

Leveraging -omics for asthma endotyping



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Activity Objectives:

1. To understand the role of asthma endotyping on directing treatment approaches.
2. To understand the immunologic factors that promote an eosinophilic or T_H2 type of asthma endotype.
3. To discuss the efforts of emerging -omics for classification of asthma endotypes along with clinical and laboratory features.

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Asthma is a highly heterogeneous disease, often manifesting with wheeze, dyspnea, chest tightness, and cough as prominent symptoms. The eliciting factors, natural history, underlying molecular biology, and clinical management of asthma vary highly among affected subjects. Because of this variation, many efforts have gone into subtyping asthma. Endotypes are subtypes of disease based on distinct pathophysiologic mechanisms. Endotypes can be clinically useful because they organize our mechanistic understanding of heterogeneous diseases and can direct treatment toward modalities that are likely to be the most effective. Asthma endotyping can be shaped by clinical features, laboratory parameters, and/or -omics approaches. We discuss the application of -omics approaches, including transcriptomics, epigenomics, microbiomics,

metabolomics, and proteomics, to asthma endotyping. -Omics approaches have provided supporting evidence for many existing endotyping paradigms and also suggested novel ways to conceptualize asthma endotypes. Although endotypes based on single -omics approaches are relatively common, their integrated multi-omics application to asthma endotyping has been more limited thus far. We discuss paths forward to integrate multi-omics with clinical features and laboratory parameters to achieve the goal of precise asthma endotypes. (*J Allergy Clin Immunol* 2019;144:13-23.)

Key words: Asthma, endotype, -ome, -omic, transcriptome, epigenome, microbiome, metabolome, proteome, multi-ome, phenotype, cluster, integrate

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Terms in boldface and italics are detailed in the glossary on page 14.

Asthma is a heterogeneous disease with varied contributing mechanisms and diverse clinical presentations. Despite variations in underlying molecular biology and phenotypic manifestation, there is often a convergent suite of symptoms including wheeze, dyspnea, chest tightness, and/or cough.

Many efforts have gone into subtyping asthma. **Phenotypes** are classifications based on common sets of observable characteristics that are often a downstream result of genetics and the environment. Disease severity, response to treatment, and exacerbating factors have long been used to delineate asthma phenotypes. Phenotypes might not reflect unified molecular and cellular mechanisms underlying disease.

Abbreviations used

BAL:	Bronchoalveolar lavage
FENO:	Fraction of exhaled nitric oxide
NK:	Natural killer
NMR:	Nuclear magnetic resonance
SARP:	Severe Asthma Research Program
SNF:	Similarity network fusion
T2:	Type 2
U-BIOPRED:	Unbiased Biomarkers in Prediction of respiratory disease outcomes

Endotypes, on the other hand, are subtypes of disease based on distinct pathophysiologic mechanisms. Endotypes can be clinically useful because they help organize our mechanistic understanding of heterogeneous diseases, such as asthma, directing treatment toward modalities that target specific pathways that might be disrupted within a given endotype. This is especially important in asthmatic patients, who respond to

medications with varying efficacy. The growing availability of biologics that modulate particular pathways makes it increasingly important to accurately characterize asthma endotypes so that therapy can be appropriately prescribed to improve asthma control.¹

Approaches to asthma classification and endotyping can be organized into 3 broad categories, including strategies based on clinical features, laboratory parameters, and molecular profiling by using -omics approaches (Fig 1). Although clinical features and common laboratory parameters have long been used to classify asthma, more recent -omics approaches offer the potential to refine current paradigms and identify novel endotypes. Integration of these approaches is emerging as a way to coalesce strategies toward more meaningful asthma endotypes.

Here we first briefly describe classification of asthma based on clinical features and laboratory parameters and then focus on how -omics approaches have contributed to endotyping thus far. We then discuss emerging efforts toward integrating -omics and

GLOSSARY

CLUSTERING: An approach to segregating samples of a data set into discrete groups based on how similar samples are to one another. In the context of -omics and disease, clustering algorithms can be used to group subjects into different subgroups that can demonstrate distinct characteristics within the data set.

CpG SITES: Regions of DNA where a cytosine nucleotide is followed by a guanine nucleotide in the linear sequence of bases along its 5'→3' direction. CpG sites occur frequently in genomic regions called CpG islands, where methylation of the cytosine within a gene can change its expression.

DNA METHYLATION: A process by which methyl groups are added to DNA, often modifying gene expression.

ENDOTYPE: Subtypes of disease based on shared pathophysiologic mechanisms. Although a disease label can be given to subjects with a given suite of symptoms and signs, the underlying mechanisms that lead to these symptoms and signs might not be uniform across subjects. Endotypes can help organize mechanistic understanding of diseases that have heterogeneous pathophysiology.

EPIGENOME: Modifications made to the genome, including chemical modifications to DNA or modifications to the chromatin packaging of DNA. These can include but are not limited to DNA methylation or hydroxymethylation and posttranslational modifications to histones. These modifications frequently alter transcription levels of nearby genes.

FRACTION OF EXHALED NITRIC OXIDE (FENO): Nitric oxide is an exhaled gas that is produced by cells involved in inflammation associated with allergic or eosinophilic asthma. FENO values are often checked to assess inflammation and response to steroid medications.

GAS CHROMATOGRAPHY MASS SPECTROMETRY (GC-MS): An instrumental technique that incorporates a gas chromatograph coupled to a mass spectrometer through which complex mixtures of chemicals can be separated, identified, and quantified. This is a reliable means to analyze hundreds of relatively low-molecular-weight compounds found in environmental materials.

GENOME: The complete set of genetic information for an organism, including genes and noncoding sequences. The genome contains information needed to build and maintain the organism. The human genome is more than 3 billion DNA base pairs.

INNATE LYMPHOID CELLS: Innate immune cells from the lymphoid lineage that produce cytokines associated with T_H cell subtypes; however,

these cells do not express antigen-specific T-cell or B-cell receptors. Innate lymphoid cells include those in group 1 (ILC1s) that produce T_H1 cytokines, group 2 (ILC2s) that produce T_H2-related cytokines, and group 3 (ILC3s) that produce similar cytokines to T_H17 cells.

INFLAMMASOME: A multiprotein oligomer of the innate immune system that is responsible for activation of inflammatory responses primarily through promoting maturation and secretion of the proinflammatory cytokines IL-1 β and IL-18, leading to pyroptosis, a form of programmed cell death.

LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY (LC-MS): An analytic chemistry technique that combines physical separation of liquid chromatography (or HPLC) with mass analysis of mass spectrometry. Liquid chromatography separates mixtures with multiple components, and mass spectrometry provides structural identity of the individual components with high molecular specificity and detection sensitivity.

METABOLOME: The set of all metabolites found in a sample. Nuclear magnetic resonance, gas chromatography mass spectrometry, or liquid chromatography mass spectrometry is typically used. Metabolites can be specific to certain body fluids, such as urine, serum, plasma, and exhaled breath condensate.

MICROBIOME: The sum of microbes and their genomic elements in a particular environment.

MULTI-OMICS: Combined measures and/or analyses of multiple system-wide molecular profiles.

NATURAL KILLER (NK) CELLS: Cytotoxic immune cells and small granules in their cytoplasm contain special proteins, such as perforin and proteases known as granzymes, which induce apoptosis in the target cell. A type of innate lymphoid cell, NK cells rapidly contain viruses while the adaptive immune system is producing antigen-specific cytotoxic T cells.

-OMICS: The study of the role, relationships, and actions of a system-wide measure of a given molecular type; for example, transcriptomics measure all transcripts in a sample, and proteomics measure all proteins in a sample.

PHENOTYPE: Classification based on a common set of observable characteristics that is often a downstream result of genetics and the environment.

PROTEOME: The set of all proteins expressed in a sample.

TRANSCRIPTOME: The set of all RNA molecules in a sample.

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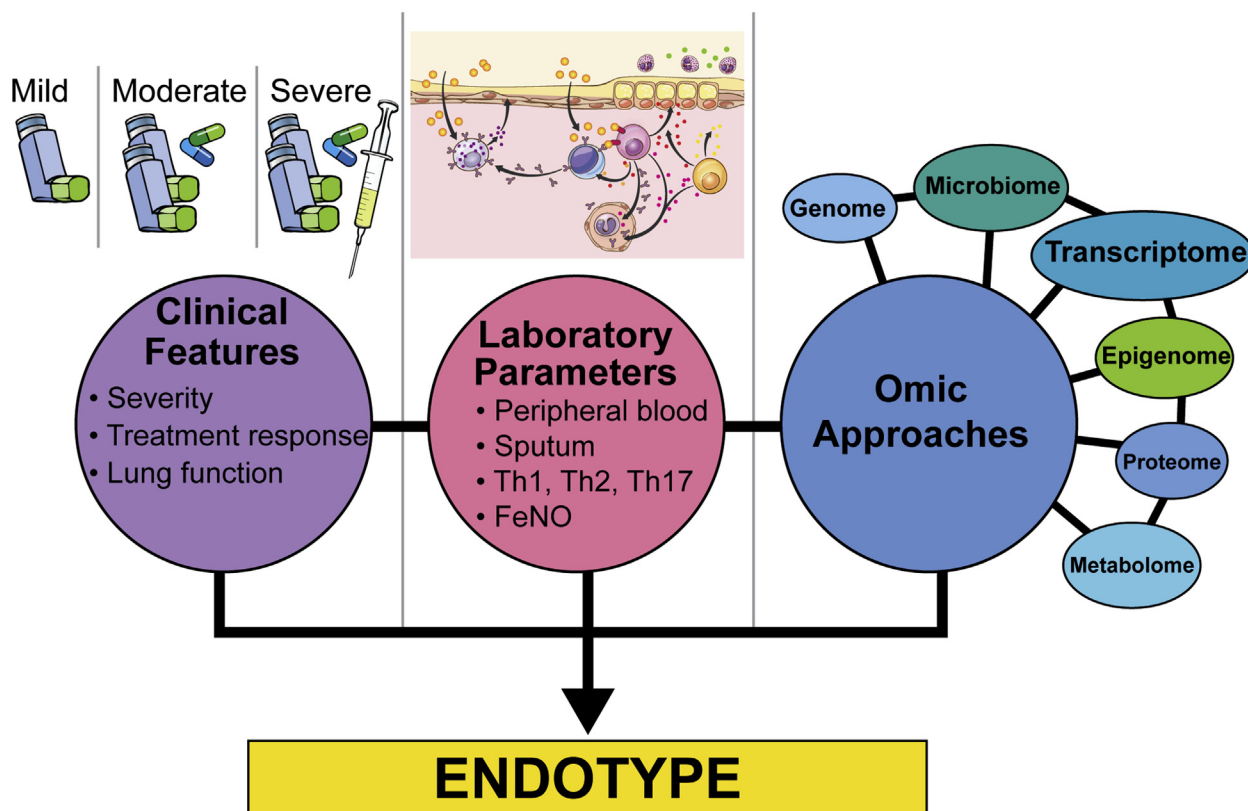


FIG 1. Approaches to endotyping asthma. Endotyping, or subtyping of asthma based on pathophysiologic mechanisms, can be informed by clinical features, laboratory parameters, and -omics approaches. Clinical features often serve as the basis for phenotyping asthma. Asthma endotyping based on laboratory parameters is common, spanning the well-recognized T2-high and T2-low endotypes based on induced sputum cells, F_{ENO} values, or both, as well as eosinophilic, neutrophilic, mixed granulocytic, and paucigranulocytic endotypes based on sputum, BAL fluid, and/or blood inflammatory cells. Application of -omics approaches to asthma endotyping has provided supporting evidence for many existing endotyping paradigms but also suggested novel ways to conceptualize asthma endotypes. Although -omics approaches to asthma endotyping have been largely based on transcriptomic data thus far, there is emerging evidence that other -omics modalities can inform endotyping. Successful integration of clinical features, laboratory parameters, and -omics approaches will advance asthma endotyping.

incorporating them with clinical features and laboratory parameters to better identify asthma endotypes.

the case, likely because of the multidimensional heterogeneity in clinical presentations, as well as their underlying mechanisms.

CLASSIFICATION OF ASTHMA BASED ON CLINICAL FEATURES

Asthma is often classified based on clinical characteristics, such as disease severity, treatment response, exacerbating factors, age of onset, and comorbid conditions (Fig 2). This classification into clinical phenotypes provides a first step to addressing heterogeneity in asthma. For example, the Severe Asthma Research Program (SARP), a multicenter network studying asthma in adults and children, used unsupervised cluster analysis to identify 5 strata of asthma based primarily on lung function and age of onset.² However, the overlap between these phenotypically defined strata and underlying pathophysiologic mechanisms (endotypes) was not clear. For example, although adults in SARP with a combined increase in sputum eosinophil and neutrophil counts had the most severe asthma, these subjects were spread among SARP phenotype clusters.² Because the underlying molecular cause of a disease is the mechanism through which phenotypes emerge, one might expect greater overlap between endotypes and phenotypes. However, this is often not

ENDOTYPING OF ASTHMA BASED ON LABORATORY PARAMETERS

Although clinical features can sometimes offer clues toward underlying mechanisms of asthma, the depth of mechanistic insight from them alone is limited. Laboratory parameters can provide deeper evidence for the mechanistic underpinnings of an endotype. Laboratory parameters used for asthma endotyping include type 2 (T2)-related attributes, airway and peripheral blood inflammatory cell patterns, and T_H cell subtype involvement.

T2-high versus T2-low asthma represents one of the most recognized endotyping strategies for asthma.³ In patients with T2-high asthma, genetic susceptibility and allergen exposure lead to skewing toward T_H2 cytokine-related inflammation, which upregulates airway inflammation and airway hyperresponsiveness, causing asthma (Fig 3). Several laboratory parameters can provide evidence for the T2-high versus T2-low endotype, including peripheral blood eosinophilia, bronchoalveolar lavage (BAL) fluid eosinophilia, sputum eosinophilia, serum total and allergen-specific IgE levels, and *fraction of exhaled nitric oxide*

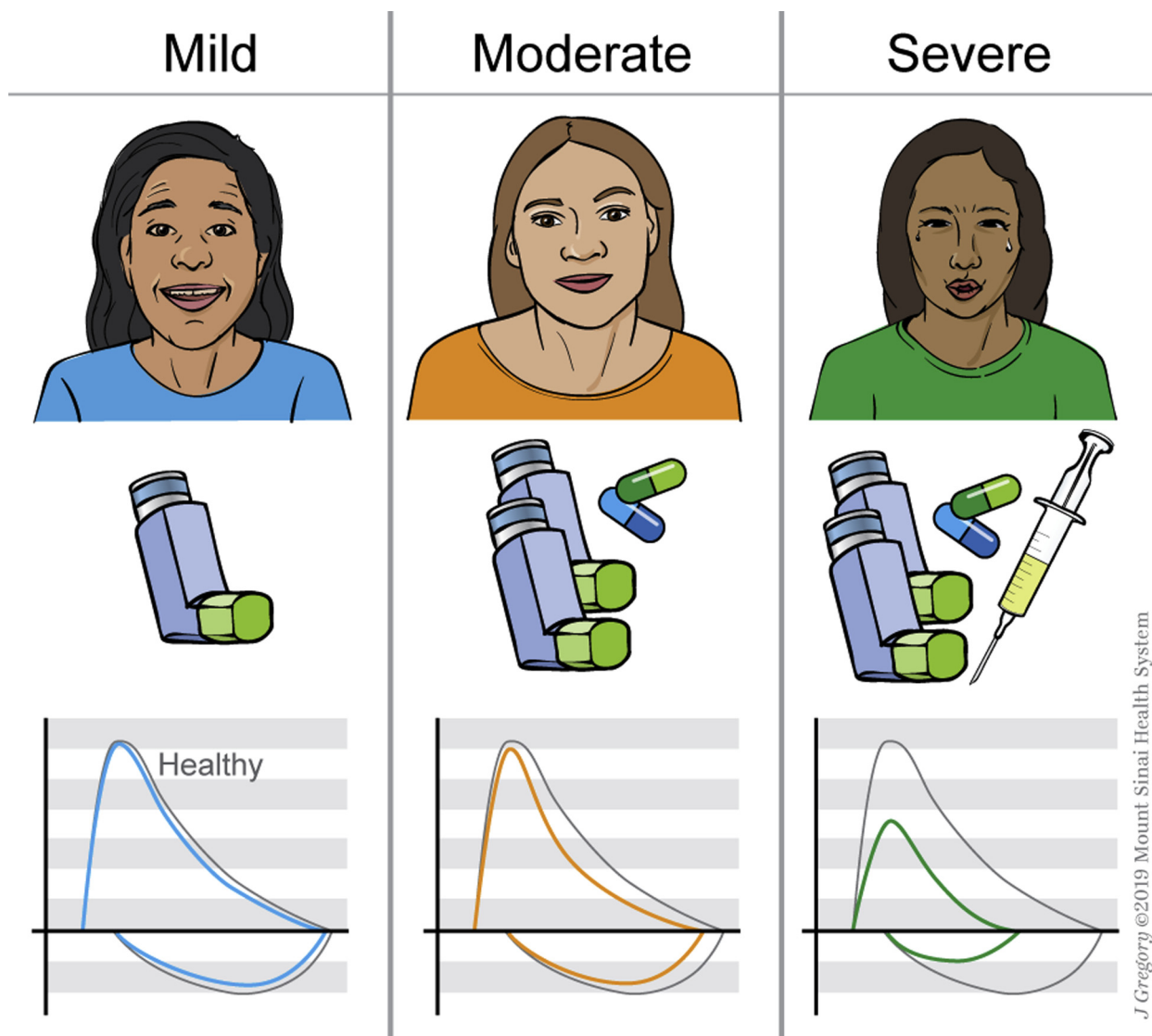


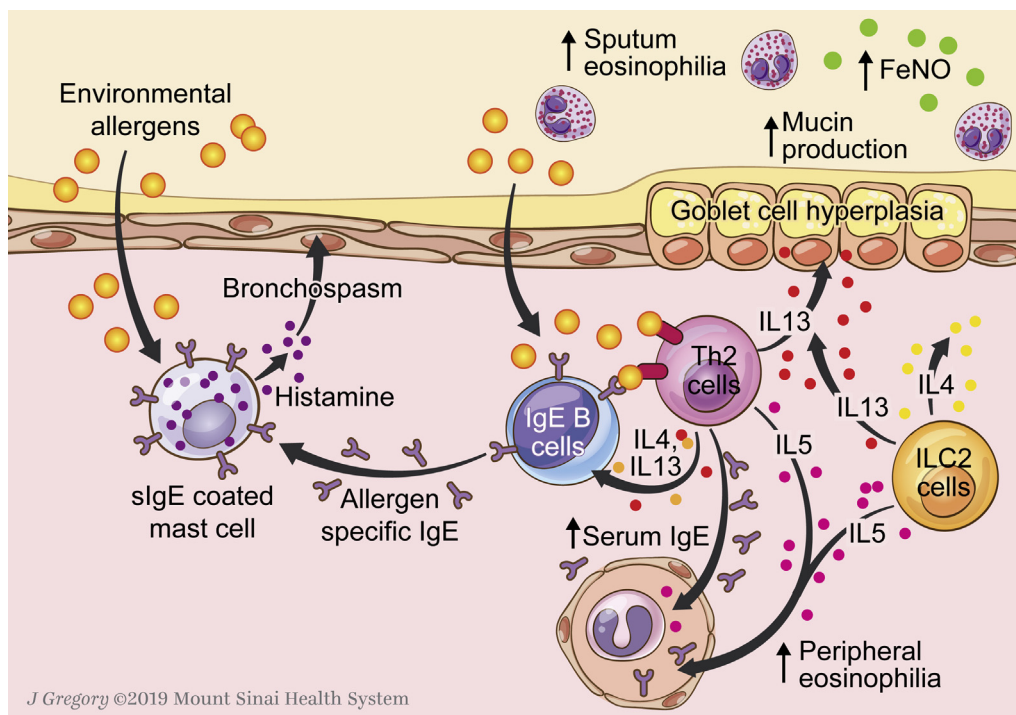
FIG 2. Clinical features can be used to classify asthma. Phenotypes of asthma are often based on clinical features, including severity of disease (eg, mild intermittent, moderate persistent, severe persistent), response to treatment, and exacerbating factors.

(*FENO*) values.^{4,5} Although these measures are often associated, a subject with T2 asthma might not have high levels in all of these dimensions.

Similarly, there is a growing appreciation for the role that *innate lymphoid cells* (ILCs) play in patients with T2 asthma. In particular, group 2 innate lymphoid cells secrete a T_H2 -related milieu of cytokines, including IL-4, IL-5, and IL-13.⁶ The relative abundance of group 2 innate lymphoid cells is highly concordant with the relative abundance of sputum eosinophils, a hallmark of T2-high asthma.⁷

The cellular pattern of inflammation can provide an alternate asthma endotyping strategy beyond the binary T2-high versus T2-low endotypes.⁸ BAL fluid, induced sputum, and peripheral blood can show varied cellular compositions in patients with asthma. The cellular pattern of these samples can be eosinophilic, neutrophilic, mixed eosinophilic/neutrophilic, or can demonstrate few cells of either class (ie, paucigranulocytic).⁹ Although there is no consensus on specific definitions for asthma endotypes based

on inflammatory cell patterns, one schema classifies the eosinophilic endotype based on increased sputum ($>3\%$) and/or blood (≥ 400 cells/ μL) eosinophils on at least 2 occasions and a positive response to treatment strategies that suppress eosinophils.⁸ This eosinophilic endotype highly overlaps with T2-high and allergic asthma. The neutrophilic endotype is based on increased sputum neutrophil counts ($\geq 64\%$) but not eosinophil counts ($<3\%$), with an increased total cell count (≥ 9.7 million cells/g) on at least 2 occasions with poor response to treatments that suppress eosinophils.⁸ Neutrophilic asthma tends to be more severe and refractory to corticosteroids than nonneutrophilic asthma.¹⁰ The mixed eosinophilic/neutrophilic endotype is defined by evidence for both neutrophils and eosinophils independently or concurrently on at least 2 occasions.⁸ Paucigranulocytic asthma is an endotype of exclusion when neither increased sputum eosinophil counts ($<3\%$) nor neutrophil counts ($<64\%$) were observed and treatment targeting eosinophils and neutrophils was ineffective.⁸



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FIG 3. Asthma endotypes based on laboratory parameters. Laboratory parameters, such as inflammatory cell fractions in sputum, BAL fluid, and peripheral blood and FeNO values, have been used to define asthma endotypes. As an example, the T2-high endotype is shown here, reflecting a pathophysiologic mechanism driven by T_H2 and group 2 innate lymphoid cells (ILC2s). In this scenario T_H2 cells and ILC2s produce IL-4, IL-5, and IL-13, leading to goblet cell hyperplasia, increased mucin production, local and systemic eosinophilia, and B-cell production of IgE. IgE coats mast cells that are then primed to release histamine on IgE cross-linking induced by environmental allergens, leading to the bronchospasm and airway inflammation that characterizes T2-high asthma. Signs of this pathophysiologic mechanism can be detected by measuring inflammatory cell patterns in sputum, BAL fluid, and peripheral blood; serum total and specific IgE levels; and cytokine levels in sputum and peripheral blood. Such laboratory parameters are often used to define asthma endotypes.

Although the T2-high versus T2-low distinction represents a classical approach for asthma endotyping, classification based on other T_H cell classes (eg, T_H17 and T_H1) has more recently come to the fore.^{11,12} T_H17 cells are a subset of T cells that are often involved in humoral response to extracellular pathogens, such as bacteria and fungi; these cells can also recruit neutrophils to the site of inflammation.¹³ There is evidence that IL-17F, a key cytokine produced by T_H17 cells, has a direct involvement in the development of asthma. Mutations in the *IL17F* gene increase the probability of having asthma, whereas overexpression of IL-17F results in neutrophil recruitment to the lung, amplifying allergic pulmonary inflammation in an animal model.¹⁴ The T_H17 -related neutrophilic asthma endotype can arise from treatment of T2 asthma with corticosteroids or anti-IL-13, which in turn induces a T_H17 profile marked by corticosteroid resistance.¹⁵ The most notable clinical differences observed between patients with T2 and those with T_H17 asthma were greater FeNO values and sputum eosinophil counts in those with T2 compared with T_H17 asthma. Diagnostically, it has been suggested that a select set of marker genes can define the T2 versus T_H17 asthma endotypes, including *POSTN*, *CLCA1*, and *SERPINB2* for T2 asthma⁴ and *CXCL1*, *CXCL2*, *CXCL3*, *IL8*, and *CSF3* for T_H17 asthma.¹⁵ In addition, high relative protein expression of carcinoembryonic antigen-related cell adhesion molecule 6 (CECAM6) in bronchial epithelium and neutrophils is associated with T_H17 versus T2 asthma.¹⁶

Another T_H cell type, T_H1 cells, can also play a role in asthma pathobiology. Typically, T_H1 immunity is associated with the intracellular pathogen response. The most notable cytokine produced by T_H1 cells is IFN- γ , and CCR5 is a surface marker of T_H1 cells.¹⁷ A T_H1 asthma endotype has recently been proposed based on numbers of $CD4^+/CCR5^+$ T cells in the lower airways of children with severe asthma.¹⁷ T_H1 cells, as well as high neutrophil counts, were found in the BAL fluid of both allergic and nonallergic children with asthma, suggesting that this feature is associated with severity independent of allergen sensitivity.¹⁷ Additionally, $CD8^+$ cytotoxic T-cell counts were increased in the BAL fluid of subjects with the highest levels of IFN- γ .¹⁷ Because this cohort was comprised exclusively of patients with severe asthma, follow-up studies quantifying the absolute number of T_H1 cells across a wider range of asthma severities are needed.

In a separate study of SARP subjects not stratified by IFN- γ levels, $CD8^+$ T-cell counts were not significantly different across healthy control subjects and patients with nonsevere and severe asthma.¹⁸ However, differences associated with T_H1 cells were still observed across severities.¹⁸ This study found that BAL fluid from patient with severe asthma demonstrated a higher ratio of T_H1 -enriched T cells ($CD4^+CCR6^-CCR4^-$) to **natural killer (NK) cells** ($CD3^-NKp46^+$ cells with lymphoid morphology) compared with that seen in patients with nonsevere asthma or healthy control subjects (>14% vs 4% to 14%).¹⁸ This ratiometric

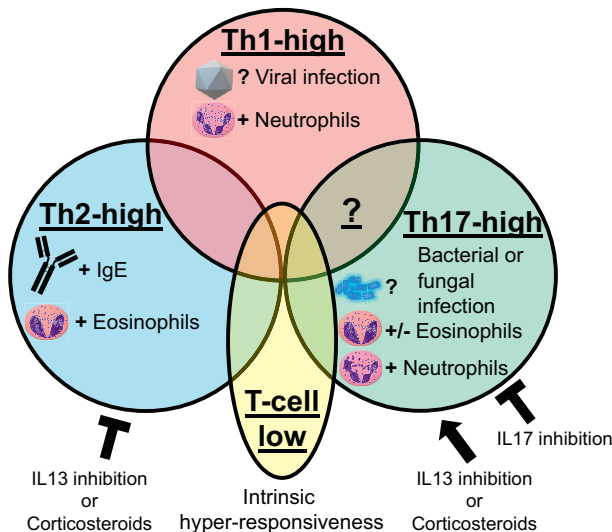


FIG 4. Asthma endotypes based on T_H cell subtype involvement. Asthma endotypes based on the relative signal strengths of T_H2 , T_H17 , T_H1 , and T cells overall have been described and associated with eosinophil, neutrophil, and serum total and specific IgE levels, as well as variable treatment response. These T_H cell-based endotypes likely have some overlap between one another.

measure also suggested the potential involvement of T_H1 cells in patients with severe asthma with concordant NK cell depletion. Given that T_H1 cells mediate immune response to viruses, this might fit with clinical observations that many asthma exacerbations are triggered by viral infection.¹⁹

Whether asthma endotypes are defined on laboratory-based quantification of airway and peripheral blood inflammatory cell patterns or T_H cell subtype involvement, it is clear that the characteristics of such endotypes overlap to some degree (Fig 4). Whether endotypes are stable over time is also not known. In theory, for example, a patient with T2-high asthma could have characteristics of a T_H1 -high endotype during a viral infection and then return to a T2-high-only endotype after resolution of the infection. Longitudinal studies of deeply phenotyped subjects can help determine whether these or other related scenarios elicit endotype changes over time.

ENDOTYPING OF ASTHMA BASED ON -OMICS

Studies using -omics approaches can provide unbiased molecular evidence for refining existing endotypes and defining new ones. In this section we will review how -omics approaches have contributed to asthma endotyping (Fig 1). We begin by discussing transcriptomics, the -omics approach that has provided the largest contribution to asthma endotyping thus far.

Transcriptomics

Transcriptomics is the systematic, quantitative, and qualitative characterization of RNA transcripts across the *genome*.²⁰ Because RNA reflects dynamic processes at play that underlie pathophysiology in a given tissue, profiling its expression through transcriptomics in disease-relevant tissue, such as airway brushings, endobronchial biopsy specimens, BAL fluid, and peripheral blood, can help with accurately distinguishing asthma status,^{21–23} classifying asthma phenotypes,²⁴ and defining its endotypes.^{25–29}

Transcriptional profiles for the T2-high versus T2-low endotypes (Fig 3) were first identified in 2009 by Woodruff et al⁴ through microarray analysis of airway brushings from 42 patients with mild-to-moderate asthma and 28 healthy control subjects. T2-high and T2-low profiles among asthmatic patients were determined based on relative expression of IL-13-inducible genes.⁴ Compared with their T2-low counterparts, patients demonstrating the T2-high gene expression signature had greater expression of *IL5* and *IL13* in bronchial biopsy specimens and significantly greater serum IgE levels, blood and airway eosinophilia, subepithelial fibrosis, and airway mucin gene expression.⁴ Lung function of subjects belonging to this T2-high endotype improved with inhaled corticosteroid treatment, whereas it did not in those belonging to the T2-low endotype. The fact that the efficacy of inhaled corticosteroids differed among endotypes suggests clinical utility in considering the T2-high versus T2-low endotype when selecting asthma therapy.⁴

More recent transcriptomic studies of asthma have supported T2-based endotypes,³⁰ as well as suggesting other endotyping paradigms.^{25–29} Among 299 young adult participants of the Childhood Asthma Management Cohort, Howrylak et al²⁵ identified 5 longitudinally stable phenotypic clusters of asthma. Their examination of gene expression profiles from peripheral blood $CD4^+$ cells revealed that each of these 5 phenotypic clusters was associated with a corresponding $CD4^+$ gene expression pattern.²⁵ Reorganization of these 5 clusters into 2 subgroups (ie, more atopic versus less atopic, which are akin to T2-high versus T2-low asthma) helped to identify a $CD4^+$ gene signature based on 5 genes (*SRM*, *HDAC2*, *SLC33A1*, *P2RY10*, and *ADD3*) that could classify atopic patients in an independent cohort with high accuracy.²⁵ Expression of *HDAC2*, *SLC33A1*, *P2RY10*, and *ADD3* was relatively upregulated in the more atopic subgroup compared with the less atopic subgroup, whereas *SRM* expression was relatively downregulated. Serum total IgE levels, serum eosinophil counts, and methacholine PC_{20} values were significantly different between the more atopic versus less atopic subgroups defined by $CD4^+$ gene signature.²⁵ Furthermore, serum IgE levels were positively correlated with all 5 genes in the $CD4^+$ gene signature, and serum eosinophil counts were positively correlated with *SRM* expression levels.²⁵

Studies from the Unbiased Biomarkers in Prediction of Respiratory Disease Outcomes (U-BIOPRED) cohort similarly suggest the possibility of new endotype clusters based on transcriptomics in addition to support for T2-based endotyping. Among 120 U-BIOPRED subjects, semisupervised **clustering** of 508 genes differentially expressed in sputum between healthy volunteers and patients with eosinophil- and non-eosinophil-predominant inflammation identified the expected 3 **transcriptome** clusters.²⁶ Although these 3 clusters did not cleanly separate paucigranulocytic, eosinophilic, mixed, and neutrophilic asthma phenotypes, there were differences in granulocytic content across these clusters. The first cluster overlapped most with the T2-high endotype and was characterized by sputum gene expression of *IL33R*, *CCR3*, and *TSLPR* and association with sputum eosinophilia, high $FENO$ values, and severe asthma with oral corticosteroid dependency, frequent exacerbations, and severe airflow obstruction. The second and third transcriptome clusters exhibited T2-low traits. Cluster 2 was identified by expression of interferon, TNF, and **inflammasome**-related genes, and subjects in this cluster demonstrated high sputum neutrophilia, increased serum

C-reactive protein levels, and atopic dermatitis. Cluster 3 was identified based on genes related to metabolic and mitochondrial pathways and included subjects with better lung function; subjects with paucigranulocytic inflammation mainly clustered to cluster 3, but cluster 3 also included subjects with eosinophilic, neutrophilic, and mixed inflammatory sputum composition.²⁶ More recent U-BIOPRED cohort studies have explored sputum, nasal brushing, endobronchial brushing, and biopsy transcriptome data together.^{27,28}

Based on these data, a gene signature for adult-onset severe asthma was identified and found to be associated with eosinophilic airway inflammation, mast cells, and group 3 innate lymphoid cells, whereas a gene signature for childhood-onset severe asthma was found to be associated with induced lung injury.²⁸ The gene set variation analysis in this study was based on a mix of data from *in vivo* and *in vitro* human sample studies, as well as murine models; therefore these inferences should be validated directly in asthmatic patients.

The concept of a “T2-ultra-high” endotype was suggested by a study of sputum transcriptomics from 84 patients with asthma and 27 healthy control subjects.²⁹ Consistent with other studies, T2-high and T2-low clusters were identified, but the investigators also highlighted a subgroup of T2-high subjects with especially high T2 signal based on their analysis. Clinically, these subjects were older and had more severe airflow obstruction.²⁹

Transcriptomic studies have also enhanced our understanding of asthma endotypes defined by airway inflammatory cell patterns. Using microarray-based gene expression profiles of sputum from 47 asthmatic patients, Baines et al³¹ identified a 6-gene (*CLC*, *DNASE1L3*, *IL1B*, alkaline phosphatase, *ALPL*, and *CXCR2*) signature that could discriminate eosinophilic asthma from noneosinophilic, paucigranulocytic, and neutrophilic asthma, as well as control subjects.³¹ Targeted quantitative RT-PCR measures of this 6-gene signature in sputum to predict future asthma exacerbation demonstrated that this signature performed better (ie, had greater areas under the receiver operating curves) than predictions based on peripheral eosinophil counts, sputum neutrophil counts, or combined sputum eosinophil and neutrophil counts.³²

Epigenomics

Although transcriptomic approaches have contributed the most to asthma endotyping thus far, epigenomic approaches might also have a role to play because they can characterize interactions between the environment and transcriptome.³³ Because asthma is a disease in which environmental factors influence pathophysiology and natural history, leveraging epigenomics toward endotyping is likely to become increasingly important.

Epigenome-wide studies have identified peripheral blood methylation loci associated with serum IgE concentration in populations with asthma.^{34,35} Liang et al³⁴ found that 13% of variance in circulating IgE levels could be explained by the peripheral blood methylation status at 3 loci (*SLC25A33*, *LPCAT2*, and *L2HGDH*). Several of the identified IgE-associated methylation loci annotated to genes that encode proteins characteristic of eosinophils, including *IL5RA*, *CCR3*, *IL1RL1*, *PGR2*, *PGR3*, and *GATA1*.³⁴ Given the central roles that IgE and eosinophils play in patients with T2-high asthma (Fig 3), these epigenomic data could be used to support T_H-related endotypes of asthma.

In line with this, Yang et al³⁶ identified 81 loci with differential methylation in PBMCs of patients with atopic asthma (n = 97)

versus healthy control subjects (n = 97). Loci relevant to T_H2 immunity tracked with IgE content and expression of T_H2-related genes, including *IL13*, *RUNX3*, *CAT*, *PRF1*, and *TIGIT*.³⁶

Epigenomic studies of tissue beyond blood also suggest that epigenomics can contribute to asthma endotyping. A **DNA methylation** study of airway epithelial cells used correlation-based network analysis to classify **CpG sites** differentially methylated between 74 asthmatic patients and 41 nonasthmatic subjects into 4 modules.³⁷ These 4 modules were associated with clinical features and laboratory parameters commonly used for asthma endotyping. Specifically, one module was associated with eosinophilia in BAL fluid, and another module was associated with FENO values; the remaining 2 modules were associated with the clinical feature of inhaled corticosteroid use.³⁷

Microbiomics

Given the recognized roles for viruses¹⁹ and bacteria³⁸ in the development and course of asthma, it is logical to consider **microbiome** data when conceptualizing asthma endotypes. Upper and lower airway microbiota are known to vary by asthma status and disease activity,^{38–40} and there is some evidence from microbiome studies that there could be distinct microbiota associated with particular endotypes of asthma. However, results to date have been heterogeneous in nature.

In a study of bronchial brushings from 40 patients with severe asthma, no taxa were found to be associated with a T2-related epithelial gene signature.⁴⁰ *Proteobacteria* abundance was found to be negatively correlated with biopsy eosinophil abundance, whereas *Proteobacteria* abundance was positively associated with expression of T_H17 genes.⁴⁰ This also further supports the negative correlation between T2 and T_H17 asthma previously described by Choy et al.¹⁵

Separately, Sverrild et al⁴¹ examined the relationship between BAL fluid inflammatory patterns and airway microbial diversity in a study of 23 steroid-free nonsmoking asthmatic patients and 10 healthy control subjects. They examined airway microbial composition differences in relation to eosinophil and neutrophil content.⁴¹ Specifically, airway microbiome composition among asthmatic patients with low BAL fluid eosinophil counts significantly differed from those of both healthy control subjects and patients with high eosinophil counts. Unexpectedly, however, there was no significant difference in composition when comparing asthmatic patients with high BAL fluid eosinophil counts with healthy subjects.⁴¹ Asthmatic patients with low BAL fluid eosinophil counts had high abundances of *Neisseria*, *Bacteroides*, and *Rothia* species compared with their counterparts with high BAL fluid eosinophil counts and healthy subjects.⁴¹ They also observed lower β -diversity in patients with greater BAL fluid neutrophil counts, suggesting lower bacterial diversity within patients with neutrophilic asthma.

More recently, the microbial composition and inflammatory patterns of induced sputum samples collected from 167 adults with severe asthma were studied.⁴² The investigators found that airway microbiota were less diverse and more dissimilar in patients with neutrophilic versus eosinophilic sputum inflammatory patterns. Diversity measures were significantly correlated with sputum neutrophil proportions but not eosinophil proportions; furthermore, the sputum neutrophil proportion was the strongest predictor of microbiota composition.⁴² In patients with neutrophilic asthma, there was reduced relative abundance

of *Streptococcus*, *Gemella*, and *Porphyromonas* species, whereas *Haemophilus* species was negatively correlated with eosinophil percentage.⁴²

The bronchial microbiota of adults with steroid-naïve atopic asthma (n = 42), patients with atopy without asthma (n = 21), and healthy control subjects (n = 21) has also been studied within the AsthmaNet clinical research network.⁴³ Microbial composition differed significantly between these groups. Asthmatic patients showed greater enrichment of several taxa, including *Haemophilus*, *Neisseria*, *Fusobacterium*, and *Porphyromonas* species, and the bronchial microbiome of asthmatic patients at baseline differed by responsiveness to treatment with inhaled corticosteroids.⁴³ Additionally, subjects identified as having T2-high asthma based on expression levels of 3 bronchial epithelial genes (*CLCA1*, *SERPINE2*, and *POSTN*) were found to have lower bronchial bacterial load.⁴³

The findings from these and other studies suggest that airway microbiome composition can be associated with inflammatory cell phenotypes, with measures of microbial diversity and composition more frequently associated with neutrophilic asthma thus far. The mechanistic links between the airway microbiome and asthma endotypes warrant further investigation.

Metabolomics

Metabolomics provides a snapshot of active physiology that has been used to classify patients with asthma and asthma phenotypes.^{20,44,45} Metabolomic profiling involves the systematic assessment, identification, and quantification of low-molecular-weight compounds in biological samples, typically by using *liquid chromatography mass spectrometry*, *gas chromatography mass spectrometry*, or nuclear magnetic resonance (NMR).²⁰ Biospecimens for which metabolites are frequently measured in studies of asthma include plasma, serum, urine, and exhaled breath.^{20,44}

A study of exhaled breath samples from 35 patients with asthma and 23 control subjects showed that a model based on 15 volatile organic compounds in exhaled breath samples could classify asthma as the eosinophilic (>2% sputum eosinophilia) endotype with an area under the curve of 0.98 or the neutrophilic ($\geq 40\%$ sputum neutrophilia) endotype with an area under the curve of 0.90.⁴⁶ Separately, a study of exhaled breath and steroid responsiveness in 25 patients with mild-to-moderate asthma found that exhaled breath volatile organic compound levels could predict steroid responsiveness with greater accuracy than FENO values or sputum eosinophil counts.⁴⁷

Using NMR spectroscopy and machine learning, another group of investigators found that NMR spectra of exhaled breath condensate could cluster 89 asthmatic patients into 3 phenotypic groups, one of which was consistent with the neutrophilic endotype with low peripheral blood eosinophil and high neutrophil counts.⁴⁸ More recently, cluster analysis based on metabolomic data from exhaled breath from 78 subjects identified 3 clusters of patients with significant differences in peripheral blood eosinophil and neutrophil percentages, as well as differences in oral corticosteroid needs.⁴⁹

Because of heterogeneity in findings and the relatively small sample sizes studied thus far,⁴⁴ it is not yet clear the extent to which metabolomics will affect asthma endotyping. However, the fact that metabolomic profiling can be done on exhaled breath is an attractive characteristic, especially for endotyping asthma in children and subjects who might be averse to more invasive sampling.⁵⁰

Proteomics

Proteomics can offer a descriptive window into the current inflammatory state of the airway. Within 240 U-BIOPRED cohort subjects, proteomic profiles were recently used to identify 10 clusters with distinct proteomic signatures that were dubbed “proteotypes.”⁵¹ Overlay of these proteotypes with sputum granulocyte counts showed strong associations. Specifically, proteotypes 1, 2, and 3 were associated with high sputum eosinophilia (mean count, 19%, 23%, and 17%) and were thought to represent a common and highly eosinophilic phenotype; transcriptomic study of sputum cells showed cytokine gene expression patterns consistent with eosinophilia.⁵¹ Proteotypes 5 and 6 had atopy as a common feature, and proteotypes 8, 9, and 10 were associated with high neutrophil counts (mean count, 64%, 82%, and 73%).⁵¹ Proteotypes 4 and 7 were less defined by granulocyte count and had the greatest levels of smoking (current or former) with the least aberrant inflammatory cell proportions relative to healthy control subjects.⁵¹ There was low concordance between the eosinophil-high proteotypes and atopy. Proteotypes with the highest sputum eosinophil counts showed high IL-13 and periostin levels but low total serum IgE levels. However, the most atopic proteotypes showed moderate levels of eosinophilia, IL-13, and periostin but high levels of total IgE.⁵¹ This suggests that differing underlying mechanisms might exist for some forms of T2 asthma. Further study of the identified proteotypes will be needed to assess the relationship to treatment response, and validation of proteotypes, such as these, will be needed to assess reproducibility and temporality.

MULTI-OMICS ENDOTYPING

-Omics approaches systematically capture a dimension of molecular processes underlying disease, whether at the genome, epigenome, transcriptome, *proteome*, *metabolome*, or microbiome level. Although comprehensive at each level, each single -ome can be thought of as capturing just one of many dimensions underlying disease pathology. In some ways this is akin to the parable of the blind men and the elephant, where none has encountered an elephant before and each must learn and conceptualize what the elephant is by touching a different part of the elephant's body. Although each claims a truth based on their individual touch, none can accurately describe the actual elephant. Such an analogy might be relevant to asthma endotyping, where we can learn a part of “truth” by each -omics approach (and deeply so), but accurately defining robust asthma endotypes will require integration of multiple -omics.

To this end, there have been limited *multi-omics* studies relevant to asthma endotyping. In a small study of 20 asthmatic patients, McGeachie et al⁵² integrated metabolomic data from plasma samples with a genome-wide genotype, peripheral blood CD4⁺ T-cell methylation profiles, and peripheral blood CD4⁺ transcriptomes to generate a conditional Gaussian Bayesian network of asthma control. Analysis of this network implicated 2 pathways (arachidonic acid metabolism and linoleic acid metabolism) in asthma control and altered sphingolipid metabolism as an underlying feature of uncontrolled asthma and cellular response to albuterol.⁵²

A U-BIOPRED study sought to assess the relationship between asthma clusters identified based on clinical features and sputum transcriptomic and proteomic profiles.⁵³ Among 266 subjects, cluster analysis based on 8 clinical features (age of onset, pack years of smoking, body mass index, FEV₁ percent predicted,

FEV₁/forced vital capacity ratio, Asthma Control Questionnaire–related score, number of exacerbations in the past year, and daily dose of oral prednisolone) identified 4 clusters of asthmatic patients.⁵³ The investigators found significant differences in sputum proteomics and transcriptomics between these clusters, with higher sputum eosinophilia in cluster 1 compared with the other 3 clusters of patients with severe asthma.⁵³ Interestingly, there were no differences in sputum neutrophil counts, FENO values, or serum IgE levels between the clusters.⁵³

In a subsequent study of sputum transcriptomics and proteomics among U-BIOPRED subjects, investigators found that neutrophilic asthma was associated with NLRP3 inflammasome expression and that *IL1R1* gene expression was associated with eosinophilic severe asthma, leading them to suggest that T_H2-driven eosinophilic inflammation and neutrophil-associated inflammasome activation are interacting pathways in patients with severe asthma.⁵⁴

PAVING A PATH TO MULTI-OMICS FOR ASTHMA ENDOTYPING

Although there is a limited track record to date of integrating multiple -omics to define asthma endotypes, the raw materials and tools for doing so are growing. These methods help provide a path forward to maximize the utility of the high dimensional data sets that are being generated in asthma studies. Numerous asthma cohorts and asthma research networks have gathered and continue to build rich phenotypic and molecular data, including -omics profiles of their participants. Strategies for integrating multi-omics data from cohorts are also becoming increasingly available, many of which have been developed in the cancer field, where -omics data have been more widely available. In envisioning next steps for how multi-omics can play a more prominent role in asthma endotyping, we describe some important considerations and approaches for multi-omics analysis below.

Successful generation and analysis of meaningful multi-omics data hold the promise of revealing systems-level insights but carry several challenges.²⁰ As is true for all studies, multi-omics, single-omics, and non-omics alike, careful study design is important to ensure the validity and generalizability of the results generated. Temporality, potential confounders, missing data, and comparability across cohorts and populations must all be considered. For -omics and multi-omics studies in particular, we previously reviewed several current challenges of high-throughput approaches, including access to systems-wide profiling technologies, cost of system-wide profiling, data transfer and management, data quality and control, accurate modeling of biological systems through data integration, and acceptance by communities accustomed to reductionist approaches.²⁰ Increasing accessibility to core facilities, high-performance computing systems, software innovations, standardization of data and data sharing, and iterative development of tools and software platforms are some solutions to these challenges.²⁰ Working toward these solutions will enable more multi-omics studies of asthma that can inform on endotypes and are reproducible across studies and populations.²⁰

A common first step to explore data is cluster analysis, in which subjects are clustered into groups based on similarities of features of interest. Within a multi-omics study, cluster analysis could be used to identify subjects who are similar to one other within and across -omes. For example, within a cohort in which both the

bronchial transcriptome and bronchial microbiome were profiled, cluster analysis could reveal 4 clusters: a cluster of subjects with a T2-high bronchial epithelial transcriptomic signature and a bronchial microbiome that differs from healthy subjects, another cluster also with a T2-high transcriptomic signature but a “healthy” bronchial microbiome, and 2 additional clusters that each had a T2-low bronchial epithelial transcriptomic signature yet show distinct microbiome signatures. Although clustering single -omics can reveal data patterns, as several studies of asthma have already shown, clustering of multiple -omics profiles generated from the same subject can reveal findings not evident from a single data type.⁵⁵

Multi-omics clustering methods can themselves be classified into 3 broad categories based on the stage at which each -ome is integrated for clustering.⁵⁵ Late integration methods treat each -ome separately and then integrate the clustering results of each individual -ome.⁵⁵ Intermediate integration performs some form of -ome specific calculation, integrating these for clustering.⁵⁵ Early integration methods often perform clustering only once on a combined multi-omics data set.⁵⁵

Late integration methods for multi-omics clustering involve first clustering on each -ome independently, allowing the user to specify various clustering approaches tailored to each -ome. The results of the per-ome clustering are then combined into a net cluster result.⁵⁵ However, methods for integrating these per-ome clustering results can vary. The Cluster-Of-Clusters Assignments algorithm concatenates binary vectors representing cluster membership for each -ome and then performs final clustering on this matrix.⁵⁶ In contrast, the Perturbation clustering for data Integration and disease Subtyping algorithm creates a connectivity matrix for each -ome in which each sample is compared with all other samples, creating a binary matrix, with 1's indicating that the samples were clustered together and 0's representing that samples were not clustered together in the given -ome.⁵⁷ The connectivity matrices for all -omes are then averaged for the final connectivity matrix, which is then used for clustering.⁵⁷

Intermediate integration of -omics data involves processing each -ome independently and then integrating these processed representations for clustering.⁵⁵ In some methods, within-ome similarity is calculated, and then each is dimensionally reduced by matrix decomposition, taking the first *k* eigenvectors for concatenation across -omes and clustering; this can be seen as a form of combined spectral clustering.^{58,59} Similarity network fusion (SNF) is a method that builds similarity networks within each -ome and then uses message passing for clustering; this ensures that any low-level similarity between subjects that is conserved across -omes is taken into account, as well as high similarity within a single -ome.⁶⁰ This approach creates a weighted network graph from a *k*-nearest neighbor embedding of subject-subject similarities for each -ome. SNF then merges all of the network graphs from each -ome for a “fused” final graph, which is then used for clustering.⁶⁰ SNF has been used successfully to identify substrata of glioblastoma multiforme through integrating mRNA, methylation, and micro-RNA measures.⁶⁰ The algorithm could meaningfully stratify subjects of several cancer types, as measured by differences in survival across identified groups.⁶⁰

Many early integration methods are performed on a matrix of data from each -ome concatenated together; this combined matrix is then deconvolved through some form of dimension reduction to orthogonal components in which each component captures a correlated suite of features, including those across -omes.⁶¹ Although high-

level representation can vary (ie, correlation,⁶² Bayesian networks,⁶³ decomposed matrix components⁶⁴),⁵⁵ the overarching theme between these methods is similar: find the correlated variables and represent them as a lower dimensional “hub,” eigenvector, or correlated component on which to perform clustering. Alternatively, correlations between variables from all combined -omes can be used to build a network graph model of combined -omes, as in the xMWAS data tool.⁶⁵ Many of these approaches will be effective when -omics data are all continuous (rather than categorical or ordinal) in nature. These approaches might not be as appropriate when trying to integrate categorical information, such as allele status, or ordinal variables, such as copy number variation, with continuous variables, such as gene expression, microbial abundances, and metabolite levels. Therefore there currently exists a need for methodologies that will, in an automated fashion, appropriately integrate these different data types for unsupervised clustering.

CONCLUSIONS

With asthma embodying such a heterogeneous disease, the quest to subtype it into endotypes based on cohesive underlying pathophysiologic mechanisms will not only organize our thinking on this complex disease but also help target treatment toward precision medicine.⁶⁶ Clinical features, laboratory parameters, and -omics approaches all have a role to play in defining and refining asthma endotypes toward robust subtypes that can meaningfully contribute to our understanding and treatment of asthma (Fig 1). Transcriptomic identification of T2-high asthma⁴ and identification of other endotypes of asthma through -omics approaches, as reviewed here, reveal the potential for multi-omic defined endotypes to inform treatment approach and candidacy for targeted biologics.^{67,68} Further refining existing endotypes and discovering new endotypes through multi-omics methods can lead to improved asthma outcomes if they can inform treatment beyond the T2-centric paradigm.

Because multi-omics studies continue to develop to inform asthma endotypes, it will be important to keep several considerations at the forefront. First, careful study design, well-characterized cohorts, appropriate address of confounding variables, and reproducibility across cohorts will be needed to ensure the robustness of the data generated and quality of results produced. Rigorous methodology and attention to data quality and control through standardization and sharing can advance this. Tissue specificity and relevance of the profiles generated is also important to consider; thus far, -omics studies of peripheral blood in asthmatic patients have predominantly yielded findings related to atopy,^{25,34,35} whereas studies of local samples (eg, airway brushings and biopsy specimens, BAL fluid, and sputum) in asthmatic patients have enabled more refined endotyping.^{4,17,18,26-28,40-43,51} Additionally, longitudinal studies can help provide evidence for the relative stability of endotypes; this will help clinicians decide on the temporal appropriateness of endotyping efforts and endotype-tailored treatments.

Although -omics approaches, including transcriptomics, epigenomics, microbiomics, metabolomics, and proteomics, have each been applied as modalities to support existing endotypes and to conceptualize new endotypes, their integrated multi-omics application has been more limited thus far. Although we have discussed paths forward for expanding multi-omics approaches for asthma endotyping, this will have to be combined with clinical features and laboratory parameters to achieve the goal of precision medicine for asthma.

Key concepts and therapeutic implications

- Although reversible airway obstruction is a defining feature of asthma, the eliciting factors, natural history, underlying molecular biology, and clinical management of asthma vary greatly among affected subjects.
- Endotypes are subtypes of disease based on pathophysiologic mechanisms.
- Endotypes help organize mechanistic understanding of heterogeneous diseases, such as asthma, and can direct treatment toward modalities that are likely to be the most effective because of their targeting of specific pathophysiologic pathways.
- Approaches to asthma classification and endotyping can be organized into 3 broad categories, including strategies based on clinical features, laboratory parameters, and molecular profiling by -omics approaches.
- -Omics approaches, including transcriptomics, epigenomics, microbiomics, metabolomics, and proteomics, have supported and refined current endotyping paradigms while also suggesting novel ways to conceptualize asthma endotypes.
- Application of multi-omics, including their integration with clinical features and laboratory parameters, can help coalesce strategies toward robust asthma endotypes that will enable precision medicine.

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