response to (targeted) asthma treatments. This approach will provide a key step to personalised medicine [12–14].

Generally, an ideal biomarker should possess the following key characteristics: clinical relevance, adequate sensitivity and specificity for (targeted) treatment effects, repeatability, simplicity and cost-effectiveness [10].

3. Promising single non-invasive biomarkers of asthma

The concept of asthma has undergone considerable changes throughout the years, from a disease mainly manifesting by variable symptoms and bronchoconstriction to airway inflammation and remodeling. More recently, heterogeneity has gained an outstanding position in asthma definition. So far, one of the most important steps in asthma history, bringing significant reduction in morbidity and mortality, was the recognition of airway inflammation in asthma and the introduction of efficacious and safe anti-inflammatory therapy for asthma control. Despite ongoing developments, current guidelines for both diagnosis and follow-up of patients with asthma are still grounded on clinical and lung function parameters. Thus, functional biomarkers were the first objective measures coming forward into clinical practice and, in general, the promise of delivering valuable molecular, cellular or histological biomarkers to daily clinical practice has not yet been met. However, intense research in asthma has brought together scientists from academia, research institutes, the pharmaceutical industry and patient organisations, with significant progress taking place in the recent years. In this section, we discuss the currently available and more advanced non-invasive biomarkers in asthma.

Clinicians and researchers dedicated to asthma may benefit from a direct analysis of the airways, profiting the patients. In fact, non-invasive airway assessment is possible through lung function tests (LFTs) and airway sampling. Furthermore, other "more distant" to the airway biomarkers (such as blood or urinary biomarkers) can also be regarded as potentially useful, considering the systemic properties of asthma.

3.1. Functional biomarkers

LFTs are essential in routine clinical practice. They are non-invasive, well validated and reproducible. At present, LFTs provide the only generally accepted functional biomarkers to objectively aid in the diagnosis, risk assessment and monitoring of asthma. Thus, asthma definition currently implies the objective detection of variable airflow limitation, while the "best personal lung function" is a hallmark of asthma monitoring and future risk assessment.

LFTs provide relative features (phenotypes) that aid in differential diagnosis, namely in the distinction from chronic obstructive pulmonary disease (COPD), but are not diagnostic in its use. For instance, neither post-bronchodilator airway obstruction, lack of bronchodilation response or hyperinflation can be used to rule out asthma.

Presently, LFTs patterns alone are not considered to define disease subsets that respond to particular therapies. However, lung function has been shown to be predictive of clinical outcomes and provide complementary information to subphenotype asthma. For instance, variability measures of lung function can predict the loss of asthma control and response to long-term beta2-agonist treatment [15].

Airway hyperreactivity (AHR) is a basic pathophysiological hallmark of asthma, but remains a complex component of this disease. A growing number of variable airway smooth muscle (ASM) and non-muscle factors contributing to AHR has been recognised. Besides its high negative predictive value in the diagnosis of asthma, AHR has been advocated as a surrogate biomarker related to airway inflammation to guide asthma management. It has been shown that anti-inflammatory therapy directed at reducing AHR may imply higher corticosteroid doses, but leads to improved lung function and better control [16, 17]. AHR evaluation has also been suggested useful in back titration of inhaled corticosteroids. However, the reduction in AHR with higher doses appears targeted to the persistent structural component of AHR (defined as opposed to the variable inflammation component of AHR). Emerging data support that it is the structural changes of the airway that mainly contribute to AHR (i.e. reticular layer thickness and ASM hypertrophy) [16]. This effect also depends on the type of challenge used: assessing AHR to indirect bronchoconstrictor stimuli is superior in the detection of changes associated with airway inflammation, while direct stimuli, mediated through direct interaction with ASM, better reflect the structural changes. Assessment of AHR is a useful non-invasive tool providing complementary information, though its routine feasibility in general practice can be hard to settle.

Summing up, lung function measurements may not, per se, reflect the precise underlying pathological processes responsible for different phenotypes. However, in a multidimensional approach to evaluate asthma as a complex dynamic disease, functional biomarkers and their variability must definitely be part of future composite parameters in asthma.

3.2. Exhaled air biomarkers

Exhaled breath can be sampled in a fully non-invasive manner across all age groups. However, exhaled breath analysis is not useful for analysing cellular or histological biomarkers and, in general, the search for useful molecular biomarkers has been hampered by methodologic difficulties mainly dealing with very low molecular concentrations, variability and lack of sampling and analysing methods standardisation [10].

FeNO is so far the most commonly used molecular biomarker in exhaled air. Nitric oxide (NO) is a gaseous chemical compound, which can be measured in exhaled breath either by chemiluminescence and electrochemical analysers. The American Thoracic Society and the European Respiratory Society recommendations for standardised procedures for the FeNO measurement have been published [18]. Accordingly, FeNO is measured at a flow rate of 50 mL/s, thus reflecting NO production from the central airways. Currently available devices allow accurate and highly reproducible measurements, through simple, fast and non-invasive methodology. Hand-held devices are now widely available in clinical practice and used in both adults and children (since preschool age, usually above the age of 4 years) [10].

Evidence-based guidelines for adequate interpretation of FeNO measurement have been developed [19]. This biomarker can be affected by several perturbing factors, mainly age, height and recent active or passive smoking. Other variables that have been reported to affect FeNO levels include weight, gender, race, atopic status, diet or alcohol intake [20]. Large variation of normal FeNO values exists, with wide inter-individual differences and significant overlaps between healthy/non-asthmatic and asthmatic populations. Intriguingly, the aforementioned confounding factors explain few of the substantial variations within the general population [20]. For these reasons, guideline-recommended cut-points are supported for routine interpretation of FeNO levels [19].

Presently, there is evidence to support the use of FeNO thresholds essentially for assessing the likelihood of Th2-mediated airway inflammation and responsiveness to corticosteroids [19]. Low FeNO levels do not rule out asthma [19].

Persistently high FeNO levels may be attributed to poor adherence to corticosteroid therapy, poor inhaled drug delivery or persistent/high allergen exposure [19]. This has also been suggested to reflect a highly reactive asthma phenotype [21]. Although FeNO may be indicative of loss of disease control or exacerbation, some patients remain with high FeNO despite good clinical asthma control, and clinical trials of FeNO-guided management have yielded conflicting results [22–24]. Increased knowledge on asthma pathophysiology and the source and biochemistry of FeNO may help to further understand these findings. Traditionally, FeNO is known to originate in the airway epithelium as a result of inducible nitric oxide synthase (iNOS) upregulation, which occurs with inflammation [19]. Recent data give further support to this view by showing iNOS overexpression in the airway epithelium of patients with asthma [25]. However, it is interesting to note that despite the strong association between FeNO and Th2-mediated/eosinophilic inflammation and atopy, eosinophils are not the principal cells in the airways that express iNOS and this enzyme is upregulated by Th1 cytokines [26]. Anti-IL-5 and anti-IgE therapy for asthma reduced sputum eosinophilia without affecting FeNO, contrary to IL-13 inhibition that significantly decreased FeNO [27]. Studies have shown that FeNO levels are not elevated in many patients with severe asthma, compared to mild and moderate asthma, despite evidence of airway inflammation [13, 28]. Other sources of FeNO need also to be considered. For instance, as NO is a highly reactive molecule, it can be trapped and directly regenerated by abundant free thiol-containing biomolecules [26]. One of these thiols is S-nitrosoglutathione, which has been shown to be depleted in severe asthma, possibly contributing to comparative lower FeNO levels in these patients. Another important reservoir of nitrogen species is nitrite/nitrous acid. These agents are physiologically recycled in blood and tissues to form NO and other bioactive nitrogen oxides. When airway pH increases, more nitrite is formed and FeNO levels fall. On the other hand, FeNO may be high with acidification [26]. Still, many questions regarding the source of FeNO and its specific role need to be explored. Another area of research that may bring additional knowledge and clinical usefulness is dedicated to partitioning of FeNO. In particular, alveolar FeNO can be obtained by measuring FeNO at multiple flow rates and has been shown to be an independent parameter that is putatively associated with increased distal lung inflammation and more severe disease [29].

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In summary, the clinical importance of FeNO as a marker of Th2-mediated airway inflammation that is likely to respond to corticosteroid treatment may be "indirect," but is well established. Further analysis is needed to address the possible need to define FeNO levels cut-points in different situations, according to the presence or absence of pertinent confounders. The application of FeNO measurement to identify particular asthma phenotypes or as part of a more comprehensive panel of biomarkers including also other "Th2 type" biomarkers may allow taking better profit of this readily available biomarker [30]. Partitioning of FeNO is a promising area of research, whose clinical usefulness is yet to be established.

Other biomarkers have been studied in exhaled breath vapor namely *volatile organic compounds* (VOCs). In general, reactive oxygen species result from inflammation and promote polyunsaturated fatty acids degradation, originating volatile hydrocarbons. These VOCs are subsequently excreted in exhaled breath. Thus, exhaled VOCs may originate from systemic metabolism or from local airway inflammation. It is important to consider also that VOCs in exhaled breath may also be originated from pathogenic bacteria or from exogenous sources such as ambient air pollution [31]. Some studies have suggested that single VOCs such as pentane or ethane could be significantly higher in patients with asthma. However, VOCs profiles analyses bring significant additional value [31].

Another potential single biomarker in exhaled air is *exhaled breath temperature* (EBT), which reflects heat, a cardinal sign of inflammation. EBT has been shown to correlate with bronchial blood flow [32], which is advocated as the main mechanism to explain EBT changes in disease status.

Several studies have shown that EBT is higher in patients with asthma [32–34]. Conflicting data have been reported regarding a possible association between EBT and asthma control, with several studies supporting [34, 35], and others rejecting this relation [36, 37]. Correlation between EBT and other biomarkers, such as sputum eosinophils and FeNO, has resulted in inconsistent reports [32, 37]. Furthermore, EBT has been shown to increase after eucapnic voluntary hyperventilation, methacholine challenge test or exercise, but no difference was found between asthmatics and healthy individuals [38], suggesting this increase in EBT to be physiologic.

However, it is important to stress that different methods have been used to measure EBT. Some studies used a flow and pressure-controlled maximal slow continuous exhalation to residual volume to measure EBT, while others measured EBT in tidal volume until a temperature plateau was reached. Different variables have been analysed: plateau EBT, rate of temperature increase, time to achieve plateau EBT. These different methods preclude results comparison and, to our knowledge, no study has analysed both methods simultaneously. The recent development of improved, easier-to-use, portable devices has improved feasibility, including in children and in the elderly [34, 36, 39].

Moreover, further studies are needed when it concerns interpretation of the results. Variables such as room temperature and relative-ambient humidity may influence the results [39]. Some studies point a correlation between gender [37, 39], age [36, 39] and lung volume [36], which

needs to be addressed. No significant correlation has been documented between EBT and auricular temperature, suggesting EBT to be a distinct variable and not just another measurement of body temperature [33, 34].

Conclusively, EBT assessment may be an appealing method enabling completely non-invasive and patient-friendly evaluation and deserves further standardisation and validation as a potentially useful biomarker in asthma.

3.3. Exhaled breath condensate biomarkers

Exhaled breath has been a source for intense research in the latest years and many other biomarkers have been studied. *Exhaled breath condensate* (EBC) has the advantage of being a more stable matrix than exhaled breath vapor, including volatile and also non-volatile compounds. It is obtained by cooling exhaled air and is thought to reflect the composition of the airway lining fluid. Many molecules have been analysed in EBC, including metabolites and also proteins. Although methodological recommendations for exhaled breath sampling and analysis have been published [40], the procedures for EBC collection and biomarker detection are not fully standardised and there is significant heterogeneity between different working groups yielding (highly) variable data.

Many biomarkers analysed in EBC reflect oxidative stress. Among these, the most extensively studied include H_2O_2 and isoprostanes.

H_2O_2 is a reactive oxygen species that contributes to oxidative stress within the airways. A meta-analysis has reported that EBC H_2O_2 concentrations were significantly higher in adults with asthma, and associated with disease severity and control [41]. This has also been reported in children. Of importance, smoking increases H_2O_2 levels. EBC H_2O_2 levels were inversely correlated with lung function parameters and improved with inhaled corticosteroids [41]. Thus, EBC H_2O_2 has been suggested a promising biomarker for asthma control monitoring.

Oxidative stress can also be assessed through the determination of lipid peroxidation-derived products. *8-isoprostane* derives from arachidonic acid peroxidation. Increased levels of 8-isoprostane have been found in EBC in patients with asthma, correlating with disease severity [42]. EBC 8-isoprostane levels have been shown to be particularly useful to indicate asthma control and severity in childhood when combined with different markers [30]. Increased 8-isoprostane levels in EBC of children with exercise-induced bronchoconstriction (EIB) have been described, suggesting a role for oxidative stress in EIB [43].

Markers of inflammation have also been addressed. *Leukotrienes (LT)* are important mediators of airway inflammation in asthma, and the most extensively studied molecular biomarkers of inflammation in EBC. Increased levels of LTs have been detected in EBC of patients with asthma, correlated with disease severity and were effectively reduced by oral corticosteroids or LT receptor antagonist [44, 45]. However, the reported effect of inhaled corticosteroids on LTB4 EBC levels is controversial [46]. LTs have been suggested as markers of asthma severity [42]. Likewise, LTs have been associated with EIB severity [47].

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Various *cytokines* and other molecules have been analysed in EBC. In particular, IL-4 has been found to be higher in EBC of patients with asthma, especially in asthma associated with atopy [30, 42]. Cytokine ratios and biomarker panels in EBC including cytokines have been suggested to be useful to assess asthma control (including IL-4 and interferon-gamma) and to predict asthma exacerbations (e.g. IL-5) [30, 48].

Last but definitely not least, the measurement of *pH* is one of the simplest and most technically validated biomarkers in EBC. EBC pH reflects airway acidification [49]. Several research groups have found higher pH levels in healthy subjects, compared to patients with asthma [10]. Significant decline in EBC pH occurred during asthma exacerbations. EBC pH shows good reproducibility, having low running costs and normal data sets have been published in self-reported healthy subjects [50].

Although some biomarkers may be useful to measure in EBC, samples are highly diluted, biomarker concentrations are difficult to measure, require specialised equipment, laboratory techniques and normalisation standards are lacking. Unfortunately, EBC has been hampered by serious drawbacks in the methodology, detection techniques and result interpretation, all consistent with large intra and intersubject variability, precluding validation for most single biomarkers.

3.4. Biomarkers in non-respiratory specimens

Other non-invasive matrices have also been analysed in search for biomarkers in asthma. *Saliva* is a readily available specimen and allows metabolites, proteins and also deoxyribonucleic acid (DNA)/ribonucleic acid (RNA) extracting (although buccal swabs perform better), including also oral microbiota assessment. *Cotinine* in saliva has been one of the most extensively studied biomarkers, with interest in asthma as a measure of tobacco exposure. Salivary *cortisol* has also been used for the evaluation of adrenal function. Morning salivary cortisol was significantly lower in patients with asthma than in healthy individuals, and poor asthma control has recently been associated with lower salivary cortisol levels [51]. Preliminary data have suggested that *inflammatory salivary markers* may also be associated with asthma control, including eosinophil-related (such as eosinophil cationic protein) and myeloid/innate mediators [52]. Additionally, a significant decrease in salivary antioxidant enzyme-peroxidase activity was observed in children during asthma exacerbations [53]. A salivary pH decline has also been associated with asthma and AHR [54]. Another area of research includes the analysis of oral microbiota, which may change in asthma, either through disease status or its pharmacotherapy. The interest in saliva studies in relation to asthma is still preliminary and the role of many possible confounders needs to be considered.

Although *urine* does not directly reflect the airways, samples are easily obtained across the full age spectrum. Several urine molecular biomarkers have been described to be associated with asthma. Here, we focus on four molecules which have been studied in more detail.

Of the potent lipid inflammatory mediators comprising the cysteinyl *LTs*, only LTE4 is stable, making this molecule the dominant LT detectable in biological fluids. Urinary levels of this end product of LT metabolism have been shown to be elevated in asthma, both in children and

adults, and in patients with aspirin-exacerbated respiratory disease [55, 56]. It has been associated with the degree of airflow limitation and acute exacerbations [55, 57]. Although inhaled corticosteroids are the most effective treatment for asthma, they do not alter LTE4 excretion. Urinary LTE4 levels have been suggested as potential predictors of better response to anti-LT therapy compared to other therapeutic approaches, though further studies are needed, including other biomarkers, to predict individual responses.

As LTs, *prostaglandins* (PG) are the end products of arachidonic metabolism. PGD2 results from cyclooxygenase pathway and is excreted in urine after being metabolised to 9α,11β-PGF2. Increased urinary excretion occurs in patients with asthma and after challenge tests, and a negative association has been found with lung function [58].

Bromotyrosine is another molecule with possible interest in asthma. It is generated from protein oxidation by eosinophils. The oxidised amino acid is stable and excreted in urine. Urinary bromotyrosine levels are higher in patients with asthma and have been associated with asthma control and lung function, predicting exacerbations [59]. Its levels have been shown to reduce during inhaled corticosteroid therapy. High urinary bromotyrosine levels could predict a favorable clinical response to inhaled corticosteroid therapy, especially in combination with high FeNO values [59]. These results warrant further developments.

Though urinary biomarkers may become useful tools, many require specialised equipment and their measurement is not fully validated or standardised. There is a current need for normalisation standards and assessment of intra and inter-individual variation to select the potentially useful biomarkers. It is also important to address urine dilution when reporting quantitative absolute results.

3.5. Airway imaging biomarkers

Airway imaging biomarkers are also emerging, offering the potential of adding complementary information, namely on small airways function and remodeling. High-resolution computed tomography (HRCT) images are used to measure airway narrowing, wall thickening, air trapping and ventilation inhomogeneity [27]. The first two measures have been correlated with lung function and asthma severity. Increased parenchymal lucency has also been associated with severe asthma exacerbations, lung function and neutrophilic inflammation. HRCT is easily performed though it requires that lungs are scanned at a standard volume for validity and reproducibility. The risk of exposure to significant ionising radiation needs to be considered and normal ranges have not been established.

4. Composite biomarkers in non-invasive sampling: what is known and what could be useful in the future?

The complexity and dynamics of asthma drive the need to establish distinct disease phenotypes and endotypes. There are several different triggers in asthma, with various pathophysiological pathways in parallel resulting in clinical expression that may be rather similar. Therefore,

repeated multiscale, multidimensional measurements may be needed to capture this complexity, which may yield more useful information than single or even panels of combined biomarkers [60]. In this view, molecular composite signatures may be obtained by high-throughput "omics" technologies, which are increasingly standardised. Several large-scale studies of the genome, transcriptome, proteome or metabolome have produced an enormous amount of data and it is pivotal to follow the available guidelines in order to avoid false discoveries [60]. The composite high-dimensional signatures or fingerprints are based on pattern recognition underlying complex non-linear biology systems. Some evidence that this approach may be successful in asthma has already emerged concerning differential diagnosis [61]. Regarding non-invasive, direct assessment of exhaled breath, it is interesting to note that while many problems arise in specific molecular biomarkers validation, recent studies have shown encouraging results with the application of metabolomics strategies to study exhaled biomarkers [60, 62, 63].

Among "omics" systems biology, metabolomics is considered the one that comes closest to phenotype expression. It involves the identification and quantification of small molecular weight metabolites. Real-time metabolomics measurements are already feasible for several clinical applications with electronic noses (eNoses). These handheld, portable devices can capture various combinations of VOCs in exhaled breath, with nanosensors arrays. The nanosensors are based on 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 and gas-chromatography (GC) coupled with mass spectrometry (MS) or flame ionisation detection [60, 64]. The pattern recognition algorithms using various eNose sensor systems indicate fingerprints of exhaled VOCs, called breathprints, which have shown to discriminate patients with asthma from healthy subjects and COPD with accuracies between 80% and 100% [65]. Breathprints have also been studied to phenotype asthma. Recent studies indicate that eosinophilic and non-eosinophilic asthma can be distinguished when using a composite eNose platform. Breath analysis by eNose could also predict the response to corticosteroids with greater accuracy than sputum eosinophils or FeNO [66]. These data suggest that composite signatures of breath analysis could be used for assessment and monitoring of airway inflammation. Important methodologic issues of technique optimisation and standardisation deserve deeper analysis, from breath sampling, to modulating factors including comorbidities and incompatibility between eNoses. These should enable external validation to determine possible disease-specific breathprints with clinical applicability.

Besides the analysis of exhaled breath vapour with GC-MS and eNoses, the novel metabolomics approach has also been applied to EBC. It has been shown to enable characterisation of metabolic compounds in even small EBC volumes, using high-resolution proton nuclear magnetic resonance (NMR) or MS. This has proved capable of discriminating healthy individuals from those with asthma [62, 63, 67, 68]. It could also discriminate between severe and non-severe asthma [63], supporting the hypothesis that severe asthma has specific metabolic features.

Interestingly, the metabolomics analysis of urine also discriminated healthy individuals from those with asthma [69], and could distinguish patients with stable asthma from those with acute exacerbations based on profiles [69, 70]. Metabolomics analysis of urine samples has also been recently suggested as a useful clinical tool to differentiate asthma from COPD [71].

Pinkerton et al. [72] demonstrated for the first time that differences between healthy controls and asthma patients could be detected via micro-RNA (miRNA) expression in EBC, and suggest that different types of inflammation may have unique miRNA signatures. These small non-coding RNAs are known to be important in the post-transcriptional regulation of inflammation, thus opening a new research field using non-invasive direct air sampling.

Proteomics has also recently been applied to EBC. Liquid chromatography (LC)-MS has been used to separate and detect proteolytic peptides present in EBC with differentiating profiles based on asthma status [73]. However, this preliminary study faced several problems such as insufficient sample volume, possible salivary contamination and difficulties in peptides identification due to their low concentration.

Besides allowing an overview of molecular signatures, the "omics" approach may potentially lead to new knowledge regarding asthma pathophysiology, due to its untargeted, hypothesis-generating approach. All biomedical researchers are facing not only the opportunities but also the challenges in accessing, managing, analysing and integrating diverse data sets that are larger, more diverse and more complex than ever before, and that exceed the abilities of current management and analysis approaches [60, 74]. Composite biomarkers research such as that coming from molecular profiling assays including various "omics" is a live example that needs to be critically interpreted and cautiously validated to yield truly significant advances in personalised medicine.

5. Non-invasive biomarkers limitations: can more invasive sampling do better?

Asthma syndromes are characterised for being dynamic, with varying changes in symptoms pattern, lung function, inflammation and remodelling throughout time. In this setting, non-invasive direct airway sampling, such as exhaled breath analysis, seems especially appealing, allowing easy and repeatable measures over time. However, low molecular concentrations and variable sample dilution lead to difficulties in methods sensitivity and validation, with consequent issues in replication of biomarker findings (**Table 2**). In comparison, bronchoscopy allows direct visual examination of the airways and direct collection of fluid (bronchoalveolar lavage, bronchial washing) and tissue (brushing, biopsy). These techniques are mostly impractical because they are invasive, require specialised equipment and qualified personnel, have contraindications and carry potential risks / complications. Therefore, ethical issues preclude bronchoscopic sampling broad use in asthma, even less when repeated samplings are needed, thus being mainly reserved for selected severe patients and for research purposes. Apart from practical issues, standard bronchoscopy techniques hold several other limitations, including lack of reproducibility and sample dilution effect, despite recently proposed

improvements (Table 2) [75]. In between invasive and non-invasive airway samplings, semi-invasive induced-sputum analysis may also reflect the airways and is easier to perform. Moreover, although indirect, blood sampling is minimally invasive and is a known relevant biomarker source in asthma.

Biomarker source	**Pros**	**Cons**
Exhaled breath	Totally non-invasive Validated for FeNO measurement Portable (FeNO, eNose, EBT, EBC) Direct results (FeNO, eNose, EBT) Multiple molecular biomarkers May be collected across all ages May be collected in severe patients Allows serial measurements	Validation not complete (except FeNO) Many perturbing factors Upper airways/salivary possible contamination Require expertise, expensive and time-consuming specialised lab assays (EBC) Soluble markers subject to dilution
Induced-sputum	Semi-invasive Validated tool Molecular and cellular biomarkers Useful to guide treatments (sputum-eosinophils)	Impossible in young children Contraindicated in severe bronchoconstriction / active cardiovascular disorders Rescue medication / procedures needed Non-repeatable over short time-period (<12 to 18 h) Procedure itself may induce changes in airways/lab results Upper airways/salivary possible contamination Require expertise, expensive and time-consuming specialised lab assays Soluble markers subject to dilution
Bronchoscopy	Direct airway assessment	Invasive Several medical contraindications Rescue medication/procedures needed Non-repeatable in many patients Expertise and experience required for procedure Require expertise, expensive and time-consuming specialised lab assays BAL markers subject to dilution Procedure itself may induce changes in airways/lab results (BAL)
Blood	Minimally invasive Some biomarkers routinely available (e.g. eosinophil counts) Useful to guide treatments (e.g. eosinophils counts) Molecular and cellular biomarkers May be collected across all ages May be collected in severe patients Allows serial measurements	Not directly reflecting the airways Not patient-friendly in all subjects (e.g. children) Require expertise, expensive and time-consuming specialised lab assays (some biomarkers)
Urine	Totally non-invasive May be collected across all ages May be collected in severe patients Allows serial measurements	Not directly reflecting the airways Require expertise, expensive and time-consuming specialised lab assays

BAL: bronchoalveolar lavage; EBC: exhaled breath condensate; EBT: exhaled breath temperature; eNose: electronic nose; FeNO: fractional exhaled nitric oxide.

Table 2. Pros and cons of main biomarker sample sources in asthma.

In this section, we will discuss these sampling methods and related current main biomarkers for asthma management.

5.1. Sputum biomarkers

Induced sputum is a validated sampling method of the more central airways. Sputum is collected after inhalations of hypertonic saline. Although relatively safe, induced-sputum requires specialised training, equipment and laboratory processing. Monitoring lung function during the induction procedure reduces the risk of excessive bronchoconstriction. Patient's active cooperation is needed for collection, making this technique unsuitable for some patients, especially for children below the age of 7 years [76].

Induced-sputum provides a rich source of soluble and cellular biomarkers and has exceptionally allowed a successful single biomarker-based clinical management approach in asthma. This is the case with sputum eosinophil percentage, which identifies patients who have eosinophilic and non-eosinophilic asthma phenotypes and can be predictive of poor asthma outcome and targeted treatment response, with demonstrated treatment-guided superior efficacy in reducing asthma exacerbations in adults [2, 27, 77, 78]. Thus, sputum eosinophil percentage acts as a key marker and correlates with severe exacerbations and AHR. It has also been useful in a panel of biomarkers to select patients who may benefit from IL-5 targeted therapies, including mepolizumab (anti-IL-5), reslizumab (anti-IL-5) and benralizumab (anti-IL-5R). In contrast with adults [77, 78], eosinophil sputum-guided therapy was not associated with decreased asthma exacerbations or improved asthma control in school-aged children and adolescents [79]. Sputum inflammatory phenotype was shown to be unstable in children with asthma, and this was not related to treatment or disease control [80].

Besides eosinophils, other sputum biomarkers are currently in research. Sputum neutrophils are often related to severe non-eosinophilic asthma with fixed airway obstruction. Soluble sputum biomarkers have been associated with asthma severity (e.g. eosinophilic cationic protein, LT, IL-4, IL-5, IL-13, IL-6, IL-12, tumour necrosis factor-α, granulocyte-macrophage colony-stimulating factor), exacerbations (e.g. IL-8, neurokinin A) or remodelling (procollagen synthesis peptides, tissue inhibitors of metalloproteinase or transforming growth factor-β) [10]. Many biomarkers can be measured, but most require highly sensitive detection methods and results may be affected by sputum processing or variable dilutions. These factors need to be taken into account to select and validate useful biomarkers in sputum.

Induced sputum may also be an interesting source for composite biomarkers. Unsupervised clustering of induced-sputum gene expression profiles identified three transcriptional asthma phenotypes that related to clinical and inflammatory parameters (resembling eosinophilic, neutrophilic and paucigranulocytic asthma) [81]. Differentially expressed genes were related

to immune and inflammatory responses, proving a framework to investigate asthma endotypes.

In summary, logistic and practical difficulties have precluded the wide use of induced sputum in clinical practice, but sputum eosinophil percentage is recommended as a supplemental measure in future asthma clinical research studies to identify specific cellular profiles and to predict or to monitor a treatment response in adult patients [27]. It is important to highlight that sputum eosinophils and FeNO are not duplicative outcome measures, even though low sputum eosinophil and low FeNO are strongly linked [27].

5.2. Blood biomarkers

Peripheral blood can be collected across all age groups, with minimal risk. Some biomarkers are routinely standardised in medical institutions and therefore readily available, such as eosinophils, total serum IgE and allergen-specific IgE. The latter are used to define atopy, which can be accurately, easily and more readily detected by skin prick test. Atopy modestly increases the probability of asthma, but is not essential for diagnosis. Though it is useful to characterise patients, atopy itself is recognised to be heterogeneous, including both "Th2-high" and "Th2-low" phenotypes [5]. Specific sensitisations are useful in clinical practice to suggest clinically relevant allergen avoidance and consider allergen-specific immunotherapy. However, total IgE or allergen-specific IgE quantification cannot predict the response to treatment and are otherwise weak biomarkers in asthma.

Blood eosinophil absolute count has long been associated with asthma and remains a recommended supplemental asthma biomarker [27]. Although it may not reflect the airways and be unspecific, blood eosinophilia supports asthma diagnosis and is an independent risk factor for exacerbations and fixed airflow limitation. Blood eosinophil counts are useful to subphenotype asthma and to monitor systemic biologic effects of pharmacologic interventions in patients with asthma, including (inhaled) corticosteroids, anti-IgE, LT antagonists and 5-lipoxygenase inhibitors [27]. Furthermore, blood eosinophil counts emerged as predictive biomarkers of clinical benefit from IL-5- and IL-13-targeted therapies, being associated with a "Th2 bronchial signature" [82].

Another promising "Th2-high" serum biomarker is the extracellular matrix protein periostin. The expression of periostin is upregulated by IL-13 in bronchial epithelial cells and, unlike IL-13, is abundant and readily detectable in peripheral blood [82]. Interestingly, a multi-centre study collecting matched sputum, bronchoscopy and peripheral blood samples from patients with asthma showed that serum periostin was the best single predictor of airway eosinophilia, with a further advantage of lower intrasubject variability over time than FeNO or blood eosinophilia [82]. However, conflicting results have recently been reported [83, 84]. Nevertheless, periostin levels have been associated with asthma severity and its levels have also been shown to be important to predict lebrikizumab (anti-IL-13) clinical benefit, with greater reduction in severe exacerbations and greater improvement in lung function in the "periostin-high" patients [85]. A greater decrease in exacerbations with anti-IgE therapy has also been reported in "periostin-high" patients. Healthy subjects and lebrikizumab-treated patients still

have measurable levels of serum periostin, thus other systemic sources of periostin than IL-13 need to be explored [82].

Overall, blood eosinophils, serum periostin and FeNO reflect "type 2" airway inflammation in different ways and are only weakly correlated; therefore, combinations of these biomarkers obtained with minimally or non-invasive samplings may further enable optimisation of treatment benefit [82, 86, 87].

Recently, application of "omics" technologies to peripheral blood and invasive sampling with unsupervised clustering are yielding crucial data to capture the complexity of various asthma phenotypes and add new insights on asthma endotypes and treatment response. Given its maturity, transcriptomics analysis using microarrays is the current state-of-the-art method for asthma signature discovery [60]. For instance, gene expression profiling of bronchial epithelium identified distinct subtypes of patients with asthma with "Th2-high" or "Th2-low" phenotype [5], supported the involvement of endotoxin and macrophage activation in corticosteroid resistance, and suggested that corticosteroids also exert their beneficial effects through activity on bronchial smooth muscle [60]. "Omics" technologies developments, with data comparison and validation, will lead to the integration of composite signature biomarkers in phenotyping asthma and improvements in our understanding of asthma. Ultimately, breakthroughs in asthma treatment may be reached through the development of innovative targeted therapies [12, 60].

Non-invasive procedures for biomarker analysis form the backbone for day-to-day clinical asthma management. However, invasive tests may provide important information to phenotype and direct therapy in patients with severe refractory asthma [88]. These techniques bring significant additional knowledge in asthma research that needs to be integrated with non-invasive procedures outcomes to allow truly innovative steps in biomarker discovery for asthma management.

6. Asthma phenotypes based on cluster analyses

In general, milder asthma phenotypes respond well to standard therapy with corticosteroids (with or without long-acting beta2-agonists), while those with more severe disease urged the development of new therapeutic modalities. To enable the development of effective (targeted) therapies, it is crucial to understand the pathophysiological mechanisms driving these subsets of asthmatic patients. Haldar et al. performed a cluster analysis on baseline data of 184 patients with mild to moderate asthma coming from different general practitioners (GP) and baseline data of 187 patients with refractory disease from specialist settings [3]. Additionally, a third dataset comprised baseline and longitudinal data of 68 patients with refractory disease followed for 12 months. Hierarchical cluster analysis revealed five different clusters, with some overlapping features between patients from GP and specialist origins. Most importantly, patients with concordant symptoms and (eosinophilic) inflammation (based on sputum analysis) were mostly coming from GP and were characterised by overall milder, often atopic, well-controlled disease, with a benign disease course. Alternatively, patients with uncontrol-

led disease, characterised by either discordant symptoms (i.e. many symptoms, little airway eosinophilia or non-eosinophilic inflammation) or discordant inflammation (few symptoms, prominent airway eosinophilia) mostly originated from the specialist settings. Commonly found confounders consisted of obesity and non-compliance. Overall, these findings supported a symptom-guided management for mild-moderate "concordant"-type asthma, while "discordant"-type refractory asthmatics might benefit from inflammation-guided therapy [78].

Using unsupervised hierarchical cluster analysis in a group of 726 patients from the Severe Asthma Research Program (SARP) revealed five distinct clinical subphenotypes within this population [4], showing some overlap with the findings by Haldar et al. [3]. The results of both cluster analysis studies underscore disease heterogeneity, even in subsets of patients with similar clinical characteristics, with potentially different pathophysiological and immunological mechanisms, requiring different therapeutic approaches.

Further analysis into the molecular mechanisms underlying different asthma phenotypes revealed at least two distinct subsets with a "Th2-high" and a "Th2-low" profile, respectively [5], based on the expression of IL-13 inducible airway epithelial genes (POSTN (periostin), CLCA1 (chloride channel regulator 1) and SERPINB2 (serpin peptidase inhibitor clade B, member2)) as previously described by this research group. Not unexpectedly, patients with Th2-driven asthma responded well to inhaled corticosteroids while those with a "Th2-low" profile did not. Hence, there is an urgent need for effective therapeutic options for "Th2-low" asthmatic patients that appeared to comprise approximately 50% of the study population, and hence, in reality may be larger than originally thought.

Additionally, these findings urged phenotyping of patients (i.e. including an adequate target population) and/or using an appropriate disease model [8], for adequate interpretation of effectiveness data in targeted intervention studies. So far, several applicable (surrogate) biomarkers have been validated to phenotype potential responders and to monitor the effects of currently available (or under development) targeted therapies, i.e. anti-IgE, and Th2-pathway targeted therapies (anti-IL-5, anti-IL-4 and anti-IL-13) [86]. Presently, biomarkers including blood eosinophils, FeNO and serum periostin thus moved the first steps to personalised medicine [87]. Further insight into the heterogeneity of Th2-driven/type 2 asthma, "Th2-low" subsets, as well as further refinement of sensitive (composite) biomarkers should be considered the next steps in this promising direction to optimise and personalise asthma management.

7. Conclusions

The complex heterogeneity and dynamics of asthma with varying response to standard treatment is driving the search for distinct asthma phenotypes and endotypes. While inhaled corticosteroids can effectively control asthma, therapeutic responses are individualised (though clinical manifestations may match), can be incomplete in a significant number of patients and no curative treatment exists.

In this setting, biomarkers are needed to innovate asthma management. As indicators of pathophysiologic processes or pharmacologic responses, biomarkers can be useful for asthma diagnosis and phenotyping, prediction of future risk or treatment selection or evaluation of response. Non-invasive sampling has the advantage of being patient-friendly and allowing repeatable measurements across all age and severity groups. More direct airway or distant assessment non-invasive sampling and analysis are currently possible, yielding molecular, cellular, functional and imaging potentially clinically useful biomarkers.

For the promise of delivering valuable new biomarkers to the clinic to come forward, it is mandatory that standard optimised procedures are set for sample collection and analysis, and that resulting data are critically processed, explored and cut-off values are well-defined. This will allow comparison of results and replication, with external validation in different population settings.

Though relevant single biomarkers have been found in asthma, increasing evidence shows that biomarker panels do better and composite signatures may indeed soon be integrated in phenotyping/endotyping of asthma. Multiscale, high-dimensional biological, together with standard clinical measures are adding new relevant knowledge. This systems medicine approach is helping to generate new hypotheses and (re)discover pathways and related biomarkers, linking phenotypes to endotypes and ultimately leading to truly innovative treatments for patients with asthma syndromes.

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