

A translational perspective on epigenetics in allergic diseases



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Overall Purpose/Goal: To provide excellent reviews on key aspects of allergic disease to those who research, treat, or manage allergic disease.

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1. To understand the concept of epigenetics and the role of epigenetic modifications in shaping the incidence and phenotype of allergic diseases.
2. To know the cellular and molecular processes involved in modification of the epigenome.
3. To understand the ways by which the environment, nutrients, and the microbiome can affect the development and course of allergic diseases through alterations in the epigenetic code.

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The analysis of epigenetic modifications in allergic diseases has recently attracted substantial interest because epigenetic modifications can mediate the effects of the environment on the development of or protection from allergic diseases. Furthermore, recent research has provided evidence for an altered epigenomic landscape in disease-relevant cell populations. Although still in the early phase, epigenetic modifications, particularly DNA methylation and microRNAs, might have potential for assisting in the stratification of patients for treatment and complement or replace in the future biochemical or clinical tests. The first epigenetic biomarkers correlating with the successful outcome of immunotherapy have been reported, and with personalized treatment options being rolled out, epigenetic modifications might well play a role in monitoring or even predicting the response to tailored therapy. However, further studies in larger cohorts with well-defined phenotypes in specific cell populations need to be performed before their

implementation. Furthermore, the epigenome provides an interesting target for therapeutic intervention, with microRNA mimics, inhibitors, and antisense oligonucleotides being evaluated in clinical trials in patients with other diseases. Selection or engineering of populations of extracellular vesicles and epigenetic editing represent novel tools for modulation of the cellular phenotype and responses, although further technological improvements are required. Moreover, interactions between the host epigenome and the microbiome are increasingly recognized, and interventions of the microbiome could contribute to modulation of the epigenome with a potential effect on the overall goal of prevention of allergic diseases. (*J Allergy Clin Immunol* 2018;142:715-26.)

Key words: Epigenetics, DNA methylation, microRNA, biomarker, allergy, asthma, forkhead protein 3, epigenetic editing, antagomirs, antisense molecules, oligonucleotide therapy

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Abbreviations used

EV:	Extracellular vesicle
EWAS:	Epigenome-wide association study
FOXP3:	Forkhead protein 3
HDAC:	Histone deacetylase
ICS:	Inhaled corticosteroid
miRNA:	MicroRNA
Treg:	Regulatory T
TSDR:	Regulatory T cell-specific demethylated region

There has been a rapid increase in the prevalence of allergic diseases, including IgE-related asthma, in the Western world. For example, severe asthma is now reaching a prevalence of 8%, and challenge-proven food allergy has reached a prevalence of up to 10% in Australia and 2% to 4% in the United States and European countries.¹⁻⁴ Allergic diseases are nowadays a major cost factor for the health care system, with estimated worldwide costs for asthma close to 100 billion US dollars per year and 25 billion US dollars for food allergy in the United States only.^{5,6}

Epigenetics is “the study of mitotically and/or meiotically heritable changes in gene function that cannot be explained by changes in DNA sequence” or, as defined more recently, “the structural adaptation of chromosomal regions so as to register, signal or perpetuate altered activity states.”⁷ Epigenetics determines which regions of the genome will be accessible and expressed with changes or altered plasticity leading to potentially disease-predisposing physiologic states. Recently, the analysis of epigenetic modifications, including DNA methylation, posttranslational histone modifications, nucleosome occupancy, and small and long noncoding RNAs, has attracted much interest in the field of allergic disease. Epigenetics might indeed hold the key to explaining the high degree of plasticity of the immune response throughout life. Epigenetics can also mediate the effects of environmental protective and risk factors on the development and course of asthma and allergic diseases.⁸

Although still under debate, a number of explanations for the increased prevalence of allergic diseases have been proposed, including increased hygiene, insufficient exposure to microbes, and Western diet.⁸ Of note, many of these factors can exert their effects on cellular homeostasis through alteration of the epigenetic code. In this review I will briefly describe the potential applications using epigenetic modifications, including DNA methylation, histone modifications, and small noncoding RNAs (microRNAs [miRNAs]) for asthma and allergic diseases (Fig 1).

DNA METHYLATION

A number of epigenome-wide association studies (EWASs) have been performed and have identified, at least in asthmatic patients, common themes, such as the importance of eosinophils and regulatory T (Treg) cells and probably an altered epigenetic state of these cells.⁹⁻¹¹ Large-scale EWASs have also shown that disease-predisposing environmental factors, such as prenatal maternal smoking or prenatal or postnatal air pollution, lead to DNA methylation changes at genes with relevance for asthma and allergic diseases.¹²⁻¹⁵ Although most studies have focused on analysis of blood-based immune cells (reviewed by Potaczek et al⁸), it has also been shown that respiratory epithelial cells from the nose or the bronchium show differential methylation between asthmatic patients and control subjects, with differences in DNA methylation levels exceeding those normally observed in

blood cells.^{16,17} In addition, nasal epithelial cells might represent a good proxy for upper airway epithelial cells.¹⁸ However, a number of challenges remain for the biological interpretation and clinical implementation of EWAS-identified DNA methylation changes, such as the choice of the best tissue and cell type for analysis, the lack of reproducibility of identified changes between studies, and concerns on the functional relevance caused by the small magnitude of the detected changes. Furthermore, coverage of the human methylome is still limited in most studies because epigenotyping arrays, the most commonly used tools for genome-wide DNA methylation analysis, cover only between 450,000 and 840,000 CpG positions of the 29 million CpGs present in the human genome, corresponding to only 1.6% to 2.9% of all CpGs. Although a major criticism of DNA methylation analyses has been that changes will only reflect variations in proportions of the analyzed cell populations, recent results have also shown that not only are cell proportions changed but also the epigenomic landscape is modified in specific cell populations.^{10,19}

Nonetheless, EWASs have great potential for explaining phenotypic variability, as exemplified by a study on DNA methylation levels associated with serum IgE levels, which showed a 10-fold greater capacity of genome-wide DNA methylation patterns to explain the observed variability in IgE concentrations compared with genetic variation.²⁰ EWASs might not only deepen our understanding of the underlying disease etiology by pointing to disease-relevant pathways, such as the T_H1/T_H2 pathway²¹ and other immunologically relevant pathways in patients with cow's milk allergy²² but could also provide a multitude of other target genes that need to be further investigated in functional studies, including transcription factors, mitochondrial proteins, and proteins involved in T-cell maturation or oxidative stress. However, despite common themes, there is currently a lack of consistency between the findings from different studies, which might be due to differences in the analyzed populations, the definition of the underlying phenotype, and statistical methods used for analysis. Signatures of differentially methylated positions are in some cases already present at birth and predict future onset of the allergic disease.²³ However, in a recent large-scale study on childhood asthma, none of the asthma-associated CpGs were found to be differentially methylated at birth, pointing rather to the postnatal environment as the critical period. However, heterogeneity of the different analyzed populations, as well as unknown confounders, might limit the ability to replicate the findings of the discovery cohorts in the birth cohort.¹⁰

DNA methylation changes as potential biomarkers for allergic disease

Different allergic diseases manifesting at the same epithelial barrier organ do not show a homogeneous phenotype but constitute a highly heterogeneous group of diseases with different molecular endotypes. Phenotyping of inflammatory profiles has allowed for patient stratification and promoted the idea of personalized management of allergic diseases. However, there is currently a lack of robust, easy-to-measure, and preferentially nucleic acid-based biomarkers to tailor therapies to individual patients. The analysis of epigenetic changes has the potential to assist in the detection, management, and possibly prevention of allergic diseases as diagnostic tools; to assess tolerance after

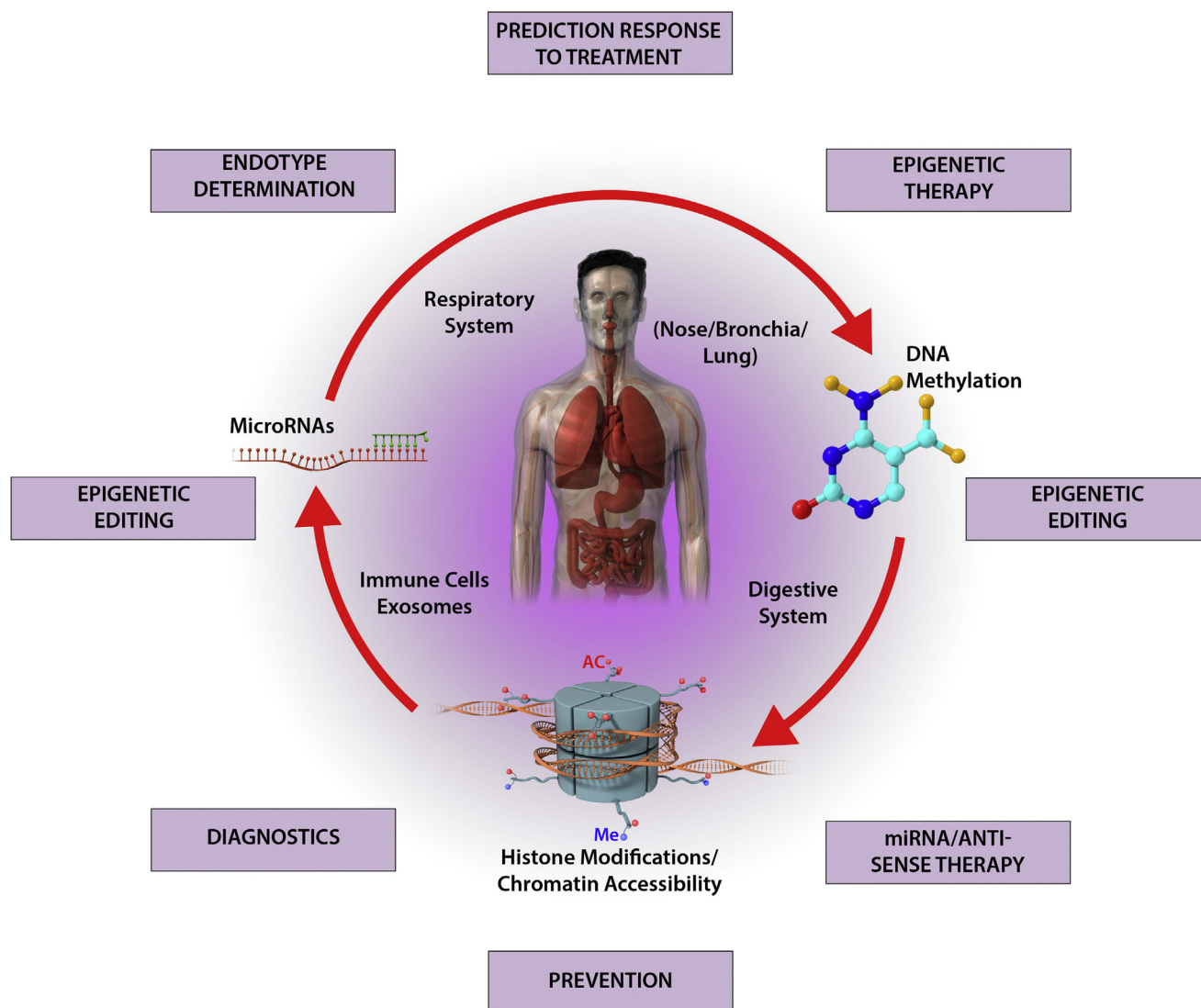


FIG 1. Schematic overview of translational applications using epigenetic modifications in patients with asthma and allergic diseases showing target organs and cells in the *inner circles*, different interacting epigenetic modifications (DNA methylation, histone modifications, and small noncoding RNAs [miRNAs]), and their potential applications, as detailed in the main text.

immunotherapy and potentially predict the success of therapy at an early time point; or to interfere with disease-associated pathways.

Analysis of blood, immune, and epithelial cells has shown differences between allergic/asthmatic patients and control subjects in a number of studies.^{8,16,17} EWASS in patients with allergic diseases have notably shown that DNA methylation signatures can separate allergic patients (seasonal allergic rhinitis) from healthy control subjects and show increased discriminatory power compared with gene expression-based signatures both during and outside the allergic season.²⁴ Development of food allergy could be detected based on a 92-CpG signature in cord blood samples of children before onset of clinically detectable food allergy in the first year of life.²³ These findings, if validated in larger cohorts, suggest that DNA methylation could be used for early detection of allergy, identifying children for early immunotherapeutic intervention.

Furthermore, DNA methylation signatures have been proposed as an alternative diagnostic test to reduce the need for oral food challenges, which are time-consuming, resource intensive, and

carry risks for the subject. Furthermore, they could help to simplify and standardize allergen testing in general because the variety of assays currently performed might lead to quantitatively different or even discordant results. A DNA methylation signature consisting of 96 CpGs predicted the response to multiple food allergens and outperformed the conventional skin prick test, as well as allergen-specific IgE tests, demonstrating the potential of epigenetic modifications for correctly diagnosing allergic diseases.²⁵ In a replication cohort the signature showed an accuracy of close to 80%.²⁵ However, replication in larger independent cohorts will be required to translate these findings into clinical practice.

DNA methylation as a biomarker for treatment and immunotherapy

Despite significant progress over the last decade, there is still an urgent need for novel drugs and treatment regimens for asthma and allergic diseases to be developed. Although asthma symptoms can be well controlled in the majority of patients by using standard

treatment options, a subset of patients, mainly those with severe and/or uncontrolled asthma, require additional treatments, such as mAbs (biologicals). Although biological agents, such as the anti-IgE antibody omalizumab, have proved effective in both adults and children with severe asthma and might reduce the severity and frequency of adverse events during oral immunotherapy for food allergy,^{26,27} the cost of these novel therapies approved or under development that target IgE, IL-5, IL-4, IL-13, or thymic stromal lymphopoietin, among others, is expected to be significant for public health systems.²⁸ Although some biomarkers correlate with response, improved classification of allergic diseases based on molecular alterations will be important and help us better tailor drugs to specific patient subgroups in which they will be most effective and to advance precision medicine in patients with allergic diseases. Although evidence is currently limited to the field of cancer, with the promoter methylation of *MGMT* being a predictive marker for the response of the alkylating drug temozolomide in patients with glioblastoma as a prime example,²⁹ analysis of epigenetic modifications might contribute to a better definition of the diseases and their underlying molecular actions and permit a better classification of patients and stratification for novel therapeutic regimens thus maximizing the chances for a positive outcome while maintaining costs for the health care system. Furthermore, additional epigenetic analyses might also help to define groups of children who might be good responders.

Available data on response to biological agents in children is rather scarce and contradictory. In a recent multicohort study from the MEDALL consortium, childhood asthma was associated with a number of differentially methylated CpG positions, especially in whole blood and eosinophils.¹⁰ In particular, analysis of a subset of subjects for whom purified circulating eosinophils were available showed an altered DNA methylation profile, which suggested a differential activation state. These findings provide an interesting basis to investigate how eosinophil-targeting/depleting therapies with anti-IgE, anti-IL-13, or anti-IL-5 receptor antagonist-based antibodies^{30,31} will modify the DNA methylation landscape in eosinophils and whether there is any correlation between the response to therapy and the pretreatment epigenetic profile.

Systemic corticosteroid treatment is currently the standard of care for asthma. There is preliminary evidence that DNA methylation changes might contribute to treatment response because methylation changes in genes, including the *OTX2* and the *VVN1* promoter, were observed in good but not poor responders in nasal epithelial cells during treatment.^{32,33} However, in contrast to gene expression signatures, no DNA methylation-based biomarker has thus far been identified to be predictive for corticosteroid response.^{34,35} Nonetheless, these DNA methylation markers might assist in molecularly defining patients unresponsive to corticosteroids having difficulties controlling their asthma symptoms.

In the field of food allergy, induction and maintenance of tolerance to antigens requires the generation of antigen-specific Treg cells. Demethylation of the regulatory T cell–specific demethylated region (TSDR) of forkhead protein 3 (*FOXP3*) is a prerequisite for stable maintenance of the suppressive properties of Treg cells.^{36,37} Demethylation is induced by means of immunotherapy, and methylation levels remain lower in subjects with sustained unresponsiveness to allergens, such as peanut or milk.^{38,39} Therefore demethylation of *FOXP3* might be a prerequisite for successful immunotherapy. Although the number

of subjects analyzed was low in both studies, DNA methylation analysis of the TSDR can be considered a promising biomarker for monitoring response to immunotherapy, as well as induction of potential tolerance.

Similarly, recently, we have shown in a mouse model of epicutaneous immunotherapy for peanut allergy that *Foxp3* methylation was reduced on successful epicutaneous immunotherapy, whereas methylation of the T_H2 key transcription factor *Gata3* was specifically increased in splenic $CD4^+$ $IL-4^+$ T cells.¹⁹ In contrast, oral immunotherapy induced only demethylation of *Foxp3* but not methylation of *Gata3*, suggesting that the latter might be important to maintain the level of sustained unresponsiveness and protection against sensitization to a second allergen observed in epicutaneous immunotherapy.

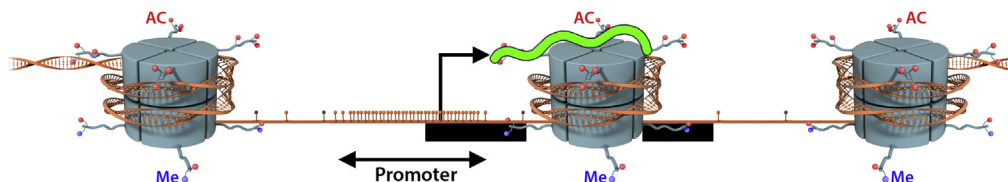
In addition, oral immunotherapy to peanut allergy has shown to induce the differentiation of novel $CD4^+$ T-cell subsets.⁴⁰ Although these have thus far only been characterized at the transcriptional level, it is highly likely that these novel $CD4^+$ T-cell subsets also contain distinct epigenetic profiles that could provide further markers correlating with sustained unresponsiveness.

The histone code in patients with allergic diseases

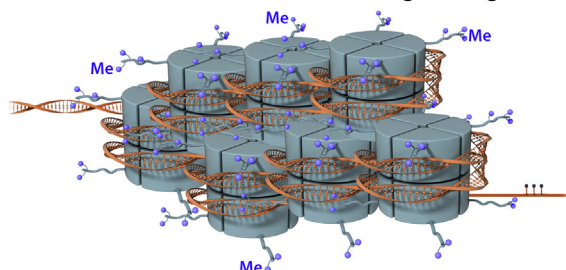
The balance between acetylation and deacetylation, as well as the methylation and demethylation of specific residues of the N-terminal tails of the histones, the main component of chromatin and the basic packaging unit for DNA, is critical for regulating and controlling gene expression. The interplay between multiple histone modifications (the so-called histone code) and DNA methylation determines accessibility of the transcriptional machinery to specific genomic elements and contributes to the cellular identity of specific lineages, including different T-cell lineages (Fig 2).^{8,41,42} Although studies are much more rare compared with the analysis of DNA methylation, deregulation of the balance between histone modifications, leading to an opening or compaction of chromatin, has been observed in patients with allergic diseases and asthma.^{8,43,44} Notably, the enhancer-associated histone marker H3K4Me2 was found to be enriched in T-cell subsets from asthmatic patients at gene-regulatory regions implicated in T_H2 development and at loci genetically associated with susceptibility to asthma.⁴⁵ In a mouse model the protective effects of maternal intranasal exposure to *Acinetobacter lwoffii* against the development of allergic airway inflammation in offspring were shown to be IFN- γ dependent, which was, at least in part, mediated by the *A lwoffii*–induced preservation of histone H4 acetylation at the *Ifng* promoter of $CD4^+$ T cells of mouse offspring, whereas DNA methylation analysis showed no differences.⁴⁶

Most studies analyzing histone modification have analyzed the profile at specific genes, especially those involved in T_H subset differentiation, whereas systematic genome-wide studies are rare.⁴³ Inflammation is in general correlated with increased transcription of immune-related genes and histone acetylation, which has been shown in both bronchial biopsy specimens and alveolar macrophages in asthmatic patients.⁴⁷ In addition to histones, histone acetyl transferases and histone deacetylases (HDACs) target a number of other proteins, including transcription factors with great relevance to allergic diseases, such as GATA-3, FOXP3, the glucocorticoid receptor, and nuclear factor κB . Reduced levels of sirtuins, class III HDACs, have been found in patients with severe asthma and lead to hyperacetylated

EUCHROMATIN (Potentially transcriptionally active gene regions)



HETEROCHROMATIN (Closed inactive gene regions)



POSTTRANSCRIPTIONAL GENE REGULATION BY miRNAs

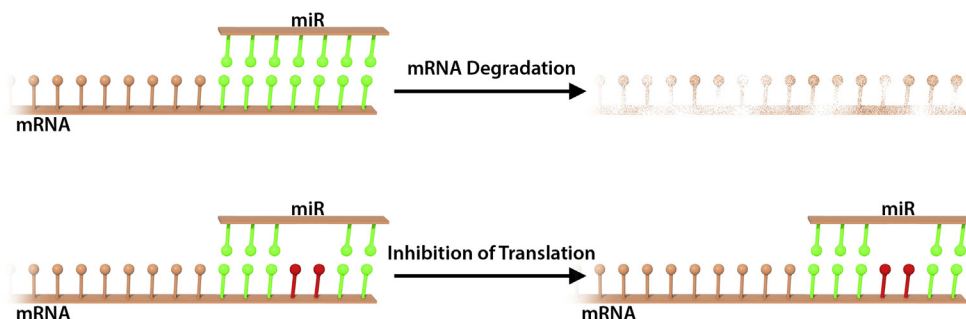


FIG 2. Epigenetic modifications associated with open (euchromatin) and closed (hetero) chromatin. Overall, CpG dinucleotides throughout the genome are methylated (*solid circle with a line*), except for CpG-rich regions (CpG islands) associated with the promoters of about two thirds of all genes. In euchromatin RNA polymerase II can access the promoter CpG island (*open circle with a line*), which corresponds to a nucleosome-free region, and the region is permissive to transcription. Histones are hyperacetylated (*circle with Ac*), including lysine 9 of histone H3 (*H3K9*) and lysine 4 of H3 (*H3K4*) is methylated (*Polygon with Me*). In contrast, the DNA and nucleosome structure is compacted in heterochromatin. H3K4 is demethylated, and H3K9, H3K27, and H4K20 are methylated. DNA is also hypermethylated, and the region is transcriptionally silent. For reasons of clarity, only selected histone modifications associated with the chromatin state are shown. miRNAs anneal to the 3' untranslated region as their mRNA target. In case of complete complementarity, the target RNA is degraded. However, in most cases binding occurs with several mismatches blocking the translation of the mRNA into proteins but not modifying mRNA abundance.

GATA-3 driving T_H2 cytokine expression and airway inflammation, which can be reversed with a sirtuin agonist⁴⁸ or forced expression of other members of the sirtuin family in mouse models.⁴⁹ Although other histone modifications have been little studied in patients with allergic diseases, there is some evidence that chromatin-modifying enzymes are altered in airway epithelial cells.⁵⁰

The methylome and histones code as a target in patients with allergic diseases

Epigenetic changes constitute heritable but reversible modifications of gene expression patterns and could be targeted to modulate the epigenome toward a lower risk of atopic diseases

or at least toward development of less pronounced allergic phenotypes. Nonspecific DNA methylation inhibitors, such as 5-azacytidine, have been little investigated in patients with allergic diseases, and available data are currently conflicting. Some studies found beneficial actions, notably through induction of Treg cells⁵¹ or demethylation of *IFNG*,⁵² whereas in other studies their use led to worsening of the allergic conditions.⁵³ Glucocorticoid treatment, the standard anti-inflammatory treatment for asthma, leads to global loss of histone acetylation through activation of several HDACs and displacement of nuclear factor κB from glucocorticoid receptor binding sites.^{54,55} Decreased sensitivity to synthetic glucocorticoids has been linked to decreased levels of HDAC2, which deacetylates the glucocorticoid receptor, and might be worsened by passive

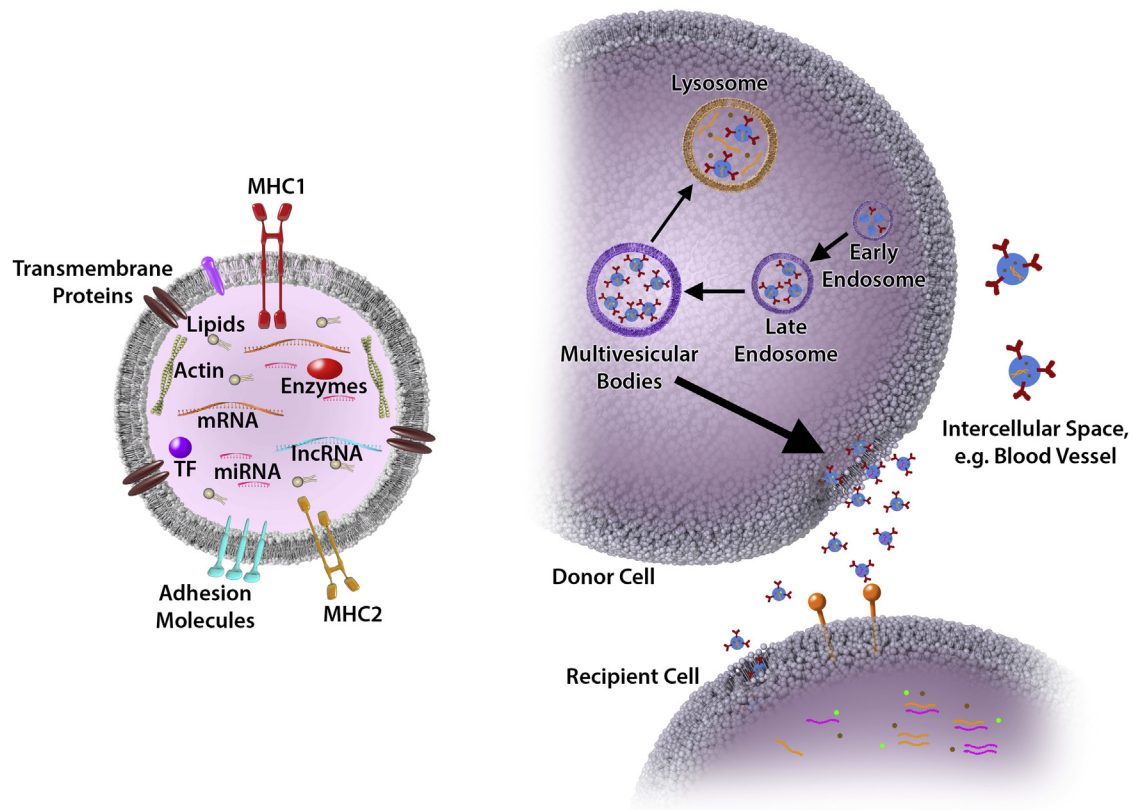


FIG 3. Exosome content and mechanism of intracellular signaling by exosomes. *Left panel,* Exosomes contain a number of proteins, lipids, and nucleic acid molecules, including cytoskeletal proteins (actin and tubulin), enzymes (glyceraldehyde-3-phosphate dehydrogenase and elongation factors), components of the endosomal sorting complex required for transport (ESCRT), transcription factors (TF) and proteins involved in signal transduction, miRNAs and other small noncoding RNAs, and mRNAs and long noncoding (lncRNAs). On the bilayer lipid membrane, antigen-presenting MHC class 1 and 2 molecules can be found, as well as molecules involved in cell adhesion (tetraspanins and integrins) and other transmembrane proteins. *Right panel,* Small vesicles are generated by means of inward budding of the inner membrane of endosomes and charged by processes, including the ESCRT, tetraspanins, and lipids, to form multivesicular bodies, which subsequently fuse with the plasma membrane to release exosomes. Exosome content is taken up by recipient cells, which might be at large physical distances from donor cells through specific ligand-receptor interactions, as well as through fusion with the plasma membrane or endocytosis.

smoking.^{56,57} Thus increasing HDAC levels in therapeutic interventions might constitute a new way to maximize treatment efficacy.

On the other hand, the use of HDAC inhibitors has yielded conflicting results, with some studies showing anti-inflammatory effects and others pointing to enhanced inflammation, thus requiring further investigation of the use of this treatment.^{44,58,59} However, because HDACs and histone acetyl transferases (de)acetylate a large number of targets and are involved in a multitude of cellular pathways, inhibition or modulation of these processes might provoke undesired adverse effects, requiring development of more selective HDAC inhibitors targeted to specific cell populations.

Because most studies only investigated the effect of epigenome-wide acting treatments on a single target gene, it is difficult to draw any conclusions on the potential use of these nonspecific genome-wide epigenetic modifiers, but caution is warranted. Recently, major technological advances using the CRISPR/dCas9 system have allowed targeted engineering of the epigenome.⁶⁰⁻⁶² For the first time, these approaches allow us to functionally investigate and validate the importance of

epigenetic modifications at any locus in the genome and might provide novel alternatives to modulate the epigenome in allergic diseases. The combination of CRISPR/dCas9, which does not introduce double-strand breaks in the genome through use of a nuclease-deficient Cas9 enzyme, allows us to guide epigenetic enzymes to specific loci in the genome, where they can specifically (de)methylate DNA or (de)methylate/(de)acetylate histones.^{63,64}

Although only recently devised, this technology bears great promise for the treatment of diseases without clearly defined underlying mutations in the future. Because epigenetic modifications are in general reversible, identification of key molecular changes induced by environmental exposure might provide new treatment alternatives with which changes can be reversed before occurrence of allergic conditions. More importantly, the altered T-cell polarization observed in patients with allergic diseases is largely driven by epigenetic modifications at promoters and binding sites of key transcription factors, cytokines, and conserved noncoding sequences.

Epigenetic editing provides potentially the tools to alter the balance between different T-cell populations. This could be achieved by using 2 vectors, each containing a cell

type-specific promoter driving expression of the key components of the editing complex, thus leading to the expression of the editing complex in T cells only.⁶⁵ However, although targeted engineering of the epigenome is an important advance on understanding the functionality of epigenetic changes and first experiments have been performed *in vivo*,⁶⁶ their potential use for therapeutic means remains uncertain because of the challenges associated with the safety and efficiency of the delivery of the constructs *in vivo*. Furthermore, questions on the efficacy of changes, the stability of the induced changes, and the observation of a high number of induced unspecific alterations (off-target effects) call for further investigation before clinical use in patients with allergic diseases can be envisioned.⁶⁷

Interactions between the microbiome and the epigenome

The microbiota influences cellular homeostasis, immune cell differentiation, and polarization through multiple mechanisms, including epigenomic regulation.⁶⁸ A number of studies have now reported altered diversity or prevalence of the microbiota in barrier tissues, including the skin, upper airways, and gut, in patients with allergic diseases.⁶⁹⁻⁷¹ A number of questions remain yet unanswered because the results of thus far published studies are not consistent in identifying changes in the presence or prevalence of different species, and both increased and decreased diversity of the microbiome were found to be associated with allergic diseases.

Some bacterial species that have been shown to be altered in patients with allergic diseases, such as the *Clostridia* species. These species can produce epigenetically active immunomodulatory molecules, such as short-chain fatty acids (SCFAs) from dietary fibers, including butyrate, that will inhibit HDACs, leading to global hyperacetylation of intestinal macrophages and systemically contribute to a T_H1/T_H17 effector T-cell polarization.⁷²⁻⁷⁴ Low levels of SCFAs have been associated with allergic diseases, and the increase in SCFA levels improved epithelial barrier function and alleviated the disease.⁷³ Similarly, butyrate was shown to inhibit proliferation of type 2 innate lymphoid cells and reduce the expression of proinflammatory T_H2 cytokines, as well as GATA-3, in mouse models of allergic inflammation with a mode of action that could be recapitulated by using a pan-HDAC inhibitor.⁷⁵ Of note, SCFAs producing *Clostridia* species have been associated with resolution of food allergy⁷⁶ and are also important for induction of colonic Treg cells through histone H3 acetylation of the locus, leading to the selective demethylation of the TSDR, which protect against food allergen sensitization and create a tolerogenic environment.^{77,78} Similarly, acetate produced by *Bifidobacterium* species leads to a hyperacetylated *Foxp3* promoter.⁷² In addition, microbial colonization induces DNA methylation through Uhrf1 in colonic Treg cells.⁷⁹ In summary, these results suggest that modification of the microbiome with epigenetically active metabolites might allow improvement of the disease state in allergic subjects.

The major aim of intervention in patients with a risk of developing allergic diseases is disease prevention.⁸⁰ Although the beneficial action of some protective environmental and dietary factors has been shown, more research is required to define changes in the epigenome that correlate with the induction of

tolerance. Exposure to probiotic or pathogenic bacteria leads to induction of exposure-specific DNA methylation changes in immature intestinal epithelial cells.⁸¹ Several probiotics have been shown to increase the epigenetic plasticity of the T-cell response and might contribute to protection against allergen sensitization or facilitate outgrowth of allergies.^{39,46} Other dietary interventions, such as polyunsaturated fatty acids, have shown beneficial effects,^{82,83} and these might be at least partially mediated through or correlate with epigenetic changes.⁸⁴⁻⁸⁶ However, epigenetic analyses have thus far only been performed in small cohorts, requiring further research and mechanistic studies to elucidate how polyunsaturated fatty acids influence epigenetic modifications.

miRNAs AND EXTRACELLULAR VESICLES

miRNAs constitute an additional level of epigenetic regulation influencing gene expression without altering the genomic sequence. miRNAs are small noncoding RNA molecules (22-25 nucleotides) that posttranscriptionally regulate gene expression by blocking mRNA translation and/or altering the stability of the mRNA by binding perfectly or with mismatches to the 3' untranslated region of the mRNA (Fig 2).⁸⁷ Each miRNA can potentially directly or indirectly regulate many target genes, and each gene can be regulated by a number of different miRNAs building up a complex gene-regulatory network. miRNAs are key players in the development, differentiation, maturation, and activation of immune cells, airway remodeling and deregulated in patients with allergic and inflammatory diseases.⁸⁸ In analogy to the results on DNA methylation patterns, there is accumulating and convincing evidence that miRNA expression profiles differ between allergic patients and healthy subjects in tissues, cells, or biofluids.⁸⁹⁻⁹³ Allergen challenge leads to further alterations in the miRNA expression profile in blood cells,⁹⁴ and at least in patients with eosinophilic esophagitis, the deregulated miRNA profile can be reversed with glucocorticoids.⁹⁵

Furthermore, expression of some miRNAs has been shown to distinguish different asthma subtypes and correlate with the severity of the disease, which might allow their future use for risk stratification.^{91,96,97} However, the cell-type specificity of miRNAs, the variety of detection technologies used, the differences between experimental animal models and human patients, the small sample size for heterogeneous phenotypes, and in many studies the absence of replication cohorts makes it currently difficult to draw definite conclusions from these data and advance miRNA-based analysis and diagnostics in the clinics.

Although facilitating clinical studies because of its low invasiveness, analysis of circulating miRNAs has been hampered by the low concentration of miRNAs in bronchoalveolar lavage fluid, serum, plasma, or other sources, such as exhaled breath. Extracellular vesicles (EVs) constitute a heterogeneous group of small membrane-coated vesicles, including exosomes, and microvesicles containing RNA, proteins, metabolites, and small regulatory RNAs, such as miRNAs, which constitute an essential component of eukaryotic cell-to-cell communication (Fig 3).⁹⁸ The content of EVs, which differs between parental cell types, is shaped by the cytokine environment and actively selected from the content of the original cell (Fig 3). EVs are subsequently taken up through endocytosis by recipient cells, which can be at a considerable distance from the originating cell. This uptake is at least partly determined by the proteins present at the EV surface,

and the released cargo can stimulate or inhibit in a context-dependent manner both the innate and adaptive immune response in the recipient cell.^{99,100}

Thus the content of exosomes has a potent reprogramming capacity of recipient cells^{101,102} and might play an important role in interactions between allergy-mediating cells and, depending on their content, might alleviate or exacerbate allergic reactions.^{93,103} Allergen sensitization leads to a strong increase in EV numbers¹⁰⁴ and in a mouse model of allergic bronchial asthma leads to selective sorting of T_H2-inhibitory miRNAs into EVs and reduced eosinophil accumulation and inflammation in the airways.¹⁰⁵ The miRNA content of EVs isolated from bronchoalveolar lavage fluid distinguishes patients with mild asthma from nonasthmatic subjects with a predictive power of 72%, contains multiple miRNAs regulating potentially disease-relevant cytokines,¹⁰⁶ and presents a large overlap with miRNAs deregulated in airway epithelial cells of asthmatic patients.¹⁰⁷

Very few studies have analyzed miRNAs in relation to treatment in patients with allergic diseases. Higher levels of miR-21, a well-known miRNA in patients with allergic diseases, were found in children resistant to inhaled corticosteroids (ICSs) compared with children sensitive to ICSs.¹⁰⁸ However, because miR-21 levels in ICS-resistant children were similar to those in patients without ICSs, the decreased levels in ICS-sensitive patients are probably a result of improvement of their asthmatic status rather than predisposing therapy to success. Similarly, miRNA changes correlated with the use of oral steroids or antileukotriene therapy.⁹⁷

miRNAs as a target in patients with allergic diseases

miRNAs present an interesting target for intervention using synthetic miRNA mimics or antagomirs (miRNA inhibitors) to modulate the expression of disease-relevant endogenous miRNAs. Some data have been obtained *in vivo* in mouse models of allergic diseases. Exogenous administration of let7-a, which targets the T_H2 cytokine IL-13, reduced cytokine levels and resolved airway inflammation.¹⁰⁹ Similar inhibition of miR-221 reduced eosinophil and leukocyte airway infiltration in an OVA-induced asthma mouse model,¹¹⁰ whereas inhibition of miR-145 in house dust mite-treated mice reduced IL-5 and IL-13 levels in T_H2 cells and mimicked the effects of corticosteroid treatment, although with lower efficacy.¹¹¹

Because of the ubiquitous expression and multiple targets of miRNAs, therapeutic agents need to be delivered in a cell type-specific manner, and predicting off-target effects requires a more profound understanding of the targets and complex mechanisms of cytokine production. Furthermore, because transcripts of interest can be targeted by a number of miRNAs, modulation at the level of single miRNAs might only have a limited effect.

Although not yet used in patients with allergic diseases, specific treatments and vectors have nonetheless been designed and are under investigation in clinical trials to increase (miR-34, miR-29, and miR-16) or decrease (miR-122 and miR-103/107) specific miRNA levels.¹¹²⁻¹¹⁴ Of special interest for patients with allergic diseases is a clinical trial (NCT02580552) targeting miR-155 in cutaneous T-cell lymphoma because miR-155 is specifically expressed in T_H2 CD4⁺ cells, and mice with an miR-155 deficiency have less severe asthma.^{115,116} With miRNA-based treatments advancing in other fields, mainly cancer, and cell type-specific delivery strategies being constantly

refined, there is a good hope that such treatments might soon also be evaluated in patients with asthma and allergic diseases.

Furthermore, long noncoding and circular RNAs have been shown to be potent miRNA sponges, reducing the level of specific miRNAs in cells.^{117,118} Although under physiologic conditions miRNA levels will adapt to this depletion, synthetically designed sponges have potential as therapeutics for depletion of disease-associated miRNAs.¹¹⁹ However, their development and the corresponding methods for *in vivo* delivery are far behind those of the miRNA mimics and antagomirs.

In the absence of known natural small RNAs targeting a transcript of interest for the treatment of allergic diseases, another option is modulation of differentiation of the cells playing a major regulatory role in the immune system (ie, T cells). This could be achieved, for example, by reducing expression of the transcription factors promoting T_H2 responses, such as GATA-3, which could be achieved by using antisense strategies. Antisense molecules prevent translation of those mRNAs to proteins through specific binding and subsequent degradation of mRNAs.¹⁵

The possibility that antisense approaches could be used to edit the phenotype of developing T cells is supported by recent studies on hgd40/SB010, a DNAzyme-type antisense molecule against the T_H2 key transcription factor GATA3.^{120,121} DNAzymes are antisense molecules possessing internal catalytic activity to deactivate targeted mRNAs on specific binding without requiring accessory molecules possessing enzymatic activity.¹⁵ Hgd40/SB010 has demonstrated in phase I clinical trials its safety and tolerability in human subjects¹²⁰ and showed in a randomized, placebo-controlled, multicenter phase II clinical trial to significantly attenuate late and early clinical asthmatic responses after allergen provocation in patients with atopic asthma.¹²¹

Preliminary data also demonstrate the potential of EVs for clinical use. Exosomes from bone marrow-derived mast cells have been shown in mouse models to reduce circulating IgE levels and to inhibit mast cell activation in ovalbumin-induced asthma, reducing inflammation.¹²² Manipulation of EVs expressing immunosuppressive or stimulatory cytokines or other molecules of relevance in patients with allergic diseases targeting immune cells *in vivo* or the use of custom-designed clinical-grade EVs targeting immune cells for therapeutic interventions might constitute novel promising approaches for immune-related diseases, as pioneered by the cancer field, where purified exosomes derived from dendritic cells have been used for immunotherapy in clinical trials.^{123,124} It can be hypothesized that administration of tolerogenic EVs could inhibit allergy and hypersensitivity responses. There is also great interest in engineering EVs to deliver biomolecules or drugs to specific cell populations through coating of the exosome surface with cell type-specific antibodies. Nonetheless, reconstituting the complexity of the content of EVs is challenging and requires a better understanding of the cellular processes associated with loading and secretion. In the short term it might be more practical to further characterize exosome subpopulations to identify naturally occurring EVs of a specific origin that can be harnessed for therapeutic interventions through either enrichment or depletion.

Which epigenetic modification might be best suited for translation?

Advances in the tools to analyze DNA methylation patterns both genome wide and at specific loci combined with the stability of DNA methylation marks in biological samples during storage

facilitate implementation of DNA methylation–based biomarkers in patients with complex diseases compared with analysis of histone modifications for which procedures, including chromatin isolation, remain more challenging. Mature and standardized technologies for the analysis of small RNAs, including miRNAs, have been developed, and the similarity to gene expression analysis will facilitate their implementation in clinical laboratories. The cell-type specificity of all epigenetic modifications, including miRNAs, remains a major hurdle requiring analysis in well-defined cell subsets. However, the gene-regulatory networks of miRNAs are far more complex to interpret than DNA methylation or histone modification changes at specific loci. If the cell type of interest is known and cells of interest can be relatively easily isolated, DNA methylation fulfills all the criteria of a powerful biomarker.¹²⁵ Otherwise, analyses of circulating miRNAs or EV content might overcome this problem, allowing repeated minimally invasive sampling of patients.

Another point that deserves attention is the stability versus dynamics of different levels of epigenetic gene regulation. DNA methylation is responsive to stimuli, although at a much slower pace, contributing to maintaining the epigenetic landscape in its altered state compared with histone modifications or changes in miRNAs, which will change strongly shortly after an external or internal stimulus, but changes might fade away over time once the altered state has been stabilized. Therefore if the biomarker predicts long-term outcome or a robust diagnosis, DNA methylation might be the marker of choice, whereas for rapid decision making (ie, predicting treatment response after a single administration), miRNA might be better suited. In addition, from an interventional point of view and despite our limited knowledge, modulation of miRNA levels using exogenous synthetic mimics or inhibitors is far more advanced in clinical trials (mainly cancer) than targeted alterations of DNA methylation or histone modifications patterns.

CONCLUSIONS

Investigating epigenetics will further our understanding of the disease mechanisms, and some epigenetic marks might be useful as biomarkers to guide clinical decisions for personalized management of allergic diseases, including the detection, management, and ideally prevention of these diseases. Because most published studies are limited to small exploratory cohorts and therefore had low statistical power, systematic inclusion of the analysis of epigenetic modifications, in particular DNA methylation and miRNAs, in large-scale clinical trials would greatly improve our capacity to define biomarkers correlating with treatment response or even predict response before the start of the treatment. However, the cellular phenotype is determined by a number of gene-regulatory mechanisms, including epigenetic modifications. Thus it is unlikely that a single change in DNA methylation levels or microRNA expression will yield sufficient sensitivity and specificity to be of clinical utility. Multilevel signatures similar to those currently emerging in the personalized management of patients with cancer will be required to obtain robust biomarker panels of sufficient performance.¹²⁶

Furthermore, the redundancy of mechanisms that maintain the epigenomic landscape within its limits of plasticity might be challenging to overcome. Consequently, a systems biology

approach is required to assess the complex interplay of the different levels of epigenetic modifications and gene regulatory mechanisms and better identify therapeutic targets.

The field of epigenetics in patients with allergic diseases is still at a very early stage. Because awareness on the importance of epigenetic changes in patients with complex diseases has increased and technologies for analysis of epigenetic modifications have matured well over the last years, a large expansion of our knowledge can be expected in the next few years with first examples of the use of epigenetic changes entering clinical practice.

What do we know?

- Epigenetics, including DNA methylation, histone modifications, and small noncoding RNAs, is a key mechanism in immune cell differentiation and plasticity.
- Profiles of epigenetic modification are altered in patients with asthma and allergic diseases in immune cell subsets and various biofluids and correlate in some cases with disease endotypes and/or disease severity.
- Demethylation of *FOXP3* in Treg cells correlates with their activation and is linked to allergen unresponsiveness.
- EVs, including exosomes, contain molecules able to alter the cellular phenotype at distant sites.
- miRNA analogues and inhibitors, as well as artificial anti-sense molecules, show promising results in clinical trials, although many are outside the field of allergic diseases.
- The host epigenome and microbiome interact closely, and modulation of the microbiome has an effect on global and gene-specific epigenetic patterns.

What we do not yet know?

- Most epigenetic changes in patients with allergic diseases are purely correlative. It is unclear which of the many changes are disease causing or might have functional relevance.
- Because epigenetic changes are cell-type specific, which is the most relevant cell type to study and to target?
- Personalized therapies, such as eosinophil-depleting strategies, have shown clinical utility, but it is unclear whether they just deplete eosinophils (corresponding to improve disease symptoms) or are able to revert the epigenetically altered state of eosinophils back to the “healthy” state.
- Epigenetic changes have been identified, but with the field still in its infancy, many studies have limited power and problems of replication of the findings in other cohorts/model systems.
- Epigenetic editing yields great promise; however, many challenges on cell-type specificity, off-target effects, and durability remain to be solved before its use in clinical interventions can be envisioned.
- Further understanding of the origin, loading mechanisms, and cell-type specificity for delivery of EVs is required before they can become usable in clinical practice.

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