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Epigenetics and childhood asthma: current evidence and future research directions

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Abstract

Asthma is the most common chronic disease of childhood, affecting one in eight children in the USA and worldwide. It is a complex disease, influenced by both environmental exposures and genetic factors. Although epigenetic modifications (DNA methylation, histone modification and miRNA) can affect transcriptional activity in multiple genetic pathways relevant for asthma development, very limited work has been carried out so far to examine the role of epigenetic variations on asthma development and management. This review provides a brief overview of epigenetic modifications, summarizes recent findings, and discusses some of the major methodological concerns that are relevant for asthma epigenetics.

Keywords

asthma; DNA methylation; epidemiology; epigenetics; genetics; histone modifications; miRNA; natural history; risk factors

Childhood asthma remains a major clinical and public health problem as it affects nearly one in eight children in the USA and worldwide [1,2]. The economic, social and public health burdens of asthma on families and healthcare organizations are substantial. During the last few decades, identification of the factors involved in asthma etiology has remained a major goal of research. A large body of evidence indicates that asthma etiology is complex and has strong environ mental and genetic components. Studies to date have documented that DNA sequence variants (SNPs) in multiple genes in innate and adaptive immune pathways are either independently [3,4] associated with asthma risk in children or synergistically interact with prenatal and early-life exposures (e.g., tobacco smoke, endotoxins, air pollution and pesticides) to affect asthma risk [5–7]. However, much of the variability of asthma still remains unexplained. While the enthusiasm in and expectations from genome-wide association studies have been slowly fading in the scientific community, findings that environmental exposures could affect epigenetic profile have brought a new era in asthma research in the recent years by examining epigenetic mechanisms as mediators of these exposures for occurrence and clinical course of asthma.

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Asthma is a chronic inflammatory disease of the airways, and as such immunologic and inflammatory mechanisms play a major role in asthma etiology and natural history. Many epidemio logical studies have found environmental exposures could modulate immune development (e.g., endotoxins) and/or impart oxidative/nitrosative stress in airways (e.g., tobacco smoke and air pollution), both of which could affect asthma risk [8]. One of the first immunologic mechanisms known as the 'hygiene hypothesis' proposed that lack of early postnatal exposure to infectious agents in children born in developed nations affects the normal development of the immune system and as such increases the risk of asthma. An increase in the prevalence of asthma and atopic disorders in the past few decades, paralleled with a reduction in infectious disease prevalence in developed countries, had led Strachan to hypothesize that early-life infection might be protective for asthma and other atopic disorders [9]. Further work showed that two major helper T lymphocyte subtypes (known as Th1 and Th2 cells) are critically important in immune development. The Th1 cells produce IL-2, IL-12 and IFN-γ, whereas Th2 cells preferentially produce IL-4, IL-5 and IL-13. In asthma, the Th2 cytokines are often elevated, and many epidemiologic studies have found that farm exposures reduce asthma risk in children. Additional work identified T-bet (also known as T-box 21) and GATA3 as the master regulator transcription factors of Th1 and Th2 cell differentiation, respectively.

Although the hygiene hypothesis formed the framework for investigating the immunological basis of asthma, further work showed that this hypothesis is a simplified approach to a much more complex biological pathway that is involved in asthma. In the last decade, Tregs have been implicated in asthma development [10], as these cells play a major role in immunological tolerance and homeostasis. The Tregs are identified as $CD4^+CD25^+$ T cells, and are best characterized by their expression of transcription factor FOXP3 [11]. In addition, a growing body of literature indicates that Th17 (helper T cells producing IL-17) and Th9 (helper T cells producing IL-9) cells are involved in asthma pathobiology [12,13]. A compre hensive review of the immunological basis of asthma is beyond the scope of this review; however, many excellent reviews have been published on this topic [8,10,14,15]. In the present article, the authors will discuss some emerging evidence that indicates that environmental exposures modulate epigenetic profiles in multiple tissues, and how such altered epigenetic profiles are linked with asthma.

The purpose of this review is to discuss direct and indirect evidence of the role of epigenetics on asthma etiology and natural history, while briefly describing epigenetic mechanisms. Because few papers directly examine the effects of epigenetics on asthma etiology, the authors discuss few studies that focused on understanding the role of environmental exposures on specific immune cell types or on animals. A comprehensive review of environmental influence on epigenetics is beyond the scope of this review and an excellent review on this topic has been published recently [16]. The present article will focus more on exposures that have been linked with asthma and provide some examples how such exposures modulate epigenetic profiles. Through PubMed and Medline searches, the authors reviewed original articles in peer-reviewed journals that were published in the past 5 years. For epigenetics, the search terms used were 'epigenetics', 'DNA methylation', 'histone modification', 'micro RNA'. For outcomes, 'asthma' and 'wheeze' were used as search terms.

DNA methylation

In DNA methylation, there is a covalent addition of a methyl group to a cytosine residue (5-methylcytosine) which lies next to a guanine residue in the DNA sequence. Because DNA methylation occurs in these particular C–G areas, they are described as CpG sites. These CpG sites are usually clustered in high frequency near gene promoters and these regions are

referred to as CpG islands (CGIs). The CGIs are found in the promoter region and first exons of many genes near the transcription start sites where they modulate gene expression. Gardiner-Garden and Frommer defined CGI as a region spanning at least 200 bp with GC content (proportion of Gs or Cs) greater than 50%, and observed-to-expected CpG ratio greater than 0.6 [17], whereas Takai and Jones defined it as a region of 500 bp or more in length with GC content greater than or equal to 55% and the observed:expected CpG ratio greater than or equal to 0.65 [18]. Since these conventional algorithms are arbitrarily defined, they exclude CpG clusters that have been associated with health outcomes [19], other approaches that overcome this problem with the traditional definition of CGI has been proposed [20–22]. In addition to CGI, Irizarry and colleagues identified regions of comparatively low CpG density that are located within 2000 bp of traditional CGI, which they termed as CGI shores [19]. They found that CGI shore methylation marks are evolutionarily conserved across species, have strong inverse association with gene expression, and could discriminate tissue types across mouse and human tissue samples.

DNA methylation is mediated by members of the DNA methyltransferase family. DNA methyl transferases transfer methyl groups to the cytosine bases in the context of CpG dinucleotides. DNA methylation can occur either in specific genes or in repetitive DNA sequences such as the long interspersed nuclear elements (LINEs) and Alu, referring to a global methylation pattern. While DNA hypermethylation in the promoter region of specific genes is often associated with gene silencing, effect of hypermethylation in intragenic regions (i.e., gene body regions that include exons and introns) on transcription is complex and less clear. In a study, the hypermethylation in the first exon was correlated with promoter hypermethylation resulting in transcriptional silencing [23]; however, another study reported that intragenic DNA methylation was positively correlated with transcription level in human $CD19^+$ B lymphocytes [24].

DNA methylation & asthma risk

Although there is a growing interest among researchers in exploring the role of epigenetic variation in asthma etiology [25], only one study has documented the relationship between gene-specific DNA methylation and asthma [26]. In that study, Perera and colleagues first used umbilical cord blood (UCB) samples and found that exposure to polycyclic aromatic hydrocarbon (a constituent found in diet, tobacco smoke and traffic-related pollution) was associated with DNA methylation level in a CpG site in *ACSL3* in UCB white blood cells [26]. Subsequently, these authors found that DNA methylation level in *ACSL3* was associated with increased asthma risk in children (odds ratio: 3.9; 95% CI: 1.14–14.3). Using this same study sample, these authors recently reported that subjects with higher benzo[a]pyrene concentration in UCB had higher DNA methylation in IFN-γ promoter in UCB white blood cells and lower expression of IFN-γ; however, the authors did not report whether variation in IFN-γ promoter was associated with asthma [27].

The role of DNA methylation in the previously reported association between prenatal exposure to dichlorodiphenyldichloroethylene (DDE) and asthma in children [7] has recently been reported by Morales and colleagues [28]. The authors used a discovery sample that included a subset of the population that was investigated for the effects of DDE on asthma (i.e., INMA Menorca pregnancy cohort in Spain that was enrolled in 1997) and a replication sample that included children who participated in the INMA Sabadell pregnancy cohort in Catalonia, Spain and were recruited in 2004–2006 [29]. Illumina GoldenGate Methylation Cancer Panel I array (Illumina, Inc., CA, USA) was used to identify CpGs that are differentially methylated in whole blood collected at 4 years of age between children with and without persistent wheeze (defined as any wheeze by age 3 years and wheeze or doctor diagnosed asthma by age 6 years). The authors found 54 CpGs that were

differentially methylated by wheeze status, and at four CGIs (*ZNF264*, *ALOX12*, *EPO* and *PDGFB*) the difference was more than 12% between children with and without persistent wheeze. The methyl ation levels were lower in children with persistent wheeze at *ZNF264* and *ALOX12*, and higher in *EPO* and *PDGFB* compared with children who never wheezed. As *ALOX12* belongs to the arachidonic acid metabolic pathway, the authors attempted to validate and replicate the findings using pyrosequencing. The validation in the discovery sample showed that the Illumina panel overestimated the methylation level and pyrosequencing-based *ALOX12* methylation level is no longer significantly associated with persistent wheeze. However, at two of the four investigated *ALOX12* CpGs, lower methylation level in cord blood DNA was significantly associated with increased risk of persistent wheeze in the replication sample. In the Menorca cohort, levels of DDE in cord blood were inversely correlated with DNA methylation in one of the *ALOX12* CpGs (labeled as E85); however, DNA methylation at this site was not associated with DDE levels in maternal serum collected in the first trimester in the replication sample. This paper provides the first epigenetic link between exposure to pesticides and asthma and suggests that *ALOX12* DNA methylation could be an epigenetic biomarker of susceptibility to asthma.

Another study conducted recently reported association between another gene in the arachidonic acid metabolic pathway and allergy. Among 36 Caucasian subjects (18 allergic and 18 controls), 22 CpGs in *PTGDR* were evaluated. DNA methylation was significantly lower in one promoter CpG and significantly higher in three intragenic CpGs in subjects with allergy compared with nonallergic subjects [30]. Although one promoter SNP (rs34236606) was within the promoter CpG that was differentially methylated by allergy status, the SNP was not associated with asthma in the overall study population (n = 637). Because the DNA methylation assay was carried out on a very small subset of the sample, the investigators were unable to examine the joint influence of genetic and epigenetic variation in *PTGDR* on asthma. Although measurement of epigenetic profile after asthma/allergy diagnosis may not imply a causal role, the findings of this paper raise the possibility that a therapeutic intervention that could modulate DNA methylation pattern in *PTGDR* may benefit allergic subjects.

Environmental exposures & DNA methylation

More studies have examined the influence of environmental exposures on DNA methylation than other epigenetic alterations. Emerging evidence indicates that some of the exposures that have been associated with asthma risk (e.g., maternal smoking, ambient air pollution, diet and microbial exposure during pregnancy) modulate DNA methylation at several CpG sites. In addition, low birth weight and maternal allergy have been found to modulate DNA methylation. However, whether the effects of these exposures and risk factors on childhood asthma are mediated by epigenetics remains largely unknown.

Several studies have documented that prenatal tobacco smoke exposure affects global DNA methylation (i.e., LINE-1, Sat2 and Alu repeats) in multiple tissues (placenta, peripheral blood and buccal cells) [31–35]. Recently, the effect of smoking on gene-specific methylation has been reported. In a genome-wide methylation study where Infinium 27K methylation array (Illumina, Inc.) was used, tobacco smoke exposures resulted in differential CpG methylation and placental expression of more genes in the oxidative stress pathways compared with the expression and DNA methylation in placenta from nonsmokers [34]. In a study conducted in the southern California CHS, exposure to maternal smoking *in utero* was associated with hypermethylation of CpGs in *AXL* and *PTPRO* in buccal DNA of the offspring [32], and the association with maternal smoking and *AXL* methylation in buccal DNA of the offspring was replicated by the investigators in an independent population [35].

Particulate air pollution has also been associated with global and gene-specific DNA methylation. While short-term (hours to days) air pollution (black carbon and particulate matter (PM) with aerodynamic diameter up to 10 μm [PM_{10}]) has been associated with global DNA (LINE-1 and Alu) and gene-specific iNOS (encoded for by *NOS2*) methylation in elderly subjects [36–38], a recent study conducted in children who participated in the CHS also documented that 7-day cumulative average exposure to PM with aerodynamic diameter up to 2.5 μm was significantly associated with lower methylation in a CpG site in the promoter region of *NOS2* [39]. Interestingly, this study also reported a statistically significant joint effect of short-term PM with aerodynamic diameter up to 2.5 μm exposure, one common promoter haplotype and promoter methylation in *NOS2* on exhaled nitric oxide (FeNO). In this sample, higher DNA methylation in *ARG1* and *ARG2* were also associated with lower FeNO, with stronger associations in children with asthma [40]. A recent study also documented that lower methylation in *NOS2* and *IL6* promoter in nasal epithelium was associated with higher FeNO among children with asthma [41]. These findings suggest a role of DNA methylation in genes in the nitric oxide synthesis and acute inflammatory pathways that regulate nitric oxide synthesis in airways.

In addition to effects on genes in the nitrosative stress pathway, air pollution exposure also influences DNA methylation in genes in the immune pathways. In a study among children with asthma, a 3-year average exposure to polycyclic aromatic hydrocarbon was associated with higher methylation of *FOXP3*, reduced Treg function and increased asthma severity [42]. In a murine model, exposing *Aspergillus fumigatus*-sensitized animals to *A. fumigatus* protein extract and diesel exhaust particles (DEPs) resulted in hypermethylation at several IFN-γ promoter CpG sites and hypomethylation at one IL-4 promoter CpG site that resulted in increased and reduced IgE levels, respectively [43]. In this study, the combined effects of allergen and air pollution were synergistic in affecting the IgE level that affected gene-specific DNA methylation. Earlier work among asthmatic subjects have shown that exposure to house dust mite (HDM) allergen results in hypomethylation in several CpG sites in IL-4 promoter in $CD4^+$ T lymphocytes and increased IL-4 levels in supernatant [44]. However, whether exposure-mediated epi-genetic changes in genes in the immune pathway affect asthma risk needs further exploration in prospective studies.

Maternal diet during pregnancy has been investigated as a potential risk factor of a child developing asthma or other allergic diseases. Recent evidence indicates that maternal folate intake could influence the epigenome. In a murine model, *in utero* exposure to dietary methyl donors was associated with increased allergic airway inflammation, whereas postnatal exposure was not significantly associated with airway inflammation [45]. One potential mechanism of the prenatal dietary exposure on airway inflammation was mediated by hyper-methylating *RUNX3* with concomitant reduced gene expression. This study demonstrated that a maternal high-methyl diet during pregnancy could be a potential mechanism of increasing the risk of allergic airway disease through epi-genetic regulation. Because serum IgE levels are elevated and airways are hyper-responsive to metha choline in *RUNX3* knockout mice [46], suggesting that suppression of *RUNX3* by increased level of methylation may be associated with asthma.

Another study demonstrated that cord blood homocysteine level was associated with lower LINE-1 methylation level in cord blood [47]. In a follow-up study, these investigators found additional evidence that gene-specific DNA methylation in some CpG sites correlates with cord blood homocysteine level, LINE-1 methylation and birth weight [48]. Another study documented that neonates with low (<2500 g) and high (≥4000 g) birth weights had significantly lower cord blood LINE-1 methylation than those with normal birth weight [49]. While insertion of LINE-1 in a transcript inhibits transcriptional elongation and reduces gene expression [50], which genes are affected by these repetitive elements and whether

methylation levels in repetitive elements affect asthma risk are currently unknown, and deserve further investigation.

Because farm-related exposures have been associated with reduced asthma risk, effect of the exposure on DNA methylation in immune cells has been investigated in few studies. In one study conducted in rural southern Germany, cord blood from neonates born to mothers who lived and worked in a farm before and during pregnancy had higher Treg cell numbers and activity, *FOXP3* hypomethylation and increased *FOXP3* gene expression compared with the levels observed in neonates born to nonfarming mothers [51]. Another study documented that *FOXP3* in cord blood $CD4^+$CD25hi Treg cells are hypomethylated in unstimulated and microbial stimulus lipid A (LpA) stimulated cord blood [52]. In addition, such *FOXP3* hypomethylation was associated with increased Th2 (IL-5 and IL-13) cytokines with LpA stimulation and with Th2 and IL-17 cytokines when stimulated with LpA and *Dermatophagoides pteronyssinus* (Derp1) allergen. In addition, such *FOX3* hypomethylation was associated with increased Th2 cytokines (IL-5 and IL-13) with LpA stimulation and with Th2 and IL-17 when stimulated with LpA and Derp1. Because Tregs are implicated in asthma and *FOXP3* is the master regulator gene for its development and function, these findings are promising to further evaluate the role of prenatal and early-life exposures on *FOXP3* methylation and how the latter influence asthma occurrence in children.

Histone modifications

A brief overview on histone modifications is presented in this article, as excellent reviews on this topic have been published elsewhere [53,54]. DNA is tightly packaged by histone proteins to form a highly organized chromatin structure. Post-translational modifications such as acetylation, methylation, phosphorylation and ubiquitination on the tails of core histones are important epigenetic modifications for gene transcription. Numerous studies have been performed on the lysine modification of histone tails. Acetylation of histone tails often results in a relatively loose structure of chromatin, which increases the accessibility of binding sites of transcription factors and thus the activation of gene expression. The status of histone acetylation is reversibly regulated by two distinct families of enzymes, histone acetyltransferases (HATs) and histone deacetylases (HDACs). Increased histone acetylation by HATs leads to the unwinding of chromatin structure and transcriptional activation, whereas removal of acetyl groups by HDACs causes chromatin condensation and transcriptional silencing.

Histone modifications & asthma

HDACs are implicated in the development and regulation of T lymphocytes, the latter implicated in asthma etiology. In a mouse model, conditional deletion of HDAC1 in T-cell lineage resulted in increased eosinophil recruitment into the lung, mucus hypersecretion, parenchymal lung inflammation and increased airway resistance, with parallel increase in Th2 cytokines (IL-4 and IL-13) in HDAC-deficient Th2 cells [55], suggesting that HDAC inhibition may result in allergic airway inflammation. Although no work detailing the role of histone modifications in the development of asthma in children has been published to date, some studies have documented the effect of HDAC inhibitors (trichostatin A [TSA] and sirtinol) on airway inflammation in humans and in animal models with conflicting results. An *in vivo* study in a murine model showed that (a reversible HDAC inhibitor), attenuated allergen-induced airway inflammation and airway hyper-responsiveness (AHR) and reduced IL-4, IL-5 and IgE in bronchoalveolar lavage fluid [56]. Mammalian SIRT1 is a class III HDAC that deacetylases several transcription factors (i.e., PPARγ, p53 and NF-κB) and plays a role in inflammation and aging [57,58]. In another murine model, OVA inhalation resulted in increased SIRT1, HIF-1α and VEGF protein levels in lung tissues and increased

AHR [59]. These effects were significantly attenuated by administration of sirtinol (inhibitor of SIRT1).

Contrary to these potential benefits of HDAC inhibitors, other studies have shown effects that could be detrimental in subjects with asthma. For example, in a study where the human alveolar epithelial cell line (A549 cell) was used, IL-1β-induced transcription of TGF-β1 and resulted in increased histone H4 and H3 acetylation in distinct promoter regions of TGF-β1 [59]. Prevention of histone deacetylation by administration of HDAC inhibitor TSA further increased TGF-β1 expression in this study, further supporting the role of histone acetylation in activation of TGF-β1. However, HDAC inhibitor-mediated increase in TGF-β1 concentrations in airway could have detrimental effects in asthma. A large body of evidence indicates that in inflammatory milieu (i.e., in the presence of IL-6 or IL-1β), TGF β1 plays a critical role in the development of proinflammatory Th17 cells [60,61]. Earlier studies have also shown that the variant -509T allele in a promoter SNP is associated with increase *TGFβ1* gene transcription, increased plasma TGFβ1 concentrations and increased risk of asthma [62,63].

In addition, earlier studies have shown that theophylline in therapeutic doses increased HDAC activity in epithelial cells and macrophages and promoted corticosteroid-mediated anti-inflammatory effects [64]. Finally, there are conflicting reports on the role of HDACs on steroid-resistant asthma. Earlier studies documented that downregulation in HDAC2 expression is one reason for steroid resistance in asthma [65,66]. However, data from a recent study suggests that downregulation of HDAC1 and HDAC2 expression does not occur in severe asthma and the earlier finding may have resulted due to clathrin cross-reactivity with commercial antibodies [67]. Based on these contradictory findings, the therapeutic potential of HDAC inhibitors in asthma management remains questionable. Additional studies are needed to fully appreciate the effects of HDAC inhibition in the different pathophysiological processes that are involved in asthma.

Environmental exposures & histone modifications

Few studies have documented associations between histone modifications and environmental exposures that are risk factors for asthma. A significant reduction of HDAC2 expression level was detected in the lungs of rats and different strains of mice exposed to cigarette smoking, and similar results were observed in asthma patients who smoked [68–70]. To study the mechanisms of cigarette smoking-induced degradation of HDAC2, Adenuga and colleagues treated human monocyte/macrophage cells, bronchial and primary small airway epithelial cells, and mouse lungs with cigarette smoke extract and found that HDAC1 and HDAC2 activity decreased in time-dependent manner [71]. The authors found induction of serine/threonine phosphorylation and proteasomal degradation was involved in tobacco smoke mediated HDAC2 downregulation. Other pathways are also implicated, as in another study, MSK1, a transcriptional activator of NF-κB was found to be an important downstream kinase that medicated tobacco smoke-mediated phospho-acetylation of histone H3 (Ser10/Lys9) and acetylation of histone H4 (Lys12) [72].

Another study documented that DEPs induced *COX2* gene expression (which plays an important role in inflammation) in human bronchial epithelial cell line through the acetylation of histone H4 associated with the *COX2* promoter and degradation of HDAC1 [73]. In addition, DEP exposure was associated with recruitment of HAT p300 to the *COX2* promoter, suggesting that acetylation is also important in regulating *COX2* expression in response to DEP exposure. The literature linking exposure to histone modifications and the latter with asthma phenotypes is quite sparse and warrants further investigation.

As mentioned earlier, exposure to farm-environment in early life has been associated with reduced asthma risk. Although histone modification and DNA methylation in genes encoding the Th1 cytokine (IFN-γ) and Th2 cytokines (i.e., IL-4, IL-5 and IL-13) have been associated with differentiation of Th1 and Th2 cells [74], whether such epigenetic mechanisms are involved in the protective effects of farm-related exposure on asthma remain largely unknown. To address this question, Brand and colleagues conducted a study in pregnant mice and their offspring [75]. They exposed pregnant mice to the farm-derived Gram-negative bacterium *Acinetobacter lwoffii* F78, and subsequently challenged the offspring with OVA (sensitized group) or phosphate buffered saline (sham-treated group) at 4 weeks of age. They found that OVA-challenged mice that were exposed to the bacterium *in utero* had significantly higher IFN-γ and lower IL-4, IL-5 and IL-13 in cell culture supernatants from splenic mononuclear cells compared with the levels in OVA-sensitized mice that were prenatally unexposed to the bacterium. In further analysis, the authors found the protective effect of *in utero* exposure to microbes on asthma phenotype was mediated by histone modification. First, the authors found that postnatal challenge with OVA reduced acetylated histone 4 (H4ac; a transcriptionally active chromatin) in mice that were unexposed to the *A. lwoffii* prenatally; however, prenatal exposure to *A. lwoffii* prevented the reduction of H4ac in OVA-challenged mice. Second, inhibition of H4ac by garcinol (a natural inhibitor of HAT [76]) opposed the protective effect of *in utero* exposure of *A. lwoffii* on asthma phenotypes in OVA-challenged mice by increasing airway inflammation and airway reactivity and significantly reducing IFN-γ production. The findings of this paper provide a link between the hygiene hypothesis and epigenetics, and suggest that the protective effects of prenatal microbial exposures on asthma could be mediated by epigenetic mechanisms.

miRNA

miRNAs are small (~22 nucleotides), non coding, ssRNAs that bind to target mRNA through complementary sequences and negatively regulate gene expression at a post-transcriptional level. The majority of miRNAs repress gene expression by mRNA destabilization and translational inhibition. Biogenesis, targets, mechanisms of action and role of miRNAs in innate (Toll-like receptor signaling) and adaptive (T-cell development) have been reviewed elsewhere [77–82]. In brief, the miRNA genes are usually transcribed by RNA polymerase II to generate a hairpin loop of primary miRNA (pri-miRNA). The resultant transcript is capped at the 5′ end, polyadenylated to confer a (poly)A tail and spliced. The RNase III endonuclease Drosha and the dsRNA-binding domain protein DGCR8/Pasha cleave the pri-miRNA to produce a precursor miRNA (pre-miRNA). The pre-miRNA is then transported into the cytoplasm by Exportin-5, where it is cleaved by RNase III endonuclease, Dicer to produce miRNA:miRNA* duplex. While the miRNA strand is incorporated into the argonaute-containing RNA-induced silencing complex, the miRNA* strand is degraded [77].

miRNAs & asthma

Many miRNAs are implicated in key patho-physiological aspects of asthma such as immune development and differentiaion, AHR and airway inflammation. A comprehensive review of such findings is beyond the scope of this article. While the role of some candidate miRNAs are reviewed here, other candidates (i.e., miR-9 [83], miR-155 [84–87], miR-127 [88], miR-147 [89] and miR-221 [90]) that are involved in immune development are not described, as these candidates have yet to be linked with asthma in humans or animals.

DNA sequence variations in miRNA genes (including the pri- and pre-miRNAs) have been found to influence miRNA function [91]. Few studies have evaluated the impact of

polymorphisms in miRNA target sites on asthma. Following up on the earlier finding that *HLA-G* as an asthma susceptibility gene [92], Tan *et al.* found that the +3142C/G SNP (*rs1063320)* in the *HLA-G* 3′-UTR affects binding of miR-148a, miR-148b and miR-152 to the gene, and the SNP interacted with maternal asthma to influence asthma risk in the offspring [93]. The GG genotype was associated with reduced risk of asthma in children born to asthmatic mother, whereas the GG genotype was associated with increased risk of asthma in children born to nonasthmatic mothers. In another study, SNPs in pre-miRNA sequence (miR-146a rs2910164 and miR-149 rs2292832) were associated with a reduced risk of asthma in a Chinese population [94].

In an experimental study, stretch stimulation of human airway smooth muscle cells induced transcription of miR-26a and resulted in hypertrophic responses in human airway smooth muscle cells [95]. The miR-26a targeted the mRNA 3′ untranslated repeats of GSK-3β and suppressed GSK-3β protein expression. However, using tissues collected by airway biopsy, no significant difference was detected for the miR-26 family and more than 200 other miRNAs (including let-7, miR-125 and miR-30 families) between normal and mild asthmatic subjects [96]. The investigators also compared miRNA profiles between airway biopsy samples before and after budesonide treatment; however, no differential expression pattern was revealed.

Animal models of asthma have implicated several miRNAs. In one study, upregulation of miR-21 was observed in three asthma models (OVA, *A. fumigatus* and induced IL-13 transgenic mice) compared with control mice [97]. The authors also found that miR-21 was predominantly expressed in the cytoplasm of cells in monocyte/macrophage lineage, with the highest expression in dendritic cells in bone marrow. IL-12p35 mRNA was found as the potential target gene of miR-21, and IL-12p35 mRNA was decreased in all three asthma models. Recently, miR-21 is implicated in allergic rhinitis (AR) in children. In a study where a panel of 157 miRNAs were interrogated using mononuclear leukocytes from human UCB, miR-21, miR-96 and miR-126 were significantly downregulated in neonates with high cord blood IgE (CBIgE), whereas the expression of miR-21 and miR-126 was significantly lower in children with AR [98]. The authors found that miR-21 targeted TGFBR2, and low miR-21 expression in cord blood was associated with significantly higher TGFBR2 expression in cord blood leukocytes in children with high CBIgE and those with AR, compared with children with low CBIgE and those without AR, respectively. Because rhinitis is a significant risk factor for asthma [99], further studies could examine whether miR-21 expression is associated with asthma risk.

The therapeutic potential of miRNA inhibitors have also been investigated in animal models of asthma. In OVA-sensitized female BALB/c mice, exposure to low levels of OVA resulted in a marked increase in miR-126 expression in exposed mice compared with control by two weeks; however, the expression was reduced to baseline level by 6 weeks [100]. Inhibition of miR-126 by administration of an antagomir suppressed eosinophil recruitment into the airways, but had no effect on chronic airway inflammation and remodeling. Another study also documented change in miRNA profiles by lipopolysaccharide exposure in male BALB/c without any significant modulating effect of dexamethasone [101]. In contrast to these findings, another study documented that exposure to HDM increased expression of miR-145, miR-21 and let-7b by fivefold in BALB/c mice [102]. Furthermore, inhibition of miR-145 by using an antagomir reduced eosinophilic inflammation, mucus hypersecretion, Th2 cytokine production and AHR; however, inhibition of miR-21 or lel-7b had no significant influence on HDM-mediated airway inflammation. The authors concluded that the anti-inflammatory effects of miR-145 were comparable to steroid treatment. The emerging evidence suggests a complex role of miRNAs in mediating the effects of exposure on underlying pathophysiological mechanisms involved in asthma.

Airway hyper-responsiveness is one of the pathologic hallmarks of asthma. In animal models of asthma, upregulation of RhoA (a monomeric GTP-binding protein) has been associated with increased contraction of bronchial smooth muscle. Using human bronchial smooth muscle cells (hBSMCs), Chiba *et al.* found that miR-131a negatively regulated expression of RhoA in hBSMCs [103]. Downregulation of miR-131a and upregulation of RhoA occurred when hBSMCs were treated with IL-13 and in airway tissues of OVA-challenged mice. Inhibition of miR-131a by treatment with antagomir-133a also resulted in upregulation of RhoA in this study.

Environmental exposures & miRNAs

Few human and animal studies have examined the role of environmental risk factors of asthma on miRNA expression. Among workers at an electric-furnace steel plant, Bollati *et al.* examined the association between PM and PM metal components on miR-222, miR-21 and miR-146a expression [104]. They measured miRNA expressions in leukocytes from blood samples collected on the first day of a work and after 3 days of work. Expression of miR-222 and miR-21 were significantly increased following 3 days of work, and the latter expression was associated with increased systemic oxidative stress as measured by 8-hydroxyguanine in blood. Baseline and postexposure miR-146a expression level were not significantly different; however, lead and cadmium levels in the PM_{10} fraction were significantly associated with lower miR-146a expression. On further analysis, the association remained significant in nonsmoking subjects but not in smokers. Lead and chromium exposure in the PM_{10} fraction upregulated miR-222 in non-smoking subjects but no significant association was found in smokers. The authors suggested that modulation of miRNA expression due to exposure to PM metal components may be one mechanism to enhance inflammation. Another study found that exposure to DEPs results in significant upregulation of miR-513a-5p, miR-494 and miR-923, and downregulation of miR-96 in normal human bronchial epithelial cells [105].

Although the effect of smoking on these miRNAs was not discussed by Bollati *et al.* [104], an earlier study documented that miR-218 and miR-128b expressions were significantly down-regulated, and miR-181 and miR-500 expressions were significantly upregulated in bronchial epithelium in smokers compared with non-smoking adults [106]. Another study documented that tobacco smoke significantly up regulated miR-31 in normal respiratory epithelium [107]. Whether secondhand smoke (SHS) affects miRNA expression in children remains to be investigated.

To date, few animal studies have reported associations between environmental exposures and miRNA expression. In Sprague Dawley rats, Izzotti *et al.* examined the effects of 4-week exposure to SHS on expression of 484 miRNAs in the lungs [108]. The exposure downregulated nearly 26% miRNAs by at least twofold and nearly 5% of miRNAs by more than threefold. Among the most downregulated miRNAs, there were some that have been found to affect pathophysiologic processes involved in asthma (e.g., let-7 and miR-146) while others were involved in regulating stress response, apoptosis, proliferation and angiogenesis. The only miRNA that was upregulated by SHS was miR-294, which is an inhibitor of transcriptional repressor genes.

Exposure to allergens such as HDM has been associated with asthma risk and exacerbation. In an animal study, Mattes *et al.* showed that HDM exposure increases miR-126 expression in the airways of mice with parallel increase in AHR and allergic airway inflammation [109]. Inhibition of miR-126 by treatment with antagomir ant-miR126 suppressed HDM-induced AHR and airway inflammation. The authors also found that expression of miR-126 requires functional *TLR4* or *MYD88* pathways, as miR-126 was not upregulated in *TLR4*-

and *MYD88*-deficient mice. In another study, the let-7 family of miRNAs was found to be the most abundant miRNAs in naive and allergen-challenged lungs [110]. While *IL-13* was found to be the target gene for let-7a, allergen-induced increase in IL-13 was not due to let-7a expression in lung, as the let-7a expression level was not different between allergen-challenged and control mice. When Th2 cells were evaluated, the authors found that let-7a was markedly suppressed, while IL-13 levels were highly elevated.

Another study documented that miR-146b was differentially expressed in the lungs of mice with and without allergen challenge [111]. In this study, three protocols of OVA inhalation challenge were performed to reflect short-, intermediate- and long-term exposures in OVA-sensitized male BALB/c mice. Among multiple miRNAs that were modulated by exposure, only miR-146b was consistently upregulated at all exposure levels. Based on a bioinformatics tool, the authors predicted miR-146b could target genes in cell signaling, immune response, inflammatory and apoptotic pathways.

These findings provide evidence that air pollutants and allergens modulate miRNA expression. However, the findings have been inconsistent across studies. Some reasons for these inconsistencies could be due to evaluation of different exposures (PM and DEPs), tissues (i.e., blood and airway epithelium) and species (human and several species of mice). In order to address these inconsistencies and to account for the adjuvant effects of pollutants on allergen-mediated effects [112], additional studies are required to examine the independent and combined effects of pollutants and allergens on miRNA profiles in cells of interest.

Conclusion & future perspective

A large body of evidence indicates that some of the environmental risk factors that have been associated with asthma risk in children are also associated with global and gene-specific epigenetic variations in multiple tissues. Furthermore, some epigenetic variations within genes that are implicated in asthma development have been associated with airway inflammation and remodeling. Although epigenetics may be one of the missing links between exposure and asthma, few studies have actually linked the exposure-mediated effects to epigenetic profiles on the risk and natural history of asthma in children. While assays performed on mixed cell populations have provided some intriguing data, more work is urgently needed to overcome some of the major challenges facing research on asthma epigenetics.

Some of the major questions that remain to be addressed in the field of asthma are:

- Do environmental exposures have relatively stable effects on some epigenetic profiles and dynamic effects on other?
- Do epigenetic changes on individual cell types that have been found to be involved in asthma predict asthma risk and modulate natural history of asthma?
- Are there critical developmental windows that modulate exposure-mediated asthma susceptibility?
- Are there synergistic effects of environmental exposures, and genetic and epigenetic variations on asthma risk and natural history of asthma?
- Can intervention strategies modulate epigenetic profiles to affect asthma risk and exacerbations in children?
- Are there transgenerational effects of exposures that are epigenetically mediated that affect asthma risk?

These research questions need to be critically evaluated in future studies to advance our understanding of the role of epigenetics on asthma and to find strategies to reduce the burden of asthma worldwide.

To address these research questions, appropriate study design, data collection and analytic strategies are required. First, epigenetic profiles need to be assessed in specific cell types, preferably more than one cell type that are implicated in asthma. It is possible that the findings published to date showing associations between environmental exposure and DNA methylation have resulted due to exposure-mediated differential cell counts in leukocytes. With the backdrop of this methodological limitation, studying the role of environment on epigenetic variation and how that relates to future disease risk becomes even more challenging, as a samples collected during susceptible windows (i.e., before disease onset) would be required for etiologic research. One may envision investigating multiple cell types of interest; however, collection of some of the tissues involves invasive testing (e.g., bronchoalveolar lavage and bronchial biopsy) or the tests have suboptimal performance (e.g., induced sputum) in infants and young children, not to mention isolation of specific cells from these samples. Although collection of buccal epithelium and blood (cord or peripheral) samples may be feasible on a relatively large sample of infants for a prospective study, one argument against the use of buccal and blood (cord or peripheral) in understanding asthma epigenetics is that these cells/tissues may not represent epigenetic changes in different cells (i.e., immune, airway epithelium and smooth muscle cells) within the lung tissue.

To make scientific progress in the field, one necessary first step would be clinical and epidemio logical collaboration that aims to identify the effect of prenatal and early-life environmental risk factors on epigenetic marks across specific cells of interest. The epigenetic marks thus identified could serve as potential bio-markers of susceptibility for further investigation in prospective studies. With a large number of ‘candidate’ environmental exposures, analytic methods need to be developed that could integrate mixtures of exposure, large-scale genetic and epigenetic data with phenotypes. There is some evidence that genetic variation interacts with exposure and DNA methylation to affect FeNO, a phenotype relevant to asthma [39]. In addition, repeated measure of epigenetic marks from birth to disease onset together with longitudinal evaluation of the effects of environmental exposures on those epigenetic marks could identify whether there are subsets of relatively stable and dynamic epigenetic marks, and whether different windows of exposure have differential impact on epigenetic profiles. While the less-modifiable epigenetic marks could serve as biomarkers of susceptibility, the modifiable ones could be targets for intervention to alter risk profile and/or improve natural history of disease. Furthermore, for linking potentially modifiable epigenetic marks with asthma development and progression, repeated measurements of exposures and epigenetic variations over time and appropriately integrating the data with lagged effects of exposure on epigenetic marks could be a useful strategy to examine the associations.

There are other methodological concerns that must be addressed. Multiple assay methods are available for measuring DNA methylation, which rely on enzymatic digestion, affinity enrichment and/or bisulfite conversion, discussed in more detail elsewhere [113,114]. Some of the genome-wide approaches for methylation assays are not sensitive enough to detect DNA methylation with low frequency; however, with next-generation sequencing approaches (although more expensive) could be used to detect rare CpGs [115]. In terms of assays using common DNA methylation array platforms, genetic variations (SNPs, repeats, deletions, copy number variations and so on) need to be excluded and background correction and normalization of the data are needed [116–117]. In addition, appropriate intra- and inter-plate controls should be included for assessing and controlling for batch effects. Once

epigenetic biomarkers are identified, technical validation of the most significant loci by another assay (i.e., pyrosequencing) and replication of the findings in independent samples are needed to assure data quality and to show consistency of findings, respectively. Correction of multiple testing could minimize false discovery. Although the bisulfite-based assay methods are quite accurate, incomplete bisulfite conversion could introduce measurement error in estimation of DNA methylation [113]. Monitoring of completion of bisulfite conversion is therefore needed [119]. Linking variation in epigenetic marks to difference in gene expression could provide functional relevance of the epigenetic marks.

Although some animal studies showed transgenerational effects of exposures (e.g., diet and obesity) on DNA methylation [45,120], true transgenerational effects of exposure on epigenetic profiles and on asthma have not been reported in humans to date. Although the area is of great interest, some major methodological problems exist. Evaluation of transgenerational effects of exposure on the epigenome requires at least four generations because any exposure during pregnancy (first generation) exposes not only exposes the embryo (second generation) but also exposes the germline of the developing gonads (third generation) [121]. In addition, as opposed to controlled animal studies, the exposures in humans could happen postnatally in all subsequent generations and effects of the exposure to paternal sources of chromosomes could further complicate the matter in distinguishing true transgenerational effects from pre- and post-natal exposure-mediated effects on the epigenome.

Investigation of the etiology and natural history of a complex trait, such as asthma in children, is difficult. Studying epigenetics as a mediating factor for the associations between exposures and asthma development holds promise to unravel novel biologic pathways. While a good dose of optimism will advance the field of asthma epigenetics, too much hype must be avoided as even the robust effect sizes could be small, similar to what was observed in genome-wide association studies. Because asthma often develops during early childhood, appropriately designed prospective studies (preferably a pregnancy cohort) in which biological samples are collected at multiple times can fill gaps in scientific knowledge and identify areas for further research or environmental intervention. The potentially modifiable nature of the epigenetic profiles may allow us identify intervention approaches to reduce the risk of asthma in children and improve natural history of the disease.

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Executive summary

Asthma

■ Asthma is the most common chronic disease of childhood, affecting approximately one in eight children worldwide.

Epigenetics

■ Epigenetics is the study of mitotically or meiotically heritable changes in gene expression that occur without directly altering the DNA sequence.

■ DNA methylation, histone modifications and miRNAs are major epigenetic variations in humans that are currently being investigated for asthma etiology and natural history.

■ DNA methylation results from the addition of a methyl group to the 5′ position of a cytosine ring and occurs almost exclusively on a cytosine in a CpG dinucleotide.

■ Histone modifications involve post-translational modifications such as acetylation, methylation, phosphorylation and ubiquitination on the tails of core histones.

■ miRNAs are short, approximately 22-nucleotide long, noncoding ssRNAs that bind to complementary sequences in the target mRNAs, usually resulting in gene silencing.

Epigenetics & asthma

■ Most studies have documented associations between environmental exposures that have been implicated in asthma etiology and epigenetic alterations.

■ Few studies have directly linked epigenetic variations and asthma development.

Conclusion & future perspective

■ Epigenetic variations are cell and tissue-specific and the complex interplay among these variations, environmental exposures and DNA sequence variants are poorly understood.

■ Whether prenatal and early-life environmental exposures result in irreversible changes in epigenetic profiles throughout early childhood remains largely unknown.

■ Some of the major methodological concerns in identifying epigenetic determinants of asthma in children include the use of mixed cell populations for epigenetic profiling, the lack of genetic material from a single cell type that had been collected before asthma occurrence, and the fact that many cell types are involved in asthma and collection of some these cells involves invasive testing that is not feasible in infants.

■ Future studies, preferably pregnancy/birth cohorts, in which genetic materials are collected and specific cell types are isolated hold promise to identify epigenetic biomarker for exposures and asthma development in childhood.

■ Longitudinal evaluation of the effects of environmental exposures and asthma medication use on epigenetic profiles within subjects will provide better understanding of the dynamic role of epigenetics on health.

■ Appropriate statistical methods need to be developed to integrate environmental exposures and genetic and epigenetic variations in understanding the etiology and natural history of asthma.